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Selective Autophagy: Xenophagy

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Abstract

Xenophagy is an autophagic phenomenon that specifically involves pathogens and other non-host entities. Although the understanding of the relationship between autophagosomes and invading organisms has grown significantly in the past decade, the exact steps to confirm xenophagy has been not been thoroughly defined. Here we describe a methodical approach to confirming autophagy, its interaction with bacterial invasion, as well as the specific type of autophagic formation (i.e. autophagosome, autolysosome, phagolysosome). Further, we argue that xenophagy is not limited to pathogen interaction with autophagosome, but also non-microbial entities such as iron.

Keywords

symbiophagy; inflammasome; ferritinophagy; LC3 Associated Phagocytosis; UPEC; infection

1. Introduction

Autophagy is a response of a number of cellular mechanisms focused on degradation and recycling of damaged organelles and proteins [1, 2]. Autophagy is the sum of a complex signaling pathway that leads to the generation of a double-membrane organelle [1]. This organelle called an autophagosome can consume damaged organelles and proteins [1]. These components can be degraded after fusion of the autophagosome with a lysosome (creating an autolysosome) [1]. It must be stressed that autophagic activity is measured by autophagic flux and not the overall amount of any autophagy structure alone [1]. As stated in the Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy, accumulation of autophagosomes or autophagy proteins may serve as a red herring [1]. The

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experimental steps discussed below will help to confirm not just autophagic response, but also the presence of autophagic flux.

Recently, there has been a growing interest in selective autophagy which designates specific targets for the autophagic response [2–5]. These targets include organelles such as the mitochondria (mitophagy) and peroxisomes (pexophagy) to name a few [2, 6–10]. Autophagy of foreign entities such as bacteria, viruses, and other pathogens is termed xenophagy, the focus of the current review. [2, 8–18]. Selective autophagy has grown in recent years to include very specific cellular events including symbiophagy (autophagic consumption of symbiotes) and ferritinophagy (processing of iron via autophagosomes). Since xenophagy covers a broad range of foreign pathogens, we have focused on an in-depth review of methods and techniques specific for bacteria related autophagy. We also introduce the concept of ferritinophagy as a potential subtype of xenophagy.

1.1 Definition and history of xenophagy

Xenophagy is an evolutionarily conserved mechanism classically observed to target and remove pathogens after host cellular invasion [12, 14, 19]. Though autophagy is a well-studied cellular mechanism surprisingly, xenophagy is a relatively newly observed phenomenon [13]. The use of the word can be found as early as the 1980s in literature, but concrete signaling studies came about only at the beginning of the 21st century [13, 19]. This suggests that the specific mechanisms for confirming xenophagy have not reached a full consensus or is not fully understood. Indeed, the most common means of elucidating xenophagy is utilizing the standardized experimental producers of autophagy [1]. While these steps are critical to confirm the formation of autophagosomes related to pathogenic invasion, further measures are needed to validate both the presences of the pathogen in the membrane as well as the specific type of autophagic membrane.

1.2 Different forms of xenophagy

Pathogens have evolved to evade or subvert xenophagic activity by inhibition of autophagic response [10, 11, 15, 20–23]. Interestingly, this subversion may have helped lead to the development of symbiotic bacterial relationships [24]. Many pathogens have evolved means of avoiding phagocytosis and autophagic consumption [5, 20, 21, 25–28]. *Brucella abortus*, for example, has been shown to utilize endosomal trafficking to enter the cell, but is able to avoid consumption by autophagosomes [29]. In contrast, uropathogenic *Escherichia coli* (UPEC) (the primary pathogen involved in urinary tract infections, [UTIs]) hijacks the autophagic pathway for prolonged intracellular survival within quiescent intracellular reservoirs (QIRS) [27, 28, 30]. Interestingly, these QIRs exist in autophagosomes which would traditionally seem hostile to the pathogen instead of a source of refuge. This is hypothesized to lead to recurring UTIs in patients [30]. Similarly, a number of other bacteria seek out autophagy for self-preservation [29, 31], while others avoid autophagic consumption by mechanisms including the release of toxins [32–35]. One group has found that increased stress of organisms with bacterial symbiosis leads to increased autophagic consumption of the bacteria (symbiophagy) leading to loss of symbiosis and potential cell death of the host [24, 36]. We refer the reader to an excellent and comprehensive review by Pareja et. al. for further examples [37].

Though xenophagy traditionally refers to pathogenic and viral invasion, the term “xeno-” refers to any foreign object including metals. Recently, a number of papers have shown evidence that iron regulation is processed and regulated by autophagy [7, 38–40]. This activity has recently been coined ferritinophagy [39]. We propose that this newly found autophagy activity should be considered as a form of xenophagy since it’s a response to a foreign body (xeno) and not the host (auto). Although ferritinophagy responds to a host protein (ferritin), the mechanism of activation only occurs due to an outside stimulus (iron).

Due to the large array of factors leading to induction and formation of xenophagy, it is important to experimentally elucidate which form is being observed. In this review, we will address the important mechanisms to observe and the experimental steps required to confirm xenophagy as well as the specific type. We will first address autophagy basics, followed by how to confirm a pathogen is the cause of autophagic response, and then address how to experimentally distinguish between induction of macroautophagy, autophagolysosome formation, and LC3-Associated Phagocytosis (LAP). Finally, we will briefly address how to identify ferritinophagy. It is advisable for anyone interested in autophagy research, to also utilize the review article “Guidelines for the use and interpretation of assays for monitoring autophagy” by Klionsky et. al. [1]. This incredibly detailed review is a compilation of techniques for validating autophagy agreed upon by majority of the leaders in the field.

2. Methods to detect Xenophagy

2.1. LC3 conversion (aka flux)

Pathogens by nature can enter a cell via phagocytosis [5, 41–43]. *Brucella* for example utilizes phagocytosis to gain entry to the intracellular environment [29]. This process leads to a phagosome inside the cell containing the invading pathogens [42]. Lysosomes can fuse with the phagolysosome and lead to degradation of the pathogen thus forgoing full autophagy [42]. A key difference between phagolysosomes and autolysosomes is the involvement of LC3-II proteins [42].

The critical steps in confirmation of autophagy are showing evidence of increased autophagic protein response and turnover of autophagosomes (i.e. fusion with lysosomes and degradation of the autolysosome) [1, 44]. Protein response is classically determined via LC3-I conversion to LC3-II [1, 45]. LC3 is a light chain protein involved in microtubule function and expressed throughout all tissue types [1, 44, 45]. The formation of a phagophore induces conversion of LC3-I to LC3-II which binds to the forming autophagosome providing structural stability [1, 45]. It is only after degradation of LC3-II in the autolysosome that true autophagic flux occurs [1]. After degradation of the autolysosome, LC3-II on the cytosolic side is recycled back into LC3-I and re-utilized while LC3-II from the luminal side is degraded [1, 45].

LC3 conversion upon pathogen association can be detected via western blot analysis. Samples with and without pathogenic challenge could be processed for western blot analysis. Conversion between LC3-I (18kD) protein to LC3-II (16kDa) is observable but requires careful handling. The gels should be around 12% or higher and run until the samples are near the end of the gel. This allows for full separation of the bands. To improve

band clarity, it is advisable to run the gel at a low voltage (100 volts or lower) and to use cold (4C) running buffer. Both of these steps will improve band clarity and reduce sheering. Finally, it is advisable to not freeze your protein lysates before running them on a gel. LC3-I stability is particularly sensitive to degradation and freezing of the sample will lower detection levels. It is advisable, to not rely on modulation of LC3-I when determining autophagic activity. LC3-II in contrast, is more stable due to its phosphatidylethanolamine conjugate and thus more reliable [1]. As such, increased LC3-II is a strong indication of autophagic activation.

An additional assay for LCII conversion is via an EGFP-LC3 immunofluorescence assay [1]. An EGFP plasmid can be transfected into cells and monitored before and after addition of pathogenic bacteria. A number of EGFP-LC3 plasmids are available for purchase through suppliers such as Addgene (Cambridge, Massachusetts). EGFP-LC3 will appear diffuse as LC3-I but upon conversion to LC3-II it appears as puncta. It is advisable to have a high threshold (around 20 punctae per cell) when confirming autophagy activation. One essential element is to ensure that the level of LC3 staining is significantly higher than background. Another useful assay is the use of a mRFP/mCherry-GFP-LC3 plasmid. The dual colors help to determine the location of the autophagosome. The GFP portion of the plasmid is sensitive to acidification present in the lysosome (i.e. after an autophagosome fuses with a lysosome). In contrast, the RFP/mCherry portion is stable under acidic conditions [1]. Thus co-localization of the two colors (i.e. yellow) would indicate the autophagosomes have not fused with lysosomes while only red staining would infer autophagic turnover.

2.2 Inhibition of autophagosome-lysosome fusion

Autophagy involves specific mechanisms for the formation of the phagophore (the early stage of an autophagosome) and fusion with the lysosome [1, 44]. The type of membrane formation is particularly critical in xenophagy as formation of the membrane structure around the pathogen is defined differently depending on its origin [42]. This process involves the formation of a double-membrane organelle from a phagophore. This collects damaged protein, organelles, and (in the case of xenophagy) pathogens to breakdown and recycle [2, 13, 19, 46]. Autophagosome proteins (termed ATG gene/proteins) help elongate the phagophore leading to the formation of a double-membrane organelle. This structure is composed of LC3-II that was converted from LC3-I. The final step involves fusion with the lysosome leading to the creation of an autolysosome allowing for breakdown of the engulfed components [1] (Figure 1).

It must be stressed, that increased LC-II is indicative of increased autophagosomes but not autolysosomes necessarily (Figure 1). Indeed, autophagosomes may be forming and accumulating, but not necessarily containing sequestered pathogens [14, 29, 47]. The presence or absence of microbes is important to determine for proper interpretation of data. A number of assays have been developed to help elucidate the nature of the autophagic response observed.

2.2.1—The classical technique is inhibition of lysosomal fusion via chloroquine or bafilomycin-1A [1]. A comparison between samples treated with the inhibitor alone and the

inhibitor with the pathogen of interest is required to determine the autophagic flux. Both inhibitors disrupt fusion of lysosomes and autophagosomes by altering the protein pump system of the lysosome [1]. Specifically, the inhibitors block vacuolar type H(+)-ATPase preventing acidification of lysosomes. The inhibition of fusion in turn leads to an accumulation of LC3-II (i.e. autophagosomal accumulation) [1]. Further accumulation of LC3-II in the experimental group compared to the untreated (with inhibitor only) would imply autophagic turnover was occurring.

2.3 Xenophagy Pathway markers for western blotting

Other markers of xenophagy that could be used for western blot include p62, ATG proteins, LAMP1/2, and upstream markers such as Beclin-1, mTOR, and AMPK [1, 48]. Increased LAMP 1/2 activity is indicative of increased co-localization of lysosomes with autophagosomes. This indirectly suggests an increase of autophagosome fusion with lysosomes. Lysosome detection can also be determined utilizing fluorescent tags such as lysotracker. An increase of stained lysosomes (detected through immunofluorescence imaging) would support changes observed in LAMP 1/2 [1, 48]. Alterations in p62 levels are often utilized as further confirmation of the LC3-II data [1]. p62 functions as a ubiquitin-binding protein and as such tags specific proteins for degradation via autophagy [49]. Since p62 is tagged to the damaged proteins and organelles, it is also degraded via autolysosomes [48]. The degradation leads to a decrease in p62 levels that can be observed via western analysis. An increase of LC3-II typically occurs in tandem to a decrease in p62. However, other reports have shown p62-independent autophagic induction suggesting additional roles for p62 [49, 50]. It is important to consider that activation of autophagic proteins does not necessarily infer autophagy. A number of studies have shown invading pathogens can lead to induction of individual autophagic proteins (ATGs) without inducing autophagy [30, 51–53]. It is only through rigorous experimental confirmation that autophagy can be directly contributed to bacterial invasion.

2.4 Genetic modulation of xenophagy (siRNA studies)

Autophagosome assembly proteins (ATG proteins) inherently seem useful in determining increased autophagosome activity. However, most of these proteins are stable and changes in overall level of ATG proteins often remain unchanged. A better alternative is to inhibit production of these proteins via siRNA (small interfering RNA) or shRNA (small hairpin RNA) transiently. For example, siRNAs against specific inflammatory pathways downstream of xenophagy can reveal whether xenophagic clearance of the pathogen is independent or dependent of said pathways.

Knockdown of ATG proteins (common targets include ATG5, ATG7, ATG16L1, ULK1) should inhibit assembly of the autophagosome [1, 48]. This can be visualized via decreased LC3-II levels. When performing knockdown studies, it is advisable to achieve as large of a knockout as possible. Often autophagy can still be functional unless there is nearly complete depletion of protein signal (greater than 90%). As a result, it is suggested to perform a double siRNA transfection if the first round is unsuccessful at decreasing LC3-II response. Upstream inducers of autophagy such as Beclin-1 and AMPK may also be targeted via siRNA or inhibitors (3-MA for Beclin-1) for reversal of autophagy activation (detected via

LC3-II) [1, 48]. Similarly, inhibition of AMPK or Beclin-1 may not reverse the autophagy pathway response if the pathways are not activated. AMPK initiates autophagy during period of glucose deprivation and starvation [48, 54]. This might not necessarily occur in a pathogenic induction of autophagy. Further, a number of studies have shown independence to Beclin-1 upon activation of autophagy [38, 55]. Due to these variables, it is important to stress that negative results from any individual knockdown is not enough to disprove autophagosome formation. In tandem, multiple targets are required to support autophagic induction as well.

2.5. Chemical modulation of xenophagy

One less reliable means of determining xenophagic activation is to chemically induce the process. Classical means of autophagic activation include utilization of rapamycin or starvation [1]. Rapamycin inhibits the mTOR signaling pathway which would otherwise block autophagic induction [48]. Similarly starvation induces the AMPK and mTOR signaling pathways that sense nutrient deprivation [1, 54]. The lack of essential amino acids will signal the activation of autophagy in order to break down proteins and organelles to produce nutrients. These techniques in theory should augment the xenophagic response in any given model. Inhibitors and inducers of xenophagy can also be used to determine the effects of xenophagy in model systems. Well-known inducers include rapamycin and starvation, while inhibitors include Wortmannin, chloroquine, bafilomycin, and 3-methyladenine (3-MA) [32, 56–60]. Furthermore, some inflammatory cytokines such as IFN- γ induce xenophagy in macrophages [61–63].

3. LC3 Associated Phagocytosis (LAP)

Recently, studies have revealed a more complicated process for pathogenic processing in phagosomes [64–67]. Pathogens engulfed by phagosomes may also be sequestered by autophagosomes (see Figure 1). This is then followed by fusion with a lysosome leading to the formation of an autolysosome that can breakdown the pathogen [64]. It is important to note, that engulfing a phagosome or a pathogen alone share the same terminology. Both lead to the formation of an autolysosome. In contrast, LC3 associated phagocytosis (LAP) does not involve autophagosome sequestration. Instead, LC3 binds to a recently formed phagosome. Interestingly, unlike macroautophagy, LAP LC3-II is not recycled on the cytosolic side. The LC3-II thus does not turn over after degradation of the LAP vesicle.

In terms of pathogens and foreign invaders, it is important to consider that LC3-II increase may serve as a red herring in autophagic studies. A number of studies have shown a non-canonical form of autophagy can occur upon invasion of pathogens via phagocytosis [64–67]. LC3-II can bind to a phagophore upon entrance of the cell marking it for lysosomal fusion creating an autophagolysosome. LAP is believed to occur as a means of preventing pathogenic harm to invaded cells. This mechanism of xenophagy forgoes double membraned autophagosome formation [64]. Thus it is critical to elucidate the exact form of xenophagy for any selected pathogen. Autophagosomal involvement can be confirmed or dismissed based on involvement of downstream and upstream regulators of the pathway.

There are a number paths a pathogen can take once it invades the host cell. The pathogen may escape into the cytosol or remain in the phagosome. Damaged phagosomes may be targeted by lysosomes which fuse and form a phagolysosome. This process may be inhibited by bafilomycin or chloroquine (Figure 1-1). The phagosome may also be recruited by a phagophore that develops into a double bound autophagosome. This may then fuse with a lysosome leading to the formation of an autophagolysosome. This can be inhibited by bafilomycin or chloroquine (Figure 1-1) or inhibition of autophagy regulating proteins (Figure 1-2). A phagosome may also recruit LC3-II leading to the formation of a LAP. This may then recruit a lysosome for fusion once again creating an autophagolysosome. This can be inhibited by bafilomycin or chloroquine (Figure 1-1) but not with inhibition of autophagy regulating proteins (Figure 1-2). Lastly, pathogens free in the cytosol can be engulfed by classical autophagic induction leading a double bound autophagosome, fusion with a lysosome, and then eventual autolysosome. Again, this process can be inhibited by bafilomycin or chloroquine (Figure 1-1) or inhibition of autophagy regulating proteins (Figure 1-2). Further, macroautophagy can be induced via nutrient deprivation (Figure 1-3).

Due to the similarities of LAP and xenophagy, it can be difficult to distinguish the two. Some groups have shown formation and movement of LAP vesicles via live cell imaging and TEMs [64–67]. LAP is also considered to be induced in the presence of high reactive oxygen species (ROS) [68, 69]. Recent work has shown LAP activity may be independent of ULK1/ATG [23, 65]. Further LAP vesicles do not contain p62 and other ubiquitin like substrates commonly associated with macroautophagy [2, 66]. Increased expression of endosomal markers shown via western blot analysis may also infer LAP formation, but for now visual confirmation of LAP is critical.

4. Determining Host-Pathogen Interactions

4.1 Methods to elucidate bacterial recognition

4.1.1 Transmission Electron Microscopy (TEM)—Above all else, the “gold standard” for autophagy confirmation is the utilization of TEMs [1]. TEMs provide visualization of double-membrane organelle that may occur due to experimental conditions [1]. A properly devised time course will allow detection of formation of autophagosomes (consumption of pathogens or damages organelles), double bound membrane autophagosomes, lysosomes, and autolysosomes. It is important to note that it is visually difficult to distinguish lysosomes and autolysosomes from one another. Both appear as electron dense (dark in coloration) spheres with a single membrane [70]. This labeling can tag either the pathogens of interest or a known protein consumed by autophagosomes (such as p62), allowing for a potential differentiation between lysosomes and autolysosomes

4.1.2 Fluorescence Microscopy—Induction of autophagy upon pathogenic infection (in-vivo) or challenge (in-vitro) does provide support to xenophagic activity. However, it does not confirm the autophagosome or phagocyte is consuming the invading object. The easiest way to confirm pathogenic involvement in autophagy is by visualizing the pathogen inside the autophagosome, autolysosome, and/or the autophagolysosome.

Fluorescence microscopy facilitates visualization of autophagy and host-pathogen interactions. This can be combined with flow cytometry, another fluorescence-based technique, to track fluorescently labeled targets, as discussed in section 4.1.3. For example, one can detect the pathogen by directly conjugating the bacteria to a fluorophore, or by fluorescent antibodies against the microbe. Various dyes also allow for the marking of particular host cell compartments, such as lysotracker-mediated marking of acidic vesicular compartments [29, 71–73] and marking of autophagosomal compartments with CytoID Green autophagy dye [74]. One can induce expression of these fluorescently-labeled protein markers by transfecting cells with plasmids or viruses encoding these markers. Furthermore, one can conjugate fluorophores to other markers in the host cells including LC3, late endosomal/lysosomal markers such as LAMP1, and monodansylcadaverine [MDC] [29, 56, 60, 75–77]. Co-localization of fluorescently-labeled bacteria with lysotracker measures successful trafficking of bacteria to acidic compartments, while co-localization of MDC with LC3 allows for the measurement of autophagy and co-localization of LC3 with bacteria measures xenophagy [33, 56, 59, 73, 76, 77].

Though MDC is sometimes used as a marker of autophagosomes, LC3 is a superior autophagosomal marker since LC3 also stains MDC⁻ autophagosomes, while MDC stains only acidic compartments [78]. MDC can be used as a marker for acidic autolysosomal compartments, given its co-localization with late endosomal markers such as CD63 and lysosomal markers such as lysotracker [78, 79]. Lysotracker can then be used as a lysosomal marker (autolysosomal or otherwise), while LC3 is used as a marker of autophagy. However, using LC3 as a marker of autophagy comes with certain caveats. First, LC3 may associate with phagosomes, as opposed to just autophagosomes, as discussed in section 3 in relation to LAP. Second, LC3 is not associated with some autophagosome-like vacuoles. LC3⁻ MDC⁺, double-membrane compartments may serve house bacteria such as *Brucella abortus*, which enter these vacuoles as opposed to trafficking through the classical phagocytic pathway. These compartments fail to fuse with the lysosomal marker cathepsin D, suggesting that the host cell is unable to successfully degrade the bacteria and that the bacteria may use these LC3⁻ MDC⁺, double-membrane vacuoles as an intracellular niche for evading degradation [80–82].

One can use inhibitors of autophagosome and lysosome fusion to reverse co-localization of bacteria with lysosomal markers or to observe changes in the number of LC3-II positive compartments. For example, a reduction in co-localization of *M. tuberculosis* with LC3 and MDC was observed in response to inhibition of xenophagy. This co-localization resulted from recognition of the bacterial ligand lipopolysaccharide (LPS) by the extracellular pathogen recognition receptor TLR4 on the surface of macrophages [56]. Fluorescence microscopy therefore facilitated the identification of an extracellular receptor important for the induction of xenophagy.

One can achieve similar results for markers for intracellular bacterial recognition as well [76, 83], as shown by the tracking of components of an intracellular pathway for detecting bacteria DNA in relation fluorescently-labelled *M. tuberculosis* or autophagy proteins [33, 84]. Fluorescence microscopy with green fluorescent protein (GFP) tagged LC3 also revealed other extracellular signals that promote pathogen clearance via xenophagy [85],

including entry of *Y. enterocolitica* into macrophages [35]. One can reveal other inducers of xenophagy by providing a signal, and then examining the co-localization of pathogen-containing vacuoles with late endosomal and/or lysosomal markers. For instance, treatment with CD154, an agonist of the co-stimulatory molecule CD40 on the macrophage surface, induced fusion of LAMP1-positive vesicles with compartments containing fluorescently-labelled *T. gondii* [60]. Finally, fluorescence microscopy can also reveal pathways downstream of xenophagic recognition, as in the case of co-localization of pathogen-derived antigens with molecules involved in antigen presentation [75].

4.1.3 Flow Cytometry—Flow cytometry, in contrast to fluorescence microscopy, allows one to quickly and easily to quantify xenophagy and autophagy in a large number of cells. The ImageStream-based flow cytometry assay is one particularly efficient way of measuring autophagy ([86, 87]); since the ImageStream system also takes single-cell images of thousands of cells [88, 89], it allows one to simultaneously depict co-localization in an image and quantify co-localization by flow cytometry. As with other flow-cytometry-based systems, one begins by generating host cells expressing fluorescently labeled targets such as LC3 and the lysosome marker LysoID, using the methods discussed in section 4.1.2. In order to remove cytosolic LC3-GFP and focus on lipidated membrane-associated LC3-GFP, one can permeabilize host cells with detergents such as digitonin and saponin, followed by washing [90–93]. One can also stain these host cells with antibodies against specific host molecules in order to monitor autophagy just within cells positive for these host molecules. The cells are then run through the ImageStream flow cytometer, which measures LC3 fluorescence intensity, LysoID intensity, and LC3 co-localization with LysoID within each cell, enabling one to use flow cytometry to measure not only autophagy, but also fusion of autophagosomes with lysosomes [86]. To measure LAP (discussed in section 3), one can measure the co-localization of LC3 with fluorescently labeled phagocytic receptors. This differs from regular phagocytosis, where LC3 will not co-localize with phagocytic receptors. One can also differentiate LAP from xenophagy within this system, since fluorescently labeled p62 will co-localize with LC3 during xenophagy, but not during LAP (as discussed in section 3).

One can also use flow cytometry to investigate the role of autophagy proteins in bacterial uptake in cells, particularly immune cells such as macrophages. As noted above, one first generates fluorescently labeled targets via the methods discussed in section 4.1.2. One can then measure bacterial uptake by infecting cells with fluorescent bacteria, forming a single-cell suspension from the infected cells, and then analyzing intracellular fluorescence via a flow cytometer [63, 94]. Expression of pathogen recognition receptors can also be measured applying fluorescent antibodies against these receptors. For example, flow cytometry with fluorescent antibodies against the extracellular scavenger receptors MARCO and SR-A1 revealed increased expression of these receptors by primary macrophages with genetic deficiencies in the autophagy proteins ATG3, ATG5, or ATG7 and increased uptake of bacteria such as *Escherichia coli* [94].

Xenophagic recognition of pathogens typically results in downstream effects. Flow cytometry can confirm the importance of autophagy proteins for these downstream effects and elucidate the effects themselves, including effects such as cell death and activation of

inflammatory pathways [72, 95, 96]. For example, application of a fluorescent antibody against HLA-DR, a molecule involved in antigen presentation, revealed an increase in HLA-DR levels in dendritic cells after the cells were treated with a molecule that binds Nod-like receptor 2 (NOD2), an intracellular receptor that recognizes bacteria and can induce xenophagy in response to bacterial recognition. However, this increase was absent in cells encoding a Crohn's disease-associated variant of the autophagy protein ATG16L1, thereby confirming a role for this autophagy protein in antigen presentation downstream of NOD-mediated pathogen recognition [72].

4.1.4. Inflammatory responses as measured by ELISA and tissue histology in the context of xenophagy

—Xenophagic uptake and clearance pathogens often dampen inflammatory responses to those pathogens *in vivo* [33, 58, 63, 71, 95]. One may measure these reduced inflammatory responses via a number of techniques, including ELISAs against inflammatory cytokines and histological evaluation of inflammation from infected tissue. These methods complement *in vitro* measures of inflammatory responses, such as ELISAs for inflammatory cytokines release by infected macrophages, including IL-1, TNF- α and IL-6 [58, 83, 85, 95]. For example, *M. tuberculosis* infection of mice with genomic deletions in Atg5 in their monocytes and macrophages resulted in increased lung levels of inflammatory cytokines such as IL-6 and TNF- α in comparison to mice with functional Atg5, as measured by ELISA [33]. And in mice treated with intra-tracheally with the bacteria *B. cenocepacia*, induction of autophagy via rapamycin resulted in less recruited inflammatory cells and less tissue hemorrhaging as determined by staining of lung sections with hematoxylin and eosin dyes [71]. Tissue histology results can also be supplemented by mouse survival studies in response to repressors of autophagy [57].

4.2 Bacterial survival measured by intracellular CFUs

How a pathogen responds to xenophagy influences the intracellular survival of those cells. A number of bacteria seek out xenophagy for self-preservation [29, 31], while others avoid xenophagic consumption through mechanisms such as the release of toxins [32, 33, 35]. Colony formation unit assays can help elucidate these pathogen-xenophagy relationships. Manipulation of autophagy proteins or levels of xenophagy should influence a pathogen engulfed via xenophagy both *in vivo* and *in vitro*, whether it be negative in terms of decreased CFU levels or positive in terms of increased CFU levels [33, 51, 95]. For example, xenophagy was essential for effective clearance of *M. tuberculosis* *in vivo* since mice with deletions in the autophagy gene Atg5 in their monocytes and macrophages displayed increased bacterial titers in their lungs, spleen, and liver as measured by CFUs, along with increased mortality in response to bacterial challenge [33]. Urinary and tissue CFU analyses revealed a pro-pathogenic role for autophagy and the autophagy protein, ATG16L1, as mice deficient for this protein were shown to be highly resistant to uropathogenic *E. coli* infection of the urinary tract as well as *C. rodentium* infection of the gut [51, 97].

5. Iron-induced xenophagy: Ferritinophagy

Iron is a critical element for cell survival [38, 98]. As a result, a stringent signaling mechanism has evolved to absorb and utilize iron for basic cell functions. Since free iron functions at oxidative state of +2, it can readily produce reactive oxygen species intracellularly [99–101]. As a result, cells utilize ferritin to store iron in unreactive state when not in use. It has been established, that to free iron from ferritin, lysosomes are needed to breakdown ferritin bound iron leading to release and acidification of the unbound iron (acidification refers to conversion of iron from Fe+3 to Fe+2) [38, 39, 98, 102–104]. Recently, it has been suggested that autophagy plays a role in shuttling holo-ferritin (iron bound ferritin) to the lysosome (i.e. the autolysosome). One study has reported a specific mechanism for iron induced autophagy which they coined Ferritinophagy [39]. In short, NCOA4 was identified via proteomic profiling to induce autophagy directed at processing iron [39, 40]. This novel marker is currently the only unique autophagy inducer regulated by iron. There are a number of means to detect ferritinophagy. Typically, observations of iron induction of autophagy are observed via iron overload conditions. This means iron was administered at high levels, ranging from 50uM to 500uM levels, leading to increased iron intracellularly [38, 99, 102, 105, 106]. The abundance of iron leads to a decrease in transferrin receptor levels (a cytokine involved in import of iron into the cell) and increase in the iron storage protein, ferritin [98, 104]. Both of these proteins are regulated at the transcriptional level by Iron Regulatory Proteins 1 and 2 (IRP-1, -2) [101]. Briefly, iron interacts with IRPs leading to increase of ferritin and decrease of transferrin receptor (along with a number of other iron regulatory proteins) [101]. These markers can be utilized to identify increased iron intracellularly either through western blot analysis, qPCR, or immunofluorescence as described earlier.

5.1. Identifying iron induced autophagy

Confirmation of autophagy can be performed using identical strategies to standard macroautophagy confirmation. This includes but is not limited to western blots, immunofluorescences, inhibitors, and TEMs. Circumstantial confirmation of ferritinophagy can be surmised by observing increased LC3-II after treatment with iron. Interestingly, p62 remains unchanged during treatment with iron even in the presences of increased LC3-II. This suggests p62 may not be involved in iron recruitment to the autophagosome. Iron can be tracked traditionally via radiolabeling. One way to tag iron without radiolabeling includes calcein-AM [107, 108]. Calcein-AM is a cell permeable dye that will fluoresce green in living cells [107, 108]. Interestingly, the dye is quenched preferentially by redox active metals such as nickle, copper, and iron. To determine localization of iron with autolysosomes, one could stain with lysotracker for the lysosomes/autolysosomes and calcein-AM for iron [38, 102]. Further, iron can often be identified visually in TEMs alone [38]. However, immunogold staining of ferritin can help to officially validate that iron is being released in the autolysosome via holo-ferritin breakdown [109, 110].

Conclusions and Suggestions

Recently, there has been a growing trend to subcategorize cell death and signaling responses, autophagy included. While the breakdown of autophagy into xenophagy,

mitophagy, ferritinophagy, and so on may appear to be splitting hairs, it is an important step in further understanding the nuances of the overall mechanism. Indeed, while majority of the techniques used for xenophagy confirmation are identical to autophagy validation [1], xenophagy is macroautophagy but xenophagy does not account for all macroautophagy events. It is likely, in the near future a large number of host responses will be elucidated that lead to recruitment to the autophagosome.

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Abbreviations

CFU	Colony Formation Unit
EGFP	Enhanced Green Fluorescent Protein
IRPs	Iron Regulatory Proteins
LC3	Light Chain 3
LAP	LC3 Associated Phagocytosis
MDC	monodansylcadaverine
NOD2	NOD-Like Receptor 2
QIRs	Quiescent Intracellular Reservoirs
RFP	Red Fluorescent Protein
TEM	Transmission Electron Microscopy
UTI	Urinary Tract Infection
3-MA	3-Methyladenine

Glossary of Terms

Colony Formation Unit (CFU)	An estimation of the overall number of a given pathogen occurring for a select experiment. CFUs may be utilized to determine overall levels of intracellular pathogen numbers
Uropathogenic E. Coli (UPEC)	The primary pathogenic bacteria involved in urinary tract infections. UPEC is considered to be linked to over 90% of UTIs
Quiescent Intracellular Reservoirs (QIRs)	Non-replicating bacteria that maintain survival intracellularly. UPEC has been shown to utilize autophagosomes for QIR maintenance
LC3 Associated Phagocytosis (LAP)	Phagosomes that utilize LC3 on their membrane but do not undergo autophagosome sequestration. This tags the

	phagosome for lysosome fusion leading to the formation of an autophagolysosome
Light Chain 3 (LC3)	A light chain protein involved in microtubule function and expressed throughout all tissue types. LC3-I is converted to LC3-II by conjugating phosphatidylethanolamine with LC3-I. LC3-II localized to the autophagosomal membrane
3-Methyladenine (3-MA)	An inhibitor of upstream signaling of autophagy. In particular, 3-MA targets the Beclin-1/hvps34 complex that is able to initiate autophagosomal formation
Transmission Electron Microscopy (TEM)	A microscopy technique that utilizes electrons transmitted through lasers. This allows one to view images at incredibly small sizes enabling visual confirmation of organelles such as autophagosomes
Monodansylcadaverine (MDC)	An autofluorescent marker that has been shown to localize specifically to autophagosomes
NOD-Like Receptor 2 (NOD2)	An intracellular receptor that recognizes muramyl dipeptide from bacteria such as <i>S. flexnri</i>
Iron Regulatory Proteins (IRPs)	Proteins involved in the transcriptional regulation of proteins involved in iron homeostasis
Autophagic flux	The actual conversion of autophagosomes to autolysosomes. This helps to distinguish between accumulation of autophagosomes and actual autophagy turn over
Autolysosome	The product of the fusion of an autophagosome and a lysosome. This structure has a single membrane and electron dense. As a result, it can be easily confused for a lysosome via TEM analysis
Autophagosome	A double-membrane organelle structure that consumes damaged organelles, proteins, or invading pathogens
Autophagolysosome	The product of the fusion of a phagosome and a lysosome
Symbiophagy	A form of xenophagy that is specific to the targeting of a host's symbiotic partner
Xenophagy	The specific term for autophagy targeting foreign entities such as bacteria, viruses, and other pathogens
Ferritinophagy	Autophagy that is believed to help regulate and process intracellular iron
Phagocytosis	The process of consuming extracellular material such as pathogens. This internalization occurs through the formation of an endosomal single membrane structure

Macroautophagy	The classical name for autophagy or a double-membrane organelle structure that consumes and degrades intracellular material
Rapamycin	Classically considered an immunosuppressive drug, rapamycin specifically targets the mTOR pathway. Inhibition of this pathway leads to increased production of autophagosomes and autophagy suggesting it can augment autophagic behavior
Bafilomycin/Chloroquine	Autophagy inhibitors that block the fusion of lysosomes with autophagosomes. These drugs block vacuolar type H(+)-ATPase preventing acidification of lysosomes that is crucial for membrane fusion

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Figure 1.
Model depicting multiple pathways of xenophagy.

Table 1
Chart distinguishing between Xenophagy, LC3-Associated Phagocytosis, and Phagocytosis.

Xenophagy	LC3-Associated Phagocytosis	Phagocytosis
LC3-II positive	LC3-II positive	LC3-II Negative
Double Bound membrane	ULK1/ATG1 independent	Single membrane
Lysosome fusion	Induced by ROS	Lysosome fusion can occur
ULK1/ATG1 dependent		