Regulation of innate immune responses by autophagy-related proteins

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Pattern recognition receptors detect microbial components and induce innate immune responses, the first line of host defense against infectious agents. However, aberrant activation of immune responses often causes massive inflammation, leading to the development of autoimmune diseases. Therefore, both activation and inactivation of innate immune responses must be strictly controlled. Recent studies have shown that the cellular machinery associated with protein degradation, such as autophagy, is important for the regulation of innate immunity. These studies reveal that autophagy-related proteins are involved in the innate immune response and may contribute to the development of inflammatory disorders.

Introduction

Pathogen recognition receptors (PRRs) trigger innate immune responses after sensing microbial components such as lipopolysaccharide (LPS), lipoprotein, flagellin, and nucleic acids and are necessary for the protection of a host suffering from microbial infection (Beutler, 2009; Kawai and Akira, 2009). After detecting microbial components, PRRs drive the coordinated activation of transcription factors, leading to the expression of inflammatory cytokines, chemokines, type I interferons (IFNs), and antimicrobial genes. Although innate immunity is essential for host defense, aberrant activation of innate immune responses results in the development of inflammatory diseases; e.g., autoimmune disease and septic shock. Thus, the signaling pathways of the innate immune system are tightly regulated to avoid either deficient or excessive responses.

Protein degradation is critically involved in the control of innate immune responses (Beutler, 2009; Kawai and Akira, 2009). Although the regulation of immune responses by the ubiquitin–proteasome (a selective protein degradation system) has long been studied (Liu et al., 2005; Bhoj and Chen, 2009), recent research has shown the involvement of another clearance system, autophagy, in the innate immune response. Autophagy is a bulk degradation system that delivers cytoplasmic constituents into lysosomes (Fig. 1; He and Klionsky, 2009; Nakatogawa et al., 2009). This process enables the reuse of intracellular constituents and supplies an amino acid pool during periods of starvation. Indeed, mice deficient in Atg3, Atg5, or Atg7 die within 1 d of delivery, indicating that autophagy is essential for survival during neonatal starvation (Kuma et al., 2004; Komatsu et al., 2005; Sou et al., 2008). Autophagy is also involved in the clearance of old/damaged organelles, long-lived proteins, insoluble protein aggregates, and lipid droplets, thus regulating cellular homeostasis, cell death/survival, and lipid metabolism (Yoshimori and Noda, 2008; Mizushima, 2009; Czaja, 2010). Several essential components of the autophagic machinery have been identified by yeast genetic screening, the so-called autophagy-related proteins (Atgs; He and Klionsky, 2009; Nakatogawa et al., 2009). Recent studies have identified mammalian counterparts of yeast Atgs, such as ULK1 (Atg1), Atg3–5, beclin (Atg6), Atg7, LC3 (Atg8), Atg9a, Atg10, Atg12, Atg13L, Atg14L, Atg16L1, FIP200 (Atg17), and WIPI-1 (Atg18), indicating that Atgs are phylogenetically highly conserved between yeast and mammals (Mizushima et al., 1998a,b; 2003; Liang et al., 1999; Kabeya et al., 2000; Tanida et al., 2001, 2002; Mariño et al., 2003; Proikas-Cezanne et al., 2004; Yamada et al., 2005; Young et al., 2006; Hara et al., 2008; Itakura et al., 2008; Sun et al., 2008; Chan et al., 2009; Chang et al., 2009; Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Matsunaga et al., 2009; Zhong et al., 2009). The coordinated action of Atgs mediates the membrane trafficking required for autophagosome formation (Fig. 1).

Autophagy plays a critical role in host defense responses by promoting the elimination of pathogens and the induction of acquired immunity (Deretic, 2005; Deretic and Levine, 2009; Virgin and Levine, 2009). The Rab7-dependent formation of bacteria-containing autophagosomes is critical for the killing of invading bacteria, such as *Streptococcus pyogenes* and *Coxiella burnetii*,

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Abbreviations used in this paper: Atg, autophagy-related protein; BCR, B cell receptor; CARD, caspase recruitment domain; DC, dendritic cell; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; IFN, interferon; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MEF, mouse embryonic fibroblast; MHC, major histocompatibility complex; pDC, plasmacytoid DC; PRR, pathogen recognition receptor; RIG, retinoic acid–inducible gene; RLR, RIG-like receptor; ROS, reactive oxygen species; ssRNA, single-stranded RNA; STING, stimulator of IFN genes; TLR, toll-like receptor; VSV, vesicular stomatitis virus.

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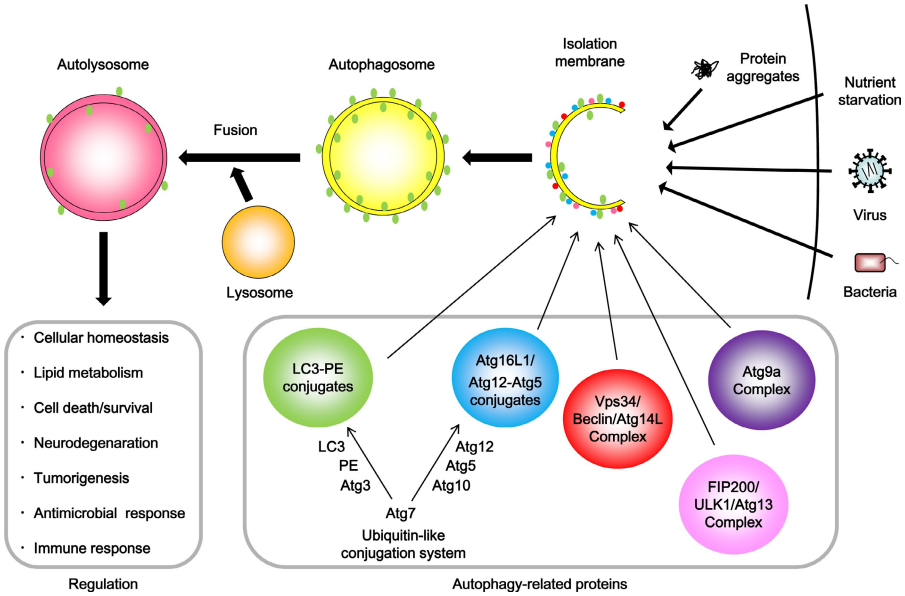

Figure 1. **Atgs are essential components for autophagosome formation.** Autophagy, a bulk degradation system, is induced after sensing various types of stress, such as nutrient starvation, the accumulation of protein aggregates, and invasion by microbes. This system enables the reuse of cellular components and the clearance of unfavorable substances, thus contributing to the maintenance of cellular homeostasis and the prevention of disease. Atgs are recruited to the isolation membrane, a source membrane of autophagosomes, and play a vital role in driving the membrane trafficking necessary for the generation of autophagosomes. Autophagosomes then fuse with lysosomes to become autolysosomes, leading to the degradation of the constituents trapped inside. PE, phosphatidylethanolamine.

in nonphagocytic cells (Berón et al., 2002; Nakagawa et al., 2004; Romano et al., 2007; Yamaguchi et al., 2009). Bacteria, such as *Shigella flexneri*, escape from the autophagic machinery via expression of the VirG protein, which is capable of inhibiting Atg5 function in nonphagocytic cells (Ogawa et al., 2005). Immunity-related GTPase family M, an IFN-γ–inducible GTPase, promotes autophagy, and the autophagic machinery effectively eliminates mycobacteria in macrophages (Gutierrez et al., 2004; Singh et al., 2006). Sequestosome 1 (SQSTM1)/p62, which interacts with both LC3 and ubiquitinated proteins, is recruited to bacteria-containing ubiquitinated vacuoles upon infection and promotes the killing of invading pathogens by autophagy (Bjørkøy et al., 2005; Komatsu et al., 2007; Yoshikawa et al., 2009; Ponpuak et al., 2010). In addition to the formation of autophagosomes, Atgs are responsible for the transport of Irga6, an immunity-related GTPase, from the ER–Golgi to the vacuoles containing invading microbes, which is a function required for the elimination of *Toxoplasma gondii* by macrophages (Zhao et al., 2008). These findings clearly demonstrate that Atgs are critical for the direct elimination of infectious agents. Autophagy also plays an indispensable role in antigen presentation to antigen-specific T cells (a process essential for the induction of acquired immunity; Gannagé and Münz, 2009). Major histocompatibility complex (MHC) class II molecules are localized on autophagosomes, and the autophagic machinery promotes the presentation of viral and self-antigens by MHC class II molecules to antigen-specific $CD4^+$ T cells (Nimmerjahn et al., 2003; Dengjel et al., 2005; Paludan et al., 2005; Zhou et al., 2005; Schmid et al., 2007; Nedjic et al., 2008; Jagannath et al., 2009; Lee et al., 2010). Upon infection by human simplex virus 1, autophagy controls the MHC class I–dependent presentation of viral antigens to $CD8^+$ T cells (English et al., 2009).

In addition to these functions, recent studies reveal that Atgs are important for innate immune responses provoked by the engagement of PRRs with microbial components. In this review, we discuss this recent evidence and its relevance to inflammatory disease.

Autophagy and inflammation

Recent genome-wide association studies have identified Atg16L1 as a candidate gene responsible for susceptibility to the inflammatory bowel disease, Crohn's disease (Hampe et al., 2007; Rioux et al., 2007). Atg16L1 forms an ∼800-kD protein complex with Atg12-Atg5 conjugates, and the self-multimerization of Atg16L1 via its coiled-coil domain is required for the recruitment of this conjugate into an isolation membrane (a source membrane of autophagosomes; Fig. 1; Mizushima et al., 2003; Fujita et al., 2008). The Atg12-Atg5–Atg16L1 complex recruits an Atg3-LC3 intermediate to the isolation membrane, and in doing so, defines the site at which LC3 is conjugated to phosphatidylethanolamine (Fujita et al., 2008). Consistently, cells lacking Atg16L1 are deficient in the conjugation of LC3 to phosphatidylethanolamine, an essential process for the elongation of autophagosomes (Saitoh et al., 2008). Mice lacking Atg16L1 cannot survive neonatal starvation periods. This phenotype is similar to that observed in other Atg-deficient mice (Kuma et al., 2004; Komatsu et al., 2005). Thus, Atg16L1 is an essential component of autophagy under both nutrient-rich and nutrient-starved conditions.

Using Atg16L1-deficient mice, we have shown that the loss of Atg16L1 enhances endotoxin-induced inflammatory immune responses (Fig. 2 A; Saitoh et al., 2008). Commensal bacteria are thought to be one of the major causative agents of bowel disease. When intestinal epithelial cells are damaged, commensal bacteria are able to pass through the epithelial layer and stimulate PRRs, leading to the induction of intestinal inflammation. Toll-like receptors (TLRs), one family of PRRs, detect microbial components and induce the production of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-18 by macrophages/dendritic cells (DCs), resulting in the infiltration of activated lymphocytes into the intestine (Beutler, 2009; Kawai and Akira, 2009). Macrophages lacking Atg16L1 produce high amounts of IL-1β and IL-18 in response to LPS, a ligand for TLR4. Macrophages from Atg7-deficient mice also show enhanced production of IL-1β, indicating the importance of Atg in

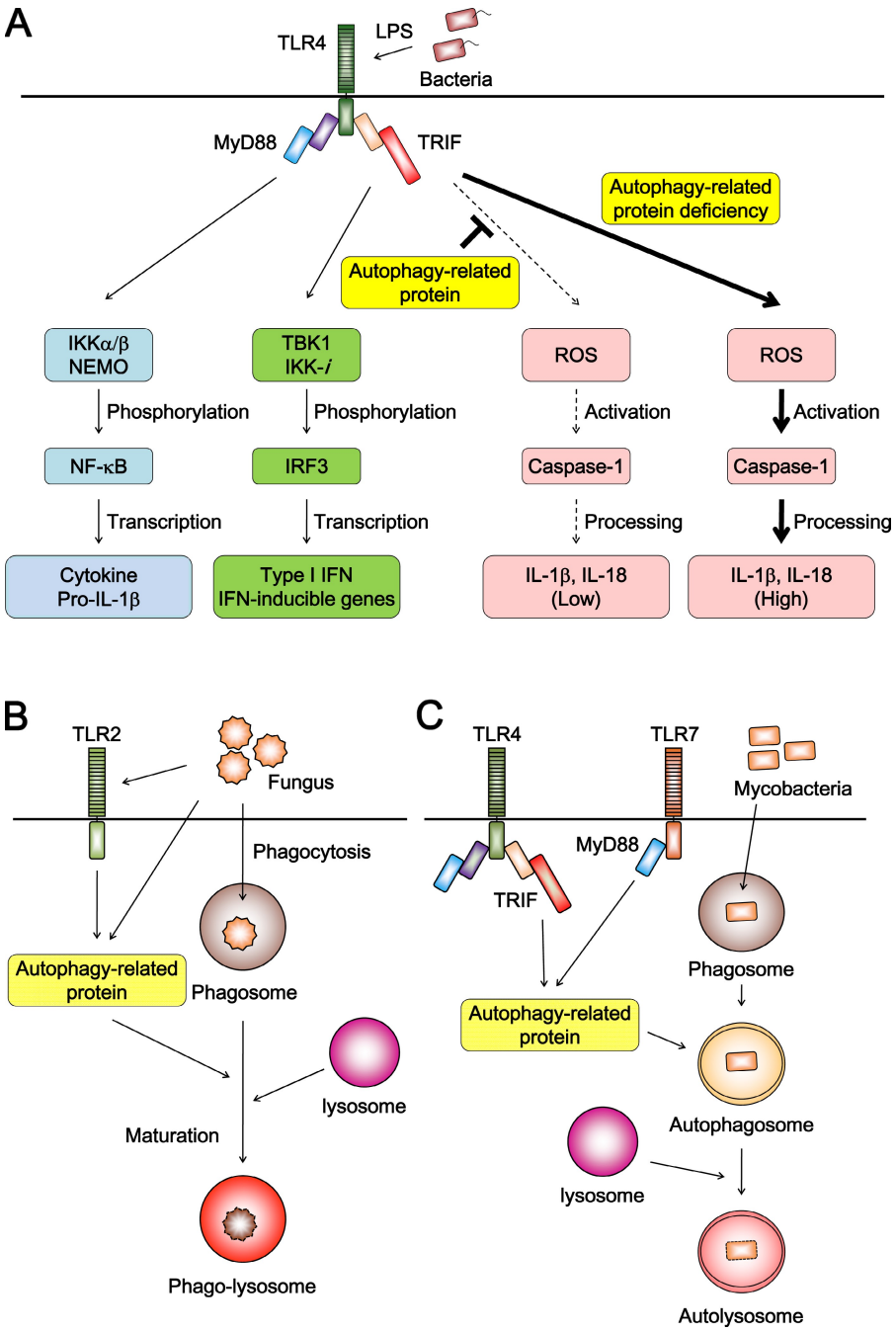

Figure 2. **TLR response and Atgs.** (A) Atgs control endotoxin-induced IL-1β production. TLR4 triggers both MyD88- and TRIF-dependent signaling pathways after sensing LPS. The IKK-α–IKK-β–NEMO complex mediates the activation of the transcription factor NF-κB, which in turn induces the transcription of proinflammatory cytokines and pro–IL-1β. The TBK1–IKK-*i* complex mediates the activation of the transcription factor IRF3, which then induces the transcription of type I IFNs and IFN-inducible genes. In autophagy-deficient cells, high levels of ROS are generated, which mediate TRIF-dependent caspase-1 activation, resulting in the processing of IL-1β. However, in wild-type macrophages, limited amounts of IL-1β are induced by LPS as the result of a lack of ROS generation. (B) Atgs contribute to TLR-dependent elimination of pathogens. After detection of the fungal cell wall component zymosan, TLR2 induces the maturation of phagosomes, leading to the elimination of the fungus. Atgs such as Atg5, Atg7, and PI3K are involved in the fusion of phagosomes with lysosomes. (C) Ligands for TLR7 such as ssRNA and imiquimod induce the formation of autophagosomes via MyD88, an essential adaptor molecule, and promote the elimination of *Bacillus Calmette-Guerin*. LPS, a ligand for TLR4, induces the formation of autophagosomes via the TRIF-p38 signaling axis, leading to the elimination of *Mycobacterial bacilli*. Atgs such as Atg5, beclin, and PI3K are required for the formation of autophagosomes by TLR stimulation.

the regulation of the inflammatory response. Stimulation of the other TLR family members (except for TLR3) fails to induce this enhanced production of IL-1β by Atg16L1-deficient macrophages. TRIF (Toll/IL-1 receptor domain–containing adaptor inducing IFN-β), the adaptor protein involved in TLR3/4 signaling pathways, also mediates IL-1β production in Atg16L1-deficient macrophages. It was shown that ATP, uric acid crystals, silicas, and asbestos induce the generation of reactive oxygen species (ROS), leading to the activation of caspase-1, an essential protease for IL-1β production (Dostert et al., 2008; Stutz et al., 2009; Zhou et al., 2010). In Atg16L1-deficient macrophages, the enhanced IL-1β production is induced by TRIF-dependent generation of ROS and is blocked by ROS scavengers. Mice with hematopoietic cells lacking Atg16L1 are highly susceptible to dextran sulfate sodium–induced colitis, which is relieved by the injection of anti–IL-1β and anti–IL-18 antibodies. These findings indicate that the loss of Atg16L1, an essential component of the autophagic machinery, results in the production of inflammatory cytokines in response to endotoxin. However, the source of the ROS has not yet been identified. One candidate source is NADPH oxidase (Dostert et al., 2008). Other possible sources of ROS include mitochondria because autophagy is required for the clearance of old/damaged mitochondria. Recent studies have shown that loss of the Atg results in the accumulation of ROS in immune-competent cells, such as T cells, as a result of the disruption of mitochondrial turnover (Pua et al., 2009; Stephenson et al., 2009). Further studies are needed to elucidate the source of ROS in macrophages deficient in Atgs.

Autophagy and TLR signaling

Recent studies have demonstrated that TLR signaling induces the maturation of phagosomes after exposure to bacteria and promotes MHC class II–dependent presentation of bacterial antigens (Blander and Medzhitov, 2004, 2006). Sanjuan et al. (2007) assessed the involvement of Atgs in the maturation of phagosomes after exposure to microbial components and found that zymosan particles (cell wall components of fungi) stimulate TLR2, thus promoting the fusion of GFP-LC3–positive phagosomes with lysosomes in macrophages (Fig. 2 B). Surprisingly, MyD88 (myeloid differentiation primary response gene 88), an essential adaptor molecule for cytokine production via TLR2 stimulation, is not necessary for the recruitment of GFP-LC3 to phagosomes by zymosan, suggesting that TLR2 triggers an as yet–unidentified pathway to induce the maturation of phagosomes. Atgs such as Atg5, Atg7, and PI3K are required for the efficient maturation of phagosomes induced by TLR2. Consistently, macrophages lacking Atg7 fail to eliminate live yeasts in phagolysosomes. Sanjuan et al. (2007) also mentioned that Pam3CSK4, a soluble TLR2 ligand, fails to increase the number of GFP-LC3–positive phagosomes, but Pam3CSK4 fused with latex beads induces the maturation of phagosomes in primary macrophages. Thus, TLR2 signaling is necessary but not sufficient for the induction of phagosome maturation. Although Atgs are important for the maturation of phagosomes after TLR2 stimulation, subsequent characterization by electron microscopy shows that the GFP-LC3–positive phagosomes do not have the morphological characteristics of autophagosomes, indicating that TLR2 stimulation induces the Atg-dependent, but autophagy-independent, exclusion of fungi in phagolysosomes. It would be of interest to examine how zymosan activates the function of Atgs via a TLR2-dependent but MyD88-independent pathway and how Atgs promote the fusion of phagosomes with lysosomes in macrophages. Because the receptor dectin-1, which recognizes the β-glucan structure in zymosan, can activate the tyrosine protein kinase Syk-dependent signaling pathway and promote uptake of zymosan (Brown and Gordon, 2001; Brown et al., 2003; Gantner et al., 2003; Rogers et al., 2005), it will be important to assess the involvement of this receptor in the Atg-dependent maturation of phagosomes after infection by fungal pathogens.

The TLR-dependent induction of autophagy in macrophages/monocytes has been reported by other groups. Delgado et al. (2008) reported that the engagement of TLR7 induces autophagy and promotes the elimination of *Bacillus Calmette-Guerin* in autolysosomes (Fig. 2 C). Both Atg5 and beclin are required for the induction of autophagy in macrophages after TLR7 stimulation. Although MyD88 is not necessary for the formation of GFP-LC3 dots after stimulation by zymosan, it is involved in the formation of GFP-LC3 dots after stimulation of TLR7 (Sanjuan et al., 2007; Delgado et al., 2008). It would be of interest to examine the reasons why MyD88 is differentially involved in the formation of GFP-LC3 dots after the engagement of TLRs and how MyD88 signals Atgs to induce the formation of autophagosomes. However, Xu et al. (2007) reported that TLR4 stimulation results in the PI3K-dependent formation of GFP-LC3 dots and enhances the elimination of mycobacteria in macrophage cell lines (Fig. 2 C). LPS stimulation increases the number of autophagosomes in primary human monocytes, although it fails to induce autophagy in primary mouse macrophages (Xu et al., 2007; Saitoh et al., 2008). The signal transducers, TRIF, RIPK1 (receptor-interacting protein kinase 1), and p38, are required for the TLR4-induced formation of GFP-LC3 dots in macrophage cell lines. Further studies are required to explain the cell type– or species-specific induction of autophagy by LPS and to identify the target of p38, whose phosphorylation is responsible for the activation of Atgs.

Autophagy and antibacterial responses

Nod1 (nucleotide-binding oligomerization domain 1) and Nod2 are intracellular sensors that recognize the unique bacterial polypeptides iE-DAP (γ-D-glutamyl-meso-diaminopimelic acid) and muramyl dipeptide (MDP), respectively (Franchi et al., 2009). Thus, Nod1 and Nod2 detect invading bacteria and play a central role in the production of cytokines and antimicrobial peptides. Importantly, a strong relationship has been found between Nod2 mutation and the development of Crohn's disease (Cho and Weaver, 2007). It has also been shown that Nod1 is a candidate gene responsible for susceptibility to Crohn's disease (McGovern et al., 2005). Indeed, immune responses to bacterial polypeptides and microbial infection are impaired in cells isolated from patients harboring Nod2 mutations (Franchi et al., 2009). In mouse models, Nod1 or Nod2 deficiency results in enhanced intestinal inflammation upon bacterial infection as a result of the disruption of innate immune responses. Furthermore, Nod2 mutant mice, whose Nod2 locus harbors the homologue of the most common Crohn's disease susceptibility allele 3020insC, are highly sensitive to chemical-induced colitis because of the enhanced production of proinflammatory cytokines such as IL-1α and IL-1β (Maeda et al., 2005). Because a single nucleotide polymorphism in the Atg16L1 gene is also associated with the development of Crohn's disease (Hampe et al., 2007; Rioux et al., 2007), the involvement of Nod1 and Nod2 in the induction of autophagy was assessed. Cooney et al. (2010) found that stimulation of Nod2 by MDP induces the formation of autophagosomes, thus promoting antigen presentation on MHC class II molecules by human DCs (Fig. 3). Atgs such as Atg5, Atg7, and Atg16L1 are required for the induction of autophagy in human DCs and the subsequent promotion of antigen presentation by MDP (Cooney et al., 2010). RIPK2, one of the downstream regulators of the Nod2 signaling pathway, is also involved in MDP-induced formation of autophagosomes. Importantly, DCs expressing Crohn's disease–associated Nod2 or ATG16L1 variants fail to induce autophagosome formation and antigen presentation in response to MDP. Travassos et al. (2010) also found that stimulation with both Nod1 and Nod2 triggers the induction of autophagy in mouse embryonic fibroblasts (MEFs), mouse macrophages, human lymphoblasts, and human cell lines (Fig. 3). However, Nod1 and Nod2 are not necessary for the formation of autophagosomes induced by nutrient starvation and rapamycin treatment, indicating that Nod1 and Nod2 are specifically involved in the formation of bacteria autophagosomes. In MEFs, Nod1, but not the downstream signaling molecules RIPK2 and IκB kinase-γ/NEMO, is required for the generation of *S. flexneri*–containing autophagosomes and promotes the elimination of

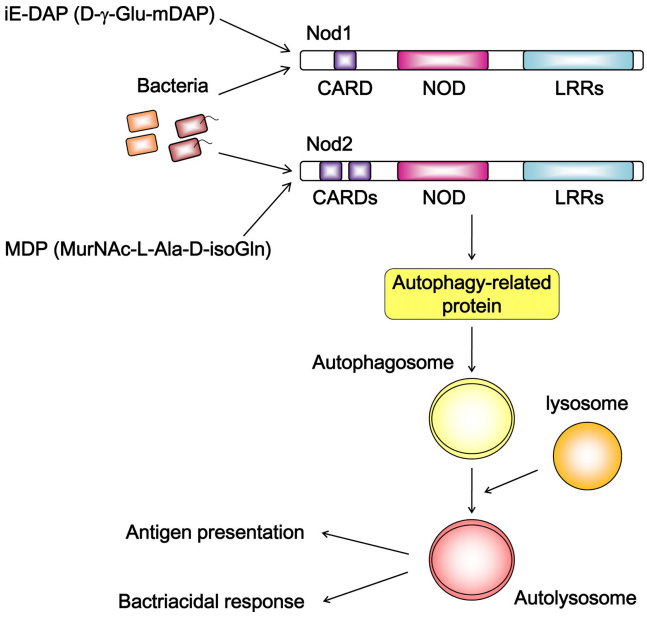

Figure 3. **Sensing of bacterial polypeptides by Nod1 and Nod2 triggers the formation of autophagosomes.** Nod1 and Nod2 are composed of N-terminal CARDs, a centrally located nucleotide-binding oligomerization domain (NOD), and multiple C-terminal leucine-rich repeats (LRRs). The leucine-rich repeats of Nod1 and Nod2 detect iE-DAP and MDP, respectively. After sensing these ligands, Nod1 and Nod2 induce the formation of autophagosomes, leading to the promotion of antigen presentation and the enhancement of bactericidal responses. Atgs such as Atg5, Atg7, and Atg16L1 are involved in Nod1- and Nod2-mediated formation of autophagosomes.

the invading bacteria. Interestingly, both Nod1 and Nod2 associate with Atg16L1 and recruit it to the bacterial entry sites after bacterial infection. The most common Nod2 mutation associated with Crohn's disease fails to induce the movement of Atg16L1 and the formation of autophagosomes. These studies indicate a role for both Nod1 and Nod2 in the formation of bacteria autophagosomes and in the regulation of antimicrobial responses. However, the signaling pathways downstream of Nod1 and Nod2 that are responsible for the induction of bacteria autophagosomes remain unclear. Further studies are still needed to clarify this issue.

Atgs and antiviral IFN responses

For host defense against RNA viruses, type I IFNs (IFN-α and IFN-β) induce the expression of a series of antiviral factors and play crucial roles in the establishment of an antiviral state (Beutler, 2009; Kawai and Akira, 2009). Plasmacytoid DCs (pDCs) detect the single-stranded RNA (ssRNA) of RNA viruses via TLR7, which is expressed on endolysosomes. TLR7 stimulates IRF7 (IFN regulatory factor 7), a transcription factor that induces IFN stimulation-responsive element-dependent transcription via the adaptor molecule MyD88 and mediates the production of type I IFNs by pDCs. In cell types retinoic acid–inducible gene I (RIG-I) and MDA-5, caspase recruitment domain (CARD)–containing RNA helicases called RIG-like receptors (RLRs) sense the cytoplasmic double-stranded RNA (dsRNA) of RNA viruses and mediate signals to IPS-1 (IFN-β promoter stimulator-1; also called MAVS/VISA/Cardif). IPS-1, a CARD-containing mitochondrial protein, activates the transcription factors IRF3 and NF-κB via the IκB kinase family, leading to the production of type I IFN and the expression of IFN-inducible genes. Recent studies have shown an important role for Atgs in both TLR-dependent and RLR-dependent antiviral innate immune responses (Jounai et al., 2007; Lee et al., 2007; Tal et al., 2009).

Two models have been proposed for the involvement of Atgs in RLR-mediated innate immune responses. Jounai et al. (2007) found a possible role for Atg12-Atg5 conjugates in the disruption of the RLR–IPS-1 signaling pathway (Fig. 4 A). The Atg12-Atg5 conjugate associates with the CARD domains of RLR and IPS-1, and ectopic expression of Atg12 or Atg5 prevents RLR- or IPS-1–mediated activation of the type I IFN promoter. Consistently, loss of Atg5 enhances the production of type I IFN by vesicular stomatitis virus (VSV) infection and dsRNA treatment and limits the replication of VSV in MEFs. Loss of Atg7, an essential requirement for the conjugation of Atg12 with Atg5, also results in enhanced type I IFN production by dsRNA (Jounai et al., 2007). However, Tal et al. (2009) showed that the disruption of cellular homeostasis by a deficiency in autophagy affects RLR–IPS-1 signaling pathways (Fig. 4 A). In cells lacking Atg5, old/damaged mitochondria accumulate because of the loss of autophagy, resulting in the elevated expression of the IPS-1 protein. RLR stimulation triggers an elevation in ROS production by the accumulated mitochondria, leading to the activation of IPS-1–dependent innate immune responses in Atg5-deficient cells. Further analysis will be required to reveal the mechanisms responsible for the ROS-dependent activation of RLR signaling.

Lee et al. (2007) reported that autophagy is necessary for the TLR7-dependent production of type I IFNs and cytokines in pDCs after RNA virus infection (Fig. 4 B). Loss of Atg5, or pharmacological inhibition of PI3K, severely impairs the production of both type I IFN and cytokine IL-12 p40 by pDC infected with VSV and Sendai virus. The number of autophagosomes in pDCs does not increase after TLR7 stimulation, indicating that basal autophagy, which constitutively occurs under nutrient-rich conditions, is responsible for the TLR7-dependent induction of innate immune responses in pDCs. These findings suggested that the viral ssRNA from replicating VSV or Sendai virus is generated in autophagosomes, or autolysosomes, in pDCs, resulting in the TLR7-dependent production of type I IFNs and proinflammatory cytokines. Lee et al. (2007) also reported that the production of type I IFN but not IL-12 p40 by A/D-type unmethylated CpG oligonucleotides (a ligand for TLR9) is severely impaired in Atg5-deficient pDCs. Because TLR7 and TLR9 share the signaling pathway that induces type I IFN, autophagy or Atgs might be involved in signaling events required for TLR7/TLR9-dependent type I IFN production in pDCs.

B cell activation by DNA-containing antigens in autophagosome-like compartments

It is known that hyperactivation of B cells by nucleic acid–containing antigens results in the development of autoimmune diseases such as systemic lupus erythematosus (Marshak-Rothstein, 2006). Although B cell receptors (BCRs) on the cell

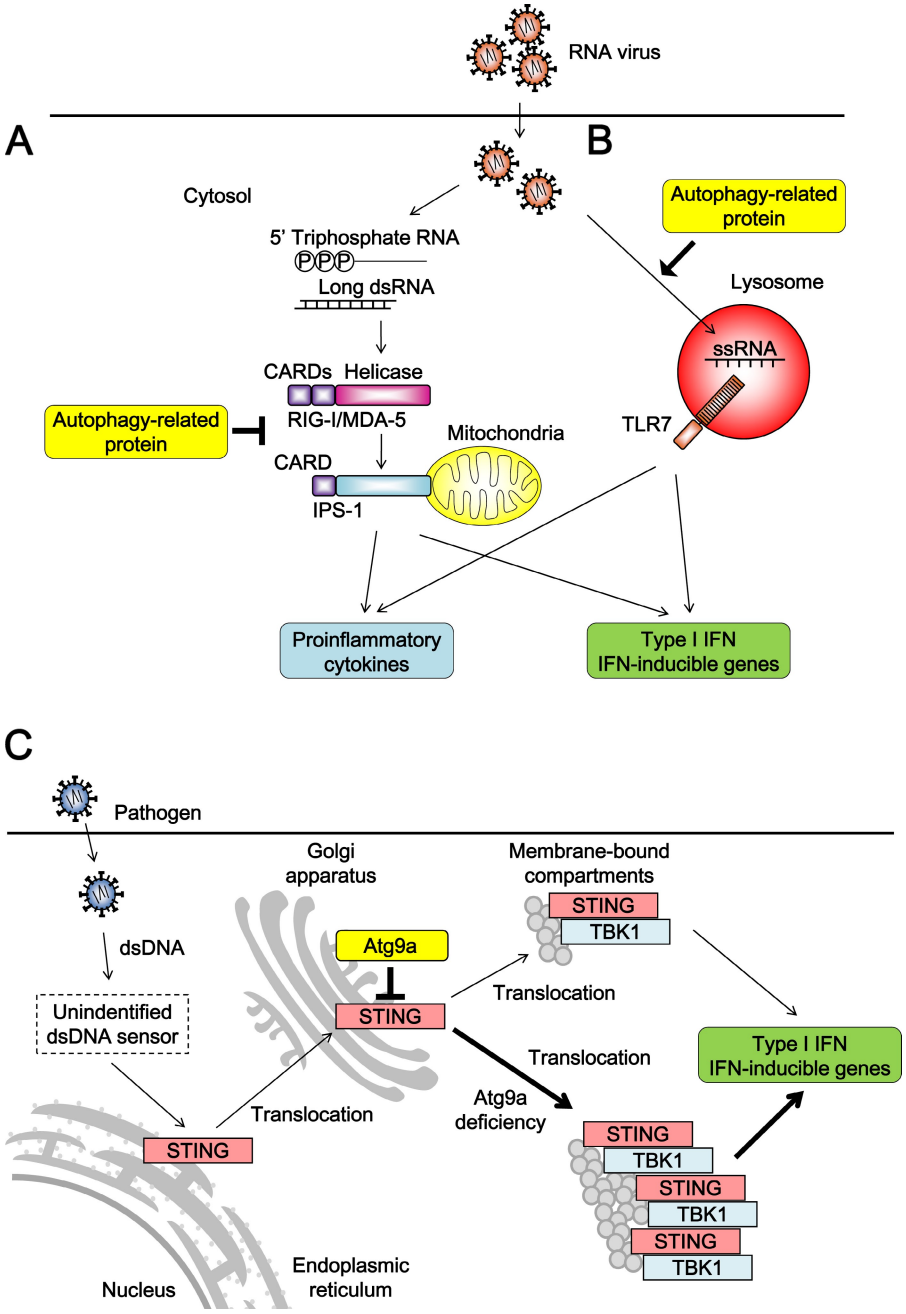

Figure 4. **Regulation of nucleic acid–induced type I IFN production by Atgs.** (A) RIG-I and MDA-5, CARD-containing RNA helicases, sense viral dsRNA and signal to IPS-1, a CARD-containing adaptor molecule, to induce the expression of type I IFN/IFN-inducible genes and proinflammatory cytokines. Atg12-Atg5 conjugates are recruited to the CARD domains of RIG-I, MDA-5, and IPS-1 and suppress the dsRNA-induced innate immune response. (B) TLR7 detects viral ssRNA and induces innate immune responses. Targeted disruption of Atg5 or pharmacological inhibition of PI3K results in the impairment of TLR7-mediated expression of type I IFN/IFN-inducible genes and proinflammatory cytokines. (C) STING, a multispanning membrane protein, and TBK1, an IRF3 kinase, mediate the induction of innate immune responses by dsDNA. After sensing dsDNA, STING moves from the ER to the Golgi apparatus and finally reaches the cytoplasmic punctate structures to assemble with TBK1, resulting in the IRF3-dependent expression of type I IFNs/IFN-inducible genes. STING colocalizes with Atg9a in the Golgi apparatus after dsDNA stimulation. The loss of Atg9a greatly enhances the dsDNA-induced assembly of STING and TBK1, leading to aberrant activation of the innate immune response.

surface and TLR7/TLR9 in the endolysosomes are involved in hyperimmune responses to nucleic acid–containing antigens, it is unclear how nucleic acid–containing antigens induce the aberrant activation of B cells via these receptors. Chaturvedi et al. (2008) found that stimulation of BCR by DNA-containing antigens results in the translocation of both the BCR and TLR9 to autophagosome-like compartments where the DNA-containing antigens are accumulated (Fig. 5). Inhibition of PLD, but not diacylglycerol kinase, blocks the translocation of TLR9 after it engages the BCR, suggesting that BCR stimulation induces the PLD-dependent generation of phosphatidic acid to promote the recruitment of TLR9 to autophagosome-like compartments. The movement of TLR9 from the endosome into the autophagosome-like compartments on BCR stimulation depends on a functional microtubular network. Treatment with nocodazole, an inducer of tubulin depolymerization, inhibits the translocation of TLR9 and the subsequent hyperactivation of MAPK by DNA-containing antigens. Thus, the movement of BCR and TLR9 induced by DNA-containing antigens and the colocalization of the receptors and DNA-containing antigens in autophagosome-like compartments facilitates the efficient engagement of the receptors, resulting in the synergistic activation of downstream signaling cascades. Study of primary B cells lacking Atgs is needed to elucidate the requirement for autophagosomes in the BCR- and TLR-dependent activation of B cells by DNA-containing antigens.

Atg9a and double-stranded DNA (dsDNA)–induced innate immune responses

Microbial DNA induces the expression of type I IFNs and proinflammatory cytokines, leading to the potent induction of innate

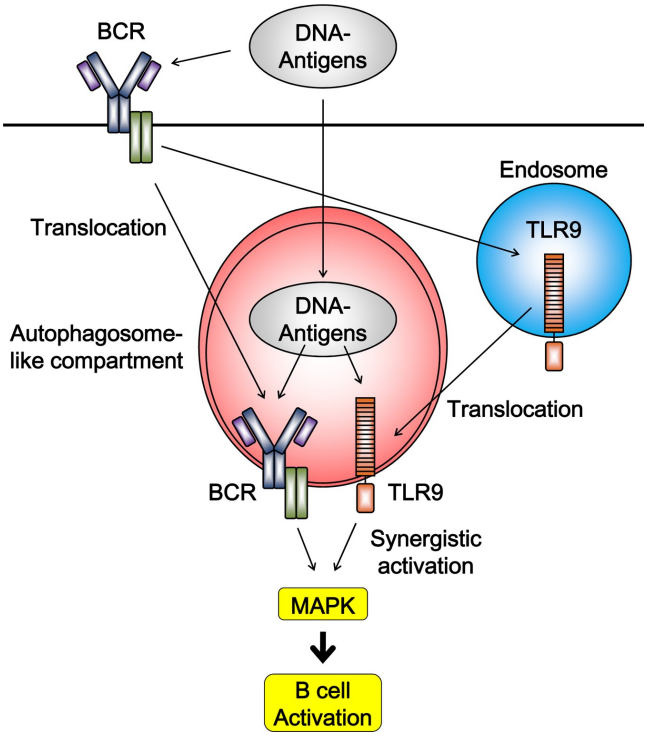

Figure 5. **DNA-containing antigens induce TLR9 signal from autophagosome-like compartments.** DNA-containing antigens stimulate BCRs, leading to the translocation of TLR9 from the endosomes to autophagosome-like compartments. DNA-containing antigens also promote the movement of BCRs from the cell surface to autophagosome-like compartments. In these autophagosome-like compartments, DNA-containing antigens trigger both BCR and TLR9 signaling pathways, resulting in the synergistic activation of MAPK. Functional PLD and microtubules are required for the translocation of these receptors and play a critical role in the hyperresponse to DNA-containing antigens.

immunity (Stetson and Medzhitov, 2006a; Kawai and Akira, 2009). Furthermore, synthesized DNA stimulates the innate immune system and acts as a good adjuvant to induce the efficient induction of acquired immune responses (Ishii et al., 2008a). Indeed, TLR9, the receptor for single-stranded DNAs containing unmethylated CpG motifs, is involved in the protection of hosts suffering DNA virus infection, and the ligands for TLR9 efficiently induce acquired immune responses upon vaccination. However, dsDNA derived from bacteria and DNA viruses, as well as host genomic DNA from dying cells, could induce the expression of both type I IFNs and IFN-inducible genes via a TLR-independent pathway (Okabe et al., 2005; Ishii et al., 2006; Stetson and Medzhitov, 2006b; Stetson et al., 2008). Although the specific sensors involved in dsDNA-induced innate immune responses are still unclear, recent studies have revealed that TBK1 (TANK-binding kinase 1) and stimulator of IFN genes (STING; also called MPYS/MITA/ERIS), function as mediators in dsDNA-induced production of type I IFN (Ishii et al., 2006; Stetson and Medzhitov, 2006b; Ishikawa and Barber, 2008; Jin et al., 2008; Zhong et al., 2008; Sun et al., 2009). Both TBK1 and STING are also required for the efficient induction of acquired immune responses by DNA-based vaccines and play a key role in host defense against the DNA virus herpes simplex virus 1 (Ishii et al., 2008b; Ishikawa et al., 2009). Upon stimulation by dsDNA, TBK1 phosphorylates the transcription factor IRF3, leading to the activation of IFN-stimulated response element–dependent transcription of type I IFN/IFN-inducible genes. STING is a multispanning membrane protein that associates with TBK1 to mediate dsDNA-induced signaling pathways. Regulators acting upstream of STING have not yet been identified. Recently, we and other groups reported that the translocation and assembly of these essential signal transducers (STING and TBK1) are required for dsDNA-triggered innate immune responses (Fig. 4 C; Ishikawa et al., 2009; Saitoh et al., 2009). After stimulation with dsDNA, STING moves from the ER to the Golgi apparatus, finally reaching the cytoplasmic punctate structures to assemble with TBK1. EXOC2/Sec5, a component of the exocyst complex responsible for targeting exocytic vesicles to specific docking sites on the plasma membrane, colocalizes with STING and regulates innate immune responses to dsDNA. Thus, it is clear that a membrane trafficking system mediates the dynamic movement of STING, leading to the efficient induction of innate immune responses to dsDNA. We assessed involvement of Atgs in the translocation of STING and found that, after dsDNA stimulation, STING colocalizes with Atg9a in the Golgi apparatus and with LC3 in cytoplasmic punctate structures but not with other Atgs (Fig. 4 C; Saitoh et al., 2009). Characterization by electron microscopy revealed that the STING-positive puncta induced by dsDNA stimulation did not have the morphological characteristics of autophagosomes but were unidentified membrane-bound compartments, suggesting a unique function of Atg9a or LC3 in the regulation of innate immune responses. Atg9a is the only multispanning membrane protein identified as an Atg in mammals and localizes to the Golgi apparatus and late endosomes (Yamada et al., 2005; Webber and Tooze, 2010). Atg9a does not reside at one site, but rather, it dynamically cycles between these organelles under starvation conditions, mediating membrane transport to generate autophagosomes. Mice lacking Atg9a cannot survive neonatal starvation periods, and cells lacking Atg9a are deficient in the generation of autophagosomes under both nutrient-rich and nutrient-starved conditions, indicating that Atg9a is an essential component of autophagy (Saitoh et al., 2009). Interestingly, the loss of Atg9a greatly enhances the assembly of STING and TBK1 triggered by dsDNA, leading to aberrant activation of the innate immune response (Fig. 4 C). However, both the localization of STING and cytokine production are almost normal in dsDNA-stimulated MEFs lacking either Atg7 or Atg16L1. Thus, deficiency of Atg9a, but not of autophagy, affects the movement of STING and TBK1 after dsDNA stimulation. Further studies are needed to reveal the unique function of Atg9a involved in the regulation of the membrane trafficking responsible for the assembly of STING and to assess importance of Atg9a in host defense against DNA viruses and pathogenic bacteria.

Conclusion

Recent advances in the study of the involvement of Atgs in innate immunity have clearly demonstrated the importance of membrane trafficking systems to host defense. However, in some cases, it is still unclear whether autophagy or Atgs are involved in the control of PRR-mediated innate immune responses. Furthermore, it is difficult to judge whether the

observed phenotypes represented by a deficiency of Atgs are caused by the inhibition of transiently induced autophagy because autophagy is constitutively induced, even under normal conditions, and maintains cellular homeostasis, the disruption of which often affects the regulation of innate immune responses. For example, SQSTM1/p62, accumulated through a deficiency in autophagy, might indirectly affect innate immune responses by inhibiting the function of the ubiquitin ligase Keap1, leading to the activation of NF-E2–related factor 2, a transcription factor involved in the regulation of PRR-mediated innate immune responses (Bjørkøy et al., 2005; Thimmulappa et al., 2006; Komatsu et al., 2007, 2010; Lau et al., 2010). Therefore, researchers must consider these issues and interpret data very carefully. In future studies, it will be important to identify the precise molecular mechanisms underlying the disruption of PRR-mediated innate immune responses because of the loss of Atgs.

Although the role of Atgs in host defense responses is extensively investigated in vitro, it is important that future studies assess its role in vivo. This is because certain cell types are difficult to maintain in vitro and because Atgs might be required for the development of unique but important cell types in vivo. Indeed, Cadwell et al. (2009) reported an essential role for Atgs in the development of Paneth cells, intestinal epithelial cells that produce the antimicrobial peptides defensin via Nod2 (Kobayashi et al., 2005; Cadwell et al., 2008). Interestingly, T300A mutation of the Atg16L1 protein disrupts the development of Paneth cells in patients suffering from Crohn's disease, suggesting that Atg16L1 is important for the development of this inflammatory disorder (Cadwell et al., 2008). Furthermore, it is possible that the loss of Atgs by a particular cell type in vivo indirectly affects innate immune responses. For example, hepatocyte-specific deletion of Atgs results in compromised lipid metabolism, which controls the level of triglycerides and cholesterol (Shibata et al., 2009; Singh et al., 2009). Altered lipid storage in the liver might indirectly affect the innate immune response because oxidized lipids and cholesterol crystals are potent inducers of inflammation (Febbraio et al., 2000; Ricci et al., 2004; Duewell et al., 2010; Stewart et al., 2010). Further studies using cell type–specific knockout mice are required for the detailed analysis of Atg-dependent regulation of the innate immune response in vivo.

Recent advances in the study of Atgs provide strong evidence that Atgs influence both the host defense response and the regulation of inflammation. Consistently, it has been proposed that the compromised function of autophagy regulators results in the development of immune-related disease such as Crohn's disease (Hampe et al., 2007; Parkes et al., 2007; Rioux et al., 2007; Fisher et al., 2008). Therefore, enhancement of autophagic activity is an attractive approach to protect the host against infectious diseases and inflammatory disorders. It would be of interest to assess the therapeutic effects of strategies to increase autophagic function, such as a hypocalorific diet and autophagy-inducible drugs, in the treatment of these diseases.

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