Published in final edited form as:
J Immunol. 2012 July 1; 189(1): 15–20. doi:10.4049/jimmunol.1102108.

Autophagy – an emerging immunological paradigm

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Abstract

Autophagy is a fundamental eukaryotic process with multiple cytoplasmic homeostatic roles, recently expanded to include unique standalone immunological functions and interactions with nearly all parts of the immune system. Here, we review this growing repertoire of autophagy roles in innate and adaptive immunity and inflammation. Its unique functions include cell-autonomous elimination of intracellular microbes facilitated by specific receptors. Other intersections of autophagy with immune processes encompass effects on inflammasome activation and secretion of its substrates including IL-1β, effector and regulatory interactions with Toll-like and Nod-like receptors, antigen presentation, naïve T cell repertoire selection, and mature T cell development and homeostasis. Genome wide association studies in human populations strongly implicate autophagy in chronic inflammatory disease and autoimmune disorders. Collectively, the unique features of autophagy as an immunological process and its contributions to other arms of the immune system represent a new immunological paradigm.

Introduction

In this review, we cover the immunological roles of macroautophagy (1), a specific autophagic process that will be referred herein as the s*ensu stricto* autophagy or simply autophagy. Autophagy is unique in its capacity to sequester, remove, or process bulk cytosol, cytoplasmic organelles (1), invading microbes, and immunological mediators (2) as depicted in Fig. 1. Another special property illustrated in Fig. 1 is that autophagy acts as a topological inverter - bringing molecules and objects from the cytosolic side to the lumenal side for degradation or processing, interaction with luminal receptors, or secretion from cells. Here, we cover the four principal manifestations of immunological autophagy (Fig. 1): (i) direct pathogen elimination assisted by sequestosome 1-like receptors (SLRs); (ii) regulation and effector functions of pattern recognition receptors (PRR); (iii) regulation of inflammasome activation and alarmin secretion; and (iv) cytoplasmic antigen processing for MHC II presentation and T cell homeostasis. We relate these processes to conventional immunological functions, defense against infectious agents, chronic inflammatory disorders and other immunological pathologies.

Autophagy pathway

The key morphological features of autophagy are endomembranous organelles, called autophagosomes (Fig. 1) whose formation is controlled by the Atg and additional factors comprehensively reviewed elsewhere (1). Briefly, the Atg system includes Ser/Thr kinases

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DISCLOSURES
The author has no financial conflicts of interest to disclose.

Ulk1 and Ulk2 (Atg1), Beclin 1 (Atg6; a subunit of the class III phosphatidylinositol 3-phosphate kinase (PI3K) hVPS34 complexes), Atg5-Atg12/Atg16L1 complex, and LC3s (multiple Atg8 orthologs), with LC3B being a commonly used marker for identification of autophagosomes (1). Ulk1/2 and Beclin 1-hVPS34 integrate upstream signals and direct the downstream Atg conjugation cascade, which involves Atg5-Atg12/Atg16L1 assembly as an "E3 enzyme" for LC3 lipidation. Lipidated LC3s in conjunction other factors assemble, elongate, and close nascent autophagic organelles. Autophagosomes interact with endosomal and lysosomal organelles to mature into autolysosomes (1), or promote unconventional secretion of cytoplasmic constituents, as first demonstrated in yeast (3) and recently shown to include immune mediators (4, 5). In addition to its immunological functions (2), autophagy plays a general cellular homeostatic role by supplying nutrients (e.g. amino acids) through cytosol autodigestion at times of starvation or growth factor withdrawal, and serves as a quality and quantity control mechanisms for intracellular organelles (1).

At the transcriptional level, regulation of autophagy is coupled to the lysosomal system via TFEB (transcription factor EB) (6) and other proteolytic systems via FoxO3A (7). However, autophagy is primarily a rapid-response remodeling of membranes that occurs in the cytoplasm under the control of the signaling systems faster than transcriptional changes. The classical nutritional/energy regulation of autophagy is via mTOR and AMPK inhibiting and activating, respectively, Ulk1/2 (1). This pathway merges with signaling via the inhibitor of NF-κB kinases (IKK), frequently involved in immune signaling. IKKα and IKKβ transduce the classical signal for autophagy induction – starvation (8) but this signaling is not based on nuclear NF-κB responses. Instead, IKK and AMPK signaling merge via TAK1 and its activators TAB2 and TAB3. Upon autophagy induction TAB2 and TAB3 dissociate from and thus activate Beclin 1 and also bind to and activate TAK1 (8), whereas TAK1 in turn phosphorylates and activates AMPK.

In T cells, autophagy is activated upon TCR engagement and CD28 co-stimulation and supports their effector functions and proliferation (9). Recently, class III PI3K hVPS34 was found to be dispensable for autophagy induction in T cells albeit required for T cell homeostasis via its regulation of receptor endocytosis (10) bringing up the possibility of alternative pathways in PI3P signaling, as suggested by the positive role of class I PI3K p110β(11).

Innate immune signaling can induce autophagy. TRAF6 downstream of TLR4 activates autophagy (12) Alarmins or damage associated molecular patterns (DAMP) induce autophagy (13, 14). HMGB1, an alarmin, undergoes translocation from the nucleus into the cytoplasm and then out of the cells by unconventional secretion (5, 15) or cell death-associated release, inducing autophagy at each stage: cytoplasmic through derepression of Beclin 1 by displacing its negative regulator Bcl-2, or extracellularly via RAGE signaling (13, 14). In addition to HMGB1, DAMPs such as ATP, IL-1β, and DNA complexes are known to induce autophagy (reviewed in (16)).

Autophagy in direct pathogen elimination

The evolutionarily most primal manifestation of immunological autophagy is direct capture and degradation of invading intracellular microbes by autophagy (Fig. 1, panel 1, left). This cell-autonomous defense function of autophagy is often countered by microbial adaptation mechanisms and a number of highly adapted pathogens can convert autophagic organelles into growth-supporting compartments (17). Autophagic capture of intracellular microbes is facilitated by autophagic adaptors, referred to as SLRs (sequestosome 1/p62-like receptors) (18). SLRs have LC3 interacting regions (LIR) and cargo-tag (e.g. ubiquitin) recognition domain and are modulated by protein kinases. *Salmonella* requires multiple SLRs (p62,

NDP52, optineurin) (19, 20), phosphorylation of at least one of the SLRs (optienurin) with an IKK-related kinase, TBK-1 (20), and an intracellular DAMP receptor (galectin 8) (21). The SLRs p62 and NDP52 are also engaged in clearance of *Shigella* and *Listeria* (22–24), whereas Streptococci are affected by NDP52 (19). Sindbis virus interacts with p62 (25). Candidate E3 ligases contributing to target ubiquitination have been identified in some instances: SMURF1 for sindbis virus (26) and LRSAM1 as a candidate for Salmonella (27).

The most recent player in these processes is galectin 8, a cytosolic lectin binding to β-galactoside glycans. The membrane glycans are normally present only on the lumenal side of parasitophorous vacuoles. However, upon membrane damage the glycans come in contact with the cytosol and thus become recognized by cytosolic gelectins (21) (Fig. 1, panel 1, hatched square). Galectin 8 is important to restrict *Salmonella* proliferation, and plays an early role until supplanted by a phase dominated with ubiquitin and ubiquitin-recognizing SLR – NDP52. It appears that the phases and the sequence of recognizing membrane damage could be ushered by the appearance of diacylglycerol (28), followed by galectin-β-galactoside recognition, followed by NDP52-ubiquitin recognition. Since galectin 8 and NDP52 interact, a sequential action is doubly ensured. Galectin 8 is important for the recruitment of NDP52, since the requirement for galectin 8 to restrict *Salmonella* proliferation could be bypassed by expressing a fusion hybrid between galectin 3 and NDP52. Galectin 3 per se is not required for restriction although it is found on *Salmonella* vacuoles, primarily since it – unlike galectin 8 - cannot interact with NDP52. Galectin 8 recognizes host membrane glycans and not directly Salmonella carbohydrates, albeit it can directly recognize blood-group-B-positive *E. coli* O86. Galectin 8 is important also for *Shigella*, *Listeria* and even recognized sterile damage to endosomes and lysosomes.

SLRs can also act in a completely different manner to promote autophagic killing of intracellular microbes (Fig. 1, panel 1, right). They gather cytoplasmic proteins (e.g. ubiquitin and ribosomal proteins) to be converted in autolysosomes into anti-microbial products that upon delivery to cytoplasmic compartments harboring microbes transform them into autophagolysosomes, organelles with enhanced antimicrobial capacities relative to conventional phagolysosomes (29–31).

Autophagy and pattern recognition receptors

Autophagy interacts with classical pattern recognition receptors (PRR), including Toll-like receptors (TLR), Nod-like receptors (NLR), and RIG-I like receptors (RLR). TLRs and autophagy intersect in two ways illustrated in Fig. 1, panel 2. Firstly, autophagy is an effector mechanism (e.g. elimination of microbes illustrated in Fig. 1, panel 1) downstream of TLR activation. TLR4 triggers autophagy via TRAF6 E3 ligase, ubiquitination of Beclin 1, and Bcl-2 dissociation from the BH3 domain of Beclin 1 (12). Secondly, autophagy as a topological inverter devise can bring cytosolic pathogen associated molecular pattern (PAMP) molecules into the lumen where they can bind the ligand recognition side of the TLR receptor. This has been demonstrated for TLR7 (32), TLR4 ligands (33) and TLR9 in the context of B cell receptor signaling (34).

NLR and autophagy interactions are evolutionarily conserved from *Drosophila* (35) to humans (16). Nod1 and Nod2 interact with Atg16L1 (36, 37), of significance for Crohn's disease (CD) since Nod2 and Atg16L1 are risk loci for CD (38). NLRC4 (Ipaf) and NLRP4 inhibit autophagy (39) and are found in macromolecular complexes with Beclin 1. RLRs activate autophagy with biologically important effects (40) but thus far more attention has been given to negative regulation of RLR signaling by autophagy factors Atg5-Atg12 (41) and Atg9 (42). Atg9 negatively regulates trafficking and activation of TBK1 in the type I interferon response to double stranded DNA (42).

Autophagy and inflammasome

Autophagy and inflammasomes interact in two ways (Fig. 1, panel 3). All reports thus far (5, 43–46) agree on the observation that autophagy plays a negative role in inflammasome activation. Autophagy lowers basal level of inflammasome activation by continually removing endogenous irritants (43, 44). For example, autophagy prevents spurious inflammasome activation by eliminating defunct mitochondria that otherwise represent endogenous sources of inflammasome agonists such as ROS and mitochondrial DNA (43, 44) (Fig. 1, panel 3). In the absence of basal autophagy, endogenous factors lead to inflammasome activation and increased IL-1β processing and represent sources of sterile inflammation. This explains how loss Atg16L1 elevates IL-1β levels in a murine model of CD (47).

On the flip side, autophagy plays a positive (but only acute, short term) role in delivering outside of the cell the effector products of inflammasome activation, such as IL-1β and potentially other alarmins, in a process referred to as the unconventional secretion of IL-1β(5). Although IL-1β and IL-18 do not have signal peptides to deliver them into the lumen of the organelles of the conventional secretory pathway (ER-Golgi-plasma membrane), they are released extracellularly upon inflammasome activation. This is at least in part supported by the topological inversion properties of autophagy, ferrying molecules from cytosolic side into lumen of putative secretory vesicles. However, this effect wanes quickly with time and the downregulation of inflammasome by autophagy becomes dominant once again (46). Thus, autophagy controls negatively inflammasome activation (5, 43–46) and positively IL-1β secretion per se (5). The topological inversion action and positive role of autophagy in secretion of alarmins is not limited to IL-1β and extends to HMGB1 (5).

Autophagy in antigen presentation and T cell homeostasis

The role of autophagy as a topological inverter (transport from cytosol to lumen) and its other functions contribute to MHC II presentation of endogenous cytosolic antigens (33, 48, 49) (Fig. 1, panel 4). The physiological role of this is manifested in immune surveillance of viral infections (48) and inhibition of this process by HIV-1 (49). Autophagy-dependent presentation of endogenous antigens plays a role in positive and negative selection of naïve T cells repertoires in the thymus (50). It has been hypothesized that peripheral tissue autophagic activities may have to be matched by central tolerance mechanisms dependent on autophagy in the thymus to prevent autoimmunity (50). Autophagy plays a role in mature T cell homeostasis, and is essential for T cell survival following exit from the thymus in part based on the requirement for autophagy to physiologically reduce the mitochondrial and ER content in maturing T cells (51–53).

Autophagy in chronic inflammatory and autoimmune diseases

Genetic links between autophagy and chronic inflammatory disorders and autoimmune diseases continue to be uncovered by genome-wide association studies (GWAS). Genetic variations in the *PRDM1-ATG5* intergenic region have been associated with rheumatoid arthritis (RA) (54). Autophagy specifically favors presentation of citrullinated proteins, which may contribute to autoimmune disorders such as RA (55). The initial GWAS linking of *ATG16L1* and *IRGM* (a modulator of autophagy (56, 57)) with CD (38) have been replicated in nearly 50 independent population studies. Polymorphisms in another autophagy gene, *ULK1*, are also associated with CD (58). Genetic associations of CD with *IRGM* have been extended to *IRGM* copy number variants in human populations (59). *IRGM* has furthermore been linked to systemic lupus erythematosus (SLE) in a recent meta-analysis of autoimmune diseases (60). GWAS in different populations link *ATG5* variants to SLE (61,

62). This genetic evidence and other studies implicate autophagy in chronic inflammatory diseases and autoimmunity disorders.

Conclusions

The initial sporadic observations that autophagy can play a role in cell-autonomous defense against intracellular bacteria such as *Mycobacterium tuberculosis* (63) and streptococci (64) have been extended in past several years to various facets of immunity. The connections of autophagy with normal function of innate and adaptive immunity at almost every level, genetic and functional associations with immunological disorders, and unique, specialized mechanisms of autophagy as standalone immune processes reviewed here and elsewhere (2) are consistent with the thesis of this review that autophagy represents a new and growing immunological paradigm.

Acknowledgments

Funding support: AI042999, AI069345, and ARRA RC1AI086845 from National Institutes of Health, Crohn's & Colitis Foundation of America CCFA2053, and Bill and Melinda Gates Grand Challenge Explorations grant.

The author apologies to the colleagues for omissions imposed by space limitations, including microbial defenses against autophagy, non-autophagic functions of the ATG factors, and roles of autophagy processes other than macroautophagy. The author is grateful to Carolyn Mold for comments on the text and to Dara Elerath for graphic design.

Abbreviations used in this article

AMPK	AMP-activated protein kinase
ATG	autophagy related genes
CD	Crohn's disease
DAMP	danger/damage associated molecular patterns
GWAS	genome-wide association studies
HMGB1	high-mobility group protein B1
hVPS34	human vacuolar protein sorting 34
IKK	inhibitor of NF-κB kinases
IRGM	immunity related GTPase M
LC3	microtubule-associated protein light chain 3
LIR	LC3-interacting region
LRSAM1	leucine rich repeat and sterile alpha motif containing 1
mTOR	mammalian target of rapamycin
NDP52	Nuclear domain 10 protein / antigen nuclear dot *52 kDa protein*
NLR	nucleotide binding and oligomerization domain-like receptors
PAMP	pathogen associated molecular patterns
PI3K	phosphatidylinositol 3-phosphate kinase
PRR	pattern recognition receptors
RAGE	receptor for advanced glycation endproducts

RLR	RIG-I-like receptors
SLE	systemic lupus erythematosus
SLR	sequestosome 1/p6-like receptors
ROS	reactive oxygen species
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
TAB2 and TAB3	TGFβ–Activated Kinase 1 (TAK1)-Binding Proteins 2 and 3
TAK1	Transforming growth factor β activated kinase 1
TBK-1	TANK-binding kinase 1
TCR	T cell receptor
TFEB	transcription factor EB

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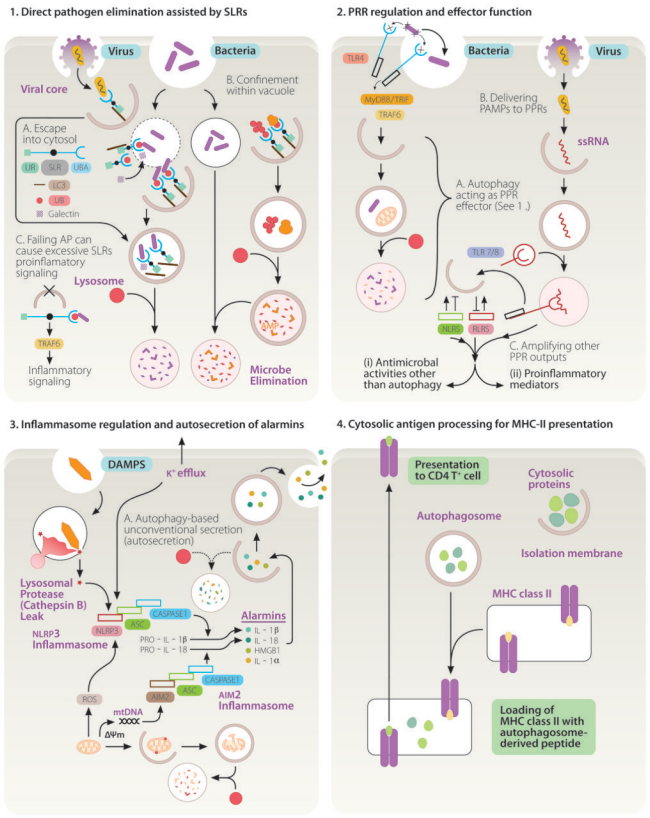

FIGURE 1.

The four principal manifestations of immunological autophagy. **1. Direct pathogen elimination assisted by SLRs and DAMP receptors.** (A) Invading microbes either escaping the endosomes or phagosome (thin outline) or remaining in phagosomes that can be partially permeabilized (dotted outline) are captured by galectins and sequestosome 1/p62-like receptors (SLR) that recognize tags such as ubiquitin (small red circles) or diacylglicerol and β-galactoside (not shown) on damaged host membranes. The captured microbes or co-captured with the earmarked membranes are delivered into autophagic organelles (thick outline) starting with phagophores (crescents) progressing through autophagosomes (full white circles) and ending in degradative autolysosomes (full pink circles). SLRs posses LC3 interacting region (LIR), phosphorylation sites (black dot, arbitrarily positioned), and tag recognition domain (UBA, depicted for p62). Galectins (hatched square), considered to be DAMP receptors, have carbohydrate recognition domains (not shown) that recognize sugars on glycans exposed on the endofacial lumenal membrane leaflet of permeabilized organelles. (B) Alternatively, autophagy can sequester cytosolic proteins such as ubiquitin and ribosomal proteins (pear shaped tan-colored objects, ribosomes) and digest them into antimicrobial peptides (AMPs) that can be delivered to pathogens confined in phagosomes. (C) SLRs can engage in pro-inflammatory signaling e.g. via TRAF6 (shown) or atypical PKC (not shown) or promote cell death by activating caspase-8 through aggregation (not shown). AP, autophagy. **2. PRR regulation and effector functions**. A. Autophagy can be activated downstream of TLR signaling upon recognition of PAMPs (x-like objects). B. As a topological inverter device, autophagy can deliver cytosolic PAMPs to the lumen of endomembranous organelles where they can interact with the receptor portions of TLRs. Known functional interactions with NLRs and RLRs are summarized by positive (arrows) and negative (lines symbolizing inhibition) effects. **3. Inflammasome regulation and secretion of alarmins.** Autophagy plays a dual

role in controlling inflammasome output: it suppresses basal levels of inflammasome activation but also assists IL-1βand IL-18 release from the cells via an autophagy-dependent unconventional secretory pathway (A; autosecretion). Inflammasomes, heteromeric protein assemblages (consisting of ASC, caspase 1, and NLRP3 or AIM2) act as platforms for activation in response to K^+ efflux or presence and action of DAMPs (silica, crystal like object; ROS, reactive oxygen species; mtDNA, mitochondrial DNA). ROS and mtDNA can be released as endogenous DAMPs by damaged mitochondria if they are not continuously removed by autophagy. This results in caspase-1 activation and proteolytic processing of pro-forms of proinflammatory cytokines (IL-1β). Whereas autophagy lowers the sources of endogenous DAMPs by disposing of depolarized ($\Delta\Psi_m$) or leaky mitochondria (sources of ROS and mtDNA), autophagy also enables secretion of the cytosolic IL-1β(and other alarmins such as HMGB1) during the very early stages of physiological inflammasome activation in response to exogenous DAMP sources (microbial or sterile). Autosecretion (autophagy-based unconventional secretion; see Fig. 2 and text for explanations) enables extracellular release of the cytosolic proteins such as IL-1β and HMGB1 per the illustrated process controlled by Atg factors and GRASP (see Fig. 2). Autosecretion occurs early in the process of stimulation and is swamped pre- or shortly post-stimulation by the anti-inflammatory effects of autophagy. The latter keep the tonic levels of inflammasome activation low and bring them back to the resting levels following stimulation. **4. Cytosolic antigen processing for antigen presentation.** Autophagy assists as a topological inversion device in delivery of cytosolic (and nuclear) proteins to MHC II processing and presentation compartments. Explanations in the text include relationships to selection of naïve T cell repertoires and citrullination of antigens.