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LETTER TO EDITOR

Role of autophagy in *Drosophila* innate immunity

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To the Editor

Autophagy is a well-conserved intracellular degradation system in which cytoplasmic components and organelles are recycled by double-membraned autophagosomes that engulf them and eventually fuse with lysosomes (Levin *et al.*, 2011). Because most of the genes involved in autophagy are conserved from yeast to invertebrates and mammals, and genetic manipulations can easily be performed in *Drosophila in vivo*, *Drosophila* is an ideal model system for studying the function of autophagy in whole animals. Studies of autophagy-related gene mutants revealed the importance of autophagic functions in larval midgut cell death during morphogenesis (Denton *et al*., 2009), and hematopoiesis in larvae (Shravage *et al*., 2013). In addition to its essential functions in development, emerging evidence indicates that autophagy functions against invasion of host cells by pathogens such as intracellular bacteria, viruses, and protozoa (Yano and Kurata, 2011). Here, we summarize and provide insight into the functions of autophagy in innate immunity in invertebrates.

The first evidence that autophagy is crucial for protection against intracellular bacterial growth came from a study of Group A *Streptococcus,* which invades mammalian culture cells (Nakagawa *et al.*, 2004). Many subsequent studies demonstrated that intracellular bacteria, such as *Salmonella typhimurium, Listeria monocytogenes,* and *Mycobacterium tuberculosis* are eliminated by autophagosomes, indicating the critical role of autophagy in innate immune responses against such bacteria (Huang and Brumell, 2014). Because studies were performed in cultured cells and autophagy-knockout animals are in most cases lethal, the critical role of autophagy *in vivo* was first demonstrated in *Drosophila* by tissue-specific autophagy knockdown (Yano *et al.*, 2008). *Listeria* utilizes the host endocytotic pathway to enter *Drosophila* hemocytes, the hematopoietic cells that function in innate immunity. When *Listeria* infects the body cavity, they invade the hemocytes because of their high phagocytic ability, but are quickly engulfed by autophagosomes for elimination. Flies in which the autophagy gene *atg5* is specifically knocked down in hemocytes are susceptible to *Listeria* infection, suggesting that autophagy is important for their survival against infection by *Listeria* (Yano *et al.*, 2008). The importance of autophagy in protecting against invasive bacteria has also been demonstrated in another insect, *Tenebrio molitor*, in which knockdown of the autophagy genes *Atg3* or *Atg5* leads to susceptibility to *Listeria in vivo* (Tindwa *et al.*, 2015).

Although basal levels of autophagy function in the basically non-selective turnover of cytosolic molecules, autophagy is selectively induced for the degradation of aggregate proteins, damaged mitochondria, or invading bacteria (Mizushima and Komatsu, 2011). Autophagosomes that surround the bacteria are spatially regulated, and autophagy is selectively induced upon the recognition of bacteria by the pattern recognition receptors (PRRs) of the host. In *Drosophila* cells, intracellular PRRs, such as peptidoglycan-recognition protein (PGRP)-LE, recognize and bind to peptidoglycans of the bacterial cell walls, and this recognition is necessary for autophagy induction (Yano *et al.*, 2008). Compared to *Listeria* infection of *Drosophila* cells, which is largely dependent on PGRP-LE for induction, mammalian cells have similar, but more complicated systems: several triggers are involved in the induction of autophagy, including recognition by PRRs such as NOD2, ubiquitination of proteins around the bacteria, and destruction of the host endosomes used by the bacteria to enter the host cells (Gomes and Dikic, 2014). Mammalian cells likely adapt to the strategies bacteria use to escape from autophagic elimination.

Ubiquitin may be one sign of autophagic engulfment (Boyle and Randow, 2013). Ubiquitin is often observed around bacteria invading the cytosol by breaking the endosomal membrane. In mammalian cells, the adaptor proteins p62, NDP52, and optineurin recognize the ubiquitinated bacteria, and recruit autophagosomes to the bacteria by binding to LC3/Atg8 protein located on the autophagosomes (Huang and Brumell, 2014). Although ubiquitinated proteins clearly colocalize with the invading bacteria, it is unknown how the ubiquitin-modified proteins are identified. The E3 ligase LRSAM1 is required for ubiquitination in mammalian cells upon *Salmonella* infection, and the enzyme is essential for colocalization of the adaptor

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proteins and bacteria (Huett *et al.*, 2012). The E3 ligase Parkin is also involved in the induction of ubiquitin-dependent autophagy upon invasion by *M. tuberculosis* (Manzanillo *et al.*, 2013). Parkin (Park2 in mammals) is an essential E3 ligase for autophagic clearance of damaged mitochondria (mitophagy), but it also functions to induce autophagy upon *Mycobacterium* infection (xenophagy), which limits bacterial replication. Parkin is essential for mitochondrial maintenance in *Drosophila* as well as in mammals, and its xenophagic function is also conserved. Flies with a mutant *parkin* gene are susceptible to *L. monocytogenes, S. typhimurium, and M. marinum* infection (Manzanillo *et al.*, 2013), indicating striking similarities between the mitophagy and xenophagy mechanisms.

In addition to its anti-bacterial functions, autophagy is important as an innate immune response against viruses in both mammalian cells and *Drosophila*. Vesicular stomatitis virus (VSV), a rhabdovirus that infects *Drosophila* S2 cells, rapidly replicates when autophagy is inhibited (Shelly *et al.*, 2009). Moreover, *Atg18* or *Atg7* knockdown flies exhibit increased susceptibility to VSV, suggesting that autophagy is essential for fly survival against VSV infection. The induction of autophagy upon VSV infection is dependent on the viral glycoprotein VSV-G, and Toll-7, a *Drosophila* Toll-like receptor that interacts with VSV-G on the plasma membrane to trigger autophagy *via* a nutrient signaling pathway for autophagy induction, the PI3K-Akt-TOR pathway (Nakamoto *et al*., 2012). Extracellular signal-related kinase signaling has a role in the anti-RNA virus activity in *Drosophila* cells and fly intestine by coupling nutrient status to antiviral defense (Xu *et al*., 2013). The precise mechanism underlying how autophagy inhibits viral replication, however, remains to be elucidated.

The *Drosophila* p62 homologue *ref(2)P* is an another candidate molecule that functions in the anti-viral response *via* autophagy. The *ref(2)P* gene is polymorphic, and some wild populations carrying restrictive alleles exhibit reduced multiplication rates of the sigma virus, a vertically transmitted rhabdovirus (Carré-Mlouka *et al*., 2007). In this case, it is not known whether the *ref(2)P*-dependent restriction of viral replication occurs *via* autophagy.

Autophagy has a critical role in anti-bacterial immunity not only in phagocytic immune cells, such as macrophages in mammals or hemocytes in flies, but also in epithelial cells. Autophagy in mouse intestinal epithelial cells (IECs) inhibits the growth of *S. typhimurium,* an intestinal pathogen that invades IECs to cause inflammation (Benjamin *et al*., 2013). Mice with IEC-specific knockout of *Atg5* are unable to eliminate *S. typhimurium*, resulting in dissemination of the bacteria to other organs. Infection by *S. typhimurium* induces autophagy, especially at the apical side of IECs. This raises the possibility that the host quickly recognizes invading *S. typhimurium* to induce autophagy. The induction of autophagy against *S. typhimurium* is independent of NOD2, an intracellular pathogen recognition protein that induces xenophagy (Travassos *et al.,* 2010), but rather depends on MyD88, a signaling molecule of the Toll pathway that leads to nuclear factor κB activation. How MyD88 contributes to autophagy induction in IECs, however, remains to be elucidated. Although in *Drosophila* less is known about the autophagic role against invasive bacteria in the intestine, the powerful genetic techniques available for *Drosophila* could provide new insight.

In addition to its role in immunity, autophagy is related to inflammation. Recent genome-wide association studies identified the autophagy-related genes *Atg16L1*, *IRGM*, and *NOD2* as risk factors for Crohn's disease, a chronic inflammatory bowel disease (Hampe *et al*., 2007; Rioux *et al*., 2007). The pathogenesis of this disease is also affected by environmental factors, such as commensal bacteria. The mechanism of inflammatory diseases caused by defective autophagy was studied in *Atg16L1* gene knockout mice: overproduction of interleukin-1β, an inflammatory cytokine, in *Atg16L1*-knockout macrophages is stimulated by commensal bacteria such as *Escherichia coli* (Saitoh *et al*., 2008), whereas dysfunction of Paneth cells, immune cells that reside in intestinal crypts and secrete antimicrobial peptides and lysozymes, occurs in *Atg16L1*-hypomorphic mice and IEC-specific *Atg16L1* knockout mice (Cadwell *et al*., 2008; Cadwell *et al*., 2010; Conway *et al*., 2013). Interestingly, this Paneth cell secretion defect is also dependent on enteric bacteria. Although these studies elucidated the importance of autophagy (genetic risk) and enteric bacteria (environmental risk) in intestinal homeostasis, the molecular mechanisms remain unknown. A *Drosophila* model with powerful genetic approaches might provide clues to the mechanisms.

As summarized here, autophagy has a critical role in anti-bacterial and anti-viral immunity. Although its role in animal survival is clear, autophagy is not a perfectly efficient mechanism for eliminating microbes, and thus some microbes evade autophagy to replicate in the host cells. The ability to avoid autophagy is not just dependent on the microbe species, but it is often observed that a portion of one kind of bacterium, such as *Listeria*, is trapped by autophagosomes, while others are able to evade autophagy. This might be because autophagy is primarily a host catabolic system that functions in the innate immune response using PRRs or other triggers for selectivity.

Because of the highly conserved systems of innate immunity between species, the genetic tools available for *Drosophila* will facilitate significant progress in understanding the role of autophagy against microbe infection. Further studies on autophagic function in innate immunity will provide new insights for clinical application.

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