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Lysine 63 Polyubiquitination in Immunotherapy and in Cancer-promoting Inflammation

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Abstract Covalent and reversible post-translational modifications of proteins are a common theme in signaling. Ubiquitin conjugation was originally described to target proteins to proteasomal degradation by ubiquitin polymerization involving lysine (K) 48 residues. Differently linked polymers of polyubiquitin have been found that modify proteins without targeting to proteasomal degradation. Instead this pathway creates docking sites for signaling scaffolds that are key to control the nuclear factor-κB (NF-κB) pathway. Indeed TRAF-2, TRAF-6, and TRAF-3 are E3 ubiquitin ligases that form K63-linked ubiquitin polymers. Therefore signaling via TNF family receptors, IL1R, IL-18R, T-cell receptor (TCR), and Toll-like receptors (TLR) use this type of post-translational modification. Specific enzymes exist (DUBs) that deactivate this system, degrading K63 polyubiquitin chains. Interestingly, mice deficient in these deubiquitinases develop autoimmunity and inflammation. In carcinogenesis, the K63 polyubiquitin pathway is possibly critical for inflammation-driven tumor promotion. The pathway is also critically involved in costimulation of tumor immunity/immunotherapy as well as in the biology of malignant cells themselves. The elements of this new signaling paradigm offer the opportunity for therapeutic exploitation and drug discovery. (Clin Cancer Res 2009;15(22):6751–7)

Cellular protein function is often regulated through post-translational covalent modifications that share common characteristics. First, they are reversible. Specific enzymes are responsible for attaching chemical groups (phosphate, acetyl), and other enzymes have the mission of removing them. Second, the modification takes place on specific amino acid residues of the target protein (serine, tyrosine, threonine, lysine). Third, post-translational modifications change the activities of the target protein.

Ubiquitin is a 76-amino acid protein abundantly present in all eukaryotic cells. Post-translational attachment of ubiquitin to target proteins is a well-known control mechanism in diverse and essential cellular activities ranging from apoptosis to intracellular protein trafficking. Ubiquitin can be linked to proteins using accessible lysine, cysteine, or the *N*-terminal amino acid. The general scheme of the ubiquitin conjugation pathway is as follows (Fig. 1A): (1) An enzyme, E1 binds to ubiquitin and activates it for reaction in an ATP-dependent fashion; (2) E2 or ubiquitin conjugase carries ubiquitin to an ubiquitin ligase E3 protein that binds to the target protein; (3) E3 ligases couple an ubiquitin moiety to the amino terminus of the substrate protein. Subsequently, the E3 ligase polymerizes additional ubiquitin molecules to form an extending polymer involving lysine side chains and the C-terminus glycine from the incoming ubiquitin monomer. E3 ligases provide substrate specificity to the pathway and are classified into two groups according to the presence of catalytic domains HECT or RING (1).

As mentioned, once a monomer of ubiquitin is attached, it can be extended to form polymers through isopeptide bonds between ubiquitin lysine amino acids and thereby generate polyubiquitin (Polyub) chains. The length of the Polyub chain is likely to quantitatively regulate the intensity of signal. Furthermore, deubiquitinating enzymes remove ubiquitins and provide reversibility to the process as modulators or terminators of signaling (2). Attachment and de-attachment of protein polymers to induce functional modifications is also observed in the case of covalent binding of the SUMO protein (3).

Ubiquitin contains seven lysine (K) residues in positions K6, K11, K27, K29, K33, K48, and K63. Formation of the ubiquitin polymer can occur by binding of the C-terminus of the incoming ubiquitin to any of these lysine residues in homogeneous and specific fashion depending on the E3 ligase that catalyzes the reaction. The nature of the specific lysine polymer involved in the linkage affects the activity of the target protein in different ways. For example, K48-Polyub tags the target protein to be degraded by the proteasome, and K29-Polyub is considered as an indicator of subsequent lysosomal degradation (1). During the last few years K63-Polyub has emerged as a novel post-translational modification of remarkable functional interest

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for fine-tuning signal transduction pathways (4). K63-Polyub has been described to create docking sites for scaffold proteins involved in the regulation of nuclear factor-κB (NF-κB; ref. 5) and mitogen-activated protein kinase (MAPK) pathways (6). These biochemical routes are of great relevance in the response of the immune system against pathogens, in inflammation, and for the biology of malignant cells.

The E2 enzyme complex that catalyzes the first step in K63-polyubiquitination is UBC13/UEV1. This E2 enzyme seems to be unique for this function in the case of K63 E3 ligases (7), thereby offering an interesting pharmaceutical target. Several E3 ligases promote the K63 polymerization presumably with highly regulated specificity for target proteins (8). This set of enzymes includes TRAF-6, TRAF-2, TRAF-5, RIP1, and TRIM25. This mechanism is considered of outstanding importance because these signal adaptors are key regulators of fundamental cascades such as those of Toll-like receptors (TLR), TNFR family pathway, T-cell antigen receptors, and the receptors for interleukin-1 and interleukin-18 (4).

K63-Polyub Pathway and Tumor Promotion

K63-Polyub in Signaling. K63-Polyub modification was first described as a mechanism for the activation of protein kinases in the NF-κB pathway (9). NF-κB is a family of transcription factors usually located in the cytoplasm in steady state conditions that after activation translocate to the nucleus where they control the expression of genes key to immunity and inflammation (5).

For NF-κB members (10) to become fully activated, they must be released from their cytoplasm-retaining inhibitors IκB (11) or the *N*-terminal segment of p100 precursor. These events are induced by the kinase activity of IKK proteins. The IKK trimeric complex is conformed by two catalytic subunits, IKK-α, IKK-β, and a regulatory and scaffold subunit, named NEMO or IKKγ. IKK complexes phosphorylate IκB and target this protein for proteasomal degradation as a result of inducing its labeling by K48-ubiquitin polymers (5). The E3 ligase that mediates this process is SCF-βTrCP. This activation scheme is known as the classical or canonical NF-κB activation pathway that is functional in almost all nucleated cells (5).

In the noncanonical pathway, the activation is initiated by the action of NIK, which phosphorylates and thereby turns on a critical function of IKK-α, which becomes capable of phosphorylating p100. This phosphorylation event induces the recruitment of a K48 E3 ligase, which marks p100 for partial proteasomal degradation until a stop signal within its sequence is reached, in such a way that the p52 transcription factor is released from its cytoplasmic localizing sequence present in the digested fragment. The resulting p52 fragment then dimerizes with REL-B forming NF-κB2 transcription factor and translocates to the nucleus where it regulates gene expression. In the process of canonical and noncanonical NF-κB activation, K63-Polyub plays an essential role (8). The mechanisms used by K63-polyubiquitination to control NF-κB activation can be described using TLRs and interleukin 1 receptor (IL-1R) pathways as an example (Fig. 1B).

After ligand binding to IL-1R or TLRs, the adaptor protein MyD88 is recruited to the cytoplasmic tail of the receptor and facilitates its interaction with IRAK4 and IRAK1 serine/threonine protein kinases. IRAK4 in turn phosphorylates IRAK1 to activate it (12). Active IRAK1 binds to TRAF6, an E3 ubiquitin ligase, capable of auto-polyubiquitinating itself and other neighboring target proteins forming K63-Polyub, which hangs from the proteins like a pearl necklace or a series of rosary beads. The K63-Polyub chain creates docking sites for proteins containing ubiquitin-binding domains (UBD). UBAN is a UBD found in NEMO, A20, and optineurin that shows specificity for K63-Polyub (13). The NZF (Npl14 zinc finger) domain present in TAB2/3 and Trabid also displays K63-Polyub binding preference (14). Recruitment by UBDs is a biological function involved in docking for signaling (i.e., TAB recruitment) or in regulatory enzymatic degradation of the ubiquitin polymers (i.e., A20, see below). Many of these E3 ubiquitin ligases self-polyubiquitinate, possibly as a result of the close molecular proximity of various identical E3 ligases that cross-ubiquitinate each other, thus suggesting a regulation of their activity by aggregation or proximity triggered by receptor cross-linking.

TRAF6 K63-polyubiquitination induces docking and activation of the TAB1, TAB2/3 adaptors, which recruit the serine-threonine kinase TAK1 into the complex. TAB2 has a UBD that exhibits K63-Polyub specificity (15, 16). The exact mechanism by which TAB2 binds to K63-Polyub and leads to TAK1 activation is not fully understood and possibly involves conformational changes. TAK1 phosphorylates and activates IKK-β and in this manner promotes IκB proteasomal degradation and translocation into the nucleus of NF-κB members. K63-polyubiquitination of NEMO (IKKγ) is critical in this process, because deletion of its UBAN domain abrogates IKK signaling (17).

The noncanonical NF-κB pathway regulation depends on the fact that NIK degradation is tightly controlled. Under baseline conditions TRAF3, TRAF2, and cIAP promote rapid proteasomal degradation of NIK that is almost undetectable. Different stimuli (CD40L, BAFF) lead to TRAF2-mediated K63-polyubiquitination of cIAP. K63-Polyub cIAP catalyzes K48-ubiquitination of TRAF3 and targets it to the proteasome, thereby releasing NIK from TRAF3 inhibition (18, 19). Sufficient accumulation of NIK results in downstream signaling toward NF-κB activation (Fig. 1B; ref. 20).

TLRs are key instruments to detect molecular patterns associated with pathogens (21) and endogenous danger signals (22). TLR3, TLR7, TLR8, and TLR9 are sensors of viral components when present in endosomal compartments of the cell. Interestingly, TLR3 interacts with the adaptor TRIF, and TRAF6-RIP1 activates the NF-κB pathway as previously explained (Fig. 1). TRIF binds to and activates TBK1 and IKK-ε. IKK-ε in turn phosphorylates IRF3 and promotes its dimerization with ATF2 (23). IRF3-ATF2 dimers critically control the expression of the IFN-β gene. This system operates predominantly in plasmacitoid dendritic cells, a minority leukocyte subset that produces high amounts of type I interferon in response to viral infection (24).

RIG-like receptors (RLR) such as RIG-1 and MDA5 recognize RNA with viral features once in the cytosol. RIG-1 and MDA-5 recruit IPS-1 (also known as MAVS or CARDIF) using CARD (caspase activation and recruitment domains). RIG-1 K63-polyubiquitination facilitates its binding with IPS-1. The E3 ligase responsible for this action is TRIM25 (25). TRAF-3 E3 ligase also participates in this process (26). IPS-1 is recruited to the outer mitochondrial membrane where it interacts with IKK-ε and TBK1 to activate IRF3 and IRF7 thereby leading to type I interferon production. Accordingly, K63-Polyub is important for the

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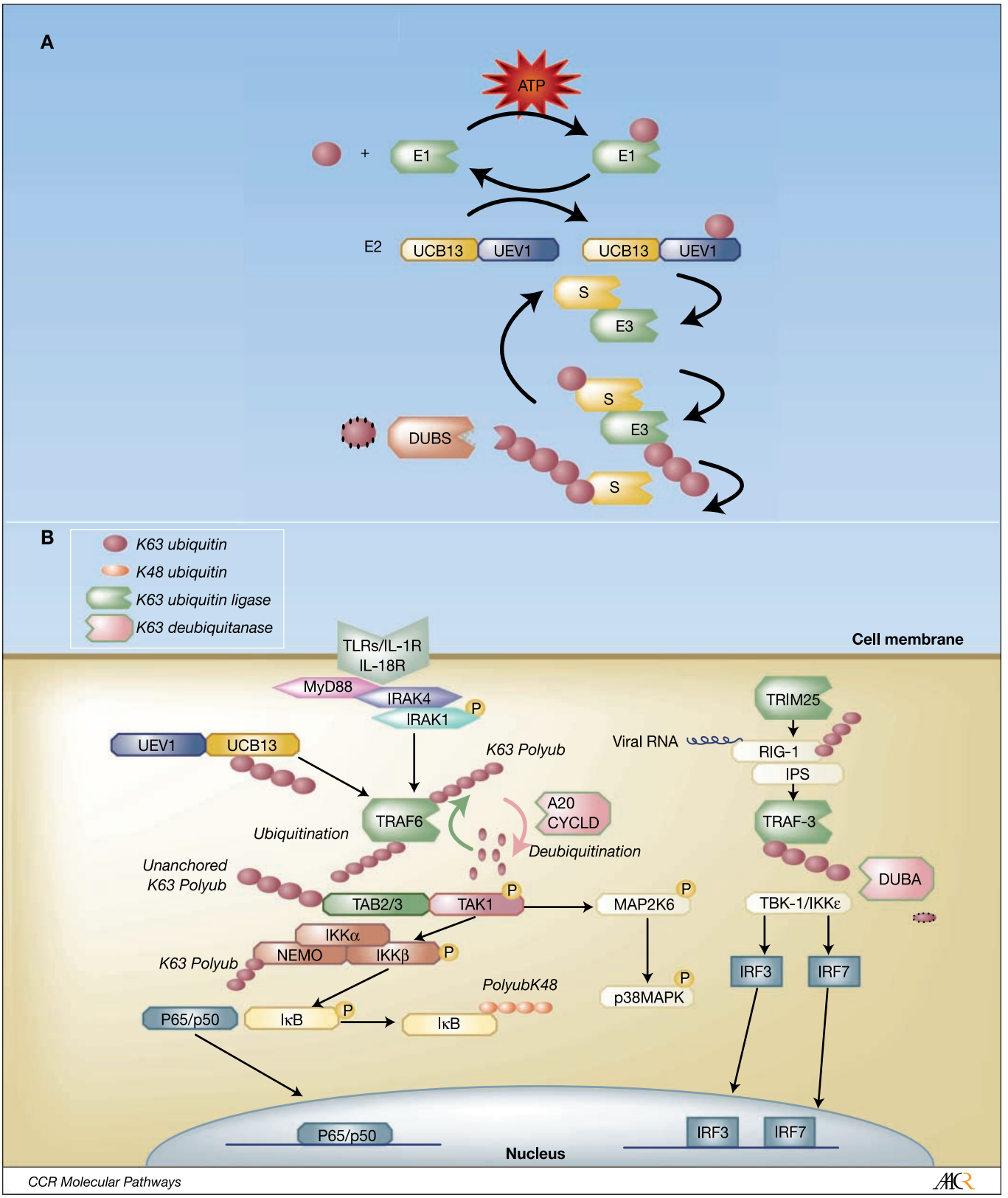

Fig. 1. K63 ubiquitin polymerization controls NF-κB activation. *A,* schematic summary of the molecular events for the ubiquitination of a substrate protein (S) by an E3 ubiquitin ligase. Some E3 ligases engage K63 from an ubiquitin to a substrate protein or to another ubiquitin to form a polymer. E3 ligases take activated-reactive ubiquitin from an E2 enzyme that in the case of K63 ubiquitin chains is UBC13/UEV1. Such a complex takes activated ubiquitin from an E1 enzyme responsible for creating the activated reaction intermediate for covalent binding. *B,* involvement of E3 K63 ubiquitin ligases in signal transduction from TLRs IL-1R, IL-18R, and RIG-1. The E3 ubiquitin ligase activity of TRAF6 and TRAF3 are critical whereas deubiquitination catalyzed by A20, CYLD, and DUBA down-regulates the intensity of the signals. Recent evidence points out that polymers of K63-Ub that are not anchored to any protein may act as second messengers inducing TAB-TAK activation (69).

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production of type I interferon as a result of intracellular and endosomal recognition of viral-denoting biomolecules. This system is present in most nucleated cells including transformed cells.

The MAPK pathway is activated downstream of TRAFs through mechanisms that are known to involve cIAP and TRAF2, and which are highly dependent on K63-polyubiquitin formation as catalyzed by TRAF2 (18). CD40 activation induces K63 self-ubiquitination of TRAF2 proteins that in turn ubiquitinate cIAP. This event recruits a multi-subunit complex composed of TRAF2, TRAF3, cIAP, and MEKK1 to the cytoplasmic end of CD40. TRAF3 maintains this multimer structure attached to the receptor cytoplasmic tail. As in the noncanonical NF-κB pathway, cIAP catalyzes K48 ubiquitination of TRAF3. TRAF3 is degraded by the proteasome allowing the TRAF2-MEKK1 complex to initiate MAPK signaling, in addition to NF-κB activation. Other connections between K63-polyubiqutin and the MAPK pathway may be discovered soon.

K63-Polyub Is Reversible and Offers Opportunity for Regulation. DUBs are the enzymes that regulate the effects of K63-polyubiquitination. CYLD and A20 proteins share the capacity of removing K63-polylinked ubiquitin molecules from target proteins (Fig. 1B). It seems that CYLD is a specific DUB for K63-Polyub chains whereas A20 can remove K63 while concomitantly displaying K48-Polyub E3 ligase activity able to target substrate proteins to proteasomal degradation (27–29). K63-deubiquitylation is a highly regulated process as evidenced by recent reports of inactivating A20 mutations believed to be pathogenic in several types of human lymphomas (30–33), and by mutations of CYLD in many malignancies that include an inheritable tumor propensity syndrome (cylindromatosis; ref. 34). A20 expression may be responsible for avoiding inflammation in tissues with microbial flora such as the gut epithelium (35) and has been used to prevent inflammation in gene therapy strategies (36).

IKK K63-Polyub-dependent activation is negatively regulated by TAX1BP1 and ITCH that operate in conjunction with A20 to down-regulate the pathway (37). It has been described that TAX1B1P1 and ITCH promote A20 association with TRAF6 to reduce K63-polyubiquitination after agonist ligand binding to IL-1R or TLRs (37). Of note, TAX, an oncoprotein from HTLV-1, inhibits TAX1B1P1- ITCH binding and thereby permits sustained NF-κB signaling (37).

DUBA is a DUB enzyme that exhibits K63-Polyub chains specificity and was identified as a negative regulator of RIG-1/MDA5 pathway. In particular, DUBA binds to TRAF3 and inhibits its E3 ligase activity. This in turn causes the disassembly of TBK1 and IKK-ε from TRAF3 and hence reduces type I interferon production (38).

Ataxin-3 (AT3) is another DUB that preferentially cleaves K63-Polyub chains. This protein is mutated in the neurodegenerative disorder Spinocerebellar Ataxia Type 3/Machado-Joseph disease (39). AT3 itself is subject of ubiquitin labeling. Ubiquitinated AT3 cleaves K63-Polyub chains more quickly and efficiently than unmodified AT3, thus conforming a negative feedback loop in which ubiquitination unleashes the process of deubiquitination (40). This could become a major common mechanism in the control of these pathways.

Unsurprisingly, aberrant NF-κB signaling has been associated with cancer development and progression. The NF-κB pathway is a key mechanism by which malignant cells evade apoptosis (41), promote angiogenesis (42), sustain inflammation, and facilitate metastatic spreading (43). As K63-polyubiquitination is a recently recognized critical event in NF-κB signaling, its role in cancer biology and treatment remain to be thoroughly explored. The recent availability of monoclonal antibodies that selectively recognize the polymers of K63-Polyub (44, 45) provides an invaluable experimental tool to study in detail the pathway both quantitatively and qualitatively. In addition, structural considerations investigated with these antibodies may help molecular modeling for drug development (45).

K63-Polyub in Inflammation Promoting Cancer. Epidemiological and experimental evidence show a link between inflammation and cancer (46–48). Subjects affected by chronic inflammatory conditions increase their risk of developing malignant tumors, and inflammatory cells of myeloid origin are often encountered in cancer biopsies and in areas of premalignant dysplasia. NF-κB is activated at these locations by diverse stimuli including tumor necrosis factor-α (TNF-α), IL-1β, and TLR ligands giving rise to inflammation (49).

TNF-α is a cytokine with pleiotropic activity (50). TNF-α binds to TNFR1 receptor, widely expressed on hematopoietic and non-hematopoietic cells. TNF-α ligation to TNFR1 induces TRADD to form complexes with the receptor and sequentially assemble complex 1 (TRAF2, RIP1, cIAP1). K63 and K48 ubiquitin polymers also modulate the TNF-α signaling pathway (Fig. 2). In these signaling complexes, both cIAP1 and TRAF2 encompass E3 ligase activity (51). K63-polyubiquitinated RIP1 activates IKK and subsequently NF-κB through a mechanism mediated by the TAB2, TAB3, and TAK1 complexes (52). TNFR signaling pathway is involved in apoptosis induction by TNF-α. TNF binding to its receptor triggers complex 2 (TRADD, FADD, RIP1 procaspase-8) assembly. In these complexes pro-caspase 8 is processed to fully active caspase-8, which initiates apoptosis. However, TNFR1-mediated NF-κB activation promotes cell survival through the transcriptional induction of cFLIP, an anti-apoptotic protein that inhibits caspase-8 from disassembling out of pro-apoptotic complex 2 (53).

In malignant lesions, TNF-α is part of an intricate network of cytokines (50) released by tumor and stromal cells with the capacity to sustain inflammation and recruit new blood vessels to support cancer growth. TNF-α is produced in the tumor by cells such as myeloid-derived suppressor cells (MDSC) or tumor-associated macrophages (TAM). These tumor co-opted leukocytes are known to support tumor growth and progression, enhancing availability of growth factors, angiogenesis, and creating an immunosuppressive microenvironment (54). As evidenced by transgenic murine tumor models, NF-κB pathways are critical for these activities involved in tumor promotion. For example, macrophage-specific gene targeting of IKKβ results in reduced tumor progression in a mouse model of colitis-associated cancer (43).

K63-Polyub in Tumor Immunology and Immunotherapy

K63-Polyub in Signaling through Antigen-specific Receptors. T-cell activation and recognition of tumor antigens is key for immune antitumor surveillance. Antigens are recognized by T cells as peptides linked to MHC molecules. The T-cell antigen receptor (TCR) is coupled to the CD3

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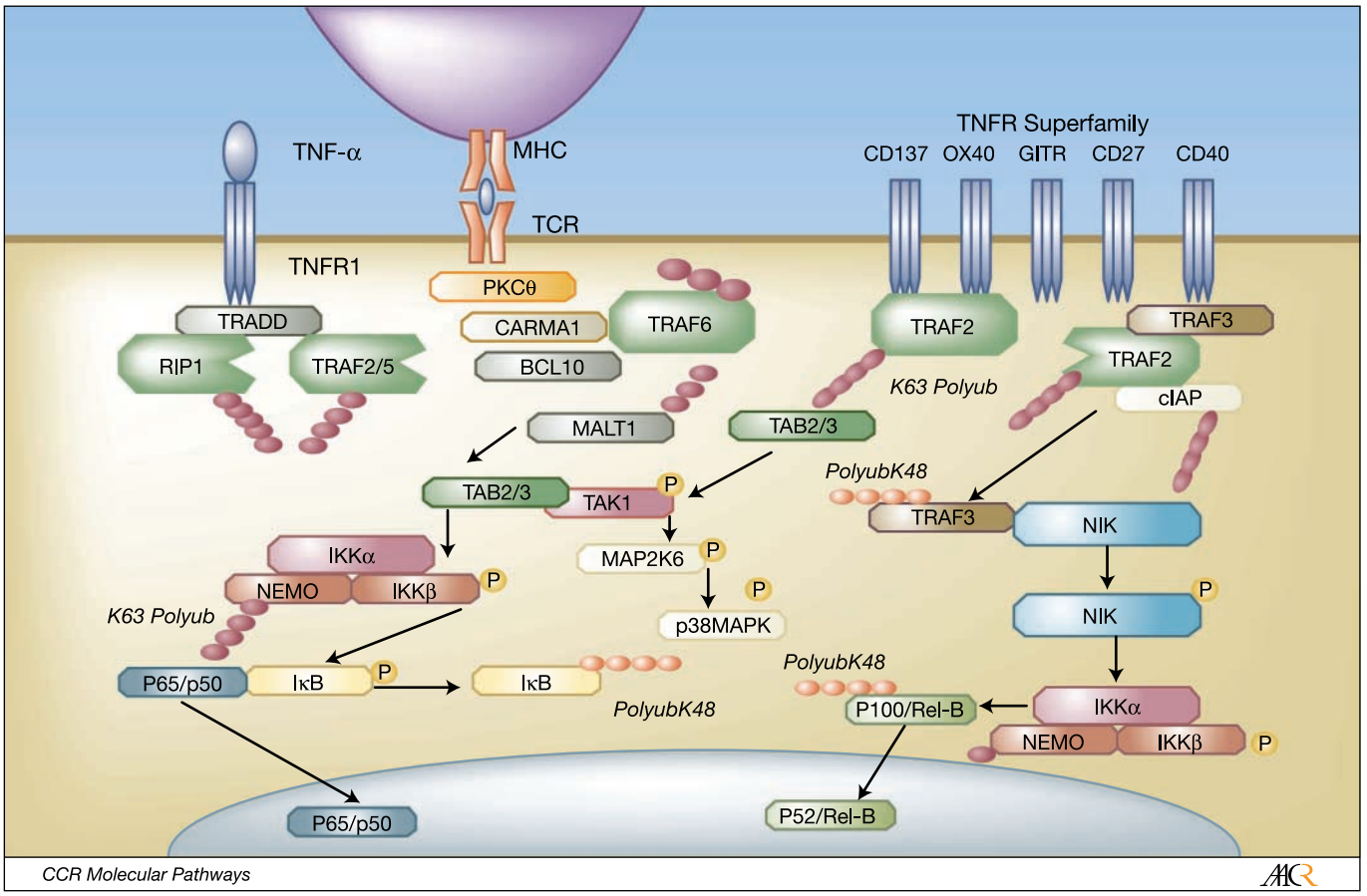

Fig. 2. K63 ubiquitin ligases in signaling pathways of immune system cells. Schematic of the signals from the TNFR1 that control NF-κB and MAPK activation through IKKα. TCR is coupled to NF-κB activation through a complex network initiated by PKCθ that involves MALT1 and TRAF6, an adaptor endowed with E3 K63-ubiquitin ligase activity. TCR-CD3 signaling is also modulated by A20 and CYLD-mediated deubiquitinations. The cytoplasmic tails of TNFR family members that display costimulatory activity bind TRAF2 (and TRAF6 in some instances such as in the case of CD40). The K63 ubiquitin ligase activity of these TRAF adaptors explains subsequent downstream activation of the NF-κB and MAP kinase routes.

signaling machinery that initiates signaling upon cross-linking and conformational changes (55) induced by the antigen. K63-Polyub formation seems to be crucial for TCR-mediated activation of the NF-κB pathway (Fig. 2).

PKCθ is well known to be activated by the TCR at the interphase between T cells and antigen-presenting cells, which is known as the immune synapse. CARMA, MALT1, and BCL10 are complexed as a result of PKCθ activity. These molecular events result in NF-κB translocation to the nucleus. MALT1 has binding sites that become K63-polyubiquitinated by TRAF6 providing docking sites for TAB1 and TAB2, thereby mediating subsequent IKK activation (56, 57). MALT1 also cleaves the DUB protein A20 during TCR activation, a process also involved in the induction of NF-κB after TCR stimulation (58).

CYLD prevents TAK1 ubiquitination and activation. CYLD deficiency in mice leads to intestinal inflammation (59). In the same way, $CYLD^{-/-}$ T cells exhibit an inappropriate hypersensitive response to anti-CD3 and anti-CD28 stimulation (60).

A K48 ubiquitin ligase termed Cbl-b is also a key regulator of TCR signaling because it K48-polyubiquitinates PKCθ to induce its proteasomal degradation. Again, Cbl-b deficiency in T cells results in widespread autoimmunity (61). K63-Polyub and K48 ubiquitination are clearly an area of research in TCR signaling that will vastly grow in the near future. Modulation of these molecular activities as seen in knock out animals, offers new opportunities for drug enhancement or inhibition of immune responses.

K63-Polyub in T-cell Costimulation. Apart from TCR signaling, other lymphocyte surface molecules play a role in T-cell activation and are termed costimulatory receptors, as they act in conjunction with the TCR by providing complementary signals (62). One important group of costimulatory proteins belongs to the TNF receptor family and includes CD137, OX40, CD27, CD40, GITR, and HVEM (63, 64). These molecules lack any enzymatic activity and rely on the use of TRAF2 for the correct propagation of their signals. TRAF2 has been shown to be an E3 ligase with K63-Polyub activity that polyubiquitinates itself and other substrates (65). TRAF2 induces the recruitment of TAK1 and TAB1. In the case of CD40, TRAF6 is an additional E3 ligase involved in NF-κB activation through a mechanism dependent on cIAP1 as detailed before (18). Agonist monoclonal antibodies anti-CD137, anti-CD40, anti-GITR, anti-CD27, and anti-OX40 induce immune-mediated tumor regression in mice (66) and some of them are in phase I and II clinical trials for cancer treatment (NCT00351325, NCT00231166, NCT00664898, NCT00900302). The accurate measurement of the K63-Polyub route of signaling may become a much-valued biomarker (67) to clinically gauge the actions of these novel therapeutic agents.

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Clinical-Translational Advances

K63-Polyub attachment defines a new paradigm in signaling with wide-ranging applications. Until recently the study of this biochemical pathway required complex proteomic approaches, but now investigation will be simplified by the availability of specific mAbs that recognize and monitor K63 ubiquitin polymers (44, 45). Our knowledge of the functions and proteins controlled by K63-Polyub is quite limited at present and thus many new elements are likely to be discovered in the near future. For example, a recent report indicates K63-Polyub involvement in chromatin remodeling via K63-polyubiquitination of hystones (68). Moreover, according to an excellent recent article, K63-Polyub chains that are not attached to any protein may act as second messengers that can be degraded by DUBs (69).

Several lines of cancer research may well undergo revolutionary advances as we deepen our knowledge of the ubiquitome and its regulation. These include:

(1) understanding tumor-associated inflammation in carcinogenesis, in the generation of tumor-promoting microenvironment, and in metastasis and/or invasion behavior in the malignant tissue; this will mainly involve the IL-1 and IL-18 receptors as well as signaling through TNF receptor family members and quite possibly via TLRs.

(2) T-cell activation as it desirably occurs in adoptive T-cell therapy and in cancer vaccination.

(3) use of artificial costimulation with the immunostimulatory monoclonal antibodies anti-CD40, anti-OX40, anti-CD137, anti-CD27, and other members of the TNF receptor family. Biomarker development involving this signaling pathway has great potential to measure and correlate biological effects in this field (67).

(4) drug discovery efforts directed at relevant enzymes for inhibition or enhancement of these signaling pathways with the aim of affecting tumor cells, stromal cells, and/or immune system cells. For instance, there is a key role for K63-Polyub in TRAF-6-mediated stimulation of AKT, thereby mediating a critical function in the anti-apoptotic resistance of tumor cells (70). Functional effects of dominant negative ubiquitin lacking K63 (44) offer a proof of concept of the druggability of the pathway. Structural evidence will be of great help for this endeavor (45).

Disclosure of Potential Conflicts of Interest

I. Melero receives honoraria from Bristol Myers Squibb and is a consultant for Pfizer, Bristol Myers Squibb, and Miltenyi.

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