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Review

Autophagy, Immunity, and Microbial Adaptations

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Autophagy adjusts cellular biomass and function in response to diverse stimuli, including infection. Autophagy plays specific roles in shaping immune system development, fueling host innate and adaptive immune responses, and directly controlling intracellular microbes as a cell-autonomous innate defense. As an evolutionary counterpoint, intracellular pathogens have evolved to block autophagic microbicidal defense and subvert host autophagic responses for their survival or growth. The ability of eukaryotic pathogens to deploy their own autophagic machinery may also contribute to microbial pathogenesis. Thus, a complex interplay between autophagy and microbial adaptations against autophagy governs the net outcome of host-microbe encounters.

Introduction

Autophagy is a fundamental biological process that simultaneously touches on multiple aspects of eukaryotic cells and metazoan organisms. In its most generic rendition, autophagy is a process that controls the quality and quantity of intracellular biomass in eukaryotic cells by targeting for autodigestion cytoplasmic components that range in complexity and size from individual proteins to whole organelles. At one end of the spectrum, a delicate process termed chaperone-mediated autophagy directly imports individual cytosolic proteins that contain specific recognition motif sequences into the lysosome. At the other end of the spectrum, macroautophagy acts as a bulk process that captures large portions of the cytosol or sequesters big organelles such as mitochondria and peroxisomes (Figure 1). The general term ''autophagy'' usually denotes canonical macroautophagy characterized by its marquee feature, the double-membrane autophagosome. It has also become increasingly evident that autophagy, through its regulators comprised of Atg (*Atophagy*) and additional factors, interacts in a number of previously unappreciated ways with other pathways and processes in the host cell that do not always easily fit under ''autophagy'' as defined above. In these secondary roles, Atg proteins interact with other systems in the cell to coordinate various cellular functions (including immunological processes) with classical autophagy functions. Of note, it has also been proposed that Atg factors may have yet a third set of functions completely unrelated to autophagy or coordination between autophagy and other systems, referred to as autophagy-independent functions of Atg genes (Virgin and Levine, 2009).

A rapidly developing area of autophagy research is the study of immunological functions of autophagy (Levine and Deretic, 2007; Munz, 2009). Since the known immunological functions of chaperone-mediated autophagy (e.g., major histocompatibility complex [MHC] II-restricted endogenous antigen presentation) overlap with a subset of macroautophagy roles, for the purpose of this review, chaperone-mediated autophagy will not be distinguished from macroautophagy. Macroautophagy, which is usually referred to simply as autophagy (with the caveat in the above paragraph), is controlled by several signaling systems relaying nutritional or stress inputs to the core executioner machinery composed of Atg factors, which in turn drive the generation of autophagic organelles to sequester and degrade cytoplasmic targets (Figures 1A and 1B). This homeostatic role of autophagy affects cell survival (Kroemer and Levine, 2008), is reflected in the complexity of its regulation (Figure 1C), and represents the underpinnings of the role of autophagy in health and disease (Levine and Kroemer, 2008; Mizushima et al., 2008), including aging, cancer, neurodegenerative disorders, immunity, infectious diseases, and chronic inflammatory conditions such as Crohn's disease (CD).

The multilayered intersections (Figure 1D) between immunity and autophagy (the term autophagy being used here in a broad sense, including all functions of Atg factors) span phenomena ranging from cell-autonomous defenses to functions of the entire immune system and can manifest themselves in adaptive and innate immunity, in regulatory and effector immune functions, and in tolerance versus immune activation and inflammation. At the level of the whole immune system, autophagy contributes to positive and negative selection of the CD4 T cell repertoire (Nedjic et al., 2008) and T and B cell homeostasis (Li et al., 2006; Miller et al., 2008; Pua et al., 2007; Pua and He, 2007). Autophagy enables endogenous MHC II antigen presentation (Schmid and Munz, 2007), thus governing thymic selection and central tolerance (Nedjic et al., 2008). This function of autophagy involves the delivery of cytosolic proteins to the lumen of MHC II antigen processing and loading compartments, extends to MHC I presentation (English et al., 2009), affects generation of optimal immune responses to pathogens, and may be of significance for vaccine development (Jagannath et al., 2009; Schmid et al., 2007). Autophagy is furthermore an effector of Th1/Th2 polarization enabling (Andrade et al., 2006; Gutierrez et al., 2004; Ling et al., 2006) or disabling macrophages to utilize autophagy in

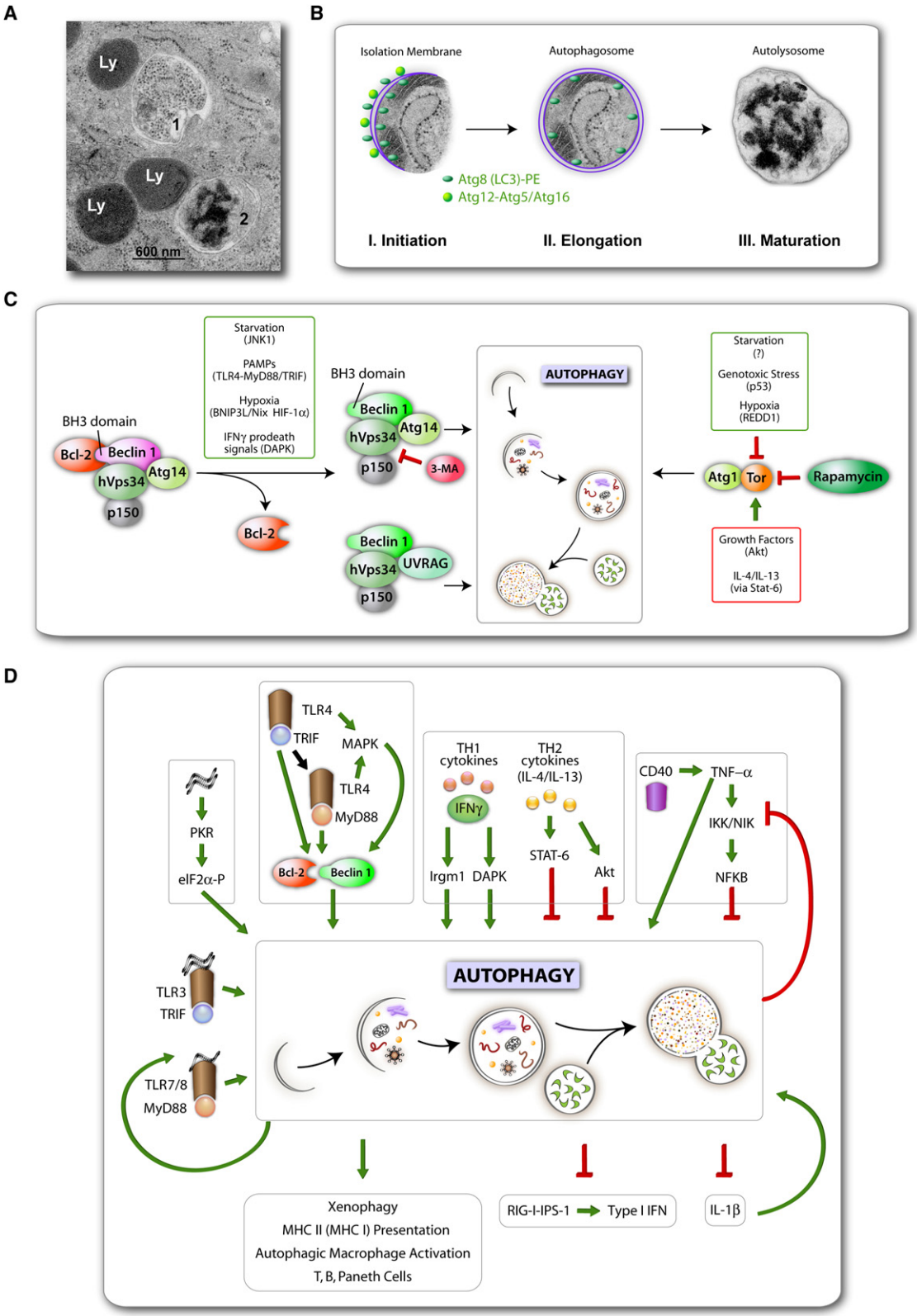

Figure 1. Autophagy Stages, Regulation, and Roles in Immunity

(A) Electron micrograph comparing the appearance of an autophagosome (1) versus autolysosome (2) with conventional lysosomes (Ly).

(B) A composite of autophagic membrane formation (schematic) with actual autophagosome from an electron micrograph. Three stages can be discerned by ultrastructural morphology: initiation (crescent membrane decorated on both sides with Atg protein-lipid [Atg8-PE; known as LC3-II] and protein-protein

control of intracellular pathogens (Harris et al., 2007). In innate immunity, autophagy is both a regulator (Jounai et al., 2007; Lee et al., 2007; Tal et al., 2009) and an effector of pattern recognition receptor (PRR) responses to pathogen- (Delgado et al., 2008; Sanjuan et al., 2007; Xu et al., 2007; Yano et al., 2008) and possibly to danger-associated molecular patterns (PAMPs and DAMPs, respectively) (Biswas et al., 2008; Saitoh et al., 2008). Furthermore, autophagy acts as a cell-autonomous defense directly eliminating intracellular microbes or their products, including bacteria, viruses, and protozoa (Levine and Deretic, 2007), in a process termed xenophagy (Levine, 2005).

Clearly, our knowledge of the immunological functions of autophagy continues to rapidly increase in scope and diversity (Levine and Deretic, 2007). This review summarizes what has been learned regarding the role of autophagy in various branches of immunity and delves into the mechanisms as they pertain to host-pathogen interactions. We explore in depth the status of autophagy as a cell-autonomous innate immunity defense against intracellular pathogens vis-à-vis adaptations that have evolved in pathogens to counter autophagy. These adaptations enable successful intracellular parasites to effectively protect themselves against autophagic inhibition or degradation and even harness autophagy for their own replication. We also highlight another emerging theme: intracellular eukaryotic parasites undergo autophagy themselves, leading to situations where autophagy contributes to both sides of the struggle in host-pathogen interactions.

Autophagy in the Host Cell

The cellular process of autophagy evolved at the beginning of eukaryotic life. The ability of viruses and bacteria to gain access to the interior of the eukaryotic cell generated selective pressures for the development of an effective cellular mechanism to dispose of such microbes. In this context, autophagy may have evolved as a primordial antimicrobial defense mechanism that can degrade intracellular parasites. At the transition to metazoan life, with the evolution of more specialized cells and immune systems, the functions of autophagy broadened so as to maintain its role at the nexus of more complex immune systems. Not only does autophagy function in a cell-autonomous manner to degrade intracellular pathogens, it also helps orchestrate the systemic immune response by functioning as a regulator of innate immunity, adaptive immunity, and inflammation. In this section, we will provide an overview of autophagy in the host cell, with an emphasis on its core molecular mechanisms, its broad roles in immunity, its newly emerging roles in the control of inflammation, and its most primal role as a cell-autonomous mechanism for eliminating intracellular microbes.

Autophagy as a Cell Biological Process

A key morphological manifestation of autophagy is the formation of autophagic organelles in the cytoplasm. They begin as short-lived membrane crescents, termed phagophores or isolation membranes, which give rise to double-membraned autophagosomes (Figure 1B). The autophagosomes undergo maturation into autolysosomes by fusion with lysosomal organelles, followed by loss of the inner of two membranes and degradation of the captured cytoplasmic target(s). While autophagosomes are considered the morphological hallmark of autophagy, their turnover is quite rapid, and often what are actually seen by electron microscopy are autolysosomes. Typically, these structures are delimited by a single membrane and contain lumens packed with remnants of cytoplasmic components, including shredded membranes and electron-dense material from decomposing ribosomes (Figure 1A). The origin of the autophagosomal phagophore membrane is not known with certainty, but both older and more recent data suggest involvement of the endoplasmic reticulum (ER) (Axe et al., 2008; Matsunaga et al., 2009).

Autophagosome formation is believed to be driven by two protein-protein and protein-lipid conjugation systems (Figure 1B). The first system yields an Atg5-Atg12 covalent conjugate that associates noncovalently with Atg16L1 (the mammalian equivalent of yeast Atg16) to form a putative E3 enzyme (terminology borrowed from the ubiquitin system) (Hanada et al., 2007), directing the site of the formation of the second protein-lipid conjugate (Fujita et al., 2008). The second system yields LC3-II (Atg8-PE), which has phosphatidylethanolamine at its C terminus that allows it to associate with or assist in autophagic membrane growth. Once formed and membrane-bound, LC3-II becomes an autophagosomal structural protein that executes other functions by interacting with the WXXL motif in the adaptor molecules p62 (Pankiv et al., 2007) and NBR1 (Kirkin et al., 2009), which capture cytoplasmic cargo earmarked for autophagic degradation, such as protein aggregates that are too large for proteasomal degradation. The partnering of LC3 with p62 and NBR1 suggests one potential mechanism by which autophagic targets may be recognized. Although it is not known whether these tag-receptor pairs are ubiquitously used to slate other targets, such as organelles, for autophagy, monoubiquitination has been shown to be sufficient to target an ectopically expressed cytoplasmic protein as well as peroxisomes for autophagic degradation (Kim et al., 2008). To date, no molecular tags of this nature have been reported on a microbe to guide autophagosomes with precision to their microbial targets. However, it is reasonable to speculate that some of the same molecular tags used to deliver cytoplasmic constituents may also be used to deliver microbes to the autophagosome. Yet PAMPs released by microbes stimulate autophagy (Delgado et al., 2009; Yano et al., 2008), and we cannot exclude the possibility that the execution stages of the process may be more stochastic and less perfectly guided toward specific microbial targets.

conjugates [Atg12-Atg5, complexed with Atg16]), elongation (growth of isolation membrane) ending in its closure to form an autophagosome, and maturation that involves the formation of degradative autolysosomes through fusion of autophagosomes with lysosomal organelles (electron-dense granular material represents ribosomal degradation intermediates). All micrographs in (A) and (B) are courtesy of Eeva-Lisa Eskalinen (reproduced with permission).
(C) Two signaling systems control induction of autophagy: left, hVps34-Beclin 1; right, Tor-Atg1. Various signals and systems transmitting them that lead to autophagy activation are shown in smaller boxes. Rapamycin induces autophagy by inhibiting Tor, and some immune signals that are transmitted via TLR adapters MyD88 and TRIF or DAPK downstream of IFNγ lead to activation of Beclin 1 (complexed with the phosphatidylinositol 3 kinase hVps34) by its dissociation from Bcl-2. Note that starvation, a classical inducer of autophagy, affects both Bcl-2-Beclin 1 complex (via JNK1) and Tor activity. Th2 cytokines and many growth factors in general inhibit autophagy via Tor (or in the case of immunologically induced autophagy, e.g., by IFNγ, via Stat-6 downstream of IL-4/IL-13).
(D) Immunological inputs and outputs of autophagy. Xenophagy, autophagic macrophage activation, additional terms, factors, and relationships are described in the text.

A multitude of nutritional and stress inputs transduced through protein and lipid kinase signaling cascades that regulate autophagy converge upon two key signaling nodes (Figure 1C): (1) Tor-Atg1 and (2) Beclin 1(Atg6)-hVps34. The Tor-Atg1 system transduces growth, nutritional, and some stress signals to initiate autophagy. The metabolic aspects of autophagy are under negative control by the growth factors, insulin receptor substrate, type I phosphatidylinositol 3 kinase (PI3K), Akt/PKB, and the downstream Tor-Atg1 signaling cascade. How Atg1 sets in motion other Atg factors and downstream morphologically distinguishable events is still not completely clear. It possibly regulates multiprotein complex formation involving a number of other Atg proteins initially recognized only in yeast (Kawamata et al., 2008), but which now appear to have counterparts in mammalian cells (Hosokawa et al., 2009).

The Beclin 1-hVps34 represents another key regulatory node centered on an ancient stress-signaling lipid kinase, known as type III PI3K Vps34. The exact role of the enzymatic product of hVps34 is not fully understood, but it likely plays a pivotal role in early autophagosomal membrane formation (Axe et al., 2008), the targeting of PI3P-binding proteins such as Atg18 to the autophagic membrane (Obara et al., 2008), the localization of LC3 lipidation (Fujita et al., 2008), and autophagosomal maturation into autolysosomes (Liang et al., 2008). Beclin 1 is a key regulator of autophagy, and it exists in functionally distinct hVps34-containing protein complexes (Figure 1C), including several modifier components: (1) Atg14, which plays a role in initiation (Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009), and (2) UVRAG/VPS38, which plays a role in maturation (Itakura et al., 2008; Liang et al., 2008) and is negatively regulated by Rubicon (Matsunaga et al., 2009; Zhong et al., 2009). Beclin 1 complexes can contain several additional factors such as Ambra1 (Fimia et al., 2007) and Bif-1/Endophilin B1 (Takahashi et al., 2007) that may further modulate its function. Many signals that lead to autophagy activation affect Beclin 1 as the "nerve center" of autophagic control. This includes stress and immunological inputs such as activation by JNK1 kinase (Wei et al., 2008), shown to activate Beclin 1 downstream of starvation (but possibly engaged during innate immune signaling); DAPK (Zalckvar et al., 2009), a kinase activated downstream of IFNγ stimulation; BH3-only proteins such as Bnip3 (in turn regulated by FoxO3 in atrophy or by HIF-1 in hypoxia) (Zhang et al., 2008); and MyD88, reported to occur downstream of PRR stimulation by microbial products (Shi and Kehrl, 2008).

Broad Connections between Autophagy and Immunity

The repertoire of autophagy's functions in immunity has been expanding at an extraordinary pace. The broad immunological roles of autophagy (Figure 1D), collectively dubbed "immunophagy" (Deretic, 2006), can be categorized as: (1) regulatory versus effector roles, (2) innate versus adaptive immunity functions, (3) Anti-inflammatory versus proinflammatory, and (4) specialized immune-cell-specific versus generic cellular homeostatic roles that are applicable to immune cells.

Among the innate immunity effector functions, the most intrinsic to autophagy (i.e., the engulfment and lysosomal degradation of cytoplasmic components) is its role in direct elimination of intracellular microbes (Figure 1D). This process can manifest itself as xenophagy (Levine, 2005), denoted to describe direct autophagy of intracellular microbes in any cell type. Here, we introduce "autophagic macrophage activation" (APMA) as a general term to denote a collection of autophagy-related processes in cells of the reticulo-endothelial system. APMA includes (1) convergence of phagocytosis and the autophagic machinery (Sanjuan et al., 2007), (2) enhanced microbicidal properties of autolysosomes in comparison to standard phagolysosomes (Alonso et al., 2007), (3) autophagic modulation of PRR signaling (Delgado et al., 2008; Sanjuan et al., 2007; Shelly et al., 2009; Xu et al., 2007), (4) cooperation between immunity related GTPases and autophagy or Atg factors in attacking parasitophorous vacuoles (Gutierrez et al., 2004; Ling et al., 2006; Singh et al., 2006; Zhao et al., 2008), and (5) enhanced antigen presentation (Schmid et al., 2007; English et al., 2009). APMA is thus recognized as a complex outcome of autophagy stimulation in macrophages, representing a unique composite process bringing about a heightened state of activation.

Autophagy in Innate Immunity

The innate immunity functions identified to date encompass both effector outputs (Delgado et al., 2008; Sanjuan et al., 2007; Shelly et al., 2009; Xu et al., 2007) and regulatory roles, some of which are in conjunction with PRRs (Jounai et al., 2007; Lee et al., 2007; Saitoh et al., 2008; Tal et al., 2009). As a regulator of immunity responses, autophagy acts in several ways (Figure 1D). Autophagy is proinflammatory, e.g., when autophagy captures cytosolic viral replication intermediates and delivers them to the lumen of endosomal compartments where they meet their cognate PRRs, as in the case with viral single-stranded RNA and Toll-like receptor (TLR) 7 (Lee et al., 2007). Complementarily, autophagy can also dampen proinflammatory responses, including IL-1β, IL-18 (Saitoh et al., 2008), and type I IFN production (Jounai et al., 2007; Tal et al., 2009) (Figure 1D). As an effector, autophagy is modulated by cytokines (Figure 1D), including IFNγ, TNF-α, IL-4, and IL-13, and acts as an output of both innate and adaptive immunity responses (Deretic, 2009).

The autophagic machinery can be activated upon detection of PAMPs by their cognate PRRs (Delgado et al., 2008; Sanjuan et al., 2007; Shelly et al., 2009; Xu et al., 2007; Yano et al., 2008) or antibody-Fcγ receptor (Huang et al., 2009). The signal transduction pathways between agonist-stimulated receptors and autophagy activation remain to be fully delineated. This is an important frontier, especially since there have been reports of the inability to detect macroautophagy downstream of TLR stimulation (Saitoh et al., 2008) and discrepancies in adaptor usage downstream of certain PRRs in the context of autophagy (Delgado et al., 2008; Sanjuan et al., 2007; Xu et al., 2007). PRR signaling to autophagy may involve the reported complex formed between the TLR adaptors (MyD88 and TRIF) and Beclin 1 and changes in the antiapoptotic protein Bcl-2's interaction with Beclin 1 upon TLR stimulation (Shi and Kehrl, 2008) (Figure 1D), akin to Bcl-2-Beclin 1 interactions observed during activation of autophagy by nonimmunological signals (Wei et al., 2008) (Figure 1C).

Autophagy can also be activated by reactive oxygen species (ROS) (Scherz-Shouval et al., 2007). Accordingly, ROS produced by NADPH oxidase downstream of TLR or Fcγ receptor stimulation in phagocytes has been shown to activate autophagy (Huang et al., 2009). The report from Sanjuan et al. that LC3-II appears on phagosomes without the appearance of conventional double membranes shortly after particle uptake when

particles costimulate TLRs (Sanjuan et al., 2007) called attention to the unconventional roles of Atg proteins. However, the coactivation of NADPH oxidase and ROS production during phagocytosis of opsonized or PAMP-laden particles may in essence mirror the observed induction of autophagy by mitochondrially produced ROS in response to starvation stimuli (Scherz-Shouval et al., 2007) and may explain in part the observations of Sanjuan et al. These events may be best understood within the concept of APMA, as proposed earlier, which is a set of linked events in macrophages and includes connections between ROS production and autophagy.

Importantly, autophagy induction downstream of PRR activation is countered by NF-κB, which is activated concomitantly (Djavaheri-Mergny et al., 2006) (Figure 1D). This may explain why PAMPs do not uncontrollably stimulate autophagy in physiological situations (Delgado et al., 2008) and a report that autophagy could not be detected upon PRR stimulation (Saitoh et al., 2008). Independent of its antimicrobial function, autophagy induced by LPS and TLR4 may act to protect against LPS cytotoxicity, which is of potential relevance for countering myocardial depression in septic shock (Yuan et al., 2009). Other innate immunity mediators such as IL-1β, a proinflammatory cytokine normally generated upon inflammasome activation with PAMPs or the host's own DAMPs, can also stimulate autophagy (English et al., 2009) (Figure 1D). Consistent with the direct involvement of DAMP pathways, ATP, which is an endogenous activator of the inflammasome, can stimulate autophagy through the P2X7 receptor (Biswas et al., 2008). Thus, both microbial PAMPs and host DAMPs appear to be linked to autophagy.

Autophagy in Adaptive Immunity

We are only just beginning to comprehend what appear to be the critical functions of autophagy in regulating adaptive immune responses, immunological tolerance, and the development and homeostasis of the immune system. At least three distinct processes contribute to these functions (Figure 1D). (1) Autophagy, by the very nature of its ability to capture cytoplasmic proteins, supports MHC II-restricted endogenous antigen presentation of cytosolic self or microbial (e.g., viral) antigens synthesized by host cells (Gannage and Munz, 2009). It may likewise influence MHC I presentation of viral antigens in a process occurring separately from and following the initial canonical ER-dependent cross-presentation pathway (English et al., 2009). (2) Autophagy shapes central tolerance via thymic selection of the T cell repertoire (Nedjic et al., 2008). (3) Autophagy also affects homeostasis of T cells (Pua et al., 2009), B cells (Miller et al., 2008), and specialized granulocytes of the intestinal epithelium known as Paneth cells (Cadwell et al., 2008).

Some additional functions of autophagy may represent more of a "fine tuning" of the immune response. For example, autophagy acts as an effector of immune phenomena such as Th1/Th2 polarization; when induced by Th1 cytokines such as TNF-α (Djavaheri-Mergny et al., 2006) or IFNγ (Harris et al., 2007), macroautophagy (i.e., xenophagy) and APMA kill intracellular microbes (e.g., *M. tuberculosis*), while Th2 cytokines such as IL-4 and IL-13 inhibit autophagy and protect intracellular pathogens form autophagic elimination (Harris et al., 2007). Additionally, in the context of vaccine development, autophagy has been used to enhance CD4 T cell responses to influenza matrix protein (Schmid et al., 2007) and to enhance BCG vaccine efficacy in animal models of tuberculosis (Jagannath et al., 2009). These studies open a new area of translational research in the application of autophagy in prophylaxis against infectious diseases.

Autophagy in Inflammation

Certain immune responses act as a double-edged sword, either resolving infection or leading to over-exuberant inflammation and tissue damage. Autophagy belongs to this category, in view of the developing connection between inflammatory bowel disease and mutations in autophagy (e.g., *ATG16L1*) or autophagy-related (e.g., *IRGM*) genes, as predisposition loci in CD that have been identified in human populations through whole genome association studies (GWAS) (McCarroll et al., 2008; Parkes et al., 2007; Wellcome Trust Case Control Consortium, 2007) (Figure 2). Genetic risks in CD were linked some time ago to innate immunity via Nod2, which like many other PRRs recognizes bacterial products of the enteric flora (Kanneganti et al., 2007). Interestingly, several risk loci identified through GWAS are common to ulcerative colitis and CD, but autophagy genes *ATG16L1* and *IRGM*, along with *NOD2*, appear to be more specific for CD (Fisher et al., 2008), and *IRGM* risk alleles may predispose even more specifically to the ileal form of CD (Roberts et al., 2008). ATG16L1 is a part of the basal autophagy apparatus, while IRGM is a member of the vertebrate family of innate immunity effectors called immunity-related GTPases (IRGs) (Bekpen et al., 2009). In the mouse, there are multiple IRG genes, while humans and chimpanzees have only one IRG, *IRGM* (Bekpen et al., 2009). The human IRGM (Singh et al., 2006) and murine *Irgm1* (Gutierrez et al., 2004) and Irga6 (Al-Zeer et al., 2009; Ling et al., 2006; Zhao et al., 2008) have all been shown to play a role in autophagic elimination of intracellular pathogens.

Functional information regarding the role of autophagy in humans in the context of CD is lacking. Nevertheless, relevant information regarding ATG16L1 has been obtained in studies at the cellular level and in vivo in mice, with three published studies identifying different effects that may be additive (Figure 2, middle panel): (1) a diminished capacity of the CD risk ATG16L1*300A to control intracellular enteric pathogens (Kuballa et al., 2008), which fits with the current focus on adherent-invasive *E. coli* (AIEC) as one of the microbial culprits in CD (Rolhion and Darfeuille-Michaud, 2007); (2) increased susceptibility of Atg16L1-deficient mice to chemically-induced colitis that is linked to elevated IL-1β signaling (Saitoh et al., 2008); and (3) direct or indirect effects in *Atg16L1* hypomorphic mice on Paneth cells (Cadwell et al., 2008), the epithelial-type stationary "granulocytes" of the intestinal crypt that guard the overhead stem cell zone from microbial penetration, a long-time suspect in the etiology of CD. The role of IRGM cannot be properly investigated in mice, as the mouse has 19 *IRGM*-like genes. Human IRGM plays a direct role in antibacterial defenses (Singh et al., 2006), which fits well with a similar role of ATG16L1 in the control of intracellular enteric bacteria (Kuballa et al., 2008). However, additional processes cannot be excluded, given that Irgm1, one of the three putative murine orthologs, shows effects on hematopoietic stem cell proliferation and T cell survival (Feng et al., 2008a, 2008b).

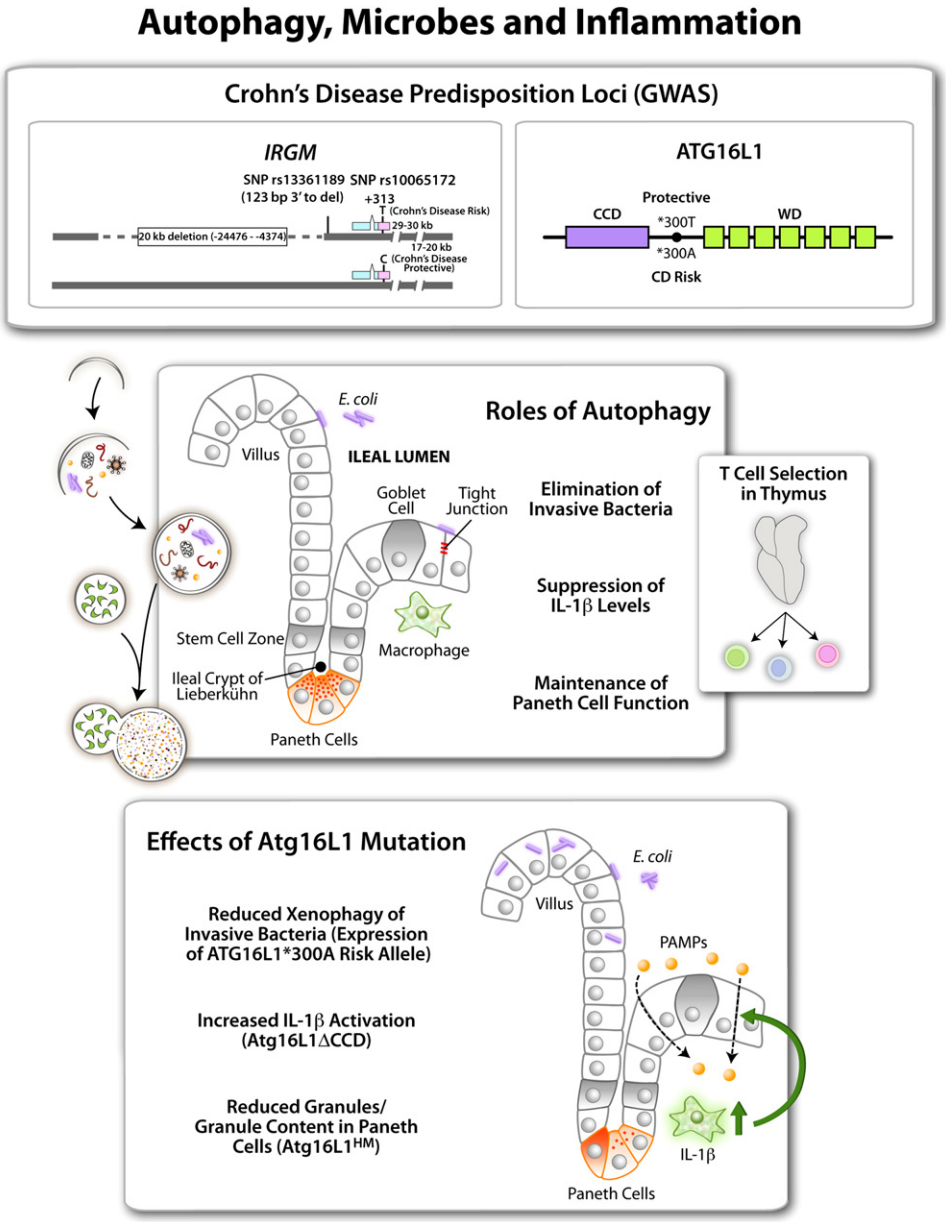

Figure 2. Autophagy in Inflammation

Top shows two autophagy factors identified as Crohn's disease (a form of inflammatory bowel disease) susceptibility loci. The *IRGM* gene has single nucleotide polymorphisms (SNP) and a 20 kb deletion in the promoter region associated with CD. *ATG16L1* alleles encode either a protective ATG16L1*300T or a risk form of ATG16L1*300A (CCD, coiled coil domain; green boxes, WD repeats domain [absent in yeast Atg16]). Middle panel shows intestinal epithelium with different cell types along with the proposed functions of autophagy in the ileal epithelium. Side box shows that thymic selection of naive T cell repertoires depends on autophagy, and autophagic anomalies may contribute to inflammation at peripheral sites such as intestinal mucosa. Bottom box lists 3 different effects reported for Atg16L1 (mouse) or ATG16L1 (human epithelial cells) mutations. ATG16L1*300A has been tested in cell lines. Atg16L1^{HM}, hypomorphic Atg16L1 allele, and Atg16LΔCCD construct have been tested in vivo in transgenic mice.

Finally, a comprehensive explanation for the role of autophagy in inflammatory processes, including CD, needs to take into account the endogenous antigen presentation process, whereby thymic epithelial cells present endogenous (self) antigens to developing T cells, influencing their positive and negative selection (Nedjic et al., 2008). Thus, autophagy, through appropriate selection of naive T cells (Figure 2, middle panel) before they exit to the periphery, serves as a guardian of immunological tolerance. This seems to be exceptionally important, as when this process is rendered dissonant between the periphery and the thymus in mice with *Atg5*$^{-/-}$ thymic implants, the animals develop multiorgan inflammation, including colitis. It remains to be seen whether aberrant autophagy (e.g., risk alleles in CD or yet to be identified potential autophagy defects in other autoimmune or inflammatory diseases) may lead to dissonant thymic selection vis-à-vis endogenous antigen presentation in the periphery.

Autophagy as a Cell-Autonomous Antimicrobial Defense System

Since autophagosomes can engulf large portions of the cytosol and digest whole organelles such as mitochondria, it is intuitively compelling to think of this process as capable of capturing and eliminating intracellular microbes. Conclusive demonstrations ex vivo and in vivo of this concept, however, have proven less than trivial. A forerunner to the development of this field was a report that ectopic Beclin 1 expression in neurons suppresses viral replication in the brain and reduces morbidity and mortality in experimental animals (Liang et al., 1998). In more recent years, a growing number of microorganisms have been demonstrated to be subject to elimination by autophagy in vitro involving one or more of the processes listed in Figure 3, upper left panel. Only very recently has the autophagic machinery been shown to protect against thus far a small number of infectious diseases in vivo (Table 1). In addition, autophagy may protect host cells against toxic products produced by pathogens, such as *Vibrio cholerae* cytolysin (Gutierrez et al., 2007), *Bacillus anthracis* lethal toxin (Tan et al., 2009), and *Helicobacter pylori* vacuolating toxin (Terebiznik et al., 2009) (Figure 3 and Table 1).

Among viruses, autophagic protection has been shown for vesicular stomatitis virus (VSV) (Shelly et al., 2009), tobacco mosaic virus (TMV) (Liu et al., 2005), herpes simplex virus 1 (HSV-1) (Orvedahl et al., 2007), and human immunodeficiency virus (HIV) (Kyei et al., 2009). HSV-1 and HIV fall prey to autophagy when they are disarmed by inactivation of their specific antiautophagy factors, ICP34.5 and Nef, respectively (Orvedahl et al., 2007; Kyei et al., 2009). Among bacteria, microbes that can invade into host cells (e.g., Gram-positive extracellular pathogens such as *Streptococcus pyogenes* [Nakagawa et al., 2004] or facultative intracellular pathogens such as *M. tuberculosis* [Alonso et al., 2007; Biswas et al., 2008; Gutierrez et al., 2004; Singh et al., 2006], *Salmonella* [Birmingham et al., 2006], and *Listeria monocytogenes* [Py et al., 2007; Yano et al., 2008; Zhao et al., 2008]) can be eliminated through autophagy. However, as with viruses, the highly evolved intracellular bacterial pathogens possess antiautophagic factors, exemplified by *Shigella*, where inactivation of the bacterial type III secretion system (T3SS)-dependent effector *IcsB* is prerequisite to elimination by autophagy (Ogawa et al., 2005). Among protozoans, in vitro and in vivo data exist to support a role for autophagy and/or the autophagic genes in defense against *Toxoplasma gondii* (Andrade et al., 2006; Ling et al., 2006; Zhao et al., 2008). These examples underscore two concurrent phenomena: (1) autophagy acts as a defense against microbes when they manage to invade the host cell interior, and (2) highly evolved intracellular pathogens have adaptations to protect themselves from autophagic elimination or even harness the host cell autophagic machinery to their own benefit, as will be covered in more detail in subsequent sections.

It is important to keep in mind the above phenomena in interpreting reports using highly adapted intracellular pathogens, as even when the specific antiautophagic adaptations are not yet known, they may exist. Until such factors are identified and inactivated, the true power of autophagic action in eliminating microbes may remain masked. In this context, experiments where pathogens and hosts are slightly "mismatched" are conducive to observing autophagy in action, which may be otherwise obscured due to evolutionary adaptations in finely tuned host-pathogen pairs. Examples of this come from using *Drosophila* to study mammalian pathogens, where infection experiments with *L. monocytogenes* (Yano et al., 2008) and VSV (Shelly et al., 2009) unambiguously demonstrate that autophagy controls these microbes in vivo. Mouse infection models of human disease have also led to the demonstration of autophagy's role in vivo, as shown in recent experiments with viruses (Orvedahl et al., 2007) and protozoans (Zhao et al., 2008). The study by Zhao et al. with *T. gondii* infection using *Atg5*$^{Flox/Flox}$ *LysM-Cre* mice (with an *Atg5* defect specifically in monocytic cells) has shown that Atg5 function is key in controlling this pathogen in vivo. The details of the study revealed a complex relationship between Atg5 and IRG (in this case Irga6)-dependent processes (Zhao et al., 2008), perhaps not unlike what has been seen with Irgm1 (MacMicking et al., 2003) and autophagy in controlling *M. tuberculosis* (Gutierrez et al., 2004). The exact details of how IRG and Atg factors work together or whether they work sequentially (e.g., with Atg5 preceding Irga6 recruitment, as implicated in the studies with Irga6 [Al-Zeer et al., 2009; Zhao et al., 2008]) remains to be delineated. Finally, whereas animal experiments remain of high significance to define further the full spectrum of autophagy in antimicrobial defense, GWAS in human populations are of equal interest in exploring whether polymorphisms in autophagy genes predispose to certain infectious diseases, as has been observed with CD.

Microbial Adaptations to Host Autophagy

Microbial pathogens that successfully parasitize eukaryotic cells (i.e., intracellular pathogens) have evolved in the setting of selective pressures imposed by cellular autophagy as a pathway central to innate and adaptive immunity. Consequently, it is not surprising that microbes have developed multipronged strategies to avoid autophagolysosomal degradation and/or to dampen autophagy-dependent activation of host immune responses (Figure 3, bottom panel). Researchers are beginning to delineate such molecular strategies and their potential roles in microbial pathogenesis, although in most cases, our understanding of this area is still quite rudimentary. The types of microbial adaptations identified to date can be broadly categorized as strategies to (1) prevent the induction of autophagy, (2) prevent the maturation of the autophagosome into an autolysosome, (3) avoid pathogen recognition by the autophagic machinery, and (4) utilize functions or components of autophagy to enhance intracellular survival, replication, or extracellular release of intracellular pathogens.

Microbial Suppression of Autophagy Induction

As discussed, the activation of autophagy in an infected cell may represent a fairly ubiquitous response triggered by PRRs that recognize microbe-specific PAMPs. While most studies have been performed using TLR ligands (rather than intact microbes) (reviewed in Delgado et al., 2009), PRR-dependent autophagy induction has recently been shown to protect against *L. monocytogenes* infection in *Drosophila* (Yano et al., 2008). It is not yet known whether pathogens possess strategies to block PRR-dependent autophagy induction or how ubiquitously PRRs are used to activate autophagy. What appears more likely, at least based on the limited research to date, is that microbial adaptations to suppress autophagy induction may be focused on targeting some of the more general (i.e., not pathogen-specific)

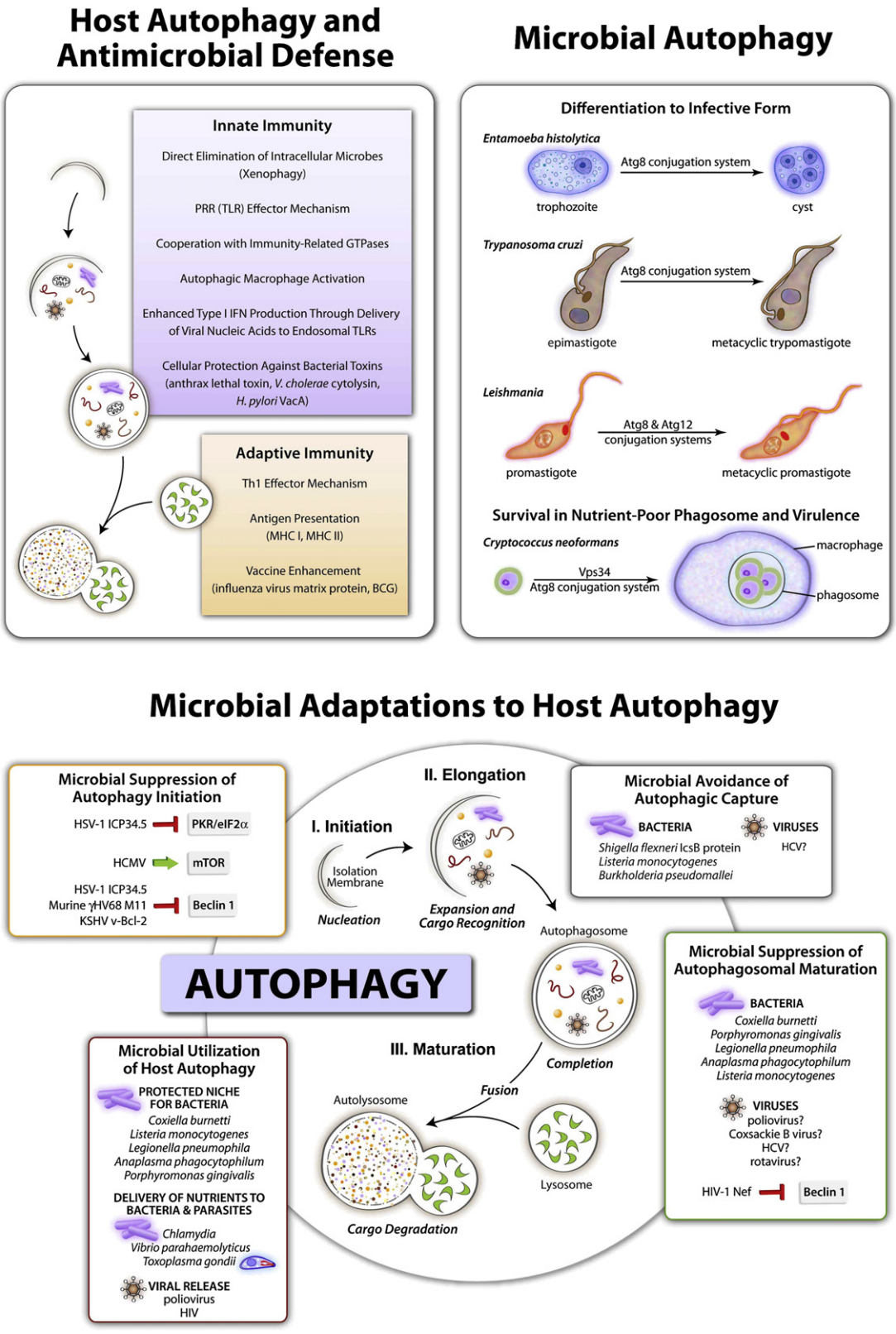

Figure 3. Schematic Depicting Functions of Host Autophagy in Antimicrobial Defense, Functions of Microbial Autophagy, and Microbial Adaptations to Host Autophagy

See Tables 1 and 2 for further details of specific host autophagy-pathogen interactions.

signaling pathways that positively or negatively regulate autophagy. This evidence stems largely from studies done with the three major groups of herpesviruses, the α-, β-, and γ-herpesviruses, although the principles learned from such studies are likely to be extrapolatable to other virus families and perhaps to other types of microbial pathogens.

Herpesviruses block autophagy induction through at least three distinct mechanisms (Table 2 and Figure 3, bottom panel), including blockade of autophagy-stimulatory PKR/eIF2α kinase signaling (Figure 1D) (through HSV-1 ICP34.5-mediated eIF2α dephosphorylation) (Tallóczy et al., 2002), blockade of the autophagy function of Beclin 1 (through HSV-1 ICP34.5, KSHV, or murine γ-HV68 viral Bcl-2 binding of Beclin 1) (Ku et al., 2008a; Orvedahl et al., 2007; Pattingre et al., 2005; Sinha et al., 2008), or activation of autophagy-inhibitory mTOR signaling (by human cytomegalovirus through an as of yet undefined mechanism) (Chaumorcel et al., 2008) (Figure 3, bottom panel). The utilization of different strategies for preventing autophagy induction by a single viral virulence protein (i.e., HSV-1 ICP34.5 blocks both eIF2α phosphorylation and Beclin 1 function), the utilization of different viral structural motifs for targeting Beclin 1 (i.e., HSV-1 ICP34.5 and viral Bcl-2 proteins both bind Beclin 1, but share no structural similarity with each other and bind to nonoverlapping regions of Beclin 1), and the prevention of autophagy induction by all three classes of herpesviruses likely underscore a critically important role for evasion of autophagy in herpesvirus pathogenesis. Indeed, for HSV-1, it has been shown that ICP34.5-mediated blockade of Beclin 1-dependent autophagy is essential for lethal HSV-1 encephalitis (Orvedahl et al., 2007). The role of CMV and γ-herpesvirus evasion of autophagy in viral pathogenesis has not yet been explored, but considering that autophagy is a tumor suppressor pathway (Levine and Kroemer, 2008; Mizushima et al., 2008), it is tempting to speculate that γ-herpesvirus evasion of autophagy might contribute to viral oncogenesis. Another open question is whether other virus families also inhibit autophagy induction; given the numerous viral proteins encoded by the diverse families of viruses that have already been shown to inhibit PKR/eIF2α kinase signaling or to activate mTOR signaling (reviewed in Cooray, 2004 and Sadler and Williams, 2008), it seems likely that suppression of host autophagy induction signaling pathways will be a fairly universal feature of viral infections.

It is not yet clear whether other intracellular pathogens besides viruses also actively suppress initiation of the autophagy pathway or rather focus uniquely on blocking pathogen recognition by autophagosomes and/or the maturation of pathogen-containing autophagosomes into acidified autolysosomes. Certain bacterial virulence factors (such as *Listeria*-encoded Prf1-regulator factors, ActA and phospholipases [Birmingham et al., 2007; Py et al., 2007], *Burkholderia pseudomallei* BopA [Cullinane et al., 2008], and *Shigella*-encoded IcsB [Ogawa et al., 2005]) are necessary for bacterial evasion of autophagy, as defined either by increased colocalization with GFP-LC3 or increased growth of replication-deficient bacterial mutants in autophagy-deficient cells. However, to date, there is no published evidence indicating that bacteria, fungi, or parasites block the induction of autophagy in infected cells (although a microarray analysis of *Francisella tularensis*-infected macrophages revealed downregulation of several autophagy genes [Butchar et al., 2008]). Moreover, in many reports, autophagy induction can be enhanced in bacterially infected cells by starvation or rapamycin, suggesting the absence of microbial mechanisms that can completely block autophagy induction. Nonetheless, it remains an open question whether nonviral pathogens suppress autophagy induction. An interesting possibility is that the known activity of *M. tuberculosis* in the inhibition of hVps34/PI3-dependent trafficking pathways in macrophages (Vergne et al., 2004) may extend to inhibition of the Beclin 1/hVps34 autophagy complex. At yet another level, highly virulent strains of *M. tuberculosis* elicit more IL-4 and IL-13 (Manca et al., 2004), the Th2 cytokines known to inhibit autophagy (Petiot et al., 2000), and suppress aspects of APMA (Harris et al., 2007). Other important and outstanding questions are whether bacteria and parasites possess mechanisms to block PRR-dependent autophagy induction and/or IRG autophagy induction.

Microbial Suppression of Autophagosomal Maturation

The greatest threat to an intracellular pathogen imposed by autophagy is not the process of autophagic sequestration per se, but rather the danger imposed by delivery to an autolysosome. Accordingly, it is not surprising that several viruses and intracellular bacteria seem to block fusion of the autophagosome or autophagy protein-dependent fusion of another pathogen-containing compartment (i.e., phagosome or pathogen-containing vacuole) with the lysosome (Table 2). For some viruses and intracellular bacteria, it is argued that autophagosome formation may enhance intracellular microbial survival, replication, or extracellular release (see section below); in such cases, enhanced autophagy is usually accompanied by a block in autophagosomal maturation (see Table 2). In this manner, microbes may "avail themselves" of promicrobial functions of autophagy while simultaneously blocking autophagy's antimicrobial functions (Figure 3, bottom panel).

Perhaps the earliest described example of this concept was with *Porphyromonas gingivalis*, a bacterial periodontal pathogen that also localizes to atherosclerotic plaques. Based largely on electron and light microscopy studies, this organism is believed to traffic to autophagosomes as a mechanism of evading the conventional endocytic trafficking to lysosomes (Dorn et al., 2001). The bacterial determinants that direct this trafficking and the precise details of the cellular trafficking events in infected cells are not yet defined. In the case of *Legionella pneumophila* infection, soluble bacterial type IV secretion products are sufficient to induce autophagy, the bacterial replication vacuoles have autophagy markers early after infection, and it is postulated that the bacteria delays autophagosome maturation, allowing time for the bacteria to differentiate into an acid-tolerant form (Amer and Swanson, 2005). A somewhat similar scenario is postulated for *Coxiella burnetii* replicative vacuoles and *Anaplasma phagocytophilum* bacterial replicative inclusions; both structures contain autophagosomal but not lysosomal markers (Beron et al., 2002; Niu et al., 2008; Romano et al., 2009), suggesting that the bacteria possess an as of yet unidentified mechanism to block or at least delay autophagosomal fusion with the lysosome. An important area of future research will be to identify specific bacterial factors that interfere with autophagosomal maturation; presumably, pharmacological inhibition of such targets would result in a substantial decrease in the survival of intracellular bacteria that seek refuge in "arrested"

Table 1. Roles of Autophagy in Protection against Microbes

Microbe	Host organism/ cell type	Autophagy genes or autophagy signals	Effects on host-pathogen interactions	References
VIRUSES				
RNA viruses				
Alphaviridae				
Sindbis virus	Mice (neurons)	*beclin 1*	Enforced neuronal Beclin 1 expression reduces CNS viral replication, neuronal cell death, and animal mortality	Liang et al., 1998
Rhabdoviridae				
Vesicular stomatitis virus	*Drosophila*	*Atg8*, *Atg7*, *Atg12*, *Atg18*	Autophagy gene silencing increases viral replication in vivo and decreases fly survival	Shelly et al., 2009
Tobamoviruses				
Tobacco mosaic virus (TMV)	Plants	*BECLIN 1*, *ATG3, ATG7*, *VPS34*	Autophagy gene silencing increases TMV local replication and spread of programmed cell death in vivo	Liu et al., 2005
DNA viruses				
Herpesviridae				
Herpes simplex virus 1 (HSV-1)	Mice	*beclin 1*	Role for autophagy gene in protection against lethal encephalitis inferred by neuroattenuation of mutant virus that cannot inhibit Beclin 1 autophagy function	Orvedahl et al., 2007
BACTERIA				
Gram-positive cocci				
Staphylococcus aureus	Mouse embryonic fibroblasts (MEFs)	*Atg5*	*Atg5* deletion inhibits bacterial degradation in autolysosomes, leading to delayed bacterial clearance and increased bacterial multiplication	Amano et al., 2006
Group A *Streptococcus*	MEFs	*Atg5*	*Atg5* deletion inhibits bacterial degradation in autolysosomes, leading to delayed bacterial clearance and increased bacterial multiplication	Nakagawa et al., 2004
Gram-positive bacilli				
Listeria monocytogenes	MEFs	*Atg5*	*Atg5* deletion increases replication of bacterial phospholipase mutants	Py et al., 2007
	Mice (macrophages)	*Atg5* (macrophages)	Macrophage *Atg5* required for in vivo resistance in mice	Zhao et al., 2008
	Drosophila	*Atg5, Atg1,* peptidoglycan-recognition protein (PGRP)	Autophagy gene silencing results in failure to control *L. monocytogenes* replication in hemocytes in vitro or in intact fly; PGRP required to signal autophagy-mediated resistance	Yano et al., 2008
Bacillus anthracis	Mouse macrophage cell line		Autophagy induction may protect cells against anthrax lethal toxin	Tan et al., 2009

Table 1. *Continued*

Microbe	Host organism/ cell type	Autophagy genes or autophagy signals	Effects on host-pathogen interactions	References
Gram-negative bacilli				
Burkholderia pseudomallei	Mouse macrophage cell lines and embryonic fibroblasts		Autophagy induction decreases intracellular bacterial survival (but no effect of *Atg5* deletion in MEFs on bacterial survival)	Cullinane et al., 2008
Helicobacter pylori	Human gastric epithelial cells		Autophagy inhibition increases vacuolating toxin stability and toxin-mediated cellular vacuolation	Terebiznik et al., 2009
	Human monocytic cells		Autophagy inhibition enhances and autophagy activation suppresses intracellular bacterial multiplication	Wang et al., 2009b
Pseudomonas syringae pv. tomato	*Arabidopsis thaliana*	*ATG6*	*ATG6* silencing in plants results in increased spreading programmed cell death and bacterial virulence	Patel and Dinesh-Kumar, 2008
Salmonella enterica	MEFs; epithelial cells	*Atg5*	*Atg5* deletion results in increased intracellular bacterial growth	Birmingham et al., 2006
Shigella flexneri	Dog kidney epithelial cells and MEFs	*Atg5* (in MEFs)	*IcsB* mutant bacteria are targeted by autophagy, leading to decreased intracellular bacterial multiplication	Ogawa et al., 2005
Vibrio cholerae	Human intestinal cell lines; MEFs	*Atg5* (in MEFs)	Autophagy protects against toxic effects of bacterial secreted pore-forming toxin, *Vibrio cholerae* cytolysin	Gutierrez et al., 2007
Mycobacteria				
Mycobacterium tuberculosis *Bacillus Calmette-Guerin*	Mouse and human primary macrophages and macrophage cell lines	*Atg5* (macrophages from *Atg5*$^{Flox/Flox}$ *LysM-Cre* mice)	Atg5 required for autophagic killing of mycobacteria in macrophages	Zhao et al., 2008
		IFNγ and immunity-related GTPases (mouse LRG-47, human IRGM), signaling through P2X7	Autophagy induction by immune signaling, starvation, or TOR inhibition increases mycobacterial targeting to phagolysosomes and decreases mycobacterial intracellular survival	Alonso et al., 2007; Biswas et al., 2008; Gutierrez et al., 2004; Singh et al., 2006
		Th1 cytokines activate and Th2 cytokines inhibit autophagy	IL-4 and IL-13 inhibit IFNγ- or starvation-induced autophagic elimination of mycobacteria	Harris et al., 2007
PROTOZOA				
Toxoplasma gondii	Mice (macrophages)	*Atg5*	Macrophage *Atg5* required for in vivo resistance in mice; mechanism believed to be autophagosome-independent recruitment of Irga6 to parasitophorous vacuole	Ling et al., 2006; Zhao et al., 2008

Table 2. Microbial Adaptations to Evade or Use Autophagy to Promote Survival or Replication

Microbe	Adaptation	Effects on host-pathogen interactions	Microbial virulence factor/mechanism	References
VIRUSES				
RNA viruses				
Picornaviridae				
Poliovirus	Infection induces formation of LC3-positive double membranes	Proposed mechanism for nonlytic virus egress	Poliovirus 2BC and 3 proteins induce GFP-LC3 colocalization with LAMP1	Jackson et al., 2005; Taylor and Kirkegaard, 2007
Coxsackievirus B3, B4 (CVB3, CVB4)	CVB3 infection induces early but not late stages of autophagy; CVB4 infection induces autophagy in neurons	May increase viral replication or yields	Calpain-dependent (CVB4)	Wong et al., 2008; Yoon et al., 2008
Flaviviridae				
Dengue virus	Infection induces autophagy	Increases viral yields; colocalization of LC3, viral dsRNA, and endosomal marker may indicate association of viral replication complex with amphisomes	Unknown	Lee et al., 2008; Panyasrivanit et al., 2009
Hepatitis C virus	Infection induces early but not late stages of autophagy in hepatocyte cell lines	Increases HCV replication (without colocalization of viral proteins and autophagosomes)	Activation of unfolded protein response	Ait-Goughoulte et al., 2008; Sir et al., 2008
Pestiviridae				
Bovine viral diarrheal virus	Incorporation of cellular LC3 into viral genome through RNA recombination	Enhances viral replication and viral cytopathic effects and causes lethal mucosal disease in cattle	Viral LC3 in genome facilitates viral polyprotein processing	Meyers et al., 1998
Orthomyxoviridae				
Influenza A virus	Infection increases autophagy and autophagic flux	May increase viral replication or yields	Unknown	Zhou et al., 2009
Reoviridae				
Rotavirus	Viral nonstructural protein NSP4 localizes with LC3 but not LAMP1	Postulated to play a role in viral morphogenesis, possibly by creating lipid membrane scaffold for formation of viroplasms	Unknown	Berkova et al., 2006
Lentiviridae				
HIV-1	Infection inhibits autophagy in primary CD4+ lymphocytes and in macrophage cell lines	Proposed mechanism of viral evasion of innate immunity	Unknown	Zhou and Spector, 2008
	Virus may utilize Atg proteins for replication in HeLa cells	Proposed mechanism of viral utilization of autophagic machinery for replication	Unknown	Brass et al., 2008
	Infection induces autophagy gene-dependent cell death in bystander cells	Proposed mechanism of CD4+ T cell depletion	HIV envelope protein triggers autophagy in bystander lymphocytes by binding to CD4 and CXCR4 through receptor signaling-independent mechanisms thought to involve fusogenic activity of gp41	Denizot et al., 2008; Espert et al., 2006

Table 2. *Continued*

Microbe	Adaptation	Effects on host-pathogen interactions	Microbial virulence factor/mechanism	References
	Infection induces early stages of autophagy and inhibits autophagosomal maturation in macrophages	Proposed mechanism for increasing HIV yields	HIV Gag interacts with the LC3 autophagy protein to augment Gag processing; HIV Nef binds to Beclin 1 and inhibits autophagosome maturation	Kyei et al., 2009
DNA viruses				
Herpesviridae				
Herpes simplex virus 1 (HSV-1)	Infection inhibits autophagy in neurons	Confers neurovirulence	HSV-1 protein ICP34.5 inhibits PKR signaling and binds to Beclin 1 to block autophagy	Orvedahl et al., 2007; Tallóczy et al., 2002
Bovine herpesvirus type 1 (BHV-1)	BHV-1 WT virus induces apoptosis in MDCK cells, whereas BHV-1 bICP0 mutant virus induces nonapoptotic cell death with autophagy	Proposed mechanism of regulating of cell death	BHV-1 bICP0 may inhibit autophagy	Geiser et al., 2008
Human cytomegalovirus (HCMV)	Infection inhibits autophagy in primary fibroblasts		Activates mTOR pathway and rapamycin-insensitive signals	Chaumorcel et al., 2008
γ-herpesviruses				
KSHV, murine γ-HV68	Viral Bcl-2-like proteins inhibit autophagy	Unknown	KSHV vBcl-2 and γ-HV68 M11 inhibit autophagy by binding to Beclin 1	Ku et al., 2008b; Pattingre et al., 2005; Sinha et al., 2008
Epstein-Barr virus (EBV)	EBV LMP1 protein induces autophagy	Proposed mechanism to decrease LMP1 levels and block its cytostatic effects on B cells	Unknown	Lee and Sugden, 2008
Hepadnaviridae				
Hepatitis B virus	HBV X protein transfection enhances autophagy in hepatocytes	Unknown	HBV X protein increases Beclin 1 promoter activity	Tang et al., 2009
BACTERIA				
Intracellular pathogens				
Gram-positive bacilli				
Listeria monocytogenes	Prevents or evades autophagic uptake	May enable cytosolic bacteria to escape lysosomal degradation	Bacterial Prf1-regulated factors, including ActA and phospholipases, function in autophagy evasion	Birmingham et al., 2007; Py et al., 2007
	Prevents acidification of autophagy-dependent spacious *Listeria*-containing phagosomes (SLAPs)	May allow persistent intravacuolar infection in host macrophages	Listeriolysin O, a pore-forming toxin and virulence factor, is essential for SLAP formation	Birmingham et al., 2008
Gram-negative coccobacilli				
Coxiella burnetii	*Coxiella* phagosomes (CPh) and *Coxiella* replicative vacuoles (CRVs) colocalize with LC3 autophagy marker	Interaction between *Coxiella* and autophagic pathway may delay lysosomal enzyme recruitment to CPh	Bacterial protein synthesis required for CRV formation and LC3 colocalization	Beron et al., 2002; Romano et al., 2009

(*Continued on next page*)

Table 2. *Continued*

Microbe	Adaptation	Effects on host-pathogen interactions	Microbial virulence factor/mechanism	References
Francisella tularensis	*Francisella* enters LC3-positive vacuoles after intracytoplasmic replication in murine macrophages	Postulated to allow cytoplasmic bacteria to regain access to endocytic compartment to promote bacterial egress through exocytosis (also proposed to be source for tularemic MHC II antigen presentation)	Unknown	Checroun et al., 2006; Hrstka et al., 2007
	Downregulates expression of autophagy genes (*ATG4A*, *5*, *7*, *12*, *16L2*, *BECN1*) during cytoplasmic replication in human monocytes	Postulated to allow cytoplasmic bacteria to escape autophagic capture	Unknown	Butchar et al., 2008
Gram-negative bacilli				
Burkholderia pseudomallei	Prevents or evades bacterial colocalization with LC3 autophagosome marker	May increase intracellular bacterial survival	*bopA* gene required for bacterial evasion of autophagy	Cullinane et al., 2008
Legionella pneumophila	*Legionella* replication vacuoles initially have the autophagy protein Atg7 and ER markers and later have LC3 and lysosomal markers	Postulated that bacteria slows autophagosome maturation, allowing time for bacteria to differentiate to acid-tolerant form	Soluble bacterial type IV secretion products are sufficient to induce autophagy	Amer and Swanson, 2005
Shigella flexneri	Evades autophagic capture of cytoplasmic bacteria in epithelial cells	Enhances intracellular multiplication in epithelial cells	Bacterial T3SS effector IcsB blocks autophagic targeting of the *Shigella* protein VirG that is required for actin-based motility, through competitive inhibition of binding to Atg5 autophagy protein	Ogawa et al., 2005
	May induce autophagy in macrophages	May prevent pyroptotic death in macrophages	Mechanism of autophagy induction in macrophages unknown and negatively regulated by caspase-1 and Ipaf	Suzuki et al., 2007
Other				
Anaplasma phagocytophilum	Bacterial replicative inclusions contain autophagosomal markers (Beclin 1, LC3) but not lysosomal markers	May shield bacteria from endolysosomal pathway and provide niche to enhance replication	Unknown	Niu et al., 2008
Chlamydia trachomatis	Chlamydial inclusion bodies juxtaposed to LC3-positive structures	Postulated that bacteria utilize nutrients derived from autophagy to promote intracellular growth	Unknown	Al-Younes et al., 2004
	Autophagy upregulated upon active bacterial growth	Postulated that bacteria have evolved to neither need to inhibit nor to utilize autophagy (since growth is unaffected by augmentation or inhibition of autophagy)	Unknown	Pachikara et al., 2009

Table 2. *Continued*

Microbe	Adaptation	Effects on host-pathogen interactions	Microbial virulence factor/mechanism	References
Extracellular pathogens				
Gram-negative bacilli				
Vibrio parahaemolyticus	Induces host autophagy	Unknown; proposed that bacteria may utilize nutrients released by autophagically active cells for their proliferation	Bacterial T3SS effectors injected into host cell required for autophagy induction	Burdette et al., 2008
Bacteria that can invade intracellularly				
Gram-positive cocci				
Staphylococcus aureus	Resides in autophagosomes after HeLa cell invasion	Postulated to promote intracellular bacterial replication, escape into host cytoplasm, and host cell killing	Bacterial localization with autophagosomes requires *agr*, a global regulator of *S. aureus* virulence	Schnaith et al., 2007
Gram-negative bacilli				
Porphyromonas gingivalis	Traffics through autophagosome-like structures in human coronary artery endothelial cells	May prevent formation of autolysosomes and pathogen destruction	Unknown	Dorn et al., 2001
PROTOZOA				
Entamoeba histolytica (*Eh*) *Entamoeba invadens* (*Ei*)	Parasite autophagy occurs during proliferation and encystation (*Ei*)	May facilitate growth of trophozoites and encystation (*Ei*)	*Eh* and *Ei* possess Atg8 but not Atg5-Atg12 autophagy protein conjugation systems	Picazarri et al., 2008
Leishmania amazonensis (*La*) *Leishmania major* (*Lma*) *Leishmania mexicana* (*Lme*)	Parasite may exploit IFNγ-induced host autophagy response (*La*)	Increased intracellular *La* (but not *Lma*) parasite load in macrophages during starvation or IFNγ-induced autophagy in mouse strain-specific manner	Mechanism of increased parasite load with host autophagy induction unknown	Pinheiro et al., 2008
	Parasite autophagy promotes differentiation and survival during starvation (*Lma, Lme*)	Parasite autophagy important for transformation to mammalian infective form and parasite virulence	*Lma* Vps and Atg proteins function in endosome sorting (Vps4), autophagy (Vps4, Atg4, Atg8 homologs, Atg12), and differentiation (Vps4, Atg4); *Lme* lysosomal cysteine peptidases (CPA, CPB) required for autophagy and differentiation	Besteiro et al., 2006; Williams et al., 2006; Williams et al., 2009
Toxoplasma gondii	Induces autophagy	Autophagy promotes parasite intracellular proliferation in nutrient-limiting conditions in vitro	Calcium-, Atg5-, and Beclin 1-dependent but Tor-independent	Wang et al., 2009b
Trypanosoma cruzi	Uses host LC3-positive membranes for cellular entry	Host autophagy enhances parasite invasion	Mechanism of host autophagic membrane recruitment to parasite unknown	Romano et al., 2009
	Parasite autophagy promotes differentiation/ development and survival during starvation	Parasite autophagy important for parasite maintenance and survival	Parasite autophagy mediated by conserved Atg proteins (Atg8) but not Atg5-Atg12 protein conjugation system and TOR inhibition	Alvarez et al., 2008

(*Continued on next page*)

Table 2. *Continued*

Microbe	Adaptation	Effects on host-pathogen interactions	Microbial virulence factor/mechanism	References
FUNGI				
Aspergillus fumigatus (*Af*)	Fungal autophagy protein functions in metal ion homeostasis		*Af* Atg1 required for metal ion homeostasis	Richie et al., 2007
Cryptococcus neoformans (*Cn*)	Fungal autophagy activated during infection in mammalian cells	Increases fungal multiplication and lethal infection in mouse model	*Cn* Class III PI3K/Vps34 and Atg8 required for fungal autophagy and virulence	Hu et al., 2008
Magnaporthe grisea (*Mg*)	Fungal autophagy activated during infection in plants (rice)	Required for fungal spore (conidium) autophagic cell death, fungal invasion, and pathogenesis in plants	*Mg* Atg8 required for conidial cell death; *Mg* Atg1 required for appressorium invasion in plants	Liu et al., 2007; Veneault-Fourrey et al., 2006

autophagosomes. It also will be important to determine whether bacterial evasion of autophagosomal maturation truly contributes to bacterial pathogenesis using in vivo models of infection with bacteria that contain mutations in putative antiautophagosomal maturation bacterial factors. Interestingly, a known *Listeria* virulence factor, the pore-forming toxin listeriolysin O is required for the formation of structures termed "spacious *Listeria*-containing phagosomes," which require the autophagy machinery for their formation and fail to acidify, thus potentially allowing persistent intravacuolar infection in host macrophages (Birmingham et al., 2008).

The literature to date suggests that several families of viruses may also block autophagosomal maturation. Poliovirus infection induces the formation of LC3-positive double-membraned structures (initially thought to serve as a scaffold for viral RNA replication and later also postulated to play a role in nonlytic viral egress from the cell) (Jackson et al., 2005; Taylor and Kirkegaard, 2007), but does not induce the formation of autolysosomes. Similarly, coxsackie B virus and hepatitis C virus induce early stages of autophagy, but not late stages of autophagy, in virally infected cells (Ait-Goughoulte et al., 2008; Sir et al., 2008; Wong et al., 2008; Yoon et al., 2008), and the rotavirus nonstructural protein NSP4 colocalizes with LC3 but not lysosomal markers (Berkova et al., 2006). One obvious interpretation of these findings is that these viruses possess specific mechanisms to prevent autophagosomal fusion with the lysosome. However, it is also possible that the lipidation of LC3 (detected by western blot analysis) or membrane localization of lipidated LC3 (detected by light microscopy), which are the usual markers of "early autophagy," do not truly represent the formation of classical autophagosomes that will invariably fuse with late endosomes/lysosomes in the absence of specific autophagosomal maturation-blocking factors. This possibility is supported by evidence that LC3 dots may represent the targeting of LC3 to structures other than autophagosomes, including phagosomes, double-membrane scaffolds for viral RNA replication complexes, or protein aggregates as well as by evidence that "autophagy" proteins may possess or may be co-opted for autophagy-independent functions (Virgin and Levine, 2009).

The identification of specific viral factors that antagonize autophagosomal fusion with the lysosome will be important to distinguish between these possibilities, and as suggested for bacteria, the identification of such factors may represent novel targets for antiviral therapeutics. Along these lines, a recent study reports that HIV-1 Nef, an important pathogenic factor required for HIV disease progression, inhibits autophagic maturation in macrophages through its interaction with the autophagy protein, Beclin 1 (Kyei et al., 2009) (Figure 3, bottom panel). This function of Nef inhibits autophagic degradation of HIV biosynthesis intermediates or virions, thereby enhancing HIV virus yields. These observations provide the first demonstration that a viral virulence factor can target autophagosomal maturation. They also underscore the importance of Beclin 1 as a core component of the autophagic machinery (Figure 1C) that functions both in autophagy initiation (which is antagonized by herpesvirus-encoded proteins) and autophagosomal maturation (which is antagonized by HIV Nef). In future studies, it will be interesting to determine whether Nef has a similar function in virally-infected CD4 T cells and whether this function of Nef contributes to HIV pathogenesis. It will also be interesting to determine whether other viral virulence proteins block the autophagosomal maturation function of Beclin 1 and/or other autophagy proteins.

Microbial Avoidance of Autophagic Capture

Another strategy employed by intracellular bacteria to escape the undesirable fate of lysosomal destruction is to avoid capture by the autophagosome (Figure 3, bottom panel). While bacteria that reside in phagosomes or other vacuolar compartments may seek to avoid lysosomal maturation, the avoidance of autophagic capture may be particularly important for intracellular bacterial pathogens that escape into the cytoplasm. As noted above, evasion of autophagic capture has been described for at least three different intracytoplasmic bacteria, including *Shigella flexneri*, *L. monocytogenes*, and *Burkholderia pseudomallei* (Table 2). The first described, most classic example is that of *Shigella* escape from autophagy, which seems to utilize a particularly intriguing scheme to escape autophagic envelopment (Ogawa et al., 2005). *Shigella* possesses a surface protein, VirG, required for actin-based motility that binds to the autophagy protein Atg5 and thereby targets *Shigella* to the autophagosome. However, the bacterial T3SS effector, IcsB, competitively binds to Atg5, thereby camouflaging its own bacterial target molecule VirG from autophagic capture. It is not yet known whether

bacterial T3SS effectors from other organisms exert similar functions or why such a seemingly inefficient mechanism—i.e., the need for one bacterial protein to essentially undo the actions of another protein—evolved. Perhaps we are seeing microbial adaptation to mammalian autophagy in evolutionary progress, and *Shigella* will ultimately undergo further adaptations in VirG itself to avoid capture by Atg5. As the specific identities of host cell-derived molecular tags for microbial targeting to the autophagosome become known, another interesting question will be whether intracytoplasmic bacteria also possess mechanisms to block such host cell tags, in addition to their own microbial tags.

Less is known about whether viruses possess specific mechanisms to avoid autophagic capture. In the case of hepatitis C virus (HCV) infection, two studies have shown that the virus induces early stages of autophagy at least in part by activating the unfolded protein response (Ait-Goughoulte et al., 2008; Sir et al., 2008). Yet, late stages of autophagy (i.e., the formation of autolysosomes) or the colocalization of HCV with markers of early autophagosomes are not observed. These observations imply that HCV may not only possess mechanisms to block autophagosomal maturation (similar to what presumably happens with poliovirus, coxsackie B, rotavirus, and HIV) but also mechanisms to block autophagic capture of HCV. Particularly for viruses such as HCV that establish persistent infection, it seems likely that future studies will identify specific molecular mechanisms by which they evade autophagic capture.

Microbial Utilization of the Host Autophagic Machinery for Intracellular Survival, Replication, or Cellular Egress

In parallel with strategies to block autophagy induction, autophagolysosomal maturation, or autophagic capture, microbes also have evolved mechanisms to utilize aspects of host autophagy to their own advantage (Figure 3, bottom panel). Postulated benefits of host autophagy for microbes include the promotion of viral replication or morphogenesis via utilization of the autophagic machinery, the shielding of bacteria from the endolysosomal pathway via the utilization of autophagosomes as a protective intracellular niche, and the enhanced survival or growth of bacteria, fungi, or parasites through the provision of autophagy-generated nutrients. Although in vitro data support some of these postulates, whether such mechanisms are important in microbial pathogenesis in vivo remains to be explored, and there is no current evidence that autophagy gene deletion in the host attenuates microbial disease. Thus, unlike the role of host autophagy in protection against infection (in which autophagy gene deletion exacerbates microbial disease [Table 1]) or microbial antagonism of autophagy (in which, for example, HSV-1 evasion of autophagy is essential for lethal encephalitis [Table 2]), the physiological significance of microbial utilization of autophagy for "promicrobial" effects remains to be established.

Several viruses are believed to induce autophagy to foster their own replication, morphogenesis, cellular egress, or pathogenicity (Table 2). One theory, originally developed in the context of poliovirus research, is that double-membrane autophagosome-like structures (that contain the autophagy protein LC3) serve as lipid membrane-scaffolds that enhance viral replication (Jackson et al., 2005). In a somewhat analogous fashion, the rotavirus NSP4 protein, a protein with pleiotropic functions in viral morphogenesis and pathogenesis, colocalizes with LC3-positive vesicular compartments and is postulated to play a role in the formation of viroplasms and/or the packaging of the rotavirus genome or transcription (Berkova et al., 2006). However, in poliovirus-infected cells, siRNA-mediated knockdown of the autophagy genes, *LC3* and *Atg12*, markedly inhibits the release of infectious virus while only minimally affecting viral replication (Taylor and Kirkegaard, 2007), suggesting that the primary function of poliovirus's utilization of the autophagic machinery may be for nonlytic viral release. A similar theme is emerging with HIV infection in macrophages, as pharmacological stimulation of autophagy increases extracellular viral yields, whereas pharmacological or genetic inhibition of autophagy decreases extracellular viral yields (Kyei et al., 2009). Interestingly, the HIV Gag precursor protein directly interacts with the LC3 autophagy protein, and this interaction may facilitate Gag processing and HIV biogenesis, suggesting a potential biosynthetic, rather than catabolic, role of the host autophagic machinery. For some other viruses, such as HCV, which induces early stages of autophagy, but which does not colocalize with autophagosomes or autophagosomal markers (Ait-Goughoulte et al., 2008; Sir et al., 2008), it is completely unknown how autophagy induction may function to increase viral RNA levels. Beyond this utilization of autophagic machinery for enhancing viral production, it is also worth noting that, in some contexts, the autophagic machinery may play a role in features associated with viral pathogenesis; for example, at least in vitro, HIV-induced death of bystander lymphocytes requires the autophagy genes *Atg7* and *beclin 1* (Espert et al., 2006).

As noted above, autophagosomes or cellular compartments that seem to require autophagic machinery for their formation may provide a safe "haven" for several bacteria, including *L. monocytogenes*, *C. burnetii*, *L. pneumophila*, *A. phagocytophilum*, and *P. gingivalis* (Table 2). In parallel with suppression of autophagosomal fusion with lysosomes and/or acidification of pathogen-containing compartments, the bacterial autophagosomal-like compartments may enable the bacteria to persist (and potentially multiply intracellularly) in a nonacidic compartment. It will be interesting to further determine the cellular fate, ability to persist intracellularly, and ability to replicate intracellularly of such organisms in target cells that lack critical components of the autophagy machinery. The enhanced pathogenicity of *L. monocytogenes*-infected mice with macrophage-specific deletion of *Atg5* (Zhao et al., 2008) and the wild-type levels of replication observed in *L. pneumophila*-infected *Dictyostelium discoideum* lacking *Atg1*, *Atg5*, *Atg6*, *Atg7*, or *Atg8* (Otto et al., 2004) suggest that speculations based upon in vitro studies regarding the "microbe-friendly" role of autophagy in microbial replication may not always correlate with the actual role of autophagy in microbial pathogenesis in vivo. Besides shielding bacteria from the endolysosomal pathway, it has also been proposed that bacterial localization to LC3-positive compartments may allow cytoplasmic bacteria to regain access to the endocytic compartment to promote bacteria egress through exocytosis (i.e., during *Francisella* infection) (Checroun et al., 2006).

Another theory is that autophagy helps "feed" intracellular pathogens, particularly those that reside in sequestered

vacuoles that lack access to cytoplasmic nutrients. This concept emerged decades ago, when it was first reported that rickettsiae induce the formation of autophagosomes in polymorphonuclear cells (Rikihisa, 1984), although a later study suggested that autophagy may be involved in killing rickettsia in endothelial cells (Walker et al., 1997). In recent years, it has been postulated that autophagy may function in infected cells to deliver nutrients to *Chlamydia* (Al-Younes et al., 2004), *Vibrio parahaemolyticus* (Burdette et al., 2008), and *T. gondii* (Wang et al., 2009a), thereby enhancing intracellular pathogen survival and proliferation. As with other beneficial functions of autophagy for microbes, the physiological relevance of these findings is not yet known. Moreover, somewhat akin to the discrepant conclusions between in vitro and in vivo studies with *Listeria* and *Legionella*, although *T. gondii* has impaired growth in *Atg5*-deficient MEFs, leading to the conclusion that host cell autophagy plays a role in promoting parasite growth through nutrient recovery (Wang et al., 2009a), *T. gondii* has increased virulence in mice with macrophage-specific deletion of *Atg5* (Zhao et al., 2008).

Microbial Autophagy

Pathogens such as protozoans, fungi, and helminths are themselves eukaryotes and hence have their own autophagic machinery (Figure 3, top right panel). An emerging area of research in microbial pathogenesis is the function of autophagy directly within such eukaryotic pathogens. The molecular machinery of autophagy was originally identified in genetic screens performed in the yeast *S. cerevisiae*, and autophagy is also present in disease-causing fungi, as well as in disease-causing protozoans and helminths. Recent analyses of autophagy in eukaryotic pathogens reveals some interesting common themes, including (1) the identification of potential differences between microbial autophagy and host autophagy in terms of what constitutes the core autophagic machinery and (2) the identification of an essential role for microbial autophagy in stress adaptation and development, both of which may be important for mammalian infectivity and virulence.

The Molecular Machinery of Microbial Autophagy

A critical question is whether the autophagic machinery truly differs in certain pathogenic protozoans from that found in yeasts and mammalian cells, or whether the lack of apparent structural homology in some autophagy genes has resulted in the mere failure of "genome mining" to identify conserved components. In yeast (*S. cerevisiae*), 17 genes encoding proteins comprising the core machinery of autophagy have been identified (Suzuki and Ohsumi, 2007), most of which are conserved in higher eukaryotes. Interestingly, two protozoan parasite pathogens, *Entamoeba* and *Trypanosoma cruzi*, are reported to contain all of the major genes of the Atg8 conjugation system (e.g., *Atg3*, *Atg4*, *Atg7*, *Atg8*) but to lack the entire Atg5-Atg12 conjugation system (Alvarez et al., 2008; Picazarri et al., 2008). This observation raises the interesting possibility that either the Atg5-Atg12 protein conjugation system is not absolutely essential for autophagy or that autophagy in these organisms is somehow qualitatively different from that in other eukaryotic organisms. However, a recent study found an atypical Atg12-like protein encoded by *Leishmania major*, which, when truncated before a scissile C-terminal glycine, complements Atg12 deficiency in yeast, as well as more typical Atg5 and Atg10 proteins that restore autophagy in *atg5*Δ and *atg10*Δ yeast (Williams et al., 2009). These three *Leishmania*-encoded Atg12, Atg5, and Atg10 proteins have syntenic homologs in *Trypanosoma bruci* and *Trypanosoma cruzi* (Williams et al., 2009), suggesting that perhaps the Atg5-Atg12 protein conjugation systems are present but have been "missed" in certain protozoan organisms. Future studies are required to address this possibility and determine whether or not fundamental differences exist between the core autophagic machinery in certain protozoan pathogens and other eukaryotic organisms.

Microbial Autophagy in Microbial Pathogenesis

Two major functions of microbial autophagy may be relevant to microbial pathogenesis: the requirement for fungal and protozoan autophagy for survival during nutrient stress and the requirement for protozoan autophagy in developmental transitions to mammalian infective forms. Of potential medical relevance, autophagy in the dimorphic human fungal pathogen, *Cryptococcus neoformans*, is important for intracellular survival in macrophages (which is postulated to represent nutrient self-supplying function in the environment of a nutrient-poor phagosome) and for virulence, since deletion of the fungal class III PI3K *VPS34* gene or *ATG8* knockdown results in decreased replication and lethality in a mouse model of cryptococcosis (Hu et al., 2008) (Figure 3, top right panel). Thus, it will be interesting to see whether it is possible to specifically target the cryptococcal but not mammalian autophagic machinery in the treatment of human cryptococcal disease. For protozoan parasites, the autophagic machinery has been shown as essential for (Figure 3, top right panel): (1) *Entamoeba* to undergo the developmental transition from the trophozoite to the cyst stage, a process essential for its transmission and reinfection (Picazarri et al., 2008); (2) *Leishmania* to differentiate into the infective metacyclic promastigote form (Besteiro et al., 2006; Williams et al., 2006, 2009); and (3) *Trypanosoma cruzi* to differentiate into the infective metacyclic trypomastigote (Alvarez et al., 2008). These high-density and/or low-nutrient-dependent stress-induced differentiation events allow the parasites to transition from a form adapted to survive in insects to a form capable of infecting mammalian hosts. While it is not yet clear whether autophagy continues to be important for parasite differentiation inside the mammalian host, *Leishmania* mutants lacking lysosomal cysteine peptidases and defective in autophagy are also defective in differentiation and lack virulence in macrophages and mice. Efforts to target lysosomal cysteine peptidases and/or the Atg4 protease as treatment of *T. cruzi* caused Chagas disease, and Leishmanias are being considered (Alvarez et al., 2008; Williams et al., 2006).

Conclusion

A number of key recent studies have expanded the scope of autophagy's role in immunity from its original incarnation as a putative antimicrobial defense mechanism to a full-range immunological process that participates in innate immunity, adaptive immunity, and inflammation. The ability of autophagy to control intracellular microbes, initially documented only in vitro or in ex vivo experiments, has now been confirmed in in vivo models of infection. In support of the importance of

autophagy as a critical defense against microbes, it is now evident that highly evolved intracellular pathogens have specialized antiautophagy adaptations that allow intracellular parasites to prevent, block, or elude autophagic elimination. Autophagy is intertwined with innate immunity regulators and can be stimulated by agonists of innate immunity receptors with feedback loops that amplify or inhibit recognition of microbial products or output signals. At an adaptive immunity level, autophagy controls T cell populations by shaping the naive T cell repertoire in the thymus and antigen-specific T cells in the periphery. B cells are likewise affected, while effector cells such as macrophages can be activated through autophagy. When autophagy is aberrant or "out of sync" in central immunological organs versus the periphery, pathological inflammation can ensue. Coming full circle, autophagy as a cell-autonomous defense of the host cell has met its adversarial counterpart in autophagy that occurs within protozoan and fungal pathogens. Apparently, autophagy works tirelessly to protect the master eukaryotic cell, often on both sides of the host-pathogen relationship.

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