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Selective autophagy: Xenophagy

Kyle A. Bauckman [a], Nana Owusu-Boaitey [a], Indira U. Mysorekar [a,b,*]

[a] Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO, USA
[b] Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA

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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.

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 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

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.

* Corresponding author at: Washington University School of Medicine, Depts. of Obstetrics and Gynecology & Pathology and Immunology, 660 S. Euclid Ave., St. Louis, MO 63110, USA. Fax: +1 314 747 0264.
E-mail address: indira@wustl.edu (I.U. Mysorekar).

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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]) highjacks 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 is a response to a foreign body (xeno) and not the host (auto). Although ferritinophagy responses 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 (18 kD) protein to LC3-II (16 kDa) 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