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# Autophagy in Innate Immunity against Intracellular Bacteria

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**Many pathogenic bacteria can invade phagocytic and non-phagocytic cells and colonize them intracellularly, then become disseminated to other cells. The endocytic degradation pathway is thought to be the only prevention against such intracellular pathogens. Autophagy, a fundamental cellular homeostasis pathway that operates with the intracellular degradation/recycling system, causes the turnover of cellular components by delivering portions of the cytoplasm and organelles to lysosomes. Recently, we reported that autophagic degradation is a previously unrecognized effector of host innate immunity. *Streptococcus pyogenes* (Group A *Streptococcus*; GAS) successfully enters human epithelial cells *via* endocytosis. GAS immediately escapes from the endosomes to the cytoplasm and gains a replicative niche, after which GAS in the cytoplasm is trapped in autophagosome-like compartments and degraded upon fusion with lysosomes. This process indicates that autophagy plays a protective role in infectious diseases. We also found that autophagic degradation was induced against *Staphylococcus aureus*, while methicillin-resistant *S. aureus* were resistant to autophagic degradation. The present review focuses on the protective function of autophagy against bacterial invasion of cells.**

**Key words: autophagy, Group A *Streptococcus*, infection, innate immunity, intracellular bacteria.**

Autophagy is a fundamental cellular homeostatic mechanism that provides for bulk degradation of organelles and cytosolic proteins (*1*, *2*), which, like the endocytic pathway, is ubiquitous in eukaryotic cells. During this process, regions of the cytoplasm as well as organelles are first engulfed by double- or multiple-membrane structures called autophagosomes, which are formed through the elongation and closure of cap-shaped cisternae termed isolation membranes. Upon completion of an autophagosome, the trapped cytoplasmic material is completely separated from the rest of the cytoplasm and delivered to autolysosomes, which are formed by the fusion of autophagosomes with lysosomes for hydrolytic degradation. This lysosomal degradation system plays a housekeeping role by promoting non-selective degradation and recycling of cellular proteins, as well as removing damaged or superfluous organelles. Autophagy also removes leaky mitochondria, excess peroxisomes, and other organelles such as endoplasmic reticulum when they need downsizing, and autophagic membranes can even sequester a whole nucleus (*3*). Under starvation conditions, autophagy is dramatically enhanced to maintain the intracellular amino-acid pool for gluconeogenesis and for synthesis of proteins essential for cell survival. The process is also known to be involved in the mediation of both healthy and diseased conditions, such as cancer, neurodegeneration, myopathies, development, and aging (*4*, *5*).

It was previously postulated that some intracellular bacteria are targeted by the autophagic degradation system (*6*). However, it has been difficult to prove the hypothesis that autophagy degrades intracellular pathogens, since individual components of the autophagic machinery have not been identified. Recently, the microtubule-associated protein 1 light chain 3 (LC3) was identified as an autophagosome-specific membrane marker in mammalian cells (*7–9*) and is the only known reliable marker for those compartments. It has also been reported that a protein complex including Atg5 is involved in autophagosome formation (*10*, *11*) and *ATG5* gene–knockout cells were unable to form autophagosomes (*12*). Using those tools, we reported that autophagy is an innate immunity effector against intracellular bacteria (*13*), while others subsequently showed protective functions of autophagy against bacteria and viruses invading cells (*14–16*). This review focuses on our continuing efforts to examine the direct implications of autophagy in innate immunity.

## Group A *Streptococcus* (GAS; *Streptococcus pyogenes*)

GAS, a Gram-positive extracellular etiological agent, is one of the most ubiquitous and versatile human bacterial

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pathogens (*17*). The bacterium colonizes the throat and skin, and is responsible for a number of suppurative infections and nonsuppurative sequelae, while it is the most common cause of bacterial pharyngitis as well as scarlet fever and impetigo. Further, GAS is responsible for streptococcal toxic shock syndrome (STSS), and it has recently been reported as a "flesh-eating" bacterium that invades skin and soft tissues, causing destroyed tissues and limbs. In a recent 5-year period (1995–1999), the number of annual cases of invasive GAS infections was reported to range from 9,600 to 9,700, including 1,100 to 1,300 that resulted in death (*18*). In addition, acute post-streptococcal glomerulonephritis is a leading cause of cardiovascular morbidity and mortality in many developing countries throughout the world (*19*).

GAS adheres to and invades a variety of types of cultured human epithelial cells (*20*–*29*), and several of its adhesive components, known as invasins, promote bacterial invasion of human cells (*24*). Thereafter, the intracellular organisms subsequently activate the focal adhesion complex (*25*) and induce cytoskeletal rearrangements (*24*, *26*), which disable cellular functions such as adhesion, migration, and proliferation. Intracellular GAS also induces proinflammatory cytokine production (*27*) and apoptosis (*23*, *29*), while such intracellular localization is thought to allow the pathogen to penetrate into deep tissues and may provide a nutritionally rich "shelter" offering protection from components of the host immune system, including phagocytes and humoral antibodies, as well as from antibiotics (*22*).

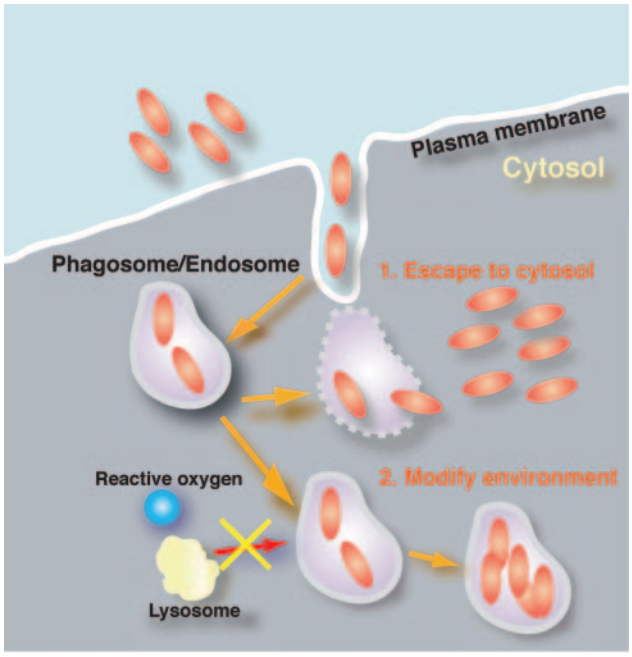


Fig. 1. **Schematic diagram of the survival mechanism of intracellular paracytic bacteria.** 1: *Shigella* species and *Listeria monocytogenes* are able to escape from endosomes to prevent lysosomal degradation. 2: Acidification of the endosomes is prevented by *Mycobacterium tuberculosis*, while *Salmonella enterica* serovar *Typhimurium* avoids or delays fusion of late endosome-like compartments and lysosome.

## Survival of intracellular bacteria

Efficient escape from the cellular phagocytic/endocytic degradation system is a crucial strategy for the survival of intracellular bacteria. A number of bacterial species are able to enter host cells through their internalization in phagosomes or endosomes (*30*). Following the maturation of these compartments, the fusion with lysosomes results in bacterial degradation, a harsh environment that provides a defense against intracellular pathogens (*31*, *32*). However, some bacteria have evolved strategies to avoid the host defense system. One technique is modification of the endocytic compartments for prevention of fusion with a lysosome or interference with its lytic action (Fig. 1), which allows the pathogen to escape from the cellular endocytic compartment to the cytoplasm. For example, *Listeria monocytogenes* normally enters host cells *via* endocytosis and escapes from the endosomes (*32*, *33*). Since these types of bacteria can replicate within the host cells, they are known as "intracellular parasites." Another technique is modification of the lysosomal environment, such as that used by *Mycobacterium tuberculosis*, which prevents acidification (*34*), and *Salmonella enterica* serovar *Typhimurium*, which resides within membrane-bound compartments called *Salmonella*-containing vacuoles of which endocytic pathway is arrested prior to fusion with degradative lysosomes (*35*).

## Autophagy and intracellular bacteria

Relationships between autophagy and bacterial infection by some pathogenic bacteria, including Rickettsiae (*36*), *Listeria monocytogenes* (*37*), *Salmonella typhimurium* (*38*), *Porphyromonas gingivalis* (*39*), and *Brucella abortus* (*40*), have been speculated. However, the methodologies used for monitoring autophagy and examining its role in those reports were unsatisfactory, as known autophagosome markers such as monodansylcadaverine and autophagy inhibitors such as 3-MA show broad specificity (*41*). Thus, conclusive proof and the significance of localization have not yet been provided using rigorous methods.

## Intracellular GAS escapes from endosomes but is trapped by autophagosomes

GAS enters human epithelial cells *via* engulfment by early endosomes, thereafter, endosomes containing GAS disappear within a few hours, indicating its escape into the cytoplasm (*13*). This displacement has been reported to be mediated *via* streptolysin O (SLO), a member of a conserved family of cholesterol-dependent pore-forming cytolysins secreted by GAS, since an isogenic streptolysin O (SLO)-deficient mutant (ΔSLO) was negligibly extricated and remained within the FYVE-positive endosomes (*42*). Following escape into the cytoplasm, GAS was found to induce the autophagic machinery and apparently became trapped by autophagosomes (*13*). In that study, epithelial cells expressing LC3 coupled with green fluorescent protein (GFP-LC3) as a marker of autophagosome membranes were infected with GAS. Confocal microscopy revealed that the majority of cellular LC3 was diffused throughout the cytoplasm in uninfected cells cultured in a nutrient-rich condition. However, under starvation conditions, LC3-positive autophagosomes (with diameters

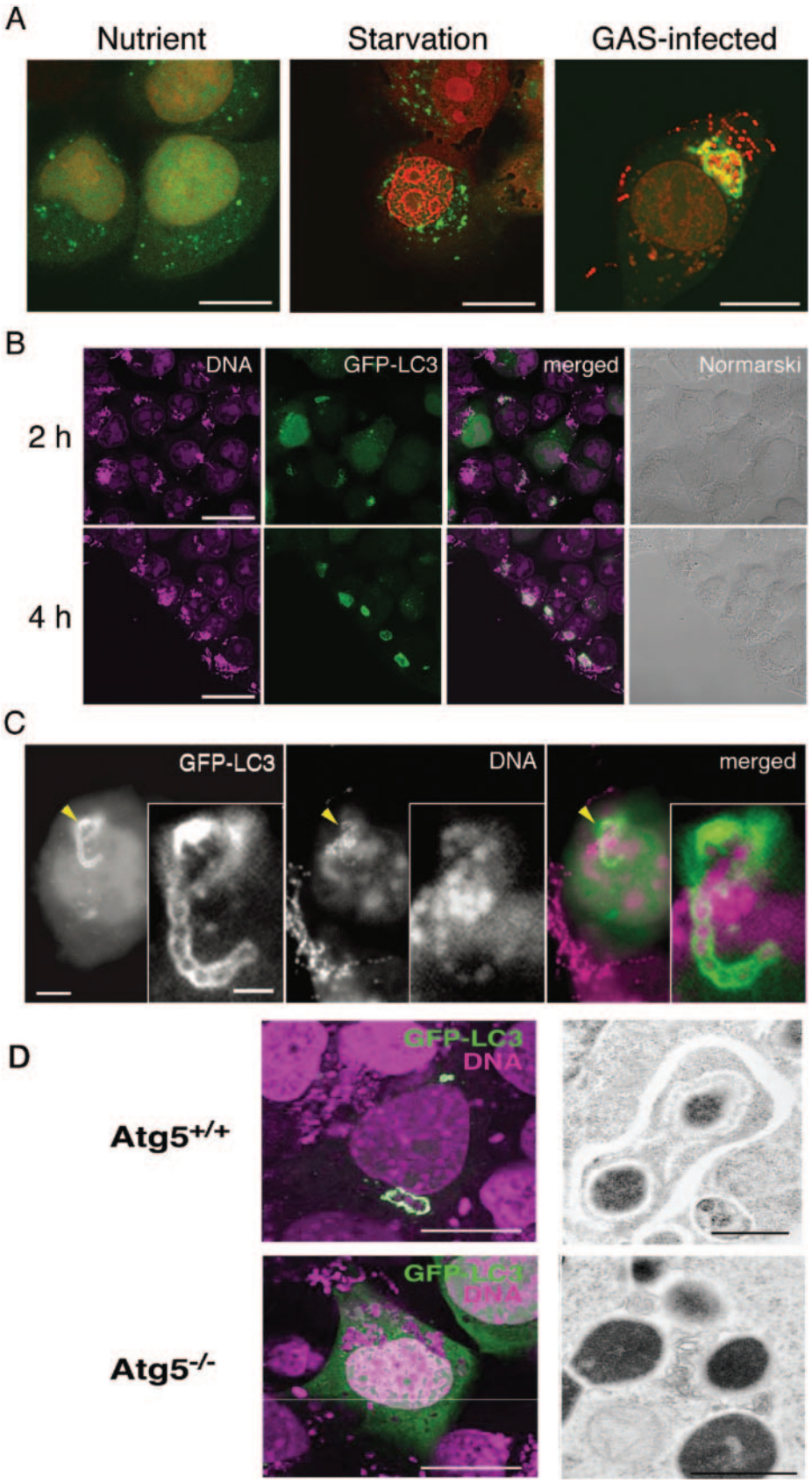


Fig. 2. **Intracellular GAS acquired by LC3-positive autophagosomes.** A: Confocal microscopic images of autophagosomes formed by HeLa cells carrying GFP-LC3 (HeLa-LC3 cells) in nutrient-rich (left), nutrient-starved (middle), and GAS-infected (right) conditions. Two hours after infection, the cells were fixed and stained with propidium iodide (red). Scale bars = 10 mm. B: Increase and growth of GAS-containing autophagosome-like vacuole (GcAV) in HeLa-LC3 cells. Scale bars = 20 μm. C: High-resolution microscopic image of GcAV (green) obtained by deconvolution. Two hours after infection, cellular and bacterial DNA was stained with propidium iodide (magenta). Arrowheads indicate the location of GAS organisms magnified and shown in the inset of each image. Scale bars = 5 μm and 2 μm in the full-scale and magnified images, respectively. Inset figures are reproduced with permission from AAAS (fig. 1D of Ref. *13*). D: Lack of autophagic ability allows GAS survival in host cells. Intracellular GAS (magenta) is shown sequestered by GcAVs (green) in wild-type cells ($Atg5^{+/+}$), but not in $Atg5^{-/-}$ cells (left side images). Bars = 10 μm. Ultrastructural observations (right side images) show no autophagosome formation in $Atg5^{-/-}$ cells. GAS is acquired with a double-membrane autophagosome in wild-type cells, whereas it exits freely into the cytoplasm of $Atg5^{-/-}$ cells. Bars = 1 μm.

of 0.5–1.0 μm) were found in the cytoplasm as punctuate structures. In the epithelial cells infected by GAS, large LC3-positive compartments that had acquired bacterial clusters were found (Fig. 2), with more than 80% of the intracellular GAS organisms in the cytoplasm trapped by those compartments. The size and morphology of the GAS-induced LC3-positive compartments were distinct from canonical starvation-induced autophagosomes, whose size sometimes exceeds 10 μm. Thus, the novel structures were designated as GAS-containing LC3-positive autophagosome-like vacuoles (GcAVs).

GcAVs express characteristic double-membrane cisternae, which resemble but are larger than the isolation membranes of autophagy (Fig. 2D). Further, single membrane-bound compartments with degraded cytosol and GAS were observed. In addition, an intracellular ΔSLO mutant negligibly induced GcAV formation, suggesting that emergence of the bacterium in cytoplasm triggers autophagic induction. SLO seems to be a critical factor for bacterial escape from endosomes, while autophagosomes are likely resistant to SLO. There is no explanation for this at present, however, SLO is known to target cholesterol (*42*) and autophagic membranes may contain less cholesterol than endosomes or none at all.

### GcAVs fuse with lysosomes for degradation

Initially, the structures classified as GcAVs did not include LAMP-1 (a lysosomal membrane protein), though they have gradually become associated with and found to be co-localized with that protein (*13*). GcAVs fuse with lysosomes, similar to that seen in the canonical autophagic pathway. Further, the lysosomal protease inhibitors leupeptin and E64d significantly suppress the killing of intracellular GAS. In Atg5$^{-/-}$ cells, an autophagy-deficient mutant, few GAS organisms are killed and protease inhibitors show no effect toward bacterial viability, implying that living GAS organisms in wild-type cells are killed by proteases provided not from the endocytic pathway, but rather from the autophagic pathway (Fig. 2D). Although certain bacterial pathogens, such as *Salmonella*, are able to survive inside lysosomes (*43*), intracellular GAS is clearly killed by autophagosomes.

### *Streptococcus aureus* eliminated by autophagic degradation

Effective autophagic elimination does occur not only with GAS, as we have found that another common Gram-positive bacterium, *Staphylococcus aureus*, is also sequestered by LC3 compartments and further degraded by autolysosomes (*44*). In addition, this pathogen is not killed, but rather multiplies, in Atg5$^{-/-}$ cells. *S. aureus* is a major cause of infections in both hospitals and care centers, and has exhibited an increasing resistance to methicillin (methicillin-resistant *S. aureus*, MRSA), while it has also been shown to be related to beta-lactams (*45*). MRSA is generally considered to be a nosocomial pathogen associated with higher morbidity and mortality than in diseases caused by pathogens susceptible to methicillin. Interestingly, among the various strains of *S. aureus*, some strains including MRSA strains have revealed a marked resistance to autophagic elimination, as they were trapped by autophagosomes, but thereafter escaped from the vacuoles to cytoplasm (*44*). It is now unclear how those strains evade autophagosomes, though demonstration that the pathogenicity of MRSA is enormously enhanced by resistance to antibiotics and also to autophagic degradation would have a global impact.

### Conclusion

The most important finding in our studies of GAS is that the autophagic machinery acts as a defense against intracellular pathogens, strongly indicating that the process of autophagy is an important, albeit previously overlooked, innate immune mechanism. An intracellular GAS organism is selectively sequestered by LC3-positive compartments, which eventually fuse with lysosomes, after which enzymes degrade the pathogen. Several different types of pathogens have been shown to be eliminated by this autophagic machinery (Fig. 3), with GAS and *S. aureus* the most ubiquitous and versatile, thus the most etiologically important. As a result, the autophagic elimination process may play a critical role in the innate human defense system. On the other hand, some bacterial species, such as Shigella and Salmonella, seem able to avoid or subvert autophagy (*14*, *38*, *46*), as secretory proteins produced *via* the type III secretion systems of those organisms might disable the autophagic as well as endocytic mechanisms. Recently, autophagy has been implicated in MHC II presentation (*16*, *47–49*) and induced by interferon-gamma (*15*, *50*), suggesting its unique role in adaptive immunity functions. Therefore, autophagy may be at the battlefront of the ongoing war between host and invading bacteria.

The autophagic machinery utilized in defense against GAS is distinct from canonical autophagy in several different points. It is specifically induced by the emergence of GAS in the cytoplasm, even under a nutrient-rich condition. Further, canonical autophagy is thought to be nonselective (*2*), and represents a random and bulk degradation of cytoplasmic contents, whereas GAS-specific autophagy appears to selectively sequester bacteria (*13*). In addition, the autophagosomes that engulf GAS clusters are extremely larger and live longer than canonical autophagosomes. These striking features imply the existence of an autophagic machinery that specializes in defense

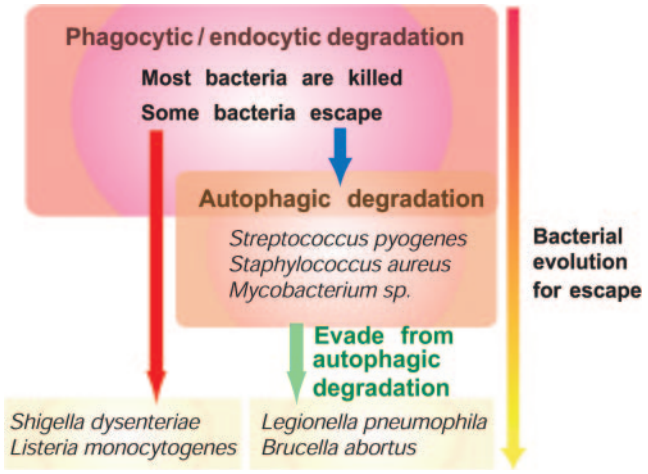


Fig. 3. **Evolution of bacterial mechanism for escape from cellular intercept machinery.**

against pathogenic bacteria. Nevertheless, extensive cell biological studies are required to uncover the full scope of these mechanisms.

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