

Review

The clearance of dying cells: table for two

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Phagocytic cells of the immune system must constantly survey for, recognize, and efficiently clear the billions of cellular corpses that arise as a result of development, stress, infection, or normal homeostasis. This process, termed efferocytosis, is critical for the prevention of autoimmune and inflammatory disorders, and persistence of dead cells in tissue is characteristic of many human autoimmune diseases, notably systemic lupus erythematosus. The most notable characteristic of the efferocytosis of apoptotic cells is its ‘immunologically silent’ response. Although the mechanisms by which phagocytes facilitate engulfment of dead cells has been a well-studied area, the pathways that coordinate to process the ingested corpse and direct the subsequent immune response is an area of growing interest. The recently described pathway of LC3 (microtubule-associated protein 1A/1B-light chain 3)-associated phagocytosis (LAP) has shed some light on this issue. LAP is triggered when an extracellular particle, such as a dead cell, engages an extracellular receptor during phagocytosis, induces the translocation of autophagy machinery, and ultimately LC3 to the cargo-containing phagosome, termed the LAPosome. In this review, we will examine efferocytosis and the impact of LAP on efferocytosis, allowing us to reimagine the impact of the autophagy machinery on innate host defense mechanisms.

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Facts

- Efferocytosis is a carefully orchestrated process by which phagocytes are recruited to sites of cell death, recognize and engulf dying cells, and clear them in an ‘immunologically silent’ manner.
- Dying cells have an active role in their own clearance; via the production of ‘find-me’ signals to attract phagocytes and exposure of ‘eat-me’ signals that engage phagocytic receptors to facilitate engulfment.
- Defects in the efferocytosis machinery are associated with inflammation and autoimmune disorders, such as systemic lupus erythematosus (SLE).
- Microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP) is required for the effective clearance of dying cells.

Open Questions

- Given the variety of ‘find-me’ and ‘eat-me’ signals, as well as their cognate receptors, how do these signals coordinate for effective efferocytosis?
- How does LAP promote the anti-inflammatory response to dying cells, and what role does macrophage metabolism have?

- Do defects in LAP contribute to inflammatory or autoimmune pathogenesis?
- What role does LAP have in oncogenesis? What role does LAP have in tumor-associated macrophages?

An Introduction: Can I Interest You in Any Appetizers?

Even from our earliest developmental stages, the process of generating and maintaining a multicellular, functional organism is characterized by the creation and unceremonious destruction of billions of cells.¹ Programmed cell death, such as apoptosis, necroptosis, or pyroptosis, are active mechanisms designed to sculpt, control, and aid the body in its development and survival. Much of our knowledge on the role of apoptosis in development comes from the study of *Caenorhabditis elegans*, wherein the first wave of cell death occurs ~4 h after fertilization, and of the 1090 cells that are generated, 131 of them are destined for death.² In mammalian embryos, apoptosis is seen during cavitation and has a dynamic role in shaping the embryo.³ It is now well understood that proper apoptosis is fundamental for the proper development of the organism, as deficiencies in mediators of apoptosis result in embryonic lethality or animals with severe malformations.⁴ Conversely, other forms of cell death, such as necroptosis and pyroptosis, are not required during

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Abbreviations: ATG, autophagy-related gene; DAMP, danger associated molecular pattern; IC, immune complex; IRF, interferon regulatory factor; LAP, LC3-associated phagocytosis; LC3, microtubule-associated protein 1A/1B-light chain 3; LXR, liver X receptor; PI(3)P, phosphatidylinositol 3-phosphate; POS, photoreceptor outer segment; PPAR, peroxisome proliferator-activated receptor; PtdSer or PS, phosphatidylserine; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SLE, systemic lupus erythematosus; TAM, Tyro-3, Axl, and Mer; TLR, Toll-like receptor

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development, as evidenced by the normal development of mice deficient for receptor interacting protein kinase3 or caspase-1/11, respectively.^{5,6} Once formed, the organism's relationship with cell death is far from over. Cellular turnover is a constant, genetically programmed process in the adult animal, and removal of these unwanted and unneeded cellular corpses is vital to prevent unwanted inflammation and immune activation.⁷ Although damage can certainly cause unwanted cellular death, most cellular death is an active process, and perturbations in the cell death programs can promote cell accumulation, autoimmunity, oncogenesis, attrition, and/or degeneration.

Within tissues, professional, non-professional, and specialized phagocytes are tasked with the clearance of dying cells. The best-characterized population of professional phagocytes, macrophages, is composed of tissue-specific, differentiated subsets of resident macrophages that clear dying cells and debris.⁸ For example, Kupffer cells in the liver clear aged red blood cells,⁹ alveolar macrophages of the lung clear apoptotic airway epithelial cells,⁸ and microglia in the central nervous system clear dying neurons.¹⁰ Other types of resident cells, such as epithelial cells and fibroblasts, have been termed non-professional phagocytes; though this designation may be a misnomer as these cells have a major role when professional phagocytes are rare, such as in the mammary gland or intestinal epithelium. In addition, airway epithelial cells are critical for the clearance of apoptotic airway epithelial cells, and epithelial cell-specific deletion of Rac1 results in increased allergen-induced airway inflammation.¹¹ Still other types of tissue-specific, multifunctional cells exist as specialized phagocytes. In the testes, Sertoli cells are responsible for clearing apoptotic germ cells that arise during spermatogenesis.¹² In the eye, retinal pigment epithelial (RPE) cells are critical for the homeostatic, daily removal of the photoreceptor outer segments (POSs), and the generation of 11-*cis*-retinal for the visual cycle. Defects in RPE cell-mediated removal of outer segments (or processing of outer segments via LAP, discuss below) can lead to a predisposition to conditions, such as age-related macular degeneration or retinitis pigmentosa.¹³

Like the death process itself, the innate immune system has tolerance systems in place to manage these morbid, yet necessary events. Although the generation and subsequent destruction of these cells is necessary for normal cellular homeostasis, wound healing, and immune responses in the adult organism, the ruin left in its wake would be catastrophic if not for the efficient work of the phagocytic system.¹⁴ Despite the constant, homeostatic turnover of cells, as well as cell death induced by stress, damage, or infection, it is rare to observe apoptotic cells under normal physiological conditions. Considering the average one million adult human cells that undergo apoptosis every second, one can appreciate the magnitude of the job facing phagocytes.¹⁵ Moreover, as this is a reoccurring and normal event in the lifespan of an organism, this process of dead cell clearance must occur in a quiescent manner, so as to not inappropriately alert the immune system.¹⁶

We now appreciate the critical role that efferocytosis has on modulating immunity, as well as the impact that different types of cell death have on the immune response. In this review, we

discuss the process of efferocytosis, chemoattraction of phagocytes, recognition of dying cells, engulfment of cellular corpses, and the processing of engulfed cellular cargo, specifically the role of LAP in clearance of dying cells and control of inflammation. Finally, we explore the effect of defective efferocytosis on pathology and disease states.

The Mechanisms of Efferocytosis: Would You Like to Hear the Specials?

As the focus of this review is the aftermath of cell death, we have summarized the four most well-defined modes of cell death (apoptosis, necrosis, necroptosis, and pyroptosis) in Table 1, as the roles and mechanisms of cell death have been studied and reviewed extensively.¹

Efferocytosis is not merely a passive event, but a carefully orchestrated process designed to efficiently eliminate cellular corpses and limit exposure to their potentially damaging components, with the goal being immunological tolerance.¹⁷ Efferocytosis can be generally categorized into 4 steps: 1) the release of 'find-me' signals by dying cells to recruit phagocytes, 2) phagocyte recognition and engagement of 'eat-me' signals on dying cells, 3) the engulfment of the cellular corpse, and 4) the processing, degradation, and immune response to the engulfed corpse. We now recognize that defects in any of these four steps can contribute to unwanted inflammation and autoimmune disorders, such as systemic lupus erythematosus¹⁸ (Table 2).

As phagocytes are often not proximal to sites of cell death or even reside in the tissues they must survey, dying cells must 'advertise' their presence to phagocytes.¹⁹ Recruitment of phagocytes to sites of cell death in *C. elegans* occurs before the completion of apoptosis, indicating that one of the first acts of a dying cell is to prepare for its own elimination.^{20,21} During this process, apoptotic cells release 'find-me' signals, distinct molecules that establish a chemotactic gradient to attract phagocytic cells.²² Nucleotides, such as ATP, are released in a caspase-dependent manner via activation of pannexin-1 channels and are perhaps the most well-defined 'find-me' signals.²³ These nucleotides are detected by phagocytes via purinergic receptors, like P2Y2, and disruption of the nucleotide/P2Y2 interaction results in an accumulation of dying cells *in vivo*.¹⁹ Apoptotic cells also release the membrane-associated molecule fractalkine (or CX3CL1), which is sensed by CX3CR1 and mediates the migration of macrophages to the dying cells. Mice deficient for CX3CR1, however, do not display a defect in apoptotic cell clearance, suggesting that multiple factors are required to recruit effectively phagocytes.²⁴ Molecules of lipid origin can also act as 'find-me' signals. Lysophosphatidylcholine is generated and released via caspase-3-dependent activation of phospholipase A, and is sensed by the G-protein-coupled receptor G2A.²⁵ Sphingosine-1-phosphate (S1P), also a lipid 'find-me' signal, is released by dying cells and sensed by multiple G-protein-coupled receptors S1P-R1-5. These lipid signals have been demonstrated to mediate phagocyte chemotaxis²⁶ (Figure 1a).

There are significant caveats to the ability of 'find-me' signals to efficiently act as chemoattractants in physiologically scenarios. The idea of an apoptotic cell's purposeful release of

a 'find-me' signal to actively recruit phagocytes is undermined by the relatively low level of signal released by apoptotic cells compared with necrotic cells. 'Find-me' signals are often

released in an active, caspase-dependent manner, yet these molecules are also released (and in greater quantities) during other forms of cell death, such as necrosis or necroptosis.²⁷

Table 1 Summary of the four major modes of cell death: apoptosis, necrosis, necroptosis and pyroptosis

Description	Characteristics	References
<i>Apoptosis</i>		
Active cellular death, largely controlled by a family of cysteine proteases called caspases Apoptotic caspases are broadly grouped into initiator caspases (caspase-8 and -9) and executioner caspases (caspases-3, -6, and -7)		
<i>Intrinsic or mitochondrial pathway</i> Activated by stress-inducing stimuli (i.e., DNA damage, accumulation of unfolded proteins, and hypoxia) and developmental signals Signals converge on the mitochondria, where pro-apoptotic and anti-apoptotic members of the BCL2 family mediate the release of cytochrome <i>c</i> , formation of the apoptosome with caspase-9 and APAF-1, which leads to the activation of the downstream executioner caspases, such as caspase-3 and caspase-7	Membrane 'blebbing,' often with separation of apoptotic bodies DNA fragmentation Chromatin condensation Considered immunologically silent due to the packaging of possible danger-associated molecular patterns (DAMPs) into discreet, tolerogenic pieces Active phosphatidylserine (PtdSer) exposure (Annexin V positive) Propidium iodide or 7-AAD negative at early stages	1,99–103
<i>Extrinsic pathway</i> Triggered by signals that engage extracellular death receptors (DR) Tumor necrosis factor (TNF) and TNF receptor-1 (TNFR1) CD95-ligand (CD95-L or Fas-L) and CD95 (or Fas) TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL-R1/2 (DR4/5) Recruitment of pro-caspase-8 to the death-inducing signaling complex (DISC) at the DR (with the adapter proteins FADD or TRADD), resulting in dimerization and activation of caspase-8, leading to caspase-3 and caspase-7 activity Caspase-8 activity can also feed into the intrinsic pathway by cleaving and activating BCL2 family proteins		
<i>Necrosis</i>		
Characterized as a passive type of cell death that occurs independently of caspase activation Triggered in response to catastrophic damage or pathology, including infarction, mechanical trauma, ischemia, frostbite, and animal venom Apoptotic cells that are not efficiently cleared by phagocytes can undergo secondary necrosis independently of any apoptotic machinery	Cellular swelling (oncosis) Organelle swelling Nuclear distention Cellular rupture Releases inflammatory cellular contents (DAMPs) or alarmins that can activate neighboring immune cells via Toll-like receptor (TLR) signaling and other mechanisms Annexin V positive due to membrane rupture Propidium iodide or 7-AAD positive	1,104–106
<i>Necroptosis</i>		
Genetically programmed cell death with the morphological features of necrosis Triggered by diverse forms of neurodegeneration, ischemia, or infection. Engagement of the extrinsic pathway (i.e., TNF–TNFR pathway) in the absence of caspase-8 can result in a necrotic cell death that requires the kinase activity of receptor interacting protein kinase1 (RIPK1) and RIPK3 RIPK3 phosphorylates and activates the pseudokinase, mixed lineage-kinase like (MLKL) Induces a conformational change that allows for its oligomerization and interaction with the plasma membrane through binding to phosphatidylinositol lipids to directly disrupt membrane integrity RIPK1 is required for a variety of innate immune signaling pathways, such as TLRs, interferons, and the RIG-I-MAVS pathway	Loss of plasma membrane integrity Swollen cellular organelles Releases inflammatory cellular contents (DAMPs) or alarmins that can activate neighboring immune cells via TLR signaling and other mechanisms Active PtdSer exposure (Annexin V positive) Propidium iodide or 7-AAD positive	5,107–112
<i>Pyroptosis</i>		
Non-apoptotic, genetically programmed cellular death mediated by excessive inflammatory caspase activity (human caspase-1, -4, and -5; rodent caspase-1 and -11) Required for cell death in a variety of experimental settings, including in the immune system, the cardiovascular system, and the central nervous system Caspase-1 is activated by dimerization at complexes termed inflammasomes that form in the cytosol and detect a diverse repertoire of pathogenic molecules, including bacterial toxins and viral RNA Activated caspase-1 in turn cleaves pro-IL-1 β and pro-IL-18, which facilitates the secretion of these pro-inflammatory cytokines Characterized by caspase-1-dependent formation of plasma membrane pores, which leads to pathological ion fluxes that ultimately result in cellular lysis and release of inflammatory intracellular contents Caspase-1 can also activate caspase-7	Cellular rupture DNA fragmentation Releases inflammatory cellular contents (DAMPs) or alarmins that can activate neighboring immune cells via TLR signaling and other mechanisms Often Annexin V positive due to membrane rupture Propidium iodide or 7-AAD positive	1,4,6,51,113,114

Table 2 Components of the efferocytosis machinery and their association with inflammatory and autoimmune diseases

Molecule	Role in efferocytosis	Disease(s)	References
<i>Molecules associated with increased incidence or risk of disease</i>			
Nucleotides (ATP/UTP)	'Find-me'	MS/EAE	115
Pannexin-1	'Find-me'	MS/EAE, seizure disorders	116,117
S1P	'Find-me'	MS/EAE	118
LPC	'Find-me'	Atherosclerosis, SLE/systemic autoimmunity	119
S1PR1-5	'Find-me'	MS/EAE	118
G2A	'Find-me'	SLE/systemic autoimmunity, atherosclerosis	120,121
CX3CR	'Find-me'	Autoimmune uveitis, MS/EAE	122,123
ICAM3	'Eat-me'	RA, SLE/systemic autoimmunity, GBS, MS/EAE	124
CRT	'Eat-me'	RA, Sjogren's syndrome, Celiac disease, SLE/systemic autoimmunity	125
C1q	'Eat-me'	SLE/systemic autoimmunity, RA, atherosclerosis	126
TIM1	'Eat-me'	SLE/systemic autoimmunity, airway inflammation	127,128
TIM3	'Eat-me'	Airway inflammation, MS/EAE	128
TIM4	'Eat-me'	SLE/systemic autoimmunity	37
BA1	'Eat-me'	Glioblastoma, neurological disorders	129
Integrins ($\alpha\beta$ 3)	'Eat-me'	Scleroderma, ulcerative colitis	52,130
MerTK	'Eat-me'	SLE/systemic autoimmunity, retinitis pigmentosa, atherosclerosis	53,131,132
MFG-E8	'Eat-me'	SLE/systemic autoimmunity, atherosclerosis	31,133
ProteinS	'Eat-me'	Thrombosis, SLE/systemic autoimmunity	134,135
CD300f	'Eat-me'	SLE/glomerulonephritis	136
ELMO1	Engulfment	Testicular pathology, impaired neurogenesis	12,137
DOCK180	Engulfment	Cardiovascular abnormalities, myogenesis abnormalities	138,139
GRK6	Engulfment	SLE/systemic autoimmunity	140
RAC1	Engulfment	RA, airway inflammation	11,141
DNase II	Processing	Polyarthritis	72
LXR α/β	Processing	MS/EAE, SLE/systemic autoimmunity, autoimmune uveitis, type I diabetes, atherosclerosis	142–148
PPAR δ/γ	Processing	MS/EAE, SLE/glomerulonephritis, atherosclerosis, osteoarthritis	95,96,149–151
ABCA1	Processing	SLE/glomerulonephritis	152
<i>Molecules associated with decreased incidence or risk of disease</i>			
Fractalkine (CX3CL1)	'Find-me'	Sjogren's syndrome, airway inflammation, RA	153–155
Purigenic receptors (P2Y2)	'Find-me'	Sjogren's syndrome, autoimmune uveitis	156,157
Integrins ($\alpha\beta$ 3)	'Eat-me'	MS/EAE	158
CD91 (LRP)	'Eat-me'	RA, SLE/systemic autoimmunity	159
RAGE	'Eat-me'	MS/EAE, DTH	160,161
GAS6	'Eat-me'	Thrombosis, nephrotoxic nephritis, SLE/systemic autoimmunity	162–164

Abbreviations: BA1, brain-specific angiogenesis inhibitor 1; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalitis; C1q, complement 1q; CRT, calreticulin; GBS, Guillain-Barré syndrome; LPC, lysophosphatidylcholine; LXR, liver X receptor; MFG-E8, milk fat globule-EGF factor 8; MS, multiple sclerosis; PPAR δ/γ , peroxisome proliferator-activated receptor γ/δ ; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; S1P, sphingosine-1-phosphate; TIM, T-cell immunoglobulin mucin receptor

Indeed, <2% of intracellular ATP is released during apoptosis.¹⁵ In addition, these released nucleotides must also survive degradation by extracellular nucleotidases, indicating that they most likely act in a short-range capacity.¹⁹ Similarly, 'find-me' signals of lipid origin are present ubiquitously in the circulation at a concentration higher than that released by apoptotic cells.⁷ The mechanisms by which 'find-me' signals, which can be recognized by a wide variety of cells, specifically recruit phagocytes, the majority of which are macrophages, are unknown. The counteractive effect of 'keep out' signals has been proposed to aid in the coordinated recruitment of phagocytes. For example, lactoferrin, a glycoprotein released by apoptotic cells, has been shown to actively exclude neutrophils and eosinophils from sites of cell death.^{28,29} Further complicating the matter is the dual role that 'find-me' signals can have, as danger-associated molecular patterns (DAMPs)³⁰ or as activating factors to prime phagocytes.³¹

Cell death does not occur in a vacuum; sites of cell death are a conglomeration of dying cells, healthy cells, and immune cells. The phagocyte must distinguish living cells from dying cells in order to maintain homeostasis, promote proper

development, and prevent unwanted inflammation. Just as dying cells must recruit phagocytes, they must also transform themselves into targets for engulfment, displaying distinct signals that differentiate them from viable cells.^{32,33} The extracellularly exposed lipid, phosphatidylserine (PtdSer), is the most well-characterized 'eat-me' signal and an essential factor in effective efferocytosis.³⁴ Normally confined to the inner leaflet of the plasma membrane lipid bilayer of living cells (and in a relatively minor amount), PtdSer is actively and rapidly externalized in a caspase-dependent manner during apoptosis.³⁴ Caspase 3-mediated cleavage of the scramblase Xkr8 facilitates exposure of PtdSer during apoptosis,³⁵ an event normally reversed by the activity of the flippase ATP11C, which is inactivated by caspase-3 cleavage.³⁶ Extracellularly exposed PtdSer is recognized by multiple, bona fide membrane receptors, such as T-cell immunoglobulin mucin receptor 4 (TIM4), brain-specific angiogenesis inhibitor 1 (BA11), and stabilin-2,^{37–39} and bridging molecules, such as milk fat globule-EGF factor 8 (MFG-E8) and Gas6, that recognize PtdSer and then engage phagocytic cell surface receptors such as integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, or Tryp3-Axl-Mer (TAM) receptors^{40–42} for engulfment.

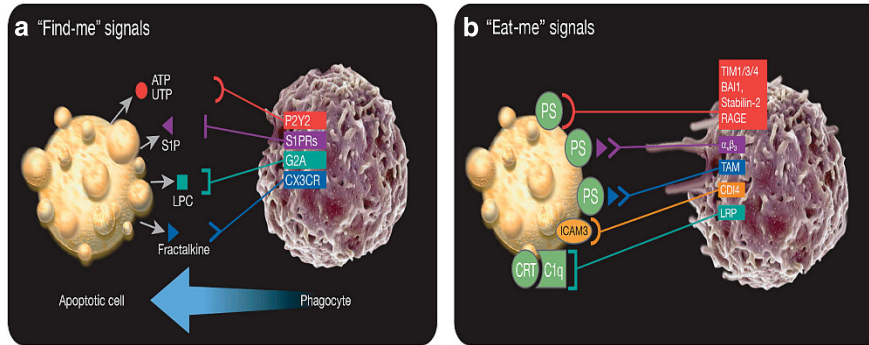


Figure 1 The recruitment of phagocytes and recognition of dying cells by phagocytes. **(a)** Dying cells release ‘find-me’ signals, such as ATP, UTP, S1P, lysophosphatidylcholine (LPC), or fractalkine, that recruit phagocytes to sites of cell death. Phagocytes sense these ‘find-me’ signals via cognate receptors (P2Y2, S1PRs, G2A, and CX3CR, respectively). **(b)** Phagocytes express a variety of receptors and bridging molecules that recognize and engage dying cells via ‘eat-me’ signals exposed on apoptotic cell surfaces. The most common ‘eat-me’ signal, phosphatidylserine (PtdSer or PS), engages the PtdSer-specific receptors, TIM1, TIM3, TIM4, BAI1, stabilin-2, and RAGE, as well as the PS-specific bridging molecules MFG-E8, Gas6, and protein S. These bridging molecules engage other surface engulfment receptors ($\alpha v\beta_3$ or TAM) to facilitate uptake. Other ‘eat-me’ signals, such as calreticulin (CRT) and ICAM3, exist and mediate recognition and engulfment via the receptors LRP (via C1q) and CD14, respectively

Other ‘eat-me’ signals have also been identified, which are likely to have a ‘tethering’ function, facilitating the above events. ICAM3 can bind to membrane-associated CD14,⁴³ and externalized calreticulin bound to complement C1q can engage CD91 (or LRP1).⁴⁴ Oxidized LDL-like moieties and glycosylated surface proteins can serve as ‘eat-me’ signals, binding to scavenger receptors⁴⁵ and lectins,⁴⁶ respectively (Figure 1b).

Similar to the ‘find-me’/‘keep out’ signal paradigm, there is evidence of a negative signal to discourage phagocytosis. Although PtdSer is considered a hallmark of cell death, forced PtdSer exposure⁴⁷ or physiologically normal exposure on activated, living cells does not mediate recognition or engulfment.⁴⁸ Thus, the simultaneous presence of ‘don’t eat-me’ signals, such as CD31, CD47, and CD61, on viable cells, may negatively regulate phagocytosis, indicating to the phagocyte that despite the extracellular PtdSer, this cell is not intended for clearance.^{18,49,50} Therefore, a coordinated effort between the dying cell and the phagocyte must exist to facilitate efferocytosis.

Although the majority of work on ‘find-me’ and ‘eat-me’ signals stems from apoptotic cells, these signals also function during other types of cell death, such as necroptosis and pyroptosis.⁷ As previously mentioned, ‘find-me’ signals, such as ATP, are released (and in greater quantities) during necrosis, necroptosis, and pyroptosis.^{6,14,51} Similarly, necrotic and pyroptotic cells also stain positive for Annexin V, although in these cases, externalized PtdSer can be attributed to rupture of the plasma membrane rather than an active exposure process.^{52–54} These dead cells can still be recognized and engaged by PtdSer receptors;⁵⁵ however, owing to the lytic nature of their demise, DAMPs have already been released into the milieu and can activate inflammatory programs. Therefore, although apoptotic cells actively coordinate their own clearance, necrotic and pyroptotic cells passively utilize these systems as well.

The tissue specificity of PtdSer receptors may help to explain why multiple receptors are required for efficient efferocytosis.^{17,18,56} Stabilin-2 is highly expressed in endothelial cells within atherosclerotic plaques,⁵⁷ although defects

in BAI1, highly expressed in glial and neuronal cells, are associated with neurodegenerative disorders.⁵⁸ Despite a common ligand and a common goal of engulfment, the mechanisms by which PtdSer receptors mediate phagocytosis are often varied. Once engaged by PtdSer-bound integrins, bridging molecules, such as $\alpha v\beta_3$ or TAM, associates with the adapter proteins ELMO1 and DOCK180 (via CrkII) at the site of phagocytosis.^{59,60} The PtdSer receptor BAI1 also requires the activity of the DOCK180/ELMO1 complex for engulfment, but BAI1 is able to recruit the complex independently.³⁸ Stabilin-2 and CD91/LRP, however, require the activity of the adapter protein, GULP, to facilitate phagocytosis.^{61–63} One of the most well-known PtdSer receptors, TIM4, contains a very short cytoplasmic region and currently its signaling components are unknown.⁶⁴

Dying cell engulfment involves active membrane ruffling by a process similar to macropinocytosis.^{65,66} Engagement of PtdSer receptors results in cytoskeletal reorganization to facilitate phagocytosis, which is mediated by the Rho family of small GTPases, including members RhoA, ROCK, Rac, Rab5, and Cdc42.⁶⁷ These GTPases cycle between the resting, inactive GDP-bound state and the active GTP-bound state, mediated by specific guanine-nucleotide-exchange factors (GEFs), such as the bipartite GEF formed by DOCK180 and ELMO1.⁶⁸ Ultimately, signaling during efferocytosis converges on the activation of evolutionarily conserved Rac1, acting at the phagocytic cup to promote actin polymerization and cytoskeletal rearrangement via the Scar/WAVE complex.^{67,69,70} Similarly, CDC42 has been linked to the engulfment of apoptotic cells, although its precise role is unclear.⁷¹ Once encased within the phagocyte, however, the dying cell is now capable of exerting its effect on critical downstream immunological and metabolic pathways.

Degradation After Phagocytosis: Did You Save Room for Dessert?

The mechanisms by which phagocytes handle the burden of processing an engulfed cellular corpse are currently of great interest. Not only must a phagocyte interpret its ingested cargo

Table 3 Components of the autophagic machinery and their association with inflammatory and autoimmune diseases

Molecule	Confirmed pathway(s)	Associated Disease(s)	References
NOX2	LAP	CGD, Alzheimer's disease, Creutzfeldt–Jakob disease	165–167
Rubicon	LAP	Ataxia	168
Beclin1	Autophagy LAP	Ovarian cancer, breast cancer, lung cancer, cystic fibrosis, Alzheimer's disease, RA	169–174
VPS34	Autophagy LAP	Schizophrenia	175
UVRAG	Autophagy LAP	Stomach cancer, non-segmental vitiligo, colorectal cancer, cardiomyopathy	85,176–178
ATG5	Autophagy LAP	Airway inflammation, SLE/systemic autoimmunity, MS/EAE, RA, Alzheimer's disease, atherosclerosis	179–184
ATG16L	Autophagy LAP	Crohn's disease, atherosclerosis	185,186
ATG7	Autophagy LAP	SLE/systemic autoimmunity, MS/EAE, type I diabetes, RA, Alzheimer's disease, cardiomyopathy	187–190
ATG4	Autophagy LAP	Otoconia	191
LC3	Autophagy LAP	Nasu-Hakola disease	192
LAMP2	Autophagy LAP	Danon disease, type II diabetes	193
ULK1	Autophagy	Crohn's disease	194
FIP200	Autophagy	Inflammatory skin disorders	195
p62	Autophagy	Huntingtin's disease	196
EPG5	Autophagy	Vici syndrome	197
IRGM	Autophagy	Crohn's disease, MS/EAE	198
SMURF1	Autophagy	Ulcerative colitis	199
WDR45	Autophagy	Encephalopathy	200
Parkin	Mitophagy	Parkinson's disease	201
PINK1	Mitophagy	Parkinson's disease	202

Abbreviations: ATG, autophagy-related gene; CGD, chronic granulomatous disease; EAE, experimental autoimmune encephalitis; LAP, LC3-associated phagocytosis; MS, Multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus
Confirmed activity in autophagy, LAP, and/or mitophagy is indicated

in an immunologically tolerant manner, it must also contend with the excess lipid, cholesterol, and protein that an entire engulfed cell brings. Acidic proteases and nucleases in mature phagolysosomal compartments degrade dying cells into their basic cellular components, including fats, sterols, peptides, and nucleotides. For example, DNase II, a lysosomal enzyme, is required for the degradation of DNA, and DNase II deficiency results in an accumulation of undigested DNA fragments within phagocytic cells, capable of activating intracellular nucleic acid sensors.⁷²

The recent discovery of LAP has shed some light on this issue. The two ancient systems of phagocytosis and autophagy represent two modes of nutrient acquisition, during abundance and scarcity, respectively. These two evolutionarily conserved pathways converge, however, during the engulfment of pathogens or dead cells.⁷³ LAP is a process that marries the processes of phagocytosis and autophagy into a fundamentally new concept, allowing us to reinterpret the impact of the autophagy machinery on innate host defense mechanisms (Table 3).

LAP is triggered when an extracellular particle, such as a pathogen, immune complex, or dead cell, is sensed by an extracellular receptor, including Toll-like receptor 1/2 (TLR1/2), TLR2/6, TLR4, FcR, and TIM4, and phagocytosed.^{55,74–76} This engulfment recruits some, but not all, members of the autophagy machinery to the cargo-containing vesicle.^{55,77} It is the activity of these autophagic players that facilitates the rapid processing of the cargo via fusion with the lysosomal pathway, which can have a critical role in the degradation of engulfed

cargo,^{77,78} as well as modulate the resulting immune response.^{55,75,78}

Despite sharing common molecular machinery, there currently exist several distinctions that differentiate LAP from canonical autophagy (Figure 2). Originally, LAP and autophagy were distinguished by the structure of the LC3-decorated phagosome (or LAPosome) and the rapidity with which LAP occurs. EM analysis revealed that LAP results in single-membrane structures,⁷⁷ as opposed to the double-membrane autophagosomes surrounding autophagic cargo.⁷⁹ Whereas LC3-decorated autophagosomes can take hours to form, LC3-II can be detected on LAPosomes in as few as 10 min after phagocytosis, and phosphatidylinositol 3-phosphate (PI(3)P) activity can be seen at the LAPosome within minutes after phagocytosis.^{55,75,77}

Recent studies have elucidated the molecular mechanisms governing LAP.⁷⁸ Although a majority of the core autophagy components are required for LAP, there exist some critical differences that can distinguish the two processes. Under basal conditions, mTOR inhibits the pre-initiation complex, comprised of FIP200, autophagy-related gene 13 (ATG13), and ULK1/2, and hence autophagy. However, the pre-initiation complex is dispensable for LAP.^{55,75,78} Furthermore, canonical autophagy requires the ULK1-dependent release of a Beclin1-activating cofactor, Ambra1, from the dynein motor complex,⁸⁰ and the function of WIPI2,⁸¹ whereas LAP does not.⁷⁸

Both LAP and canonical autophagy require the class III PI3K complex, which contains the core components Beclin1,

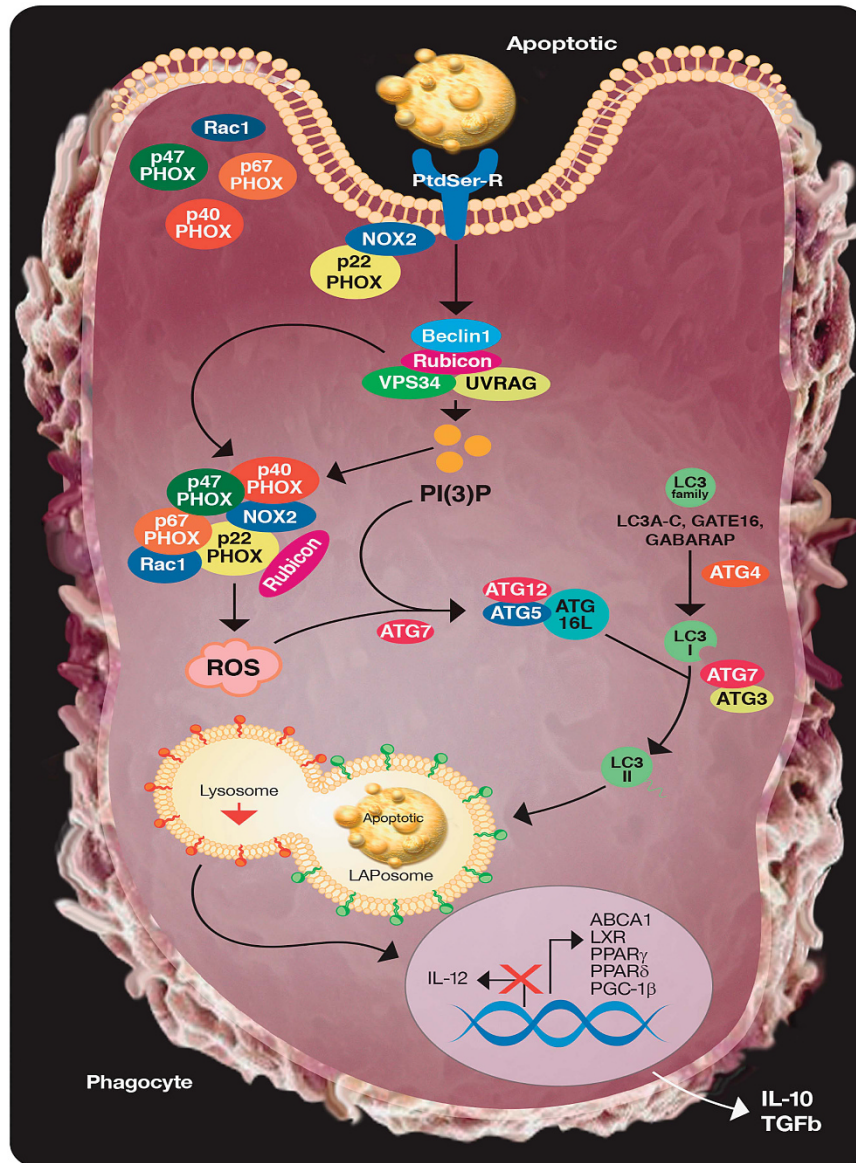


Figure 2 The processing of engulfed dying cells requires LC3-associated phagocytosis (LAP) and promotes an anti-inflammatory response. Upon engulfment of dying cells, components of the LAP pathway are recruited to dead cell-containing phagosome (or LAPosome). The class III PI3K complex, comprised of Beclin 1, VPS34, UVRAG, and Rubicon, is critical to the sustained and localized production of PI(3)P at the LAPosome. PI(3)P serves two roles – the recruitment of the downstream LAP machinery (such as ATG5, ATG12, ATG16L, and ATG7) and stabilization of the NOX2 complex for the production of ROS. Rubicon is also required for the stabilization of the NOX2 complex. Both ROS and PI(3)P are required for successful LC3-II decoration of the LAPosome, and LC3-II is required for fusion to the lysosome and maturation of LAPosome. The anti-inflammatory effects of efferocytosis are mediated by the activity of lipid and cholesterol sensors, such as ABCA1, LXR, PPAR γ/δ , and PGC-1 β , leading to the production of IL-10 and TGF β , whereas pro-inflammatory mediators, such as IL-12, are actively repressed

VPS34, and VPS15.⁸² It can, however, differ in its additional composition. ATG14 and UVRAG are mutually exclusive in their association with the class III PI3K complex during autophagy,⁸³ and silencing of either ATG14^{83,84} or UVRAG⁸⁵ inhibits canonical autophagy. LAP, on the other hand, only requires the activity of the UVRAG-containing class III PI3K complex, whereas ATG14 is dispensable.⁷⁸

Rubicon (RUN domain protein as Beclin 1 interacting and cysteine-rich containing) is a protein that associates constitutively with the UVRAG-containing class III PI3K complex.⁸⁶ Rubicon is a negative regulator of autophagy (via its inhibition

of VPS34^{84,86} or by blocking GTPase Rab7 activation⁸⁷), and silencing of Rubicon results in an increase in the number of autophagosomes.⁷⁸ During LAP, Rubicon is uniquely associated with LAPosomes (but not conventional phagosomes), and Rubicon-deficient cells are completely defective in LAP.⁷⁸ Thus, Rubicon is a molecule that is uniquely required for LAP, but dispensable for canonical autophagy.

Studies suggest that the role for Rubicon in LAP is twofold. First, Rubicon promotes the association of the active class III PI3K complex with the LAPosome, thereby aiding in the localization of VPS34-mediated PI(3)P at the LAPosome.

In both canonical autophagy and LAP, PI(3)P is required for the recruitment of the downstream ubiquitin-like conjugation systems, the ATG5-12 and LC3-PE conjugation systems.⁷⁸ In LAP, Rubicon and PI(3)P have an additional role. Rubicon stabilizes NOX2, the predominant NADPH oxidase in phagocytes, by interacting with its p22^{phox} subunit via its serine-rich domain (aa 567–625), a domain separate from the CCD domain (aa 515–550) responsible for its interaction with Beclin1⁸⁸ and the RUN domain (aa 49–180) responsible for its interaction with VPS34.⁸⁹ Moreover, PI(3)P binds and stabilizes the p40^{phox} subunit of NOX2.⁹⁰ Collectively, Rubicon promotes the association of the active class III PI3K complex with the LAPosome and the production of PI(3)P. Rubicon and PI(3)P stabilize the active NOX2 complex to promote optimal reactive oxygen species (ROS) production, which is also required for successful LAP.⁷⁸ Indeed, NOX2-deficient cells fail to undergo LAP^{78,91} and scavenging of ROS by antioxidants, such as resveratrol, Tiron, or alpha-tocopherol is also an effective way to inhibit LAP.^{78,88,91} Thus, LAP and canonical autophagy are molecularly distinct processes.^{13,55,75,76}

In addition, LAP and canonical autophagy are functionally distinct as well. There is mounting evidence that LAP is a critical regulator of inflammation *in vivo* and under physiologically relevant conditions. Not only is LAP critical for the degradation of engulfed organisms, such as intraphagosomal yeast⁷⁷ or *Aspergillus fumigatus*,⁷⁴ but LAP can have a profound effect on the immune response to the engulfed material. Upon intranasal challenge with *A. fumigatus*, a TLR2 ligand, LAP-deficient animals fail to efficiently clear the pathogen and display increased levels of pro-inflammatory cytokines both locally (lung) and systemically (serum).⁷⁴ Thus, many of the autophagic defects associated with control of pathogens could actually be defects in LAP.

LAP can also be triggered in specialized phagocytes, such as the RPE. On a daily basis and regulated by circadian rhythm, RPE cells phagocytose and digest shed POSs, a process crucial for supplying nutrients and O₂ to the retina and the metabolism of vitamin A for the visual cycle. However, the receptor(s) that recognize shed POS and trigger LAP remains unknown. What is known is the requirement for LAP in the proper processing of POS and promotion of the visual cycle, a series of biochemical reactions within the RPE and retina that ultimately results in the production of the chromophore 11-*cis*-retinal (RAL) necessary for the phototransduction signaling cascade. RPE cells deficient for LAP (ATG5, Beclin1), but not canonical autophagy (ULK1, FIP200, ATG13) displayed defective POS degradation, diminished production of 11-*cis*-retinal, and decreased visual function with age. Thus, LAP functions to support chromophore regeneration through the efficient processing of POS by the RPE.¹³

LAP is also required for establishing specific signaling compartment and is a critical regulator of the type I interferon response in some cases. In plasmacytoid dendritic cells, LAP is induced by engagement of the FcγR by immune complexes (IC), complexes of self-antigen (such as DNA) and auto-antibodies commonly found in patients with SLE. In cells deficient for LAP, failure to lipidate LC3 on the DNA-IC-containing LAPosome results in a failure to acquire a late-endolysosomal phenotype. Subsequently, these LAP-deficient cells fail to form the specialized interferon

regulatory factor 7 (IRF7)-signaling compartment required for TLR9-mediated activation of IRF7, and therefore fail to produce IFN- α . This suggests that LAP could affect the functional immune response elicited by autoantigens and have an important role in autoimmunity.⁷⁵

Unwanted inflammation and autoimmunity is held in check by the efficient clearance of dying cells every day.^{55,92} It is the responsibility of the phagocytes to first clear the dying cell from circulation and then instigate an anti-inflammatory response. Phagocytes that have engulfed apoptotic cells have been shown to secrete anti-inflammatory cytokines, such as TGF β and interleukin-10 (IL-10),⁵⁴ whereas actively suppressing pro-inflammatory cytokines, such as tumor necrosis factor, IL-1, and IL-12.⁹³ How the phagocyte achieves this feat is of great interest. LAP is triggered during efferocytosis, and apoptotic, necrotic, and necroptotic cells can engage the PS receptor, TIM4, resulting in a recruitment of the LAP machinery to the dead-cell-containing, single-membrane LAPosome. LAP-deficient macrophages fail to recruit LC3 to the LAPosome, leading to a failure in phagosomal acidification and subsequent corpse degradation. Whereas the paradigm of efferocytosis is 'immunologically silent', LAP-deficient macrophages produce markedly increased levels of IL-1 β and IL-6 when fed dying cells, yet produce significantly less anti-inflammatory cytokines, such as IL-10, upon such engulfment.⁵⁵ LAP is engaged by a variety of receptors and is critical for directing a variety of different immune response, including preventing an unwanted inflammatory response and promoting the formation of the interferon signaling compartment.^{55,75} Although these functions may appear contradictory, it suggests that the fundamental role of LAP is to shape the appropriate response, and absence of this pathway seems to result in aberrant inflammation and pathogen control.

How the LAP pathway modulates the immune response to apoptotic cells remains to be elucidated, though clues may lie in the mechanisms by which the phagocyte handles the metabolic stress of doubling its content of cellular components. The sensing of one such component, cholesterol, can have a significant effect on the phagocyte's response to engulfed dead cells and their increase in basal cholesterol efflux activity.⁹⁴ Members of the peroxisome proliferator-activated receptor γ/δ (PPAR γ/δ) and liver X receptor (LXR) families, both important regulators of cellular lipid homeostasis, are activated during efferocytosis, and results in a positive feedback signal wherein the phagocytic receptors, such as members of the TAM family, are upregulated.^{95,96} Furthermore, cholesterol efflux machinery, such as 12-transmembrane protein ABCA1 (ATP-binding cassette sub-family A, member 1), is upregulated to accommodate the increase in cholesterol load.⁹²

The non-immunogenic nature of efferocytosis of apoptotic cells is one of its key characteristics, and cholesterol homeostasis has a critical role in establishing this tolerance.^{18,22} PPAR γ/δ are central players in the polarization of anti-inflammatory ('M2') macrophages, and agonists for both PPAR γ and LXR have been shown to inhibit inflammatory responses.^{18,96} Conversely, PPAR $\gamma^{-/-}$ and PPAR $\delta^{-/-}$ macrophages are defective in efferocytosis. The dual functions of PPARs and LXRs in both lipid apoptotic cell clearance and

lipid homeostasis suggest the interconnectedness between efferocytosis and metabolism.

Despite all types of dying cells providing excess cholesterol for the engulfing cells, uptake of necrotic cells does not induce enhanced cholesterol efflux in the phagocytes, suggesting that engagement of ligands on apoptotic cells, not extra cholesterol, induces a 'prophylactic' cholesterol efflux from phagocytes.⁹⁷ Studies have shown that mere co-culture of macrophages with PtdSer liposomes can induce the cholesterol efflux, anti-inflammatory cytokine production, and suppression of pro-inflammatory genes.^{93,98} These data suggest that metabolic sensors, in conjunction with engagement of 'eat-me' signals, such as PtdSer, contribute to the immunological tolerance associated with efferocytosis.

Conclusions: Check Please!

Defects at multiple points in the efferocytosis pathway have been reported to result in unchecked inflammation or autoimmunity, and understanding the mechanisms by which dying cells are effectively cleared can pave the way for therapies that target these processes. Although many studies have examined inflammatory disorders in the context of defective attraction, recognition, and physical engulfment of dead cells, we now recognize that aberrant processing of dead cells, potentially via deviations in LAP, can also result in inflammation. Although systemic disorders, such as SLE, have been long linked to defects in dying cell clearance and the autophagy machinery, more definitive roles for these pathways in 'localized' inflammatory diseases, such as ulcerative colitis, atherosclerosis, neurodegeneration, and rheumatoid arthritis should be described. Moreover, the intricate link between inflammation and cancer raises the question of what the role of efferocytosis is during tumor development, metastasis, and chemotherapy-mediated tumor clearance. Although clearance of dying cells is a common occurrence in healthy and diseased cells, recent studies describe the process of entosis, wherein living cells are engulfed by phagocytes. Although some entotic cells can escape from their engulfment unscathed, most are targeted for destruction by LAP.⁷⁶ Entosis events are common in human cancers, but their role remains unclear.⁷⁶ The mechanisms by which entosis occurs, and its similarity to efferocytosis, implies that the burden that lays before the phagocytic system is a daunting one.

Conflict of Interest

The authors declare no conflict of interest.

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