Journal of Oral Biosciences 54 (2012) 151–154

Contents lists available at SciVerse ScienceDirect

Journal of Oral Biosciences

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

Review

Nuclear dot protein 52, an autophagy-associated protein, regulates Toll-like receptor signaling

Megumi Inomata, Takeshi Into*

Department of Oral Microbiology, Division of Oral Infections and Health Sciences, Asahi University School of Dentistry, Hozumi 1851, Mizuho, Gifu 501-0296, Japan

ARTICLE INFO

Article history:
Received 14 March 2012
Accepted 16 April 2012
Available online 10 August 2012

Keywords:
Autophagy
Toll-like receptor
Signal transduction
NDP52
A20

ABSTRACT

Toll-like receptors (TLRs) recognize molecular patterns on various microbes and serve as innate immune sensors. After cognate ligand recognition, TLRs activate signaling pathways to induce innate immune defense mechanisms, which eliminate pathogenic microbes, including periodontogenic bacteria, to a certain extent. Recent findings have shown that TLR signaling is linked to induction of autophagy to facilitate direct killing of cytosol-invading bacteria within infected cells. However, whether autophagy has any regulatory effects on TLR signaling remains unclear. Our recent study showed that the signaling molecules Toll/interleukin-1 receptor homology domain-containing adaptor inducing interferon-β and tumor necrosis factor receptor-associated factor 6 are selectively degraded by autophagy after activation of TLR signal transduction. We found that the nuclear dot protein 52 (NDP52), an autophagy-associated protein, is involved in such degradation, negatively regulating TLR signaling. However, interestingly, this activity of NDP52 is strictly restricted by the deubiquitinase A20. Here, we describe an autophagy-associated regulatory function of NDP52 in TLR signaling on the basis of our recent findings.

Contents

1. Introduction

In innate immunity, pathogen-associated molecular patterns (PAMPs), which are usually conserved in a wide variety of microbes, are recognized by various classes of pattern-recognition receptors (PRRs) [1]. Toll-like receptors (TLRs) are generally perceived as the major PRRs. In humans, 10 functional TLRs have been identified. Cell surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) mainly recognize bacterial components, such as membrane lipoproteins, lipopolysaccharides (LPS), flagella, and fimbriae. The intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize viral and bacterial nucleic acids. After recognition of these cognate PAMPs, TLRs activate cellular signaling pathways to induce various immune responses.

Periodontal diseases are characterized by various inflammatory symptoms in the periodontal connective tissue, often accompanied by destruction of the tissue. The presence of periodontogenic gram-negative bacteria, such as *Porphyromonas*

* Corresponding author. Tel./fax: +81 58 3291422.
E-mail address: into@dent.asahi-u.ac.jp (T. Into).

1349-0079/$ - see front matter © 2012 Japanese Association for Oral Biology. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.job.2012.04.004

gingivalis, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythia*, in the subgingiva is strongly associated with disease development [2]. Periodontal inflammation is at least partially triggered by recognition of bacterial PAMPs, such as LPS and fimbriae, by TLRs [3], but several periodontogenic bacteria have the capability to evade TLR recognition [4]. The mechanism of how TLR recognition of PAMPs chronically advances inflammatory processes through involvement of various cell types around the periodontal tissue has been gaining importance.

TLR signaling is activated via 2 key adaptor molecules: myeloid differentiation factor 88 (MyD88) and Toll/interleukin (IL)-1 receptor homology domain-containing adaptor inducing interferon (IFN)-β (TRIF, also known as TICAM-1) [5]. MyD88 is involved in signaling mediated by all TLRs except TLR3, whereas TRIF is only involved in signaling mediated by TLR3 and TLR4. Activation of the MyD88-dependent pathway is principally mediated by the E3 ubiquitin ligase tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 catalyzes polyubiquitination (polyUb) of its target proteins, including IL-1 receptor-associated kinase-1 and TRAF6 itself [6]. TRAF6-mediated polyUb activates nuclear factor κB (NF-κB), a transcription factor that induces a variety of genes, such as the genes coding for cytokines, adhesion molecules, and antimicrobial molecules. However, the TRIF-dependent pathway branches into 3 major pathways that are mediated by TRAF6, receptor-interacting serine/threonine protein kinase 1 (RIP1) and another E3 ubiquitin ligase, namely, TRAF3 [5]. Both TRAF6- and RIP1-mediated pathways lead to NF-κB activation. TRAF3 catalyzes polyUb of its target proteins, including TRAF3 itself and TRAF family member-associated NF-κB activator-binding kinase 1, which activates a transcription factor called interferon regulatory factor 3 (IRF3) that drives transcription of type I IFNs and IFN-related chemokines [5].

Recent findings have revealed that TLR signaling regulates the induction of macroautophagy [7–9]. Macroautophagy (hereafter, referred to as autophagy) involves sequestration of intracellular components within autophagosomes, followed by their delivery to lysosomes and eventual degradation [10]. The major functions of autophagy in mammals are nutrient supply restoration during cell starvation, cytoprotection against stressors for homeostatic quality control, and clearance of cytosol-invading microbes and viruses. In the case of TLR4-activated autophagy, TRIF, but not MyD88, serves as an essential mediator of autophagy signaling [8]. However, in the case of TLR7-activated autophagy, MyD88 serves as a critical mediator [9]. Downstream of these adaptor proteins, TLR-induced autophagy leads to clearance of cytosol-invading bacteria, such as *Salmonella* species, and phagocytosed bacteria, such as *Mycobacterium tuberculosis* [8,9,11].

In contrast to the bactericidal effect of autophagy, the effect of autophagy on TLR signaling is still unclear. Our recent study suggested that TLR3 may activate autophagy to selectively degrade TRIF and TRAF6, thus negatively regulating TLR signal transduction [12]. In addition, an autophagy-associated protein, nuclear dot protein 52 (NDP52) was found to mediate such degradation. In this review, we address how the NDP52-associated mechanism of autophagy can regulate TLR signaling.

2. A20 conceals a mechanism of selective degradation of TRIF and TRAF6

Excessive induction of TLR-mediated immune responses results in the accumulation of pathological damage and occasionally leads to the development of inflammatory disorders [13], including periodontal diseases. To prevent this, cells usually employ mechanisms to suppress TLR signaling at multiple levels. The deubiquitinase A20 is one of the major negative regulators. A20 removes polyubiquitin chains from its substrates, such as TRAF6, TRAF3, and RIP1, to inactivate TLR signaling by inhibiting signaling downstream of these substrates and NF-κB and IRF3 activation [14]. Expression of A20 is regulated by NF-κB and thus, A20 functions in a negative feedback loop in TLR signaling. Consistent with this, we confirmed that A20 expression is upregulated by the TLR3-stimulant ligand poly (I:C) in HeLa cells and human bone marrow-derived mononuclear cells (MCs) [12]. Gene silencing of A20 by small interference RNA (siRNA) promoted poly (I:C)-induced gene expression of the NF-κB-driven cytokines IL-6 and IL-8 and the IRF3-driven chemokine CXCL10; this indicated that A20, indeed, serves as a negative regulator. However, unexpectedly, the protein levels of TRIF and TRAF6, but not of TRAF3 and RIP1, were obviously decreased after poly (I:C) treatment in A20 gene-silenced cells (Fig. 1A). However, the TRIF and TRAF6 mRNA levels and glyceraldehyde 3-phosphate protein level did not change. These observations suggest that A20 can suppress selective protein degradation mechanisms. Such a function for A20 in protein degradation is completely inconsistent with that reported by a previous study showing that A20 can promote degradation of its target proteins [15].

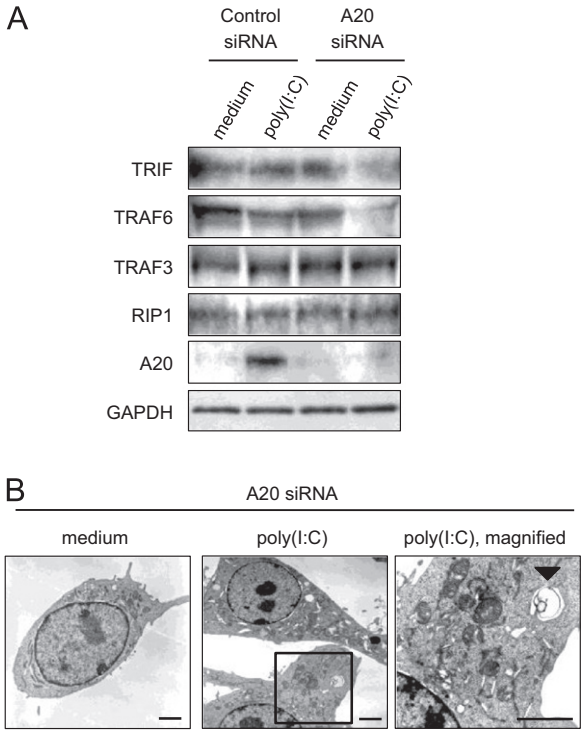

Fig. 1. A20 conceals the mechanism of degradation of TRIF and TRAF6 by autophagy. (A) TRIF and TRAF6 are selectively degraded after TLR3 engagement when A20 is silenced. Human bone marrow-derived primary mononuclear cells were transfected with control siRNA or A20 siRNA. Cells were stimulated for 6 h with 50 μg/ml poly (I:C). The cells lysates were analyzed by immunoblotting with antibodies against TRIF, TRAF6, TRAF3, RIP1, A20, and GAPDH (modified from Inomata et al. [12]). (B) Autophagosome formation after poly (I:C) stimulation in bone marrow-derived primary mononuclear cells in which the A20 gene is silenced. Human bone marrow-derived primary mononuclear cells were transfected with A20 siRNA. The cells were stimulated for 6 h with 50 μg/ml poly (I:C). The cells were evaluated by electron microscopy (modified from Inomata et al. [12]). The magnified photograph represents the area indicated in the square. Arrowhead shows autophagosome. Scale bar, 2 μm. Original magnification 3000 ×.

3. TRIF and TRAF6 are selectively degraded by autophagy

We investigated the machinery involved in the selective degradation of TRIF and TRAF6; this degradation seems to be absent in the presence of A20. The major protein degradation systems in eukaryotes are the ubiquitin–proteasome system and autophagy-mediated lysosomal system. We utilized 2 inhibitors, the 26S proteasome inhibitor MG-132 and autophagy (or class III phosphatidylinositol 3 kinase [PI3K]) inhibitor 3-methyladenin (3-MA). Interestingly, TRIF and TRAF6 degradation in A20-silenced cells was strongly inhibited by 3-MA but was unaffected by MG-132 [12].

Generally, autophagy is initiated by formation of the isolated autophagic membrane (called phagophore) around the target components at the phagophore assembly site called "omegasome-like structure" near the rough endoplasmic reticulum (rER) [16]. The elongated phagophore is closed to form a double-membrane autophagosome, and the autophagosome matures through docking and fusion with lysosomes. Subsequently, lysosomal enzymes degrade the autophagosomal inner membrane and cargo. In A20-silenced MCs, we observed increased formation of double-membrane autophagosomes after poly (I:C) stimulation (Fig. 1B). Furthermore, TRIF and TRAF6 degradation in A20-silenced cells was suppressed by the lysosomal protease inhibitors E-64, pepstatin A, and leupeptin as well as by the lysosomal vacuolar H^+-ATPase inhibitor bafilomycin A1 [12]. Thus, when A20 is silenced, TRIF and TRAF6 may be selectively delivered into autophagosomes and degraded by lysosomal enzymes via autophagy.

Formation of the phagophore is thought to occur from the membranes of rER or possibly from the Golgi complex, mitochondrion, and plasma membranes [17]. These membranes have an enzymatic complex of class III PI3K and vacuolar protein sorting-associated protein 34 that includes the essential regulatory protein Beclin-1 [18]. Initiation of phagophore formation depends on the enzymatic activity of this complex to generate phosphatidylinositol 3-phosphates. Phagophore elongation is mediated by 2 regulatory systems of autophagy proteins (ATGs). One involves the conjugation of ubiquitin-like ATG12 to ATG5 to form a large E3 ubiquitin ligase-like complex via further conjugation of ATG16L1. The other regulatory system involves the conjugation of phosphatidylethanolamine (PE) to LC3 (an ATG8 ortholog in mammals) [19]. The conversion of normal LC3 (LC3-I) to the PE-conjugated form (LC3-II) is considered a hallmark response of autophagy flux [20]. ATG7 regulates both formation of the ATG5-ATG12-ATG16L1 complex and conjugation of PE to LC3 [19]. We investigated the involvement of these autophagic processes in TRIF and TRAF6 degradation. As described above, TRIF and TRAF6 degradation was suppressed by 3-MA, an inhibitor of the class III PI3K. However, silencing of the Beclin-1 gene did not affect degradation of these molecules in A20-silenced cells [12]. In addition, silencing of the genes encoding ATG5 and ATG7 did not affect degradation of TRIF and TRAF. Furthermore, LC3 conversion was not induced after poly (I:C) treatment in A20-silenced cells. Thus, Beclin-1, ATG5, ATG7, and LC3, all of which are involved in the "canonical" autophagic processes, may not be associated with TRIF and TRAF6 degradation. We were unable to determine why the autophagic degradation of TRIF and TRAF6 does not depend on canonical autophagic processes. However, the existence of "non-canonical" autophagic processes, which do not require these autophagic proteins but are mediate d by the anti-apoptotic B cell lymphoma extra large protein, has been reported [21].

4. NDP52 mediates selective degradation of TRIF and TRAF6

Next, we investigated how TRIF and TRAF6 are selectively degraded by autophagy. Autophagy has long been considered to be nonspecific in the removal of cytosolic components. However, recent studies have revealed that cytosolic constituents can be selectively delivered into autophagosomes during a particular type of stress-induced autophagy [22]. During selective autophagy, posttranscriptional modifications of the target constituents, of which polyUb is thought to be the primary modification, is required [22]. Targets with polyUb are recognized by autophagy-associated ubiquitin-binding proteins (so-called autophagy receptors), which enable sequestration of the target to form cytoplasmic aggregates and autophagic clearance. So far, many autophagy receptors, including sequestosome 1, neighbor of breast cancer type 1 susceptibility protein 1, NIX/adenovirus E1B 19 kDa protein-interacting protein 3-like, histone deacetylase 6, B cell lymphoma 2-associated athanogene 3, optineurin, and NDP52, have been identified [22]. We tested whether autophagy receptors mediate the selective degradation of TRIF by screening the known autophagy receptors for the ability to interact with TRIF, and only NDP52 was observed to interact with TRIF [12]. Furthermore, NDP52 gene knockdown significantly restored poly (I:C)-induced degradation of TRIF and TRAF6 in A20-silenced cells (Fig. 2). Thus, NDP52 was identified to be a critical mediator of the selective degradation of TRIF and TRAF6.

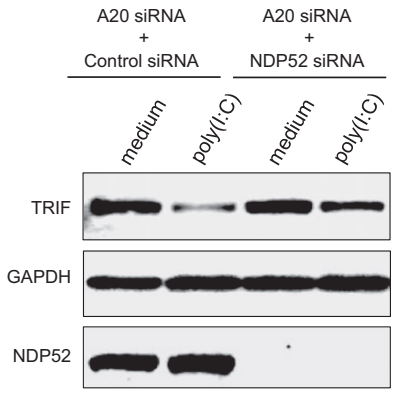

Fig. 2. NDP52 mediates selective degradation of TRIF. HeLa cells were transfected with A20 siRNA along with control siRNA or NDP52 siRNA. Cells were stimulated for 6 h with 50 μg/ml poly (I:C). The cells lysates were analyzed by immunoblotting with antibodies against TRIF, GAPDH, and NDP52 (modified from Inomata et al. [12]).

NDP52 (also known as CALCOCO2) was a recently identified autophagy receptor that eliminates intracellular bacteria [23], but its detailed function is largely unknown. NDP52 is ubiquitously expressed in various tissues and cells and is mainly a cytosolic protein [24]. Two vertebrate paralogs of NDP52, COCOA (also known as CALCOCO1) and TAX1BP1 (also known as CALCOCO3), are known. Human NDP52 contains a skeletal muscle and kidney-enriched inositol phosphatase carboxyl homology (SKICH) domain at the N terminal, an intermediate coiled-coil (CC) domain with a leucine zipper sequence, and a Lin11, Isl-1, and Mec-3 (LIM)-like domain containing 2 zinc fingers at the C terminal. All these domains are conserved in NDP52 proteins found in chimpanzees, bovines, dogs, and chickens. However, mouse Ndp52 has a highly mutated CC domain and completely lacks the LIM-like domain. Therefore, the functions of NDP52 may be considerably different in humans and mice because the LIM-like domain of NDP52 domain has been suggested to specifically bind to polyubiquitin chains conjugated to the intracellular bacterial surface [23]. We investigated which domain is required for TRIF and TRAF6 interaction and degradation. NDP52 mapping revealed that the SKICH domain was required for binding to TRIF and TRAF6, whereas the LIM-like domain was required for TRIF and TRAF6 degradation [12]. Thus, NDP52 interacts with TRIF and TRAF6 through the SKICH domain and possibly recognizes polyUb through the LIM-like domain.

Autophagy receptors mediate aggregation of their targets with polyubiquitin, and this process is required for selective autophagic removal of the targets [7,22]. We found that NDP52 markedly increased aggregated TRAF6 in the cytoplasm [12]. In addition, TRAF6 aggregates colocalized with NDP52 and proteins with polyUb. Furthermore, under the condition of A20 silencing, TRAF6 aggregation clearly increased. Thus, NDP52 plays a role in the aggregation of TRAF6 with polyubiquitin, and this process is normally restricted by A20. Next, we examined how NDP52 aggregates TRAF6. Aggregation of intracellular proteins is usually associated with accumulation of proteins with polyUb [24]. We found that TRAF6 catalyzes polyUb of NDP52 [12]. When A20 is silenced, NDP52 polyUb further increased. Thus, TRAF6-induced polyUb of NDP52 may promote TRAF6 aggregation.

5. NDP52 has the potential to negatively regulate TLR signaling

NDP52 has the capability to induce selective autophagic degradation of TRIF and TRAF6, though this induction is normally inhibited by A20. Degradation of signaling molecules is generally linked to downregulation of signal transduction. Therefore, we investigated whether NDP52 negatively regulates TLR-mediated signaling when A20 is silenced. Interestingly, silencing of NDP52 did not significantly affect poly (I:C)-induced IL-6, IL-8, and CXCL10 but silencing both A20 and NDP52 markedly induced these molecules [12]. Thus, NDP52 effectively exerts a negative regulatory affect on TLR signaling only when expression of A20 is silenced.

6. Conclusions

The deubiquitinase A20 serves as a potent suppressor of TLR-mediated signal transduction through the removal of polyubiquitin from TRAF6, TRAF3, and RIP1. Our study indicates that A20 suppresses induction of autophagy by inhibiting the autophagy receptor NDP52, which mediates selective autophagic clearance of TRIF and TRAF6 after it undergoes polyUb by TRAF6. Therefore, only in the absence of A20, NDP52 can be fully activated by TRAF6. Activated NDP52 mediates aggregation of TRIF and TRAF6. This process may be important for their selective autophagic degradation in lysosomes. In the absence of A20, NDP52-mediated autophagy ultimately leads to negative regulation of TLR signaling. Thus, the fine-tuning effect of NDP52 on TLR signaling is paradoxically downregulated by A20, which is known as a signaling fine-tuner to prevent excess TLR signaling.

Although the biological significance of such an unanticipated regulatory mechanism is currently unclear, future research should address this question. In addition, it will be important to elucidate how NDP52-associated autophagy affects TLR-induced innate immune responses in healthy periodontal tissue or inflammatory processes in infected periodontal tissue.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We are grateful to Prof. Ken-ichiro Shibata (Laboratory of Oral Molecular Microbiology, Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine), Dr. Shumpei Niida (Laboratory of Genomics and Proteomics, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology), and Prof. Yukitaka Murakami (Department of Oral Microbiology, Division of Oral Infections and Health Sciences, Asahi University School of Dentistry) for their support in this study. This work was supported by a Grant-in-Aid for Young Scientists (B: 23792158 to M. Inomata) provided by the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This work was also supported by a Grant-in-Aid for Scientific Research (B: 23390431 to T. Into, M. Inomata, E. Takayama, and S. Takigawa) from the Japan Society for the Promotion of Science (JSPS).

References

[1] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
[2] Haffajee AD, Cugini MA, Tanner A, Pollack RP, Smith C, Kent Jr RL, Socransky SS. Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. J Clin Periodontol 1998;25:346–53.
[3] Hans M, Hans VM. Toll-like receptors and their dual role in periodontitis: A review. J Oral Sci 2011;53:263–71.
[4] Hajishengallis G. Immune evasion strategies of *Porphyromonas gingivalis*. J Oral Biosci 2011;53:233–40.
[5] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373–84.
[6] Chen ZJ. Ubiquitin signaling in the NF-kappaB pathway. Nat Cell Biol 2005;7:758–65.
[7] Into T, Inomata M, Takayama E, Takigawa T. Autophagy in regulation of Toll-like receptor signaling. Cell Signal 2012;24:1150–62.
[8] Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 2007;27:135–44.
[9] Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. EMBO J 2008;27:1110–21.
[10] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451:1069–75.
[11] Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, Brady NR, Richter B, Korac J, Waidmann O, Choudhary C, Dotsch V, Bumann D, Dikic I. Phosphorylation of the autophagy receptor optineurin restricts *Salmonella* growth. Science 2011;333:228–33.
[12] Inomata M, Niida S, Shibata KI, Into T. Regulation of Toll-like receptor signaling by NDP52-mediated selective autophagy is normally inactivated by A20. Cell Mol Life Sci 2012;69:963–79.
[13] Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of Toll-like receptor-mediated immune responses. Nat Rev Immunol 2005;5:446–58.
[14] Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, Hurley P, Chien M, Chai S, Hitotsumatsu O, McNally E, Pickart C, Ma A. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol 2004;5:1052–60.
[15] Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signaling. Nature 2004;430:694–9.
[16] Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell 2011;147:728–41.
[17] Tooze SA, Yoshimori T. The origin of the autophagosomal membrane. Nat Cell Biol 2010;12:831–5.
[18] Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 2008;19:5360–72.
[19] Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol 2011;27:107–32.
[20] Virgin HW, Levine B. Autophagy genes in immunity. Nat Immunol 2009;10:461–70.
[21] Priault M, Hue E, Marhuenda F, Pilet P, Oliver L, Vallette FM. Differential dependence on Beclin 1 for the regulation of pro-survival autophagy by Bcl-2 and Bcl-xL in HCT116 colorectal cancer cells. PLoS One 2010;5:e8755.
[22] Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy 2010;7:279–96.
[23] Thurston TL, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nat Immunol 2009;10:1215–21.
[24] Ding WX, Sorting Yin XM. recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. Autophagy 2008;4:141–50.