A 'fly-by' killing with a primordial cellular weapon

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Autophagy has been suggested—on the basis of *in vitro* studies—to be involved in defense against bacterial challenge. A study in drosophila now shows the importance of autophagy *in vivo* and links a pattern recognition receptor to the autophagy pathway.

Macro-autophagy (called 'autophagy' here) is a process by which cells surround cytoplasm and cytoplasmic organelles with a double membrane–bound autophagosome. After being enveloped, the cargo contained by the inner autophagosomal membrane is delivered to the lysosomal compartment for degradation. The identification of autophagy proteins such as Atg1, Atg5, Atg7 and Atg8 (also called LC3) has led to an explosion of studies linking autophagy, autophagy proteins or defects in autophagy to a notable list of fundamental processes and diseases in mammals, including responses to nutrient deprivation, development, clearance of dead cells, cell death, protection against cell death, genome stability, aging, cancer, cardiac function, neurodegeneration, muscle disease, inflammatory bowel disease, immunity and pathogen resistance[1–3]. The race is on to determine where and how autophagy and autophagy proteins really matter in the intact host; the prize may well be ways to prevent or treat a range of human diseases, including infection. In this issue of *Nature Immunology*, Yano *et al.* study flies infected with the intracellular bacterium *Listeria monocytogenes* and make an important contribution to the field by showing that autophagy is important for survival after bacterial infection *in vivo*[4]. They also link autophagy to a peptidoglycan-recognition protein (PGRP) that senses bacterial invasion of the cytoplasm.

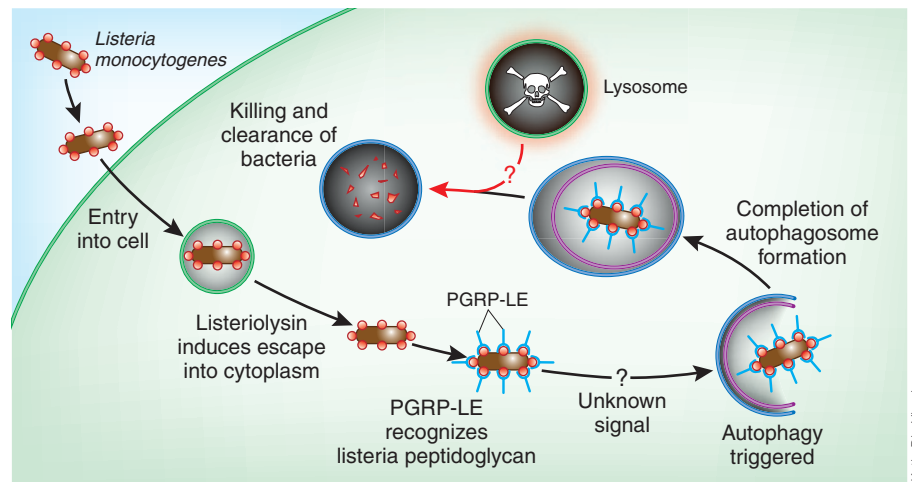Since the first demonstrations that autophagy is important for control of viral

Figure 1 Autophagy in the defense of drosophila against *L. monocytogenes*. After entering drosophila hemocytes, *L. monocytogenes* exits the vesicle by means of the pore-forming protein listeriolysin and escapes into the cytoplasm. Entry into the cytoplasm allows the detection of bacterial peptidoglycan by the drosophila protein PGRP-LE, which triggers a signaling pathway culminating in autophagosomal envelopment of *L. monocytogenes*. The bacteria are killed and cleared from the cell. One potential mechanism for killing and clearance might be fusion of the autophagosome with the lysosome and exposure of the bacteria contained in the inner autophagosomal membrane to lysosomal enzymes.

infection in mammals[5,6] and control of viral and bacterial infection in plants[7], a spate of papers using infection of cultured cells have suggested an antipathogen function for autophagy during infection of mammals with shigella, francisella, salmonella, toxoplasma, listeria, mycobacteria or streptococcus[1–3]. However, none of those studies has answered the fundamental question of whether host autophagy is important for control of such infections *in vivo*. Furthermore, many have shown relatively subtle effects of autophagy proteins on the replication or clearance of the pathogen; in addition, some have failed to distinguish between functions for autophagy as a process versus those for individual autophagy pro-

teins, and some have suggested involvement of autophagy at a single time point or have relied on pharmacologic approaches fraught with potential complications.

Thus, the time is ripe to answer two physiologic questions relevant to infection. First, does host autophagy really matter for control of bacterial infection *in vivo*? Second, what mechanisms link autophagy and antibacterial host defense in relevant cell types *in vivo*? Yano *et al.* provide some answers to these questions by infecting various strains of intact flies and primary fly phagocytic cells called 'hemocytes' with various strains of *L. monocytogenes*[4]. This work is a good example of how genetic manipulation of both the host and the pathogen can be combined to define

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important immune mechanisms operative *in vivo*[8]. Like any important work, this study opens a veritable Pandora's box of new questions that will keep researchers in this burgeoning field happily at work for some time to come.

Yano *et al.* pursue the mechanism by which the protein PGRP-LE protects flies from *L. monocytogenes* infection. PGRP-LE recognizes diaminopimelic acid–type peptidoglycans and can induce the production of antimicrobial peptides (AMPs) that are important for controlling infection in the fly hemolymph. Toll and immune deficiency (IMD) signaling pathways have already been linked to PGRP-mediated induction of AMPs. Yano *et al.* start by showing that deletion of PGRP-LE results in more growth of *L. monocytogenes* in cells in the intact fly and in hypersusceptibility of flies to lethal *L. monocytogenes* infection. Deletion of listeriolysin, a bacterial protein required for escape from vesicles into the cytoplasm, results in attenuation of *L. monocytogenes* in PGRP-LE mutant flies, which suggests that entry of the bacteria into the cytoplasm is required for virulence.

Notably, the authors find that transgenic expression of PRGP-LE specifically in hemocytes renders flies resistant to *L. monocytogenes*, which identifies these innate phagocytes as the cells in which PGRP-LE has its 'life-or-death' function. Furthermore, PGRP-LE is essential for control of *L. monocytogenes* in isolated primary hemocytes. These studies in primary cells are particularly important, as transformed or continuous cell lines or cells not normally involved in infection *in vivo* have been used in some studies of the potential function of autophagy in pathogen resistance. There is no guarantee that autophagy, a cellular process with tumor-suppressor activity and one intimately involved in cell survival and cellular stress responses[3], will be either intact or normally regulated in a continuous or transformed cell. Furthermore, the literature is rife with examples of cell type–specific interactions between pathogens and their target cells. This makes working with the relevant primary cell a key requirement for physiologically relevant studies of pathogen resistance. For example, a study using embryonic stem cells indicating that Atg5 is required for coronavirus replication has been refuted by studies of primary macrophages, which are important for the initial stages of coronavirus infection *in vivo*, lacking this protein[9]. A strength of the study by Yano *et al.* is that they 'go the extra mile' to identify the relevant cell type in the intact host and then define mechanisms operative in that cell type.

In further key experiments, they show that control of *L. monocytogenes* replication in hemocytes is independent of the Toll and IMD pathway proteins encoded by *imd*, *Relish, MyD88*, *Dif* and *dorsal*. Hot on the trail of something new, the investigators assess whether autophagy acts as an effector mechanism in the control of *L. monocytogenes* infection. They find that hemocytes lacking either Atg5 or Atg1 are unable to control *L. monocytogenes* replication *in vitro* and that knockdown of Atg5 expression specifically in hemocytes renders intact flies susceptible to *L. monocytogenes* infection, consistent with a key function for autophagy in resistance to *L. monocytogenes*. Similar experiments are needed in mammalian systems analyzing infection in the setting of hypomorphic expression, or cell type–specific deletion, of autophagy proteins. Null mutations in genes encoding autophagy proteins such as Atg5 and Atg7 cause perinatal death of mice[3]. Thus, manipulation of autophagy in relevant cells or specifically in infected cells by expression of recombinases such as Cre from the pathogen itself will be essential for assessing the function of autophagy in innate and adaptive immunity in the mammalian host.

The experiments described above led Yano *et al.* to answer a series of additional questions with a combination of studies in hemocytes and a macrophage-like insect cell line that does not express PGRP-LE. Expression of PGRP-LE results in resistance to *L. monocytogenes* that is dependent on Atg5 but is independent of *imd*, *Relish*, *Dif* and *dorsal,* which therefore confirms the presence of a previously unknown pathway for *L. monocytogenes* resistance in these cells. Further studies show PGRP-LE–dependent recruitment of the autophagy protein LC3-I to cytoplasmic bacteria and infection-induced conversion of LC3-I to its phosphatidylethanolamine-conjugated form, LC3-II. Furthermore, electron micrographs show *L. monocytogenes* contained in double-membrane vesicles in PGRP-LE-expressing cells, which indicates involvement of classical autophagy in PGRP-LE-induced resistance to infection. Additional experiments show that induction of autophagy by PGRP-LE occurs independently of the Toll and IMD pathways and that autophagy is induced in a PGRP-LE-dependent way by peptidoglycans that are recognized by PGRP-LE. Together, these studies support a model in which *L. monocytogenes* invades the cytoplasm exposing peptidoglycans that recruit PGRP-LE, which triggers, in a Toll- and IMD-independent way, envelopment and killing of the bacterium by autophagosomes (**Fig. 1**). The mechanisms

responsible for killing the bacteria remain undefined, but involvement of the lysosome is reasonable to consider.

These studies by Yano *et al.* leave many questions unanswered. The most prominent is, of course, the identification of the signaling pathway that links PGRP-LE to autophagy and whether it is also involved in mammalian cells. Answering this for mammalian cells will probably be very challenging, as a bewildering array of intracellular signaling pathways, each of which might be involved in infection, has been linked to autophagy. These include pathways involving eIF2α kinases, the serine-threonine kinase Akt and its effector mTOR, Janus-activated kinases and STAT transcription factors, Toll-like receptors, class I and class III phosphatidylinositol 3-OH kinases, DNA damage responses, antiapoptotic Bcl-2 family proteins, ubiquitination, Janus-activated kinase mitogen-activated protein kinases, AMP kinase, the p53 tumor suppressor, endoplasmic reticulum stress, GTPases, ceramide and calcium metabolism[3]. A key related future issue is whether and how autophagy is involved in cytokine-dependent immunity. Cytokines such as the interferons regulate autophagy, but the mechanisms responsible for the cytokine regulation of autophagy and the function of autophagy or other autophagy protein–dependent cellular processes in cytokine-dependent immunity are not well understood. Serious consideration is being given to treating a variety of diseases, from cancer to neurodegeneration, by manipulating autophagy. It will be essential to understand the functions of cytokines and signaling pathways in autophagy and autophagy protein–dependent immunity to predict the infection-related therapeutic effects or complications to be expected from drugs that regulate autophagy in humans.

Another fundamental issue is which mammalian proteins have a PGRP-LE-like function in triggering autophagy and/or targeting autophagy protein–related defense mechanisms in response to intracellular pathogens such as bacteria, parasites and viruses. How does the cell recognize the presence of the intracellular invader and trigger autophagy? Is autophagy or other autophagy protein–dependent cellular responses targeted specifically to invaders, as shown by Yano *et al.*? This field has a long way to go, but studies already suggest an intricate interaction between pattern-recognition receptors and autophagy in mammals. For example, studies suggest an important function for Toll-like receptor signaling in mammalian autophagy[10,11] and that autophagy is important for signaling

from endosomal Toll-like receptors during viral infection[12]. An important related issue is whether classical autophagy is responsible for all of the functions of autophagy proteins in innate and acquired immunity. Involvement of autophagy pathway proteins, but not of classical autophagosomes, has been demonstrated in the fusion of phagosomes with lysosomes[13], and other non-autophagy functions of autophagy proteins have been described. Perhaps these processes instead

of or in addition to classical autophagy are key to different aspects of immunity. Thus, there is probably more 'novelty' in store for immunologists and microbial pathogenesis researchers as the relevance and mechanisms of autophagy and autophagy proteins are defined in the intact host.

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Cat and mouse

Julio C Delgado & Peter E Jensen

New findings show that ERAAP, an endoplasmic reticulum aminopeptidase involved in antigen processing, helps mice survive encounters with a feline-derived parasite.

The endoplasmic reticulum aminopeptidase ERAAP (also called ERAP1) participates in the major histocompatibility complex (MHC) class I antigen-processing pathway through its ability to trim peptides delivered into the endoplasmic reticulum lumen by the transporter associated with antigen processing (TAP)[1,2]. ERAAP may be critical in antigen presentation to CD8+ T cells, as many peptides delivered by TAP exceed the eight- to eleven–amino acid length optimal for binding to MHC class I molecules. Thus, trimming is probably important in generating the final peptide antigens presented by MHC class I molecules. ERAAP deficiency, however, does not prevent animals from mounting protective CD8+ T cell responses to viral pathogens[3,4], and so far no evidence indicates that ERAAP is important in the host defense against pathogens. In this issue of *Nature Immunology*, Blanchard, *et al.*[5] demonstrate a critical function for ERAAP in protective immunity to an intracellular parasite.

Toxoplasma gondii is an obligate intracellular protozoan parasite that has a broad host range in mammals and birds[6,7]. However, the sexual stages of this parasite occur exclusively in cats, the definitive host, which shed oocysts in their feces. Much of the human population is exposed through contact with oocysts or undercooked meat from infected animals, but infections are generally subclinical. The para-
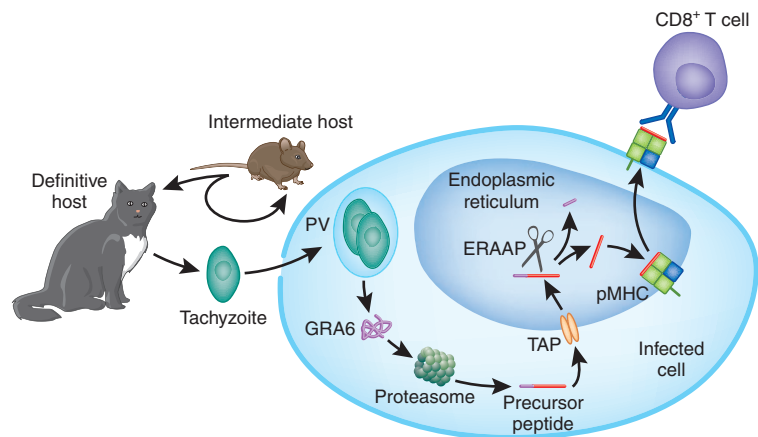
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Figure 1 Processing and presentation of an immunodominant protective CD8+ T cell epitope from *T. gondii* requires trimming by ERAAP. The protozoan parasite *T. gondii* is transmitted to intermediate hosts, including mice and humans, by ingestion of fecal oocysts from cats or of undercooked meat containing tissue cysts. Oocysts and tissue cysts transform into tachyzoites that invade a wide range of cell types and proliferate in a specialized intracellular parasitophorous vacuole (PV). In H-2d mice, control of the infection is highly dependent on CD8+ T lymphocytes that recognize a single peptide from the parasite GRA6 protein. GRA6 escapes the parasitophorous vacuole by an unknown mechanism and enters the cytoplasm, where it is cleaved by the proteasome. An amino-terminally extended precursor peptide is transported into the lumen of the endoplasmic reticulum by TAP. ERAAP is required for final trimming to generate the optimal MHC-binding peptide. ERAAP-deficient mice have a much lower immunodominant T cell response, greater mortality and higher parasite burden after infection. pMHC, peptide-MHC.

site can persist in cysts as an asymptomatic latent infection that can be reactivated after organ transplantation or infection with human immunodeficiency virus. *T. gondii* can cause life-threatening encephalitis or systemic disease in immunocompromised people and can cause severe fetal injury after primary infection during pregnancy. During acute infection, tachyzoites enter cells by phagocytosis or cellular invasion and localize in parasitophorous vacuoles that do not fuse with lysosomes. In

these vacuoles, *T. gondii* creates a secluded intracellular environment and can downregulate antigen presentation and modulate cytokine production in infected cells[8,9].

Despite its relative sequestration in parasitophorous vacuoles (or cysts), it is apparent from studies of mice, which like humans are natural intermediate hosts, that *T. gondii* elicits a potent immune response characterized by the production of T helper type 1 cytokines and antigen-specific T cells. The T helper type 1 cytokine