Immunology Letters 202 (2018) 44–51

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Review

Promising new therapeutic targets for regulation of inflammation and immunity: RING-type E3 ubiquitin ligases

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ARTICLE INFO

Keywords:
Ubiquitin-proteasome system
Ubiquitin
E3 ligases
RING
Inflammation
Immunity
T cells
Small molecule
MDM2
p53
Cullin
GRAIL
TRIM
TRAF

ABSTRACT

Ubiquitin–proteasome system (UPS) is a primary signaling pathway for regulation of protein turnover and removal of misfolded proteins in eukaryotic cells. Enzymes of the UPS pathway - E1 activating, E2 conjugating, E3 ligating - act together to covalently tag substrate proteins with a chain of ubiquitins, small regulatory proteins. The poly-ubiquitin chain then serves as a recognition motif for 26S proteasome to recognize and degrade the substrate. In recent years UPS has emerged as attractive enzymatic cascade for development of novel therapeutics against various human diseases. Building on the previous success of targeting this pathway in cancer – the broader scientific community is currently looking for ways to elucidate functions of E3 ligases, substrate-specific members of the UPS. RING-type E3 ubiquitin ligases, the largest class of E3s, represent prospective targets for small molecule modulation and their importance is reinforced by ever growing evidence of playing role in non-cancer diseases, primarily associated with inflammatory and immune disorders. In this review, we aim to briefly cover the current knowledge of biological functions of RING-type E3 ligases in inflammation and immunity.

1. Introduction

Intracellular signaling is primarily controlled by key post-translational modifications - phosphorylation, acetylation, and ubiquitination [1]. Ubiquitination is carried out by the ubiquitin-proteasome system (UPS) and is now widely recognized as the primary signaling event in regulation of inflammation and immunity. In the last two decades, scientists around the globe have witnessed increasing attention towards UPS that regulates protein turnover in eukaryotic cells. Research in this area substantially accelerated after the 2004 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose for their discovery of ubiquitin-mediated proteasomal protein degradation [2]. The currently well-known molecular mechanism of the UPS activity is regulated by consecutive action of three enzyme types (E1 activating, E2 conjugating, E3 ligating). These enzymes operate in a concerted manner to poly-ubiquitinate a substrate protein and define its subsequent fate (Fig. 1A) [3,4]. The chain of ubiquitin molecules, small 76 amino acid proteins, regulates molecular signaling depending on lysine linkage type of the poly-ubiquitin moiety, its length and additional post-translational modifications. Poly-ubiquitin chain that can be formed via seven distinct lysine residues (K6, K11, K27, K29, K33, K48, K63). For example, K48-linked chain directs ubiquitinated substrate

Abbreviations: ALS, amyotrophiclateral sclerosis; AP-1, activator protein 1; APC, antigen-presenting cell; APC/C, anaphase promoting complex/cyclosome; CLR, C-type lectin receptor; CRL, Cullin RING ligase; ERK, extracellular signalregulated kinase; FANC, Fanconi anemia ligase; FTD, frontotemporal dementia; GRAIL, gene related to anergy in lymphocytes protein; HIF-1α, hypoxia-inducible factor 1 alpha; IFN, interferon; IKK, IκB kinase; IL, interleukin; IRF, interferon-regulatory factor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signaling protein; MDM2, murine double minute 2; MHC, major histocompatibility complex; MS, multiple sclerosis; NFATc2, nuclear factor of activated T cells c2; NF-κB, nuclear factor–kappa-light-enhancer of activated B cells; NLR, NOD-like receptor; PBMC, peripheral blood mononuclear cell; PDC, plasmacytoid dendritic cell; PROTAC, proteolysis-targeting chimera; PRR, pattern recognition receptor; RA, rheumatoid arthritis; RBR, RING-between RING-RING ligase; RIG-I, retinoic acid-inducible gene-I; RING, really interesting new gene; RLR, RIG-I-like receptor; SCF, Skp1-Cullin-F-box complex; Skp2, S-phase kinase-associated protein 2; SLE, systemic lupus erythematosus; TAK1, transforming growth factor-β activated kinase 1; TAB, TAK1-binding protein; TBK, TANK-binding kinase 1; TCR, T cell receptor; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFRSF5, tumor necrosis factor receptor superfamily member 5; TNRF2, tumor necrosis factor receptor 2; TRAF, tumor necrosis factor receptor-associated factor; TRIM, tripartite motif; VHL, von Hippel-Lindau disease tumor suppressor; UPS, ubiquitin-proteasome system

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https://doi.org/10.1016/j.imlet.2018.08.001
Received 21 December 2017; Received in revised form 24 July 2018; Accepted 8 August 2018
Available online 09 August 2018

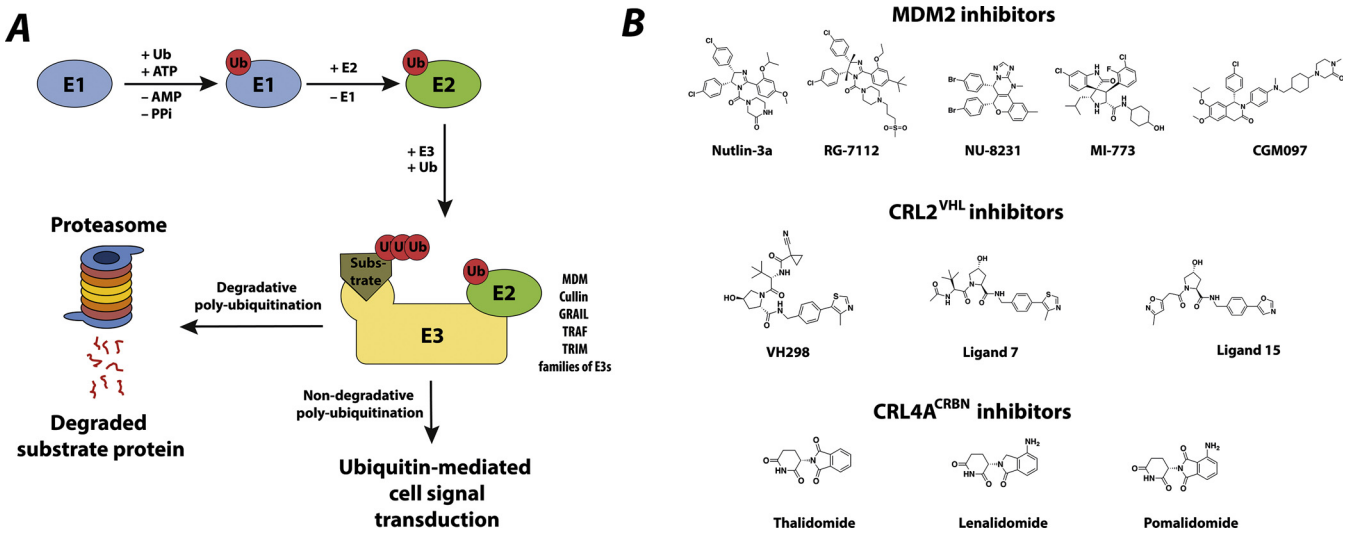

Fig. 1. (A) Schematic representation of the UPS enzymatic cascade resulting in either degradation of the substrate protein or non-degradative ubiquitin-mediated signal transduction. (B) Chemical structures of several small molecule inhibitors of MDM2, $CRL2^{VHL}$ and $CRL4^{CRBN}$ RING-type E3 ligases that could potentially be used for modulation of immune and inflammatory response.

protein for proteasomal degradation, and K63-linked chain is involved in kinase-mediated cell signaling. Ubiquitination represents an important mechanism for post-translational protein modification in eukaryotes that results in functionally distinct intracellular signals such as regulation of innate immunity and protein quality control, trafficking and degradation by 26S proteasome. Not surprisingly, enzymes of the ubiquitin-proteasome system are emerging as promising molecular targets for drug discovery - they are implicated in cellular physiology and homeostasis at multiple regulatory levels, assumed as a primary mechanism in mediation of immune response and regulation of inflammatory signal transduction [5,6].

A prominent example of such enzyme is the proteasome, a highly promising target not only in cancer, but also in inflammatory and autoimmune diseases. Circulating proteasomes and respective anti-proteasome autoantibodies were detected in serum samples from patients with autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [7]. Selective proteasome inhibition is a mechanism of action demonstrated by FDA-approved anticancer drugs Carfilzomib and Bortezomib, both of which are also being considered as potential drug candidates for modulation of immune response. For example, Bortezomib that was initially approved for treatment of multiple myeloma now undergoes clinical trials against several autoimmune diseases such as proliferative lupus nephritis, refractory cold agglutinin disease and IgA nephropathy. This prospective therapeutic strategy is also supported by clinical data showing that refractory SLE patients indeed respond to the Bortezomib-based therapy [8]. In addition, both Bortezomib and Carfilzomib were tested in SLE animal models and demonstrated decreased levels of autoantibodies and retarded disease development [9]. The therapeutic effect was accompanied by diminished number of plasma cells and suppressed production of interferon alpha (IFNα) by plasmacytoid dendritic cells (PDCs).

An emerging and highly promising drug discovery approach is to target multiple levels of ubiquitination cascade upstream of proteasome - E1, E2 and, particularly, E3 enzymes [10]. High interest towards E3 ubiquitin ligases is caused by their function to confer substrate specificity of whole UPS. E3s act via two primary mechanisms - intermediary catalytic transfer of ubiquitins from E2 ~ Ub conjugate to the substrate or, alternatively, direct ubiquitin transfer bypassing the E3 ligase itself. The first mechanism is common for Homologous to E6-AP Carboxy Terminus (HECT)-type E3s, whereas the second one is typical for really interesting new gene (RING)-type E3s. Development of potent small molecule modulators for these enzymes is a risky and challenging task complicated by diverse protein-protein interfaces of the multi-component complexes, common lack of a classical enzymatic/catalytic active site and specificity problems stipulated by a variety of potential substrates.

Conserved RING finger domain constitutes the core of E3 ligase catalytic activity by serving as docking platform for E2 enzyme. RING motif was initially identified in RING1 protein and later confirmed as a structural element of Rbx1, an E2-recruiting subunit of E3 ligase [11–14]. The canonical RING finger motif can be represented as $Cys–X_2-Cys-X_{(9–39)}-Cys-X_{(1–3)}-His-X_{(2–3)}-Cys-X_2-Cys-X_{(4–48)}-Cys-X_2-Cys$, where X is any other amino acid [15]. The motif includes two Zn^{2+}-coordinated loops and intervening central α-helix that together form a conserved structural platform for anchoring the E2 conjugating enzyme. RING E3s can be categorized into subclasses according to the form of subunit organization: 1) mono-subunit ligases, i.e. murine double minute (MDM); and 2) multi-subunit complexes, i.e. Cullin RING ligase (CRL), anaphase promoting complex/cyclosome (APC/C) [16] and Fanconi anemia ligase (FANC) [17]. Another important RING family members are RING-between RING-RING ligases (RBRs), the single subunit enzymes with multiple RING domains [18]. In addition, worth mentioning are U-box ligases, containing atypical RING domains without coordinated Zn^{2+} ions, that are often featured as separate from canonical RING E3s [19].

Inflammation and autoimmunity is often described as defective response of adaptive immune system due to deregulated activation of B or T cells, and is accompanied by immune tolerance towards self-antigens and tissue damage. Cell-mediated immunity is based on functions and signaling of T lymphocytes that includes maturation of $CD4^+$ helper and $CD8^+$ cytotoxic T cells in thymus, then migration to peripheral tissues and activation through a process in which T cell receptor (TCR) binds to antigen presented by major histocompatibility complex (MHC) of antigen-presenting cells (APCs). This is followed by $CD8^+$ cells attacking and destroying pathogen-infected host cells, $CD4^+$ T cells activating phagocytes and facilitating differentiation of various immune cells, antibody-producing memory B cells providing strong pathogen-specific immune response [20]. Understanding molecular principles underlying the initiation and progression of inflammation has long been a major priority in the field. Therefore, identification of disease-associated proteins and subsequent design of small-molecule modulators to target those proteins represents a highly attractive route to develop new treatments.

Table 1
Members of MDM, Cullin, GRAIL, TRAF and TRIM families of RING-type E3 ligases, their functions in immunity and inflammation, reported small molecule modulators.

RING-type E3	Function	Small molecule modulators
MDM family		
MDM2	Promotes proliferation of auto-reactive T cells and production of auto-antibodies [30]; Negatively regulates TCR signaling via NFAT pathway [38]; Linked to multiple sclerosis, systemic lupus erythematosus, lupus nephritis and primary Sjogren's syndrome [29]	Nutlin-3a [94], RG-7112 [95], NU8231 [96], MI-773 [97], CGM097 [98]
Cullin family		
$CRL2^{VHL}$	Essential for Treg stability, regulates IFNγ production by Tregs [51]	Ligand 7, Ligand 15, VH298 against $CRL2^{VHL}$ [104,105,106]; thalidomide and its derivatives lenalidomide, pomalidomide against $CRL4A^{CRBN}$ [107,108]
SCF^{Skp2}	Negatively regulates Tregs [50]	
CRL4	Regulates granulocyte differentiation [46]; Facilitates production of TNFα by macrophages [47]	
GRAIL family		
GRAIL	Essential component of $CD4^+$ T-cell tolerance and induction of anergic phenotype [55,56]; Linked to Crohn's disease [57]	N/A
TRAF family		
TRAF3	Negatively regulates IL-2 production by $CD25^+Foxp3^+$ Tregs [64]	N/A
TRAF6	Involved in TCR-mediated T cell activation [66]	
TRIM family		
TRIM5α	Upregulates NF-κB and drives production of pro-inflammatory cytokines [74]; Functions as viral restriction factor [75]	N/A
TRIM8	Induces TNFα and IL-1β, activates NF-κB pathway [81]	
TRIM9	Negatively regulates NF-κB [82]	
TRIM21	Linked to breakdown of immune tolerance in systemic lupus erythematosus [77,78]	
TRIM23	Essential for antiviral innate and inflammatory responses [83]	
TRIM25	Upregulates production of IFNβ [76]	

Here, we discuss implications of several major families of RING-type E3 ubiquitin ligases (MDM, Cullin, GRAIL, TRAF, TRIM) as targets in inflammation and immunity, and provide insights into potential small molecule interventions to mediate the cellular responses (summarized in Table 1). We briefly cover the role of RING-type E3 ubiquitin ligases in substrate protein stability and trafficking, T cell signaling and cytokine productions upon activating stimulus.

2. MDM family

Murine double minute 2 (MDM2) protein is mainly known as a negative regulator of primary oncosuppressor and transcription factor p53. These two proteins are linked in tightly regulated negative feedback loop whereby MDM2 ubiquitinates p53 resulting in its proteasomal degradation [21]. The importance of p53 activation in immune response and inflammation has been implicated a lot in recent years revealing that p53 is responsible for transactivating genes involved in pathogen recognition, cytokine production, immune checkpoint regulation and broadly in the maintenance of immune homeostasis [22–24].

MDM2 also regulates nuclear factor–kappa-light-enhancer of activated B cells (NF-κB), a primary transcription factor that serves as master regulator of cell signaling by coordinating immune and inflammatory responses [25]. In that capacity MDM2 functions as non-redundant co-factor for NF-κB signaling by facilitating binding of NF-κB to promoter regions of its target genes [26]. Importantly, MDM2 is transcriptional target for both p53 and NF-κB, therefore MDM2–p53–NFk-B axis is believed to function as potential link between inflammation and tumorigenesis [27]. Despite normally having antagonistic roles in cancer, p53 and NF-κB demonstrate concerted signaling in inflammation, i.e. they co-regulate transcription of inflammation-associated genes in human macrophages and T cells [28]. In that way, MDM2 promotes mostly pro-survival NF-κB signaling and blocks mostly pro-apoptotic p53 signaling, thereby small molecule MDM2 inhibitors like Nutlin-3a act as NF-κB antagonists and p53 agonists. In response to Nutlin-3a both p53 and NF-κB induce pro-inflammatory responses by binding to promoter regions of cytokine genes and promoting production of cytokines, i.e. IL-6 and IL-8.

MDM2 represents a novel and highly promising target for therapeutic intervention in multiple inflammatory autoimmune diseases, including MS, SLE, lupus nephritis and primary Sjogren's syndrome [29]. MDM2 was previously reported to be upregulated in SLE animal models and inhibition of this E3 enzyme is considered as a promising approach to suppress inflammation. Nutlin-3a was demonstrated to reduce systemic inflammation in SLE and lupus nephritis by blocking formation of immune complex and suppressing abnormal expansion of all T cell subsets linked to systemic inflammation [30]. MDM2 suppression substantially reduced lymph proliferation through depletion of auto-reactive T cells and plasma cells, and also inhibited production of auto-antibodies. Animal model experiments revealed that MDM2 upregulation induced expansion of plasma cells and double negative $CD3^+CD4^-CD8^-$ T cells that promote production of auto-antibodies. These results support the opinion that MDM2 is a valuable therapeutic target for treatment of SLE, lupus nephritis and, potentially, other autoimmune diseases associated with polyclonal antibody production. In agreement with this, substantially higher levels of anti-MDM2 auto-antibodies were detected in SLE and primary Sjogren's syndrome patients compared to healthy humans suggesting potential role of MDM2 as serological marker in pathogenesis of these diseases [31,32].

The involvement of MDM2 in mechanisms underlying development of inflammatory autoimmune diseases is additionally supported by recent data showing that MDM2 promotes rheumatoid arthritis [33]. Here, collagen-induced arthritis mouse model was used to demonstrate that high MDM2 levels correlated with disease severity and stimulates release of pro-inflammatory cytokines through activation of NF-κB and MAPK signaling pathways. Whereas, inhibition of MDM2 by Nutlin-3a reduced inflammation and suppressed propagation of immune response.

In another study, Nutlin-3a-mediated p53 activation induced strong negative impact on NF-κB signaling in LPS-stimulated neutrophils and macrophages [34]. Nutlin-3a limited cellular immune response to LPS, partly by attenuating DNA binding activity of NF-κB and impairing transcription of its target cytokine genes. At the same time p53-negative cells demonstrated increased NF-κB DNA-binding activity and were characterized with elevated levels of cytokine release upon LPS stimulation compared to p53-positive cells. Later it was shown that Nutlin-3a-driven p53 activation also modulates dendritic cell-induced T

cell proliferation [35], leads to transcriptional activation in human PBMCs and macrophages [36] and also suppresses M2 macrophage polarization by repressing transcription of c-MYC [37].

In addition, in T cells MDM2 is involved in negative regulation of TCR signaling by mediating NFAT pathway resulting in ubiquitination and proteasomal degradation of nuclear factor of activated T cells c2 (NFATc2), a crucial step in avoiding T cell-mediated immune overreaction in response to various stimuli. The study demonstrated that self-degrading auto-ubiquitination of MDM2 during T cell activation leads to nuclear accumulation of NFATc2 and followed by cytokine induction [38]. Such suicidal MDM2 auto-ubiquination can be blocked by USP15 deubiquitinase that prevents activation of NFATc2-mediated cytokine expression such as production of IFNγ by Th1 effector T cells.

3. Cullin family

Cullin RING E3 ligases (CRLs) constitute the largest family of E3 ligases with over 200 known members [39]. In certain cell types up to 20% of the proteasomal protein degradation is mediated by CRLs [40]. Assembly of the multi-subunit CRLs was originally reported for the archetypal Skp1–Cdc53–F-box Cdc4 (SCF) complex [41]. The CRL structure is based on modular organization of constituent subunits such as substrate receptors (F-box, SOCS-box, DCAF, BTB), adaptors (Skp1, ElonginB, ElonginC, DDB1, BTB), Cullin scaffolds (Cul1-Cul7, Cul9) and RING finger proteins (Rbx1 and Rbx2) [42,43]. The wide range of building blocks and their combinations enables formation of a multitude of functionally diverse E3 ligases [39,44,45].

CRLs and their constituent Cullin proteins are implicated in various types of immune cell functions and signaling outcomes. For example, Cul4A affects granulocyte differentiation and cell cycle [46]. On the other hand, its close homolog Cul4B sustains the levels of macrophage secreted cytokine tumor necrosis factor alpha (TNFα) [47] and also negatively regulates LPS-induced peritonitis [48].

FBXO1, an F-box protein family member, is a substrate-recognition subunit of the SCF-type E3 ubiquitin ligase. A recent genomic study revealed that expression of *CCNF* gene encoding FBXO1 can lead to aberrant ubiquitination and affect the mechanisms of neuronal degeneration in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [49]. In another example negative regulation of Treg functions was demonstrated by SCF with F-box protein Skp2 as substrate recognition domain (SCF^{Skp2}) [50]. Here, SCF^{Skp2} was found to be involved in regulation of Treg cell cycle control and death. Overexpression of SCF^{Skp2} suppressed Treg functions via induced loss of Foxp3, an essential transcriptional regulator crucial for immune system homeostasis. On the contrary, SCF^{Skp2} downregulation resulted in transformation of auto-reactive pathogenic T cells into Foxp3 expressing Tregs.

Von Hippel-Lindau disease tumor suppressor (VHL) is a well-described substrate recognition subunit in $CRL2^{VHL}$ E3 ubiquitin ligase that mediates proteasomal degradation of hypoxia-inducible factor 1 alpha (HIF-1α) as its primary substrate. VHL-mediated alteration of HIF-1α levels was demonstrated to be essential for Treg stability and suppressive capacity [51]. HIF-1α binds to *Ifng* gene promoter and enhances transcription of this gene, therefore directly affecting levels of IFNγ produced by Treg cells. Animal studies demonstrated that VHL-deficient Tregs produce excessive amount of IFNγ that leads to increased inflammation, whereas HIF-1α knockdown or knockout balances excessive IFNγ production in these cells.

4. GRAIL family

Gene related to anergy in lymphocytes protein (GRAIL, encoded by *Rnf128* gene) is a transmembrane RING-type E3 ligase involved in T cell anergy and tolerance, and also regulation of actin cytoskeletal organization [52]. This E3 was initially identified in $CD4^+$ T helper cells and $CD4^+CD25^+$ regulatory T cells as an upregulated transcript that was later linked to immunosuppression [53,54].

GRAIL is an essential component of $CD4^+$ T-cell tolerance and induction of anergic phenotype, a state of T cell unresponsiveness to antigenic stimulation that inhibits interleukin production [55,56]. GRAIL overexpression in $CD4^+$ T cells has been clinically linked to Crohn's disease, an inflammatory bowel disorder [57]. In addition, GRAIL regulates T cell tolerance and functions by mediating ubiquitination and proteasomal degradation of TCR-CD3 [55]. Activated GRAIL-deficient naive T cells enhance cytokine expression and proliferation, retard downregulation of TCR-CD3 expression and reduce hyper-responsiveness to TCR stimulation. Lower immunosuppressive capacity of GRAIL-deficient Treg cells was linked to overexpression of Th17-specific genes. In agreement with this, GRAIL-deficient mice had higher susceptibility to autoimmunity due to impaired induction of T cell tolerance. Another important GRAIL substrates include actin-related protein 2/3 subunit 5 (Arp2/3–5) and coronin 1 A, both associated with actin cytoskeleton organization and dynamics [58]. Arp2/3–5 and coronin 1 A levels were found to be significantly downregulated in GRAIL-overexpressing and anergic T cells. GRAIL also promotes innate antiviral immunity to RNA and DNA viruses via ubiquitination of TBK1, serine/threonine-protein kinase essential for production of IFNβ [59].

Recent data suggests that GRAIL absence can lead to anti-tumor activity of $CD8^+$ cytotoxic T cells, essential for maintenance of anti-tumor processes [60]. *Rnf128* gene (encoding GRAIL ubiquitin ligase) expression was upregulated in $CD8^+$ T cells infiltrated into lymphoma tumors. Additional experiments demonstrated that GRAIL-deficiency enhances anti-tumor potency of $CD8^+$ T cells, and contributes to long-term regulation of tumorigenesis and capability to repress existing tumors. The same study proposed IL-21R as another GRAIL substrate, confirming previous data on involvement of GRAIL in negative regulation of IL-21 mediated by NFATc1 transcription factor [55]. GRAIL overexpression and IL-21R downregulation was observed in $CD8^+$ T cells isolated from lymphoma patients as compared to samples from healthy donors. On the opposite, loss of GRAIL in lymphoma patients resulted in enhanced infiltration of GRAIL-deficient $CD8^+$ T cells with IL-21R overexpression in turn leading to high IL-21 responsiveness [60].

5. TRAF family

Tumor necrosis factor (TNF) receptor associated factor (TRAF) family of proteins includes seven members, TRAF1-TRAF7, that control many biological processes, including regulation of innate and adaptive immune functions, homeostasis, cytokine production and cell survival [61]. TRAF family members contain several zing finger motifs and share high sequence homology in C-terminal TRAF domain, also all of them (except TRAF1) contain N-terminal RING finger domain that confers E3 ubiquitin ligase activity. Adaptor functions and E3 ubiquitin-ligating activity of TRAF proteins allows them to mediate transduction of downstream signaling. Ubiquitination is considered as a primary mechanism of TRAF signaling that is conducted via catalysis of non-degradative K63-linked poly-ubiquitination of their substrates, such as various receptors, kinases and transcription factors [62].

In the past decade TRAFs have been recognized as signal transducers for a broad range of receptor families engaged in innate and adaptive immune signaling and cytokine production. Several primary receptor classes currently known to be involved in TRAF signal transduction include T cell receptor, Toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), IFN receptors, TGFβ receptors, IL-1 and IL-17 receptors [62]. TRAF-dependent signaling pathway leads to activation of mitogen-activated protein kinases (MAPKs), interferon-regulatory factors (IRFs) and, most importantly, key transcription factors such as NF-κB and activator protein 1 (AP-1) that are responsible for expression of multiple genes associated with inflammation and immune response.

TRAF1 and TRAF2 were initially identified as tumor necrosis factor receptor 2 (TNRF2)-associated components, TRAF4 was overexpressed in breast carcinoma cells, whereas TRAF3, TRAF5 and TRAF6 were discovered by their interaction with specific domains in the cytoplasmic tails of trans-membrane receptor CD40 [63]. TRAFs serve as essential adaptors for members of TNFR signaling pathway such as TNFR2 and CD40, and are involved in assembly of signaling complexes associated with intracellular domains of trans-membrane receptors.

TRAF3 is primarily involved in thymic T cell development and B cell signaling. It also negatively regulates IL-2 that is required for conversion of precursor T cells to mature $CD25^+Foxp3^+$ regulatory T cells, a major subset of immunosuppressive $CD4^+$ T cells that maintain peripheral immune system and prevent autoimmune diseases [64]. TRAF3 regulates Treg effector functions and humoral immune response, and accordingly TRAF3-deficiency leads to increased number of Tregs in thymus and peripheral tissues [65].

TRAF6 is an important mediator of interleukin-1 receptor (IL-1R)-associated activation of NF-κB signaling pathway and is essential for induction of immune response and maintenance of immune tolerance. TRAF6 also regulates development and functioning of B cells, T cells and macrophages. For example, in T cells TRAF6 is involved in TCR-mediated T cell activation, Th/Treg cells differentiation, $CD8^+$ T-cell homeostasis and memory development [66]. Whereas, in B cells it promotes signal transduction via B-cell surface antigen CD40, a.k.a. tumor necrosis factor receptor superfamily member 5 (TNFRSF5), and activates extracellular signal–regulated kinases (ERKs) resulting in induction of immunoglobulin secretion. In this respect, TRAF6-deficiency in B cells results in lower number of mature B cells in spleen and bone marrow [67]. Moreover, B cell-specific TRAF6-deficient mice were demonstrated to have impaired T cell-dependent and T cell-independent humoral immune response.

6. TRIM family

Tripartite motif (TRIM) family of proteins are key players in regulation of intracellular signaling, cell development and innate immunity. Chromosomal location of *TRIM* genes suggests their importance for immune response, primarily against viral infections. Human *TRIM* genes are transciptionally upregulated in immune cells in response to viral pathogen-mediated induction of interferons [68], and also participate at various levels of NF-κB signaling pathway implicating regulatory functions in immunity and inflammation [69]. Previous genome-wide association analysis revealed that single nucleotide polymorphisms (SNPs) in several TRIM genes (*TRIM10*, *TRIM15*, *TRIM26*, *TRIM39* and *TRIM40*) define susceptibility to MS, a chronic autoimmune disease linked to inflammatory demyelination of the central nervous system [70].

Currently there are over 70 members of TRIM family classified into 11 subgroups according to their domain architecture, as extensively reviewed in recent study [71]. TRIMs are characterized by common N-terminal tripartite RBCC motif that consists of one or two BBox domains, coiled-coil (CC) domain, and RING-finger domain that confers E3 ligase activity. The variable substrate-specific C-terminal motif includes PHD, BROMO, SPRY, COS and several others that mediate K48- and K63-linked substrate poly-ubiquition.

TRIMs play part in positive and negative regulation of pattern recognition receptors (PRRs) of the innate immune system such as RLRs, TLRs, NLRs and C-type lectin receptors (CLRs) amongst others [72]. These receptors detect viral infection and then activate TRIM-mediated antiviral response by induction of pro-inflammatory cytokines, type I interferon and TNFα. Remarkably, primary involvement of TRIMs in immune response against viral pathogens resulted in these pathogens acquiring a range of sophisticated mechanisms to overcome the host immune system that includes hijacking host ubiquitination pathway and targeting TRIM family ligases, in particular. [73].

Two primary members of the TRIM family – TRIM5α and TRIM25 – function as antiviral effector and immune regulator, respectively. TRIM5α is one of the best characterized members of the TRIM family (both structurally and functionally) and reported to be an important ubiquitin-mediated regulator of a major kinase complex consisting of transforming growth factor-β activated kinase 1 (TAK1) and TAK1-binding proteins (TABs) [74]. In turn, TAK1-TAB complex activates another heteromeric kinase complex IKKα-IKKβ-NEMO that facilitates ubiquination and degradation of NF-κB inhibitor IκBα. Resulting NF-κB upregulation and nuclear translocation drives production of IFNβ and various pro-inflammatory cytokines. TRIM5α also functions as viral restriction factor – it recognizes HIV-1 capsid and facilitates its disassembly, in this way blocking the retroviral infection [75]. Here, SPRY domain of TRIM5α specifically interacts with retroviral capsid lattice and induces NF-κB-dependent pro-inflammatory response leading to autophagy-mediated capsid degradation.

TRIM25, another major member of the family, is crucial for antiviral immune response associated with IFN signaling. TRIM25 mediates K63-linked ubiquitination and activation of RIG-I that further leads to upregulation of IFNβ [76]. As an example, influenza A virus NS1 targets TRIM25 activity to suppress IFNβ-mediated immune response and avoid recognition by RIG-I, the host sensor of viral RNA. RIG-I ubiquitination by TRIM25 results in activation of RIG-I/MAVS signaling pathway and induction of type I interferon in response to viral infection.

TRIM21 performs regulatory functions in both physiological and pathological immune responses, and was proposed as another potential target in SLE [77]. TRIM21 overexpression can result in breakdown of immune tolerance that triggers generation of TRIM21 auto-antibodies in genetically susceptible SLE patients [78]. In addition, patients with SLE and Sjogren's syndrome were demonstrated to have increased levels of TRIM21 that enables induction of autoimmune B and T cell responses [79]. The humoral immune response engages TRIM21 as cytosolic antibody receptor that interacts with immunoglobulins at the surface of internalized pathogens and facilitates propagation of diverse immune signals, i.e. TAK-1-dependent NF-κB activation [80].

Among other members of the TRIM family worth mentioning are also TRIM8, TRIM9 and TRIM23 that elicit their immune cellular stimuli primarily through alteration of NF-κB activity. Specifically, TRIM8 poly-ubiquitinates TAK1 resulting in induction of TNFα, IL-1β and activation of NF-κB [81]; TRIM9 stabilizes IκBα and in this way inhibits NF-κB [82]; TRIM23 poly-ubiquitinates NEMO (IKKγ), an important NF-κB regulator, and promotes RIG-I-associated signaling [83].

7. Small molecule targeting

Drug discovery in the UPS field keeps advancing, driven by foreseeable role of these enzymes as targets in numerous human diseases. Primary examples of FDA approved drugs include 26S proteasome inhibitors Bortezomib (approved in 2003, marketed as Velcade®) and Carfilzomib (approved in 2012, marketed as Kyprolis®) for treatment of multiple myeloma. The emerging and highly promising therapeutic strategy is based on targeting different levels of ubiquitination cascade upstream of proteasome, mainly E1, E2 and E3 enzymes [10]. Here, E3 ligases determine substrate specificity and, therefore, represent primary molecular targets for small molecule modulation. E3 ligases in general, and RING-type E3s in particular, represent lucrative yet highly sophisticated drug targets.

RING-type E3s are growing in importance as attractive targets for small molecule therapeutics that could operate not only as inhibitors but also activators of enzymatic activity, disruptors and stabilizers of protein-protein interactions, regulators of protein dynamics. However, targeting RING-type E3s for therapeutic applications is obstructed by several factors. Firstly, there is no general approach and each enzyme has to be tackled individually according to its structural and functional characteristics. Secondly, RING-type E3s are complex molecular entities that are often assembled from several independent subunits that form a

number of protein-protein interfaces and druggable pockets. Thirdly, the availability of X-ray structural data, especially for substrate-specific components remains to be one the main limitations for structure-based design of modulators for multi-subunit E3 complexes.

Surprisingly, the apparent complexity of this multi-subunit system might turn out to be an advantage – the multitude of interfaces and pockets provides additional opportunities for design of specific binders. Small molecules have potential to modulate E3 enzyme activity directly by inhibiting substrate/receptor interaction, disrupting enzyme assembly, inducing allosteric conformational shifts that lead to suppressed activity or altered ensemble dynamics [84], stabilizing specific protein-protein interactions within the RING-type E3s complex [85].

Several main strategies for targeting E3s described in the literature include: *in vitro* screening using functional assays [86]; computer programs for predicting potential druggable pockets, including those at protein-protein interfaces, and subsequent docking-based *in silico* ligand screening [87]; fragment-based design and further rational structure-based elaboration [43,88]. Although each approach can have several advantages, amongst the drawbacks and limitations are following: functional screening does not provide direct evidence regarding the binding site of the hits, computational methods for pocket prediction have limited reliability and also require available crystal structures (ideally of ligand-bound complexes), and fragment-based approach strongly relies on numerous biophysical techniques, requires laborious structural optimization, and is often limited by the low ligand-efficiency associated with small molecules targeting protein-protein interactions. Given slow yet steady increase of solved crystal structures of RING-type E3s and their ligand-bound complexes we reasonably expect that structure-based design will prove itself as one of the most promising strategies for developing specific small molecules for targeting these enzymes.

Despite the difficulties numerous RING-type E3s or their functional components have been successfully targeted in the past using small molecule modulators of protein-protein interactions, i.e. binding at substrate/receptor HIF-1α/VHL [89], p53/MDM2 and p53/MDM4 interfaces [90], adaptor/receptor interface Skp1/Skp2 [91] and several other, as reviewed in [92,93].

MDM2 is one of the most targeted E3 ligases with numerous reported synthetic inhibitors such as Nutlin-3a [94], RG7112 [95], NU8231 [96], MI-773 [97], CGM097 [98] and several others already in clinical trials as drug candidates [99] (Fig. 1B). The CRL activity modulators also emerge as a class stipulated by increasing number of solved crystal structures of CRL subunits that allows rational design of synthetic binders [92]. There is currently no information on small molecule modulators for GRAIL, TRAF and TRIM E3s, yet we anticipate such molecules to be developed soon due to highly promising nature of these enzymes as targets in cancer, inflammatory and autoimmune diseases.

A novel paradigm-shifting approach that dramatically increases the attractiveness of RING-type E3 ligases as targets for drug design is based on proteolysis-targeting chimeras (PROTACs) [100,101]. These are hetero-bifunctional compounds with bivalent selectivity, they consist of three key elements: substrate-specific component (“warhead”), short linker, and E3-specific component. PROTACs bind and bring into close proximity substrate protein of interest and E3 ligase thus facilitating E3-mediated ubiquitination of the substrate. This approach, sometimes called as “chemical knockdown”, enables ligand-induced degradation of specific endogenous proteins [102]. Despite demonstrating high potential for a novel modality of chemical intervention on E3 ligases the use of PROTACs has yet unknown effect on modulation of immune response.

8. Conclusions

Inflammation and immune responses have evolved as a complex signaling architecture to resist a multitude of external and internal causes, such as infectious pathogens or autoimmunity. Recent advances in this field asserted ubiquitin-proteasome system as one of the cornerstones of this dynamic signaling framework. Accumulating evidence indicates that E3 ubiquitin ligases rise above other components of the UPS due to their expanding role in orchestrating a plethora of cell signaling outputs. These enzymes were proposed to become the “new kinases” owing to the significant therapeutic and market potential of their small molecule modulators [103]. E3s have indeed captured a significant attention of the research community because of their essential role in binding and poly-ubiquitinating target proteins.

Being the largest class of E3s RING-type enzymes have been a particular focus of attention and received a broad recognition as remarkably promising therapeutic targets. They are implicated in cellular at multiple regulatory levels and perform critical functions in numerous human diseases. Recent results have shed light on molecular and cellular pathways linking these enzymes to MS, SLE, Crohn’s disease and other inflammatory autoimmune disorders. Amongst others, MDM, Cullin, GRAIL, TRAF and TRIM families of RING-type E3s were discovered to tightly regulate innate/adaptive immunity and mediate inflammatory response in addition to their previously known roles in cancer.

In conclusion, going forward in understanding molecular mechanisms that govern inflammatory and immune-associated cellular functions of these enzymes we must fill the gaps in our knowledge of their functional specificity, context-dependent activity and signaling pathways. We anticipate a forthcoming shift of research efforts from functional/structural characterization of these enzymes to rational design of precision molecular tools capable of modulating E3 activity in living cells and organisms. Further efforts in development of RING-type E3 small molecule modulators will provide novel therapeutic options for treatments of autoimmune and inflammatory diseases. Despite high expectations one could envisage that targeted therapeutic action upstream of proteasome could potentially lead to emerging resistance caused by feedback-driven compensatory mechanisms. However, the challenge could be overcome by administering a synergistic combination of drugs that act via distinct molecular mechanisms to achieve the required biological consequences.

Conflict of interest

None of the authors has any conflict of interest to declare.

Acknowledgements

E.B. was supported by grant 16-34-60213 mol_a_dk from the Russian Foundation for Basic Research (RFBR). R.S. and A.V. were supported by grant of the President of Russian FederationMK-4253.2018.4. The work was performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

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