Review

K63 ubiquitination in immune signaling

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Ubc13-catalyzed K63 ubiquitination is a major control point for immune signaling. Recent evidence has shown that the control of multiple immune functions, including chronic inflammation, pathogen responses, lymphocyte activation, and regulatory signaling, is altered by K63 ubiquitination. In this review, we detail the novel cellular sensors that are dependent on K63 ubiquitination for their function in the immune signaling network. Many pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), can target K63 ubiquitination to inhibit pathogen immune responses; we describe novel details of the pathways involved and summarize recent clinically relevant SARS-CoV-2-specific responses. We also discuss recent evidence that regulatory T cell (Treg) versus T helper (T$_H$) 1 and T$_H$17 cell subset regulation might involve K63 ubiquitination. Knowledge gaps that merit future investigation and clinically relevant pathways are also addressed.

Highlights

K63 ubiquitination can drive NF-κB- and MAPK-mediated immune cell activation in a cIAP1/2- and TRAF2/3/6-dependent manner.

Ubc13 catalyzes K63-linked ubiquitin chains on stimulator of interferon genes (STING) and UbcH5c catalyzes mono-ubiquitination and can have important roles in antiviral immunity, including SARS-CoV-2 infection.

Ubc13 can regulate K63-linked ubiquitination of FOXP3 and has been reported to modulate the immunosuppressive function of peripheral Tregs.

K63 ubiquitination can modulate Casitas B lineage lymphoma B- and SHARPIN-mediated T cell receptor activation while regulating the balance between Treg and inflammatory effector T cell subsets.

Biological significance of K63 ubiquitination

K63-linked ubiquitination is involved in the regulation of signal transduction by multiple receptors of innate and adaptive immunity in eukaryotes, including tumor necrosis factor (TNF) and members of the TNF-receptor (TNFR) family, T and B cell receptor (TCR/BCR), **Toll-like receptor** (TLR; see Glossary), **Nod-like receptor** (NLR), **RIG-I-like receptor** (RLR), and the Interleukin-1 receptor (IL-1R) pathways [1,2]. In this review, we focus on the emerging understanding of the role of K63-linked ubiquitination in an array of immune response mechanisms. We also highlight the important regulatory role of K63 ubiquitination in striking a balance between immune activating *versus* immune tolerance mechanisms. We review prominent examples of key signaling adaptors, including cellular inhibitor of apoptosis proteins (cIAPs), TNF receptor-associated factors (TRAFs), NLRs, RLRs, and stimulator of interferon genes [STING, also referred to as Mediator of IRF-3 activation (MITA) or endoplasmic reticulum IFN stimulator (ERIS)], that serve as docking platforms for K63-linked ubiquitin chain assembly during immune cell activation and tolerance mechanisms (Figure 1, Key figure). The complementary role of deubiquitinating enzymes is also briefly mentioned, as well as the emerging putative role of K63-linked ubiquitination in SARS-CoV-2 immune dysregulation. In addition, we summarize emerging breakthrough discoveries on SARS-CoV-2-induced specific immune responses that might be associated with K63 ubiquitination.

Ubiquitination involves covalent binding of ubiquitin to target substrate proteins in an ATP-dependent manner, a mechanism that involves isopeptide linkage between the C terminus of ubiquitin and the epsilon amino group of an internal lysine residue of the target substrate [3,4]. Ubiquitin chain formation can involve any of the possible seven lysine (LYS, K) residues (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal methionine (Met1) on ubiquitin, depending on various factors, including the type of E2, E3 ligases, and the target substrates, which ultimately dictate the functional specificity of any particular ubiquitination event [5] (Boxes 1–3). The K48-mediated ubiquitin linkages target proteins for proteasomal degradation and are typically referred to as canonical. The K63-linked ubiquitin chains do not target to the proteasome, but instead have important roles in eukaryotes, including DNA damage repair, cell

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signaling, and autophagy [1,5–7]. Ubc13 is an unusual E2 that, in complex with its non-enzymatic co-factors (either Mms2 or Uev1a), catalyzes K63-specific linkages [8,9]. Ubc13-Mms2 is essential for cellular mechanisms involving DNA damage repair, whereas the Ubc13-Uev1a complex regulates cell signaling pathways that drive immune, inflammatory, and cell proliferation/survival responses [8,9,10,11]. K63-linked ubiquitin chains can be formed anchored to the target protein or can exist as free or unanchored chains and serve as docking sites for signaling molecules that drive immune pathways, such as TLRs, IL1R, and RLRs [12–15]. Heterogeneous K63 ubiquitin chains or K48/K63 branched ubiquitin chains can preferentially target the substrates for proteasome degradation [16]. K63 ubiquitin-conjugated E3 ligases and their target substrates undergo deubiquitination in the presence of deubiquitinases (DUBs) and have an important role in modulating K63-dependent cell signaling [17,18] (Box 4). Research on Ubc13-catalyzed K63 ubiquitination has rapidly advanced over the years and the functional implications of this non-proteolytic post-translational mechanism that are of relevance to innate and adaptive immunity are summarized herein. While the focus is largely on K63-linked ubiquitination and the role of Ubc13 in immune cell signaling, it is noteworthy that other important modulators of immune responses, including other unusual E2s, UbcH5, UbcH7, other E3 ligases, and LUBAC, are not addressed and are detailed elsewhere [2,19–23].

Ubc13 turns on the inflammatory response

Studies using gene ablation in mice demonstrated the key roles of *Ubc13* in pathways for host defense, autoimmunity, and inflammatory diseases [24–26]. Given that the homozygous *Ubc13* knockout is embryonic lethal, haploinsufficient *Ubc13*^{+/−} mice were generated on a C57BL/6 and 129SV background by retroviral insertional mutagenesis that inactivated one *Ubc13* allele; these heterozygotic *Ubc13*^{+/−} mice showed a normal phenotype [24].

Comparisons of murine splenocytes and macrophages of homozygous wild-type (WT) *Ubc13*^{+/+} and heterozygous *Ubc13*^{+/−} cells stimulated with lipopolysaccharide (LPS) or TNF revealed reduced cytokine production by *Ubc13*^{+/−} immune cells relative to those from WT controls [24]. In culture, splenocytes of *Ubc13*^{+/−} mice, upon stimulation with LPS, exhibited reduced IκBα degradation, phospho-JNK and phospho-p38 MAPK expression, which were associated with diminished TRAF6 polyubiquitination relative to WT controls [24]. In addition, *Ubc13*^{+/−} mice exhibited reduced Ubc13 protein in most tissues, along with significantly impaired inflammatory signaling in response to TNF and LPS stimulation *in vivo* [24]. This decreased signaling was associated with reduced *in vivo* TRAF6 ubiquitination and reduced *in vivo* activation of NF-κB and stress kinases (p38 MAPK and JNK), demonstrating a key role for Ubc13 in inflammatory responses [24].

However, in other independent studies, tissue-specific gene-knockout studies reported in B cells, T cells, and macrophages derived from *Ubc13*-deficient mice (*Lck*_Cre *Ube2n*^{fl/fl} mice generated on a mixed C57BL/6 and 129P2/OlaHsd background) showed different results [25,26]. While homozygous *Ubc13*^{−/−} B cells and macrophages demonstrated essential roles for Ubc13 in BCR-, TLR/IL-1R-, or CD40-mediated activation of MAPKs, Ubc13 had a minor role in BCR-, TLR-, IL-1R-, or CD40-mediated NF-κB activation and IL-1β-mediated TAK1 phosphorylation in these same cells [25]. Nevertheless, conditional deletion of *Ubc13* in T cells (*Lck*_Cre *Ube2n*^{fl/fl} thymocytes) showed reduced NF-κB essential modulator (NEMO, also known as IKK-γ) ubiquitination, IκBα degradation/phosphorylation, and NF-κB activation upon phorbol 12-myristate 13-acetate (PMA)/Ionophore and anti-CD3/CD28 antibody stimulation relative to WT [26]. The authors attributed this discrepancy to *Ubc13* deletion efficiency in each conditional model and/or to a cell type-specific role of Ubc13 in the activation of NF-κB [26]. Comparison of the results from homozygous *versus* heterozygous deletion of *Ubc13* also

Glossary

Caspase recruitment domain-containing membrane-associated guanylate kinase protein1-B cell lymphoma 10-mucosa-associated lymphoid tissue protein 1 (paracaspase) [CARMA1-BCL10-MALT1 (CBM)] complex: forms a signalosome in the classical NF-κB activation pathway.

Damage-associated molecular patterns (DAMPs): include aged, dead, and damaged self-structures released from host cells in response to necrosis or apoptosis and are endogenous alarmins that trigger an immune response.

Inflammasome: multiprotein oligomeric complex assembled by some NLRs upon sensing intracellular PAMPs/DAMPs; serves as a signaling platform for the recruitment of caspase-1, which activates IL-1β from its precursor form (pro-IL-1β) to induce innate immunity.

Linear ubiquitin chain assembly complex (LUBAC): comprises the catalytically active HOIP and accessory proteins HOIP-L and SHANK-associated RH domain interacting protein SHARPIN; functions as an E3 ligase that forms linear ubiquitin chains from Met1 of ubiquitin.

Pattern recognition receptors (PRRs): present on the host cell surface, in the endosome, or in the cytoplasm; recognize a variety of conserved structural motifs present on PAMPs and DAMPs, activating immune signaling pathways that target pathogens/alarmins for their eventual clearance by the host immune system.

Pathogen-associated molecular patterns (PAMPs): conserved molecular structures present on microbes or pathogens; specific to the pathogen of interest, are carbohydrates, lipoproteins, or nucleic acids (bacterial or viral DNA and RNA) sensed by host cells to trigger an immune response.

NLRP3: also known as cryopyrin; a cytosolic PRR that forms the inflammasome upon sensing PAMPs/DAMPs to trigger an induced innate immune response.

Nod-like receptors (NLRs): a family of cytoplasmic PRR sensors that detect cytosolic PAMPs or DAMPs via LRR domains and assemble to form inflammasomes, which trigger innate immunity in response to infection or endogenous alarmin signals.

Regulatory T cell (Treg): subpopulation of CD4+ helper T cells

suggested gene dose-dependency effects and a cell type-specific role for Ubc13 [24–26]. In summary, these studies confirmed that Ubc13 could regulate immune cell signaling responses in a cell type-specific manner [24–26].

Recently, male and female haploinsufficient $Ubc13^{+/-}$ mice (on a C57BL/6 background [24]) were shown to be protected against age-related insulin resistance under normal diet (ND) and high-fat diet (HFD) conditions compared with WT controls, suggesting an additional role for K63 ubiquitination in chronic inflammation-induced metabolic syndromes [27]. Histological analysis of visceral adipose tissue (VAT) showed the adipocyte cell size to be smaller in female HFD-fed $Ubc13^{+/-}$ mice compared with WT mice [27]. Moreover, 18-week-old female $Ubc13^{+/-}$ mice showed lower inflammatory expression of the cytokine genes *TNFA*, *IL6*, and *IL1B* in VAT, secondary to reduced weight gain, compared with male $Ubc13^{+/-}$ and WT mice [27]. These results indicated that the effects of *Ubc13* haploinsufficiency were more prominent in female HFD mice compared with male mice; thus, further investigations are needed to understand the mechanism behind these mouse gender differences.

K63 ubiquitination in immune signaling
K63-linked ubiquitination involving cIAPs and TRAFs in immune cells: implications for immunomodulation

cIAPs are phylogenetically conserved proteins that have up to three Baculovirus IAP repeat (BIR) motifs. Some cIAPs are implicated in the suppression of apoptosis by virtue of their ability to bind active caspase-family proteases [28,29]. The IAP family of proteins, including cIAP1, cIAP2, and XIAP, have E3 ubiquitin protein ligase activity [30–32]. TRAF2 and TRAF6 form a complex with cIAP1/2 to conjugate K63-linked ubiquitin chains catalyzed by Ubc13, followed by NF-κB or MAPK activation in human immune cell activation via antigen and **pattern recognition receptors** (PRRs) [33,34] (Figure 2, Box 2). While IAPs activate the classical NF-κB pathway, relying on K63 ubiquitination, these E3 ligases repress the non-canonical NF-κB cascade by promoting K48-mediated degradation of NF-κB-inducing kinase (NIK); both pathways proceed in a TRAF-dependent manner. The immunomodulatory role of TRAFs is reviewed elsewhere [35] (Figure 2).

Of clinical relevance, several cIAPs are overexpressed in various types of cancer [36–39]. Human MALT lymphoma samples with the frequently observed t(11;18)(q21;q21)-positive translocation express cIAP2/MALT1 fusion protein, which shows an increase in polyubiquitinated NEMO compared with human tonsil samples, suggesting that the c-IAP2/MALT1 fusion protein drives constitutive NF-κB activation in MALT tumor-bearing cells [40]. The cIAP2/MALT1 fusion gene construct overexpressed in 293T cells as cIAP2/MALT1 fusion protein has been shown to interact with Ubc13, suggesting that this fusion protein is also involved in K63 ubiquitination [40].

In the context of TCR activation, TRAF2/TRAF6-dependent K63-ubiquitination of MALT1 in the **Caspase recruitment domain-containing membrane-associated guanylate kinase protein1-B cell lymphoma 10-mucosa-associated lymphoid tissue protein 1 (paracaspase) [CARMA1-BCL10-MALT1 (CBM)] complex** is required for NF-κB activation [32,41] (Figure 2). MALT1 K63 ubiquitination, in response to PMA/Ionomycin- and/or anti-CD3/CD28 antibody-induced human Jurkat and murine CD4$^+$ T cell activation, was associated with IκBα degradation and NF-κB activation compared with uninduced cells [41]. TRAF2-binding (WT) cIAP2/MALT1 *versus* TRAF2-nonbinding (mutant) cIAP2/MALT1 in Jurkat T cells showed elevated NF-κB induction for WT, but not mutant complexes [32]. Ubc13-Uev1a- (and UbcH5c-)catalyzed TRAF6-mediated K63 ubiquitination of MALT1 was confirmed using a cell-free system [41,42]. MALT1-deficient T cells isolated from $Malt1^{-/-}$ mice showed impaired IκBα degradation and NF-κB activation upon PMA/Ionomycin stimulation compared with WT controls [41]. These observations implied that ubiquitinated MALT1

with immunosuppressive properties that help maintain self-tolerance and immune cell homeostasis.

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs): family of RIG-I-like receptors, RNA helicases, cytoplasmic PRRs, sense cytosolic viral RNA, induce transcriptional activation of Type 1 IFN (IFN-α/β) responses, and trigger antiviral immunity.

Toll-like receptors (TLRs): family of PRRs homologous to the *Drosophila* Toll receptor; form dimers with extracellular leucine-rich repeat (LRR) domains, sense the presence of pathogenic microbes, bind to PAMP ligands and, upon recognition of PAMPs, activate downstream signaling cascades contributing to innate/inflammatory responses.

Key figure

Ubc13-catalyzed K63-linked ubiquitination can regulate immune cell activation and immune tolerance mechanisms
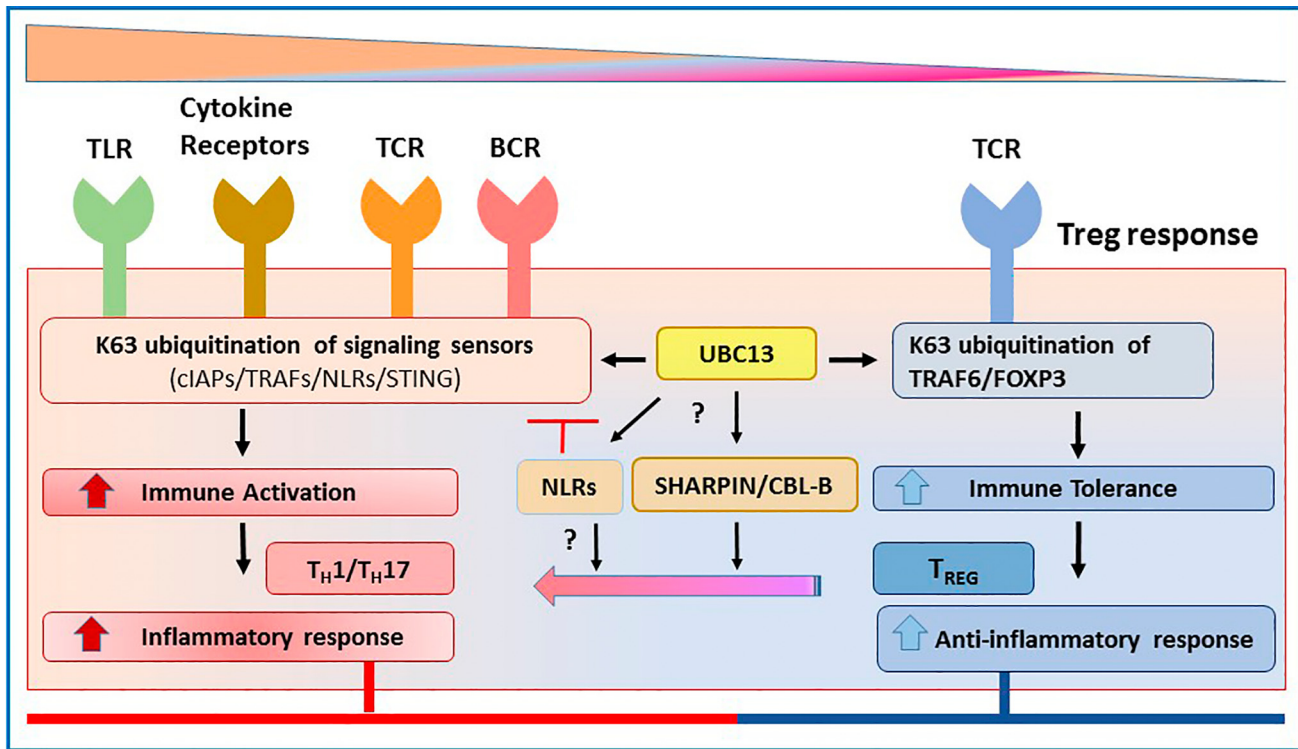
Figure 1. Signaling sensors of K63-linked ubiquitination [including cellular inhibitor of apoptosis proteins (cIAPs), TNF receptor-associated factors (TRAFs), Nod-like receptors (NLRs), and stimulator of interferon genes (STING)] activate innate and adaptive inflammatory responses (left). FOXP3-dependent K63 ubiquitination of target substrates regulates immune tolerance mechanisms that counteract the inflammatory phenotype (right). While some NLRs negatively regulate K63-linked ubiquitination to blunt inflammatory responses, the role of NLRs in regulatory T cell (Treg)- *versus* T_H1/T_H17-induced T cell responses is unknown. The mechanistic role of Ubc13 on the modulators of K63 ubiquitination [including SHARPIN or Casitas B lineage lymphoma b (CBL-B)], deficiency of which blunts immune tolerance mechanisms and causes a shift from Treg-induced immunosuppression to T_H1/T_H17-induced immune activation, needs further investigation. Abbreviations: BCR, B cell receptor; TCR, T cell receptor; TLR, Toll-like receptor.

of the CBM complex could serve as a docking platform for subsequent ubiquitination, followed by activation of the IKK complex of the classical NF-κB cascade upon PKC-theta-dependent TCR engagement (Figure 2).

Additionally, SHARPIN [a subunit of the **linear ubiquitin chain assembly complex (LUBAC)**, which promotes M1-linear ubiquitin chains] has been shown to bind K63-ubiquitinated cIAP1/2 in activated B cell-like diffuse large B cell lymphoma (ABC DLBCL) cells [43,44]. cIAP1/2 interacts with the CBM complex and inactivation of cIAP1/2 using the CRISPR/Cas9 gene-targeting system in human HBL1 ABC DLBCL cells showed a decrease in IκBα degradation and NF-κB activation relative to controls, highlighting an immunomodulatory role of cIAP1/2 in classical NF-κB activation [43,44]. Mechanistically, these results suggest that K63 ubiquitination of BCL10 and MALT1 is needed for LUBAC-mediated recruitment of IKK into the CBM complex, as well as

subsequent ubiquitination of NEMO for activation of NF-κB signaling in lymphocytes [43–45] (Figure 2).

Immunomodulatory functions of K63-linked ubiquitination in T cells

K63-linked ubiquitination has a dual regulatory role in both immune cell activation and immunosuppression, as part of immune surveillance mechanisms involving T cells (Figure 1). K63 ubiquitination of TRAF6 regulates FOXP3-dependent TGF-β-induced peripheral **Treg** function [46]. *Traf6*$^{fl/fl}$*Foxp3Cre*$^+$ mice with FOXP3$^+$ Treg-restricted deletion of TRAF6 (*Traf6*$^{-/-}$) either failed to support the growth of implanted B16 melanoma cells or delayed the implantation of MC38 colon cancer cells compared with WT mice [46]. Impaired Treg function and increased anti-tumor immunity were also associated with impaired K63 ubiquitination of FOXP3, observed in TRAF6- and FOXP3-overexpressing HEK293T cells along with a robust increase in the production of IFN-γ and IL-17 cytokines from tumor-infiltrating leukocytes or tumor-draining lymph nodes upon *ex vivo* stimulation with PMA/Ionomycin [46]. In summary, these studies show a possible role of K63 ubiquitination in immune tolerance, as evidenced by the lack of tolerance and a strong T cell activation associated with a shift toward enhanced antitumor immunity in *Traf6*$^{fl/fl}$*Foxp3Cre*$^+$ mice compared with WT controls [46].

Treg-specific *Ube2n*-deficient mice (*Ube2n*$^{fl/fl}$ *Foxp3*$^{GFP-hCre}$; known as *Ube2n*^{TregKO}), generated upon conditional deletion of the *Ubc13* coding gene (*Ube2n*), displayed autoimmune symptoms with a simultaneous large increase in T$_H$1, T$_H$2, and T$_H$17 effector CD4$^+$ T cells compared with age-matched *Ube2n*$^{+/+}$*Foxp3*$^{GFP-hCre}$ WT controls [47]. An autoimmune disease phenotype was observed in *Rag1*$^{-/-}$ mice upon adoptive transfer of Tregs from *Ube2n*^{TregKO}; these transferred Ubc13-deficient Tregs acquired a T$_H$1/T$_H$17 phenotype and were IFN-γ-IL17 double-positive under lymphopenic conditions compared with WT controls [47]. Compared with WT cells, Ubc13-deficient Tregs retained FOXP3 expression and had lower expression of the genes encoding IL10 (*Il10) and suppressor of cytokine signaling 1 (Socs1*, which prevents conversion of Tregs into T$_H$1 and T$_H$17-like effector T cells) [47]. Thus, the regulatory role of K63 ubiquitination is thought to be due to the maintenance of T cell homeostasis, as evidenced from the essential role of Ubc13 in the *in vivo* immunosuppressive function of Tregs, maintenance of Treg stability,

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Box 3. NLRs and STING activation

Some NLRs regulate STING activity (see Figure 3 in the main text). Stimulation of the immune response to viral infections involves many of the same immune pathways as immune responses to tumor cells, including Type 1 and 3 IFNs and cytokine secretion. While NLRC3 binds to STING and blocks Type 1 IFN responses [110], NLRC5 attenuates Type 1 IFN responses due to its ability to interfere with the binding of the RLRs, RIG-I and MDA-5, to MAVS [111]. NLRX1 also binds to mitochondrial adaptor MAVS and prevents its interaction with RIG-I, thus inhibiting not only NF-κB, but also IRF3 pathway-induced antiviral immune responses [112]. NLRP12 abrogates K63 ubiquitination of RIG-I, prevents RIG-I from activating MAVS, and inhibits RIG-I-induced antiviral immune responses [113,114]. NLRP12 reduces K63 ubiquitination of RIG-I, while enhancing K48-linked ubiquitination of RIG-I, as demonstrated both in an overexpressing system using HEK293 cells and endogenously in dendritic cells (derived from WT and $Nlrp12^{-/-}$ mice).

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and prevention of T cell transformation into an inflammatory CD4$^+$ T$_H$1- or T$_H$17-like phenotype [47] (Figure 1).

There are several other modulators of K63 ubiquitination (Figure 3). For instance, SHARPIN-deficient $Cpdm^{-/-}$ mice show a significant reduction in the generation of Tregs, as evidenced by the inefficient induction of Foxp3 in $Cpdm^{-/-}$ CD4$^+$ T cells upon anti-CD3 antibody stimulation, compared with $Cpdm^{+/-}$ mice [48]. The autoimmune and inflammatory phenotype of $Cpdm^{-/-}$ mice was overcome by replenishing $Cpdm^{-/-}$ neonatal mice with SHARPIN-sufficient Tregs from $Foxp3^{YFPCre}Cpdm^{+/+}$ via adoptive transfer. This was shown by a reduction in the lung inflammatory effector T cell subsets (T$_H$2 and T$_H$17-like) and their cytokines (including IL-3, IL-5, and IL-17), compared with $Cpdm^{-/-}$ mice that received no Treg cells [48]. Human Jurkat T cells that stably expressed SHARPIN and endogenous SHARPIN in mouse WT CD4$^+$ T cells assembled K63-ubiquitin chains, attenuated TCR signaling, and showed an increase in FOXP3 expression relative to controls [48]. However, CD4$^+$ T cells from $Cpdm^{-/-}$ mice that lacked K63-ubiquitinated SHARPIN showed greater association of TCRζ with the signaling kinase Zap70

Box 4. Deubiquitinases that reverse K63-linked ubiquitination

K63 ubiquitin-conjugated E3 ligases and their target substrates are also subjected to deubiquitination in the presence of DUBs [17,18]. The zinc-finger (ZnF) protein A20 and cylindramotosis (CYLD) protein are DUBs that catalyze the hydrolysis of K63-linked ubiquitin chains conjugated to target E3 ligases or substrates [115]. CYLD$^{-/-}$ T cells are hypersensitive to TCR/CD3 and TCR/CD28 activation of T cells. A20 negatively regulates NF-κB signaling by inhibiting E3 ligase or substrate activity (TRAF2, TRAF6, cIAP1, RIPK1, and MALT1) [116,117] (see Figure 2 in the main text). A20 inhibits the interaction between E2s Ubc13 or UbHc5c and, by doing so, A20 along with Tax1-binding protein 1 (TAX1BP1) targets Ubc13 or UbcH5 for K48-ubiquitin chain-conjugated proteasomal degradation [118]. A20 functions to deubiquitinate K63-ubiquitin linkages conjugated to MALT1 of CBM-Ubc13-TRAF6 complex and impairs TCR-induced NF-κB signaling; A20 also undergoes proteasomal degradation to restore TCR/CD28-induced IKK activation [119]. OTUD7B is a DUB that deubiquitinates K63-assembled ubiquitin chains from GβL, a substrate for TRAF2 E3 ligase [120]. Ubiquitin-specific protease 5 (USP5) specifically targets the free C-terminal diglycine motif of the unanchored polyubiquitin chain and deubiquitinates ubiquitin monomers from its proximal end [121]. It would be interesting to investigate whether USP5 targets the unanchored K63-linked ubiquitin chains catalyzed by Ubc13, for example in the context of RLR signaling and antiviral immunity [12–15,121].

Given the importance of K63-linked ubiquitination in mediating several innate and adaptive immune signaling pathways, it is not surprising that pathogens have evolved mechanisms to counteract this post-translational modification. For example, some bacterial virulence factors have DUB-like activity, such as SseL (from *Salmonella enterica* serovar *typhimurium*), YopJ (from *Yersinia pestis*), and ChlaDUB1 (from *Chlamydia trachomatis*). These bacterial proteins modulate host immune responses [122–124]. Macrophages infected with SseL-deficient *Salmonella* show increases in IκBα degradation and ubiquitination followed by transcriptional activation of NF-κB-dependent gene expression [122]. Although not a member of the DUB family of cysteine proteases, SARS-CoV-1 encodes a Papain-like protease protein (PLpro; derived from the larger nsp3) that inhibits STING by deubiquitination of K63 linkages [81]. USP49, a ubiquitin-specific protease, interacts with and deubiquitinates STING after HSV-1 infection. USP49 decreases antiviral responses, and knockdown or knockouts of USP49 potentiate not only HSV-1, but also cytoplasmic DNA and cGAMP-induced Type 1 IFN production [125]. The SARS-CoV-2 PLPro protein inhibits TLR-7 signaling by deubiquitinating K63 linkages from TRAF3 and TRAF6 in human promonocyte cells that were stably transfected [126]. These findings further demonstrate that K63 ubiquitination is important for Type 1 IFN production and antiviral responses due to both STING and cGAMP, helping to explain why pathogens may target STING for deubiquitination. In summary, DUBs function as a counter-regulatory mechanism to either shut down or promote signaling.
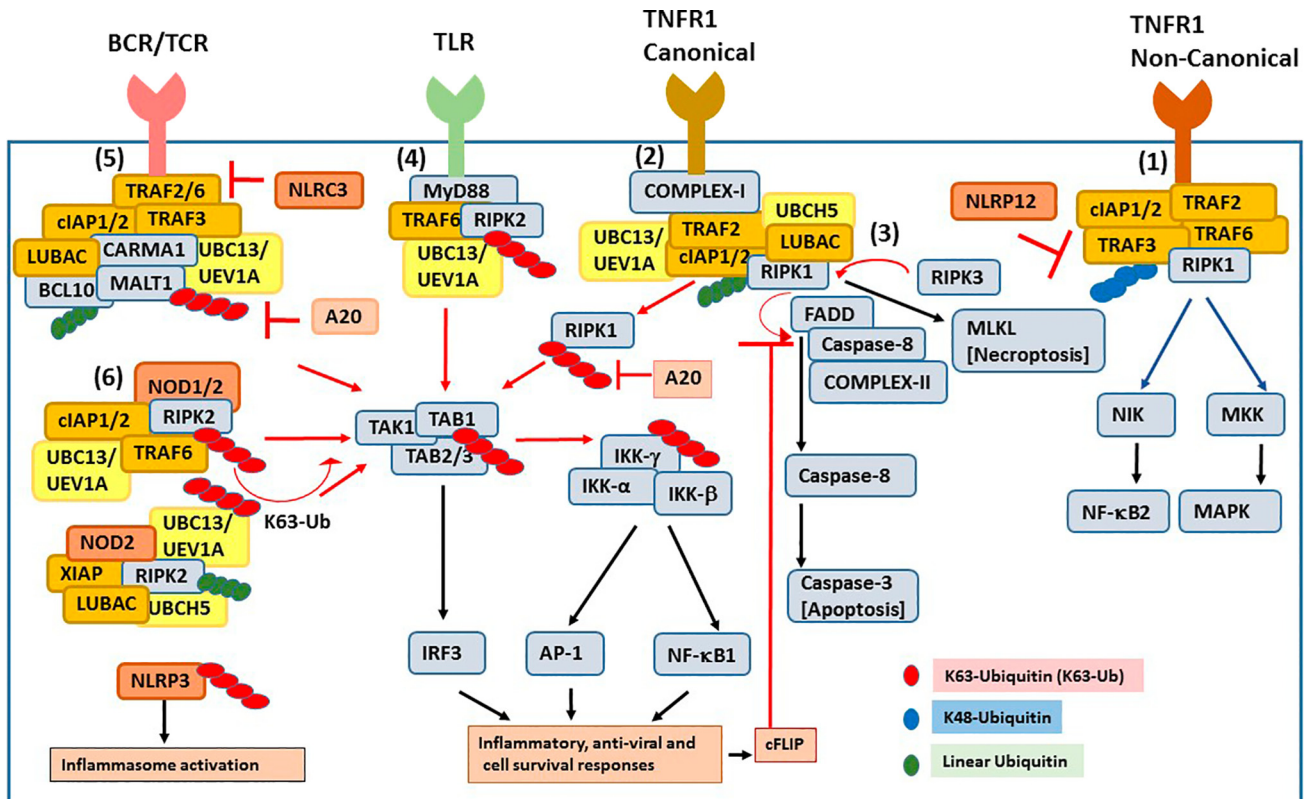
Figure 2. K63 ubiquitination in immune cell signaling cascades. K63-linked polyubiquitin chains (red ovals) and linear polyubiquitin chains (green ovals) catalyzed by Ubc13-Uev1a and UbcH5/linear ubiquitin chain assembly complex (LUBAC) are necessary for efficient activation of immune and antiviral cell signaling pathways. (1) The tumor necrosis factor receptor 1 (TNFR1)-induced non-canonical mechanism involves K48-ubiquitin chain-mediated degradation of TNF receptor-associated factor 3 (TRAF3) by cellular inhibitor of apoptosis proteins 1/2 (cIAP1/2), release of TRAF2/TRAF6 for K63 ubiquitination, and activation of non-canonical nuclear factor KappaB (NF-κB)/MAPK. (2,3) TNFR1-induced K63-ubiquitination of RIPK1 drives two opposing TNFR1 canonical signaling pathways: canonical NF-κB activation modulated by TRAF2-cIAP1-UbcH5-LUBAC-Ubc13-RIPK1 (shown as Complex-I) and classical apoptosis mediated by RIPK1, Caspase-8 and FADD (shown as Complex-II), which is regulated by RIPK1 kinase activity or necroptosis mediated by RIPK1-RIPK3 activity. (4) Stimulation of Toll-like receptors (e.g., TLR4) triggers MyD88-dependent TRAF6- cIAP1/2-Ubc13-catalyzed K63 ubiquitination of downstream substrates followed by NF-κB/MAPK activation. (5) T/B cell receptor (TCR/BCR) activation recruits the caspase recruitment domain-containing membrane-associated guanylate kinase protein1–B cell lymphoma 10–mucosa-associated lymphoid tissue protein 1 (paracaspase) [CARMA1-BCL10-MALT1 (CBM)] complex and activates E2-E3 ligase-mediated K63 ubiquitination-dependent downstream pathways. (6) Stimulation of Nod-like receptors (NLRs) recruits the adaptor proteins to trigger K63 ubiquitination of E3 ligases and their target substrates (including RIPK2) for activation of downstream pathways. The deubiquitinase A20 and some NLR family proteins [e.g., Nod-like receptor Card domain 3 (NLRC3) and NOD-, LRR-, and pyrin domain-containing protein 12 (NLRP12)] negatively regulate K63 ubiquitination-mediated immune cell activation.

upon TCR stimulation compared with WT CD4$^+$ T cells. Thus, K63 ubiquitination-deficient SHARPIN activated the TCR, inhibited FOXP3 induction, negatively modulated Tregs, and skewed the effector CD4$^+$ T cell population toward the inflammatory T$_H$17 phenotype to induce an inflammatory response upon TCR stimulation [48]. This suggests that aberrant ubiquitination can prevent lymphocyte populations from becoming self-tolerant and that, in turn, might contribute to autoimmunity.

The E3 ligase Casitas B lineage lymphoma b (CBL-B) is another example of a modulator of K63 ubiquitination (Figure 3). Deficiency in CBL-B protein expression, recently observed in patients with systemic lupus erythematosus (SLE), correlated with a decrease in K63 ubiquitination specifically in CD4$^+$CD25$^+$ Tregs but not in CD4$^+$CD25$^-$ effector T cells isolated from patients with SLE, suggesting that dysfunctional CBL-B associated with an aberrant K63-ubiquitination profile

of Tregs from such patients contributes to impaired immunosuppression [49]. This is thought to be due to the ability of CBL-B to ubiquitinate the TCRζ chain to terminate anti-CD3/CD28 antibody-mediated TCR activation [49]. Additionally, phosphorylated STAT3 is increased in $CD4^+CD25^+$ Tregs from patients with SLE, but not from healthy controls [49]. IL-6-activated STAT3 has been found to be a transcriptional repressor of *Ubc13*, as demonstrated by chromatin immunoprecipitation of STAT3 association with the *Ubc13* promoter [from *Stat3*-sufficient bone marrow-derived macrophages (BMDMs) in the presence and absence of IL-6] [50]. Deficiency in CBL-B that prevents STAT3 degradation might cause downregulation of *Ubc13* expression, indicating that CBL-B might indirectly regulate Ubc13 function [50]. These reports suggest that Ubc13 maintains the immunosuppressive phenotype of Tregs and that CBL-B or SHARPIN deficiency contributes toward skewing the balance from a Treg to an inflammatory effector T cell phenotypic subset. This is relevant because Ubc13 also has a key role in immune cell activation pathways. However, further studies are required to assess these pathways and functional putative immunomodulatory outcomes.

Role of K63-linked ubiquitination in NLR-mediated signaling

NLR family members can form a variety of '**inflammasomes**', some that stimulate activation of proinflammatory caspase and others that stimulate the MAPK, NF-κB, and endoplasmic reticulum (ER) stress pathways [51–59] (Figure 2). The NLRs, NOD1/NOD2, undergo oligomerization upon recognition of **pathogen-associated molecular patterns (**PAMPs), recruit RIPK2 via CARD-CARD domain interactions to activate K63 ubiquitination of RIPK2, mediated by TRAF2, TRAF5, TRAF6, CARD9, cIAP1/2, and XIAP (which also interacts with RIPK2, recruits the E3 ligase LUBAC and promotes linear polyubiquitination on RIPK2) and the Ubc13-Uev1a complex (which catalyzes K63 ubiquitination) to drive NF-κB activation [60–62] (Figure 2). Of note, TRAF3 mediates K63-linked ubiquitination of the inflammasome adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which, in turn, also induces inflammasome formation [63].

Recently, Ubc13 was also shown to associate with **NLRP3**, catalyze K63-linked ubiquitination of NLRP3 and activate the inflammasome [64] (Figures 2 and 3). Upon stimulation with LPS and ATP, BMDMs from $Ubc13^{deltaMye}$ mice (which specifically lack *Ubc13* in myeloid cells; $Ubc13^{+/-}$) showed diminished secretion of IL-1β and impaired caspase-1 maturation compared with WT ($Ubc13^{+/+}$) controls [64]. Endogenous Ubc13 interacted with NLRP3 in BMDMs, correlating with inflammasome activation, NLRP3 was K63-ubiquitinated in the presence of WT Ubc13 but not mutant Ubc13C87A.

Some NLRs inhibit rather than enhance inflammatory responses induced by other PRRs by modulating K63 ubiquitination (Figure 3). BMDMs from $Nlrc3^{-/-}$ mice, upon stimulation with LPS and $Nlrc3^{-/-}$ mouse $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies, had more K63-ubiquitinated TRAF6, which also formed faster, compared with WT controls [65,66]. This was correlated with higher amounts of IFN-γ/TNF-α secreted upon stimulation of $CD4^+$ T cells from $Nlrc3^{-/-}$ mice and a rapid loss of IκBα, suggesting that NLRC3, a non-inflammasome-forming NLR, attenuated the E3 ligase function of TRAF6 [65,66]. Accordingly, adoptive transfer of $CD4^+$ T cells isolated from either WT or $Nlrc3^{-/-}$ mice into $Rag1^{-/-}$ mice followed by induction of experimental autoimmune encephalitis (EAE, a model of multiple sclerosis) demonstrated that $Rag1^{-/-}$ mice that received $Nlrc3^{-/-}$ $CD4^+$ T cells exhibited worse EAE symptoms compared with those that received WT control $CD4^+$ T cells [66]. The exacerbation of EAE was associated with more IFN-γ and IL-17A, as part of TCR-induced NF-κB activation of its target genes in $Nlrc3^{-/-}$ $CD4^+$ T cells. This indicated a crucial checkpoint function of NLRC3 in preventing downstream NF-κB-induced inflammatory responses in this autoimmune model [66].
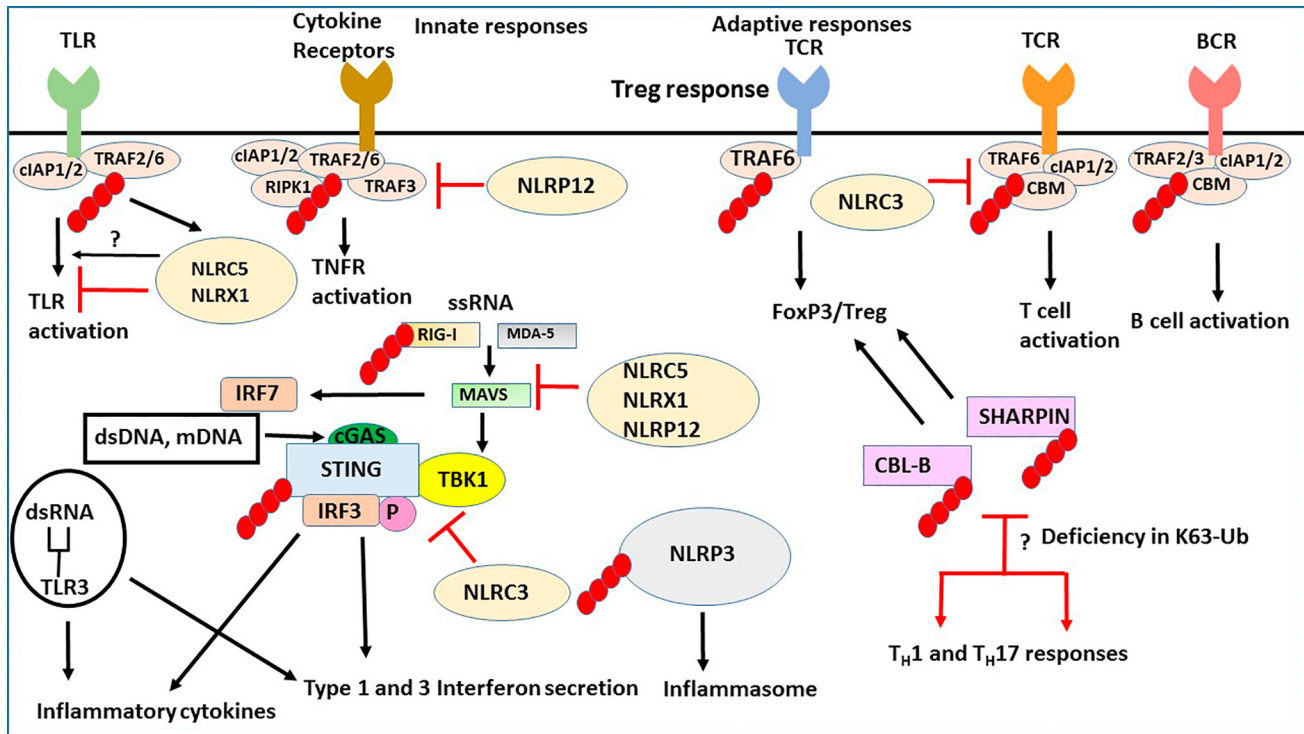
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Figure 3. Ubiquitination signaling pathways in innate and adaptive immunity. The black arrows indicate stimulation of a pathway and the red arrows denote either inhibition or functional implications due to inhibition. The small red ovals attached to proteins are K63-linked ubiquitin chains. Membrane Toll-like receptors (TLRs) and cytokine receptors [including canonical tumor necrosis factor (TNF) receptors] activate ubiquitinated TNF receptor-associated factor 2/6 (TRAF2/6) pathways to stimulate cytokine and interferon (IFN) production. Single-strand (ss)RNA, double-strand (ds)RNA and mitochondrial (mt)DNA activate cytosolic receptors that are also controlled by ubiquitination. Stimulator of interferon genes (STING) is an intermediate for activation of downstream IFN-activating genes, IFN regulatory factor 3/7 (IRF3/7) by retinoic acid inducible gene-I (RIG-I), mitochondrial antiviral-signaling protein (MAVS), melanoma differentiation-associated protein 5 (MDA-5) through TANK-binding kinase 1 (TBK1), cyclic GMP-AMP (cGAMP) sensing dsRNA. K63 ubiquitination of SHANK-associated RH domain interacting protein in postsynaptic density (SHARPIN) and Casitas B-lineage Lymphoma-b (CBL-B) promotes regulatory T cell (Treg) lineage commitment and deficiency in K63-ubiquitinated SHARPIN or CBL-B stimulates helper T cell (T_H) 1 and T_H17 inflammatory responses. K63 ubiquitination of NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) has a role in the activation of the inflammasome. Nod-like receptor card domain 3 (NLRC3) blocks both STING activation and TNF receptor-associated factor 6 (TRAF6) activation. NLRC5 and NLRX1 inhibit IKK activation, enhancement of nuclear factor KappaB (NF-κB) and inflammation signaling by TLRs. NLRP12 negatively regulates non-canonical NF-κB pathway. NLRC5, NLRX1, and NLRP12 negatively regulate RIG-induced antiviral immune responses. Inhibition of NF-κB, RIG-I, and MDA-5 signaling (by ssRNA) is blocked when NLRC5 is K63-ubiquitinated.

NLRC5 undergoes K63 ubiquitination in the presence of TRAF2 or TRAF6 and ubiquitinated NLRC5 regulates the catalytic function of the IKK complex [67]. Upon treatment with LPS, HEK293Ts overexpressing NLRC5, RAW264.7 macrophages, and mouse embryonic fibroblasts (MEFs) show increased K63 ubiquitination of NLRC5 [67]. Small interfering (si)RNA-mediated knockdown of endogenous *Traf2 or Traf6* in primary macrophages followed by stimulation with LPS attenuated NLRC5 polyubiquitination, suggesting NLRC5 K63 ubiquitination as a component of LPS signaling responses [67].

NLRX1 is another non-inflammasome NLR that binds to TRAF6 and when stimulated with LPS, undergoes K63-linked ubiquitination; ubiquitinated NLRX1 inhibits IKK and NF-κB activation, as shown in overexpressed 293Ts [68,69] (Box 3). Consistent with its inhibitory role, *Nlrx1* knockdown in mice (NLRX1-KD) show to increased plasma IL-6 and TNF-α and enhanced susceptibility to LPS-induced septic shock, as observed in peritoneal macrophages and MEFs from NLRX1-KD mice compared with WT controls [69]. NLRP12 can also suppress inflammation [70]. For example,

colon organ cultures from *Nlrp12*$^{-/-}$ mice subjected to acute experimental colitis (EC) showed increased amounts of NIK and p52, with little difference in pIκBα, pp65, IL-1β, and TNF-α compared with WT controls, indicating that *Nlrp12*$^{-/-}$ mice showed increased susceptibility to inflammation in an acute EC model compared with WT control mice [70]. Myeloid dendritic cells (mDCs) from *Nlrp12*$^{-/-}$ mice primed with Pam3Cys4 and stimulated with CD40L showed decreased TRAF3 amounts but no change in TRAF6 compared with WT controls, suggesting that, by binding to TRAF3, NLRP12 prevents the degradation of TRAF3 and negatively regulates the non-canonical NF-κB pathway [70].

Roles of K63-linked ubiquitination in signaling by STING in viral infections

STING was initially identified as a key downstream coordinating protein for the sensing of bacterial and viral DNA, promoting IFN-γ secretion [71]. STING, in collaboration with RIG-I, mitochondrial antiviral-signaling protein (MAVS), melanoma differentiation-associated protein 5 (MDA-5), cyclic GMP-AMP (cGAMP) and virus inhibitory protein, endoplasmic reticulum-associated, interferon (IFN)-inducible (Viperin) has a central coordinating role in cellular sensing of cytosolic DNA and pathogen-derived RNA [72]. While STING does not bind directly to foreign or self-DNA or RNA, STING operates as a central control point for antiviral responses from PRRs, such as TLR-7/8 [for single-strand (ss)RNA], TLR-3 [for double-strand (ds)RNA] and TLR-9 [72] (Figure 4). In addition to pathogen responses, host mitochondrial and dsDNA that enter the cytosol (e.g., during chromosomal nondisjunction events in aneuploid cancer cells) can also activate STING [73]. Thus, STING
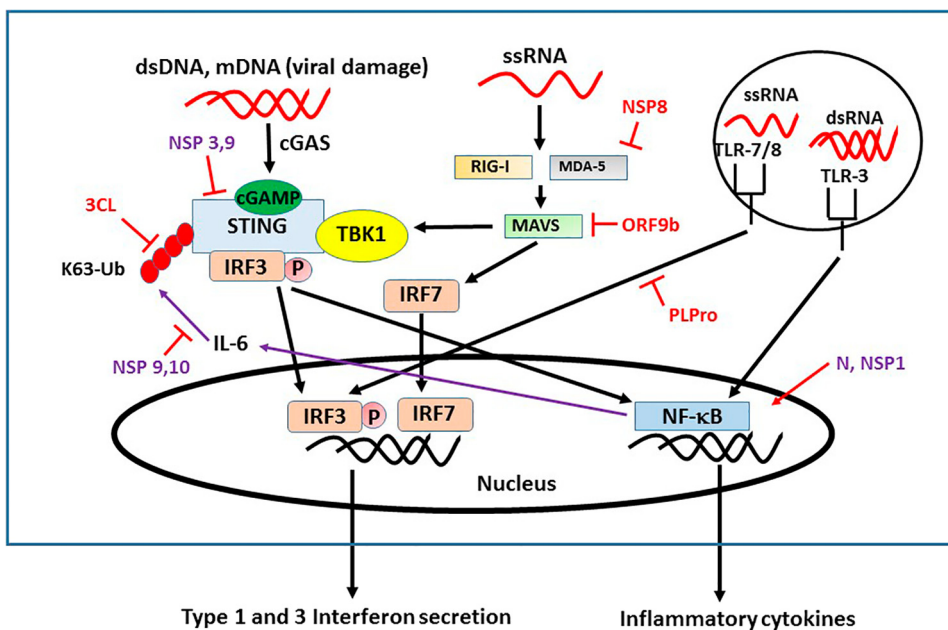
Figure 4. Stimulator of interferon genes (STING) in interferon (IFN) responses. STING has a central role in stimulating innate immunity, Type 1 and 3 IFN production, and also inflammation via nuclear factor KappaB (NF-κB). IFN production by STING is dependent on K63 ubiquitination (red ovals). STING acts as a linker for activation of downstream IFN activating genes (IFN regulatory factor 3; IRF3,) by retinoic acid inducible gene-1 (RIG-I), mitochondrial antiviral-signaling protein (MAVS), melanoma differentiation-associated protein 5 (MDA-5) through TANK-binding kinase 1 (TBK1) and cyclic GMP-AMP (cGAMP), which senses RNA viruses. Additionally, TLR7/8 and TLR3 signal IFN production by single-strand (ss)RNA and double-strand (ds)RNA and these signal through K63-ubiquitinated TNF receptor-associated factor 3/6 (TRAF3/6; not shown here). The four identified severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) open reading frame proteins that inhibit K63 ubiquitination and innate responses are 3CL, ORF9, NSP8, and Papain-like protease (PLPro) (in red). Unknown mechanisms are in purple. The purple arrows show a feedback loop that turns off STING and is inhibited by the NSP9,10 proteins, which can block the K63 ubiquitination of STING that is key for IFN 1 and 3 production but not for NF-κB activation.

responds downstream to PRRs that recognize cytosolic viral ssRNA, dsRNA, and DNA, in addition to also responding to **damage-associated molecular patterns** (DAMPs), such as mitochondrial and double-stranded host DNA [72,73].

RIG-I binds to viral RNA and activates MAVS via the CARD-like domain to aggregate and bind E3 ubiquitin ligases, which include TRAF2, TRAF5, TRAF6, and LUBAC [74]. Similarly, MDA-5 can also activate MAVS via the CARD domain [75]. The DNA and RNA sensors RIG-I and MDA-5, pair with MAVS while cGAS and nucleic acid-sensing TLRs pair with their signaling molecules STING and TRIF, respectively, activating the kinase TANK-binding kinase 1 (TBK1) to phosphorylate IRF3 and stimulate Type 1 IFN production [76]. Further research has shown that Ube2D3, together with the ubiquitin ligase Riplet, activates proteins via K63-ubiquitin anchored chains, while Ube2N preferentially activates via unanchored K63 polyubiquitin chains [13].

C-GAS recognizes dsDNA [from both viral and mitochondrial (mt)DNA] and increases the production of the novel intracellular second messenger cGAMP [77]. cGAMP directly binds and activates STING [78], which leads not only to both TBK-1 binding and IRF3 binding (and ultimately Type 1 IFN induction and secretion), but also NF-κB activation (and cytokine secretion), similar to RIG-I and TLR7/8 activation (Figure 4). Myb Like, SWIRM and MPN Domains 1 (MYSM1) is a metalloproteinase that deubiquitinates and cleaves the K63-linked ubiquitinated STING suppressing cGAS-STING signaling; *Mysm1*-deficient C57BL/6 mice exhibit a hyperinflammatory response, acute tissue damage, and high mortality when infected by viruses such as HSV-1 [79]. Additionally, MYSM1 has been reported to act as a suppressor for SLE [79].

K63 ubiquitination of STING at the K224 residue is essential for the delivery of STING to TBK1 and the binding to IFN regulatory factor 3 (IRF3) that initiates Type 1 IFN production and secretion [80]. TRAF E3 ligases catalyze the formation of K63-conjugated ubiquitin chains on STING; such K63-linked ubiquitination is involved in STING-TRAF6-TBK1/STING-TRAF3-TBK1 complex formation [81–83]. The E3 ligases belonging to the TRIM family, TRIM56 and TRIM32, also mediate K63-linked ubiquitination of STING [84,85]. The non-proteolytic E2 Ubc13-catalyzes TRAF6-mediated K63-linked ubiquitin chains on STING and UbcH5c catalyzes TRIM56-mediated mono-ubiquitination of cGAS [82,86].

Viruses, such as Human T cell lymphotropic virus type 1 (HTLV-1), can impair host immunity by inhibiting host cell IFN-γ responses by producing the Tax protein [87]. HTLV-1 Tax protein decreases K63 ubiquitination of STING and inhibits STING interaction with TBK1 [88]. Other RNA viruses, such as coronaviruses and flaviviruses, also interfere with STING to inhibit the antiviral IFN response [89]. Whereas activation of STING turns on innate immunity, antiviral responses, and Type 1 IFNs, its overactivation can also lead to autoimmunity. There is strong evidence linking excessive Type 1 IFN to the promotion and exacerbation of certain autoimmune diseases, especially SLE (although autoimmune diseases clearly have multifactorial causes and inputs) [90]. Of note, some NLRs that are activated by bacterial PAMPs also regulate STING activity as do viral PAMPs (Figure 3, Box 3).

SARS-CoV-2 infection and K63-linked ubiquitination in immune responses

Recent evidence has highlighted the crucial role of STING and Type 1 and 3 IFN secretion in the innate immune response to SARS-CoV-2 infection. Decreased Type 1 IFN (specifically low IFN-α and no IFN-β) has been strongly associated with more serious coronavirus 2019 (COVID-19) symptoms and death, while the amount of NF-κB-associated cytokines (e.g., TNF-α, IL-1 and IL-6) are elevated in patients with severe COVID-19 [91]. In human lung epithelial cells and in an animal model of COVID (K18-ACE2-transgenic mice), a STING agonist (diABZI-4) strongly inhibited SARS-CoV-2 replication for both the alpha and delta variants (B.1.135.1) [92]. The

study also showed that SARS-CoV-2 could induce a delayed immune response that could be overcome with exogenous IFN administration [92]. Two other research groups also reported that this same STING agonist (diABZI-4) can significantly inhibit SARS-CoV-2 replication in human lung epithelial cells [93,94].

Since K63 ubiquitination is pivotal in STING signaling (as described in the preceding text), several studies have examined the potential inhibitory role of SARS-CoV-2 open-reading frame proteins in K63 ubiquitination and in diminishing Type 1 IFN secretion. There are four SARS-CoV-2 proteins that have been shown to alter K63 ubiquitination, affect IFN signaling and may lead to decreased Type 1 and 3 interferon secretion (Figure 4): 3CL, ORF9b, NSP8 , PLpro. 3CL is an important protease in SARS-CoV-2, which directly inhibits STING K63 ubiquitination and causes a decrease in downstream signaling in human, mouse, and chicken lung epithelial cells [95]. The ORF9b SARS-CoV-2 protein antagonizes Type 1 IFN production after activation of RIG-I-MAVS by blocking K63 ubiquitination of NEMO in human primary epithelial cells [96]. The SARS-CoV-2 nucleocapsid (N) protein also inhibits Type 1 IFN secretion by inhibition of RIG-I, probably due to inhibition of RIG-I K63 ubiquitination by TRIM25 E3 ligase [as has been shown for Middle East respiratory syndrome (MERS)] in human embryonic kidney cells (HEK-293) [97,98]. NSP8 from SARS-CoV-2 suppresses MDA-5-stimulated IFN signaling by binding to MDA-5 CARD and blocking K63-linked ubiquitination in human embryonic kidney cells [99]. In summary, SARS-CoV-2 inhibition of Type 1 and 3 IFNs appears to be important for evading the innate immune response. Multiple mechanisms of inhibition of interferon signaling by SARS-CoV-2 proteins have been elucidated and alterations in K63 ubiquitination are prominent in this evasion of the host response (Figure 4).

Concluding remarks

K63 ubiquitination is important as a proximal event in signal transduction pathways involved in innate and adaptive immunity. Ubc13-catalyzed K63 ubiquitination has a key role in striking a balance between immune cell activation and immune tolerance mechanisms, as evidenced from murine studies. Recent findings demonstrated that the control of multiple immune functions, such as chronic inflammation, pathogen responses, lymphocyte activation, regulatory signaling pathways, and lymphocyte subset development, can be altered by K63 ubiquitination. While we have discussed some details of K63 ubiquitination-dependent regulation of immune signaling and described some of the novel cellular sensors that are dependent on K63 ubiquitination during immune signaling, numerous questions remain (see Outstanding questions). For instance, mechanistic details of SARS-CoV-2 infection and K63 ubiquitination in immune responses remain far from being understood and require robust experimentation. Therefore, although advances have been made regarding K63 ubiquitination in the immune system, the field eagerly awaits much-needed further research in this important area, particularly when considering the relevance to coronavirus infections, such as from SARS-CoV-2.

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Outstanding questions

What is the mechanistic role of cIAP/TRAF-mediated K63 ubiquitination in regulating a balance between T_H1, T_H17 *versus* Treg immune cell functions? K63-linked ubiquitination can balance on/off immune responses to preserve homeostasis. The redundant and non-redundant functions of cIAP and TRAF members mediating K63 ubiquitination in immunostimulatory *versus* immunosuppressive phenotypes needs further research.

What are the relevant E3 ligases involved in the regulation of chronic inflammation-induced metabolic syndrome? $Ubc13^{+/-}$ mice are protected from age-related insulin resistance and obesity. The involvement of relevant E3 ligases may provide further evidence of the explicit role of Ubc13-catalyzed K63 ubiquitination in chronic inflammation-induced metabolic syndrome.

What is the role of Ubc13 in K63-ubiquitinated SHARPIN- and CBL-B-mediated regulation of immunological homeostasis? Direct mechanistic information as to how K63-ubiquitinated SHARPIN and CBL-B control immunological homeostasis may be obtained from investigations on $Ube2n^{TregKO}$ mice, which acquire T_H1/T_H17 phenotypes under lymphopenic and inflammatory conditions.

Is there putative coordinated regulation between IAPs and TRAFs in K63-linked ubiquitination of the inflammasome? Some NLRs regulate TRAF-mediated K63 ubiquitination. The coordinated regulation between NLRs and TLRs, including the likely E2-E3 pairs, in K63 ubiquitination-mediated inflammasome activation needs further investigation.

What is the role of K63-linked ubiquitination mediated by NLRs in the SARS-CoV-2-induced IFN response? Deciphering K63 ubiquitination in SARS-CoV-2 infection may help characterize pathogen-specific induced signaling pathways. For example, NLRP12 reduces binding between RIG-I and an E3 ligase and functions as an attenuating factor in SARS-CoV-2-induced RIG-I-mediated IFN-γ expression.

Does viral/bacterial DNA directly interact with K63-linked ubiquitin chains to trigger an immune response? Recent

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studies reported that K63-linked ubiquitin chains specifically interacted with DNA. Investigations to assess whether K63-linked ubiquitin chains interact directly with viral/bacterial DNA in triggering immune response may be informative.

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