Mechanisms and physiology of the clearance of dead cells by efferocytosis

Emilio Boada-Romero[1], **Jennifer Martinez**[2], **Bradlee L. Heckmann**[1,†], **Douglas R. Green**[1,†]

[1]Department of Immunology, St. Jude Children's Research Hospital, Memphis, USA.

[2]Inflammation & Immunity Group, National Institute for Environmental Health Sciences, Research Triangle Park, Durham, USA.

Abstract

Multiple modes of cell death have been identified, each with a unique function and each induced in a setting-dependent manner. As billions of cells die during mammalian embryogenesis and daily in adult organisms, clearing dead cells and associated cellular debris is important in physiology. In this Review, we present an overview of the phagocytosis of dead and dying cells, a process known as efferocytosis. Efferocytosis is carried out by macrophages and to a lesser extent by other 'professional' phagocytes (such as monocytes and dendritic cells) and non-professional phagocytes, such as epithelial cells. Recent discoveries have informed this process and how it functions to maintain tissue homeostasis, tissue repair, and organismal health. Here, we outline the mechanisms of efferocytosis, from the recognition of dying cells through to phagocytic engulfment and homeostatic resolution, and highlight the pathophysiological consequences that can arise when this process is abrogated.

Introduction

Cell death and the effective clearance of dying cells are fundamental processes that maintain homeostasis in multicellular organisms. In nearly all physiological and most pathological scenarios, cells participate in their demise by a programmed cascade of signaling events ("regulated" cell death)[1] whereby damaged or obsolete cells die in a controlled manner and are replaced with new cells arising from stem cell progenitors[2]. Cell death is important for development; billions of cells are eliminated during mammalian embryogenesis and development in order to shape new structures and maintain organ function[3,4]. Large numbers of cells also die during the resolution of pathological events, including tissue damage and

infections. Cell death must be carefully controlled; extensive damage, for example caused by heat, mechanical compression or osmotic pressure, can cause cells to undergo necrosis, releasing their intracellular contents to the surrounding milieu and leading to the activation of inflammatory immune pathways that can damage surrounding healthy cells and tissues.

Removal of cellular corpses is important in both homeostasis and disease. The engulfing of dead cells by professional phagocytes, a multistep process known as efferocytosis [G], allows multicellular organisms to recycle cellular components. When disposal of cell corpses is defective, autoimmune and other pathologies can arise (Fig. 1). Whereas the degradation and the recycling of a cell's mass are common features in the clearance of any dead cell, some features of cell clearance are unique to a specific mode of cell death (Box 1) Dying cells can expose and secrete signals that attract phagocytes, favour their engulfment, or promote a return to tissue homeostasis depending on their mode of death. Different forms of cell death can also confer pro-inflammatory or anti-inflammatory signals through modulating macrophage activity following efferocytosis.

There are some situations in which cell death and corpse disposal are uncoupled phenomena, meaning disposal does not immediately follow cell death. One such example is the shedding of dead intestinal epithelial cells from the villi tip to the intestinal lumen[5], although recent evidence suggests efferocytosis of dead intestinal epithelial cells nevertheless occurs as well[6]. Likewise, cornification of the outermost layer in the epidermis constitutes an extreme instance where the presence of the cell's remnants has a biological role, serving as a physical barrier for environmental insults[7]. Conversely, in the process of entosis [G], cells engulf and kill healthy neighboring cells. Although the responsible mechanisms are typically distinct from those of dead cell removal, shared aspects have been reported[8-11]. Nevertheless, in the vast majority of cases, dying cells are rapidly cleared by efferocytosis, and such uncoupled events are not considered further herein.

Here, we summarize our current knowledge of the mechanisms of efferocytosis, and how efferocytosis affects the physiology of the organism, including effects on inflammation and the adaptive immune response. Further, we consider the consequences of defects in this critical homeostatic mechanism.

Recognition of dying cells

In early studies, cell shrinkage preceding cell death was linked to a non-inflammatory mechanism of cell clearance, whereas cell swelling prior to death was associated with an inflammatory response[12-14]. These morphologies were associated with the processes of apoptosis and necrosis, respectively. Over the past decades, more molecularly-diverse programs for regulated cell death have been identified[1], with each form greatly influencing the biological consequences of cell death (Box 1). Both apoptotic and non-apoptotic dying cells display and release molecular cues to signal to phagocytes and direct the subsequent phagocytic and immune response.

Find-me signals of apoptotic cells

Apoptosis is the archetypal form of 'silent' cell death (Box 1). Processing of the apoptotic cell by the actions of activated caspases and their substrates, encapsulation of the cell into apoptotic bodies, and its subsequent disposal and recycling by surrounding phagocytic cells prevents the release of pro-inflammatory cellular contents and inflammation. During apoptosis, the cell releases soluble signals into the milieu to attract macrophages and stimulate their scavenging potential. These 'find-me' signals are diverse, including modified membrane lipids, such as lysophosphatidylcholine (LPC)[15] and sphingosine-1-phosphate (S1P); nucleotides including ATP and UTP[16]; and chemokines such as Fractalkine (encoded by *CX3CL1*)[17]. The importance of each find-me signal depends on a number of factors, including the type of phagocyte and dead cell[18]. Moreover, the variety of find-me signals suggests that there is inherent redundancy that ensures dead cells are recognized by macrophages.

LPC and S1P are apoptosis-specific find-me signals. During apoptosis, caspase-3 cleaves and thereby activates calcium-independent phospholipase A2, which then synthesizes LPC from phosphatidylcholine[15]. Similarly, some apoptotic cells upregulate the S1P mitogen-activated protein kinases SPK1 and SPK2[19,20], which phosphorylate the membrane lipid sphingosine to produce S1P. Although the mechanism governing S1P release from apoptotic cells remains obscure, LPC is released by the ATP-binding cassette transporter 1 (ABC-1) following its activation[21].

In addition to their roles as chemotactic factors, find-me signals also modify phagocyte behavior when bound to their cognate receptors. As an example, the binding of S1P by S1P receptor (S1PR) on macrophages can protect them from cell death and promotes an autocrine signaling loop comprised of HIF1α, erythropoietin (EPO), EPO receptor, and PPARα, the latter of which upregulates the expression of receptors for dying cells[22]. Consequently, S1P may cause long-term potentiation of efferocytosis.

Signals from non-apoptotic dying cells

In contrast to apoptosis, plasma membrane integrity is compromised during execution of non-apoptotic cell death, and nearby cells are exposed to inflammatory signals released from the ruptured, dying cells. Cells infected with intracellular pathogens can release pathogen-associated molecular patterns (PAMPs) upon non-apoptotic cell death. PAMPs are pathogen-specific cues that engage pattern recognition receptors (PRR) on or in the phagocyte (Table 1); these have a variety of effects on macrophage function and immune activation that have been reviewed elsewhere[23,24].

Unlike PAMPs, which are derived from microbes, damage-associated molecular patterns (DAMPs) are of cellular origin and can be liberated upon cell death. DAMPs trigger inflammatory responses, and may also serve as chemoattractants for macrophages. DAMPs are metabolically diverse entities, including genomic and mitochondrial DNA, nuclear proteins (HMGB, histones)[25], cytoplasmic proteins (S100), cytokines (IL-1α, IL-33, IL-36), and other small molecules (ATP, UTP, uric acid crystals) (Table 1)[26]. In addition, inflammasome **[G]** -mediated caspase-1 activation generates inflammatory cytokines IL-1β

and IL-18 during pyroptosis (see Box 1) that lead to inflammatory immune activation after cellular demise.[27] Below, we review the relevance of DAMPs during efferocytosis, the ability of DAMPs to modulate inflammation, and specific DAMPs and their effects.

DNA as a DAMP.—Several mechanisms ensure low DNA burden following apoptotic death and contribute to its immune-silent phenotype. In healthy cells, caspase-activated DNase (CAD) exists in complex with its inhibitory chaperone ICAD and remains constitutively inactive in the cytosol[28,29]. Active caspase-3 cleaves ICAD[28,30], promoting CAD homodimerization, nuclear translocation, and DNA hydrolysis between nucleosomes. Nuclear pieces are then neatly packaged with cytoplasm into apoptotic bodies that are eventually digested during efferocytosis[31]. In contrast, nuclear and mitochondrial DNA (mtDNA), as well as pathogen-derived DNA molecules in those cells dying due to an infection, can be released to the extracellular environment from non-apoptotic dying cells. Toll-like receptor 9 (TLR9) is activated by unmethylated CpG sequences such as those found in mtDNA or bacterial DNA (Table 1), and activation of TLR9 triggers downstream inflammatory responses. circulating DNA DAMPs can accumulate in the body in cases where non-apoptotic cell death is widespread; for example, mtDNA was found to be elevated in the plasma of trauma patients[32], likely as a result of injury-induced cell death.

DNA in the extracellular environment is processed by DNase-I[33], while DNase-III (also known as TREX1) clears cytoplasmic DNA[34], and DNase-II processes DNA from dying cells in the phagocyte's lysosomes to help maintain negligible levels of DNA following efferocytosis. Should DNA escape to the cytosol, it can be recognized by cytosolic DNA sensors[35], including cyclic GMP-AMP synthase (cGAS) and the inflammasome component AIM2. cGAS is activated upon cytosolic DNA binding and subsequently catalyzes a reaction between GTP and ATP to form cyclic GMP-AMP (cGAMP)[36]. The newly synthesized cGAMP binds to and activates the stimulator of interferon genes protein (STING), leading to TANK binding kinase 1 (TBK1)-dependent phosphorylation of the interferon regulatory factor IRF3[37]. These events trigger the IRF3-mediated activation of a Type I interferon response.

DNase-II-deficient or DNase III-deficient mice die during embryogenesis and this embryonic lethality can be prevented if the response to misplaced DNA is abrogated through deletion of cGAS, STING, or the Type-I interferon receptor (IFNAR)[38-41]. It is possible that these DNases function to limit cytosolic DNA following efferocytosis during development, thereby preventing this lethal interferonopathy, although how the DNA of engulfed corpses might be released from the phagosome or lysosome to become cytosolic, triggering such responses, remains unknown.

Similarly, recognition of cytosolic DNA by AIM2 causes AIM2 to recruit and activate caspase-1, resulting in IL-1β processing and release, contributing to inflammation[42,43]. Again, when and how defects in the clearance of DNA during efferocytosis may engage AIM2 remains unclear.

Protein DAMPs.—High mobility group protein B1 (HMGB1) is a nuclear protein that binds to DNA and assists replication, repair and transcription[44,45,46]. Although some

HMGB1 can be released to the extracellular milieu under steady state conditions[47] or during apoptosis[48], it is predominantly released during forms of immunogenic cell death[25]. Efficient efferocytosis can thus limit the release of HMGB1. Based on its redox status, HMGB1 can function as a chemotactic agent (reduced) or as an inflammatory agent (oxidized)[49]. In its reduced form, HMGB1 can prevent the induction of immune tolerance [G] to antigens associated with the dying cell[48]. Reduced HMGB1 may bind to TLRs or the receptor for advanced glycation end products (RAGE) (Table 1)[50], leading to immune cell activation and cytokine production. Recently, binding of reduced HMGB1 to the chemokine receptor CXCR4 was observed during tissue regeneration following injury[51], highlighting the possible importance of this molecule in the response to dying cells. S100 proteins can also be released from dying cells[52], and efferocytosis can limit the release of these proteins from dying cells. Again, TLRs and RAGE appear to be the primary receptors on macrophages that promote inflammatory activation in response to S100 proteins.

IL-1-family cytokines, such as IL-1α, IL-33 and IL-36, are released from non-apoptotic dying cells and lead to activation of the MyD88-NF-κB axis (Table 1)[53,54], resulting in a sustained inflammatory response. A compelling case has been made that IL-1 family members represent the 'true' DAMPs that signal inflammatory immune responses upon non-apoptotic forms of cell death[55]. Like other DAMPs, these proteins are released only when the plasma membrane of a cell is compromised. However, unlike HMGB1 and S100 proteins, each IL-1 family cytokine is controlled at several levels, including intracellular processing and the existence of specific receptor antagonists that regulate the extent to which the cytokine acts if released. For example, mice or humans lacking the IL-36 receptor antagonist display a greatly exacerbated inflammatory response to tissue injury (reviewed in[55]).

Nucleotides.—ATP and UTP can act as find-me signals and can be actively secreted by the dying cell (Table 1)[56]. This process of ATP and UTP release is governed in apoptotic cells by Pannexin-1 (PANX1), a plasma transmembrane protein that forms constitutively inactive hexameric channels[57]. During apoptosis, PANX1 is activated by caspase cleavage[57,58]. Inhibition of PANX1 channel function does not affect cell death but does limit macrophage recruitment to the apoptotic cell[58,59]. Necroptotic, pyroptotic, and other necrotic cells[60] passively release nucleotides[60]; a PANX1-mediated mechanism for the release of nucleotides was initially proposed in pyroptosis[61], although this mechanism is under debate[59]. These nucleotides are recognized by purinergic G-protein coupled receptors on phagocytes to trigger the find-me response[62].

Display and binding of eat-me signals

In addition to generating find-me signals, dying cells display 'eat-me' signals on their cell surface (Fig. 2a). These eat-me signals allow phagocytes to discriminate dying cells from their healthy neighbors, which bear 'don't-eat-me' signals (Fig. 2B).

Phosphatidylserine.—One feature shared across all forms of cell death is the loss of phospholipid asymmetry in the plasma membrane. In most healthy cell types, phosphatidylethanolamine and phosphatidylserine reside in the inner leaflet of the plasma

membrane, whereas phosphatidylcholine and sphingomyelin are restricted to the outer leaflet. The generation and maintenance of membrane lipid asymmetry requires the ATP-dependent action of the flippase **[G]** ATP11, which confines phosphatidylserine to the inner leaflet of the plasma membrane and restricts its lateral mobility in healthy cells[63]. During apoptosis, the inactivation of ATP11 by caspase-3 cleavage, together with the caspase-3-dependent activation of the scramblase **[G]** XK-related protein 8 (XKR8),[64] promotes the exposure of phosphatidylserine on the surfaces of apoptotic cells. Additionally, this loss of phospholipid asymmetry can be achieved by the activation of the pore-forming protein MLKL **[G]** during necroptosis[65,66] (see Box 1). In other forms of cell death, the externalization of phosphatidylserine also contributes to the engulfment of the dead cells[60], although how it is exposed is less clear. Loss of intracellular ATP upon membrane rupture, such as in ferroptosis[65,67], or the activation of pore-forming proteins such as gasdermins **[G]** in pyroptosis,[60] may lead to a loss of ATP-dependent ATP11 activity and passive diffusion of phospholipids to the outer leaflet.

Phosphatidylserine exposure can also occur transiently in healthy cells through the action of the calcium-activated scramblase, TMEM16F[68,69]. Cells with such transient phosphatidylserine exposure are not engulfed; in contrast, healthy cells lacking functional ATP11 constitutively expose phosphatidylserine and are rapidly engulfed by macrophages[10]. This finding suggests that ATP11 actively prevents engulfment, perhaps via its ability to restrict the lateral mobility of phosphatidylserine even when it is exposed.

Several biologically redundant systems for phosphatidylserine recognition exist; however, not all of them are typically expressed or engaged on a given phagocytic cell[70,71]. Surface receptors for phosphatidylserine include T-cell immunoglobulin mucin-1 (TIM1) and TIM4, brain-specific angiogenesis inhibitor 1 (BAI1), stabilin-2, and members of the CD300 family. Alternatively, soluble bridging proteins can mediate the indirect binding of phagocyte surface receptors such as integrins (bridged by milk fat globule-EGF factor 8 (MFG-E8)) and TAM receptors (for example MERTK, which is bridged by dimers of Protein S and GAS6) to phosphatidylserine exposed on the surface of the dying cell[72]. The CD300 family member, CD300f, appears to have distinct functions depending on the phagocyte; on macrophages it promotes engulfment, while on dendritic cells it inhibits engulfment of dead cells[73]. The class B scavenger receptor CD36, a well-characterized lipid-binding receptor with preference for anionic phospholipids, likewise recognizes and binds exposed phosphatidylserine on dying cells[74,75].

Phosphatidylserine not only functions as a link between the dying cell and the phagocyte, but its binding to these receptors also triggers an anti-inflammatory response mediated by the secretion of IL-10, TGFβ, and prostaglandins, and the active inhibition of the production of pro-inflammatory cytokines, including TNFα and IL-1β[76-80].

Other eat-me signals.—While phosphatidylserine is a potent and well-characterized eat-me signal, other signals can have roles in the recognition and engulfment of dying cells. Exposed LPC on the plasma membrane of dying cells can bind to IgM, which in turn binds to Fc receptors on phagocytes, including macrophages[81]. Hence, LPC appears to function as both a find-me and eat-me signal. Proteins present in the lumen of the endoplasmic

reticulum (ER) such as calreticulin can be exposed at the plasma membrane of dying cells and serve as an eat-me signal in the absence of don't-eat-me signals[82] (Fig. 2a). Calreticulin is recognized by phagocytes via the receptor LRP1 (also known as CD91) in coordination with complement component C1q and mannose-binding lectin (MBL)[82,83].

Don't eat me signals.—Healthy cells express don't-eat-me signals that prevent engulfment even when eat-me signals are exposed. Like eat-me signals and receptors, there appears to be extensive redundancy in this inhibition of engulfment (Fig. 2b). Two examples of don't-eat-me signals are the cell surface proteins CD47 and CD24, which are recognized by the receptors SIRPα and SIGLEC-10, respectively, on macrophages[84,85] (Fig. 2B). Similarly, homotypic interaction of CD31 (on healthy cells and macrophages) appears to also prevent engulfment[86]. Finally, class I MHC molecules on healthy cells engage the inhibitory receptor, LILRB1, preventing engulfment and inhibiting expression of inflammatory mediators[87]. In each case, it appears that the inhibition of engulfment upon recognition of these don't-eat-me singles is mediated by the protein phosphatases, SHP1 and SHP2[88].

Disruption of this active inhibition of engulfment can promote the uptake of healthy cells, and such disruption is under investigation as potential anti-cancer therapies. Approaches to blockade of the don't-eat-me signal include antibodies, directed against the signal or receptor as well as small molecules that bind specific receptors[89-91]. Importantly, upon disruption of don't-eat-me signals, cancer cells are more likely to be engulfed than normal, healthy cells. Such engulfment appears to be promoted by calreticulin as the eat-me signal[92]. Why calreticulin might be preferentially exposed on the cell surface in many cancers remains unclear.

Since don't-eat-me signals prevent engulfment, even when eat-me signals are present, it follows that dying cells must lose this signal if they are to be effectively cleared by efferocytosis. However, very little is known about how this occurs. One study has suggested that during apoptosis, the levels of CD31 and CD47 decline in a caspase-dependent manner, which appears to be a consequence of active shedding of these proteins in released microparticles[93]. The extent to which this occurs in different cell types undergoing apoptosis, and how the disruption of these and other don't-eat-me signals occurs during other forms of cell death remain unresolved.

Mechanisms of engulfment

Uptake of dying cells

Once the phagocyte recognizes a dying cell, engulfment of the cell corpse requires rapid plasma membrane reorganization and synthesis to allow for effective phagocytic internalization of the dead cell[94]. A dynamic mesh of cortical actin fibers lying underneath the plasma membrane allows for phagocyte motility and environmental sampling. Upon recognition of a dying cell, the phagocyte initiates actin remodeling that allows for invagination and localized extravagation of the plasma membrane and ultimately formation of the phagosome. The signaling mechanisms that bridge receptor ligation to activation of actin remodeling and associated pathways, vary depending on the receptor engaged but

generally require the orchestrated activation of kinases (such as those from the Srk, Syk and protein kinase C families) and inactivation of phosphatases including SHP-1, processes that have been well reviewed previously[95]. During efferocytosis, two primary mechanisms leading to the reorganization of actin exist and both converge on a key regulator, the Rho family small GTPase Rac1. In the first system, Rac1 activation is mediated through LDL Receptor Related Protein 1 (LRP1) and the adapter protein GULP[96]; however, the precise mechanism as to how LRP1 and GULP activate Rac1 is not known. The second system leading to Rac1 activation is dependent on the guanine nucleotide exchange factor (GEF) DOCK180 and the phagocytic regulatory protein engulfment and cell motility protein (ELMO). Following receptor ligation by the dying cell, another GEF called TRIO loads GTP onto the small GTPase RhoG, leading to the recruitment of ELMO[18,97-99]. ELMO is then able to interact with the SH3 domain of DOCK180[100]. Together, the DOCK180-ELMO complex serves as a GEF for Rac1, leading to Rac1 activation. Once activated via either system, Rac1 is subsequently able to direct localized actin polymerization necessary to coat or grasp the cargo by activating nucleation-promoting factors of the WASP family, SCAR and WAVE, which recruit the ARP2/3 complex and function together to establish an actin nucleation core[95]. In addition to forming a nucleation core for de novo actin polymerization, the ARP2/3 complex binds established actin filaments, allowing for new actin synthesis while maintaining actin networking and branching, processes that are essential for phagosome formation[101].

While actin polymerization is a critical component of phagosome formation and efficient capturing of the dead cell, actin depolymerization is of equal importance for the scission of the phagosome from the plasma membrane. The processes of phagosome sealing is highly dependent on various phosphoinositides including PtdIns(3,4,5)P$_3$ which activates Rho-family GAPs leading to deactivation of the GTPases including Rac1, resulting in depolymerization[102]. A coordinated effort between actin depolymerization and dynamin exists to promote scission of the phagosome Dynamin-2 also directs downstream trafficking of the now complete early phagosome[103].

Degradation by lysosomes

Following recognition and entrapment of the dying cell, the phagosome and the cell corpse are destined for a well-orchestrated destructive end. The phagosome fuses with lysosomes, which contain a large variety of proteases, nucleases, and lipases that digest the phagosome cargo. This fusion is promoted or impaired by modifications of the phagosome, as discussed below.

The phagosome containing the cell corpse is targeted to lysosomes through a multistep maturation process, which begins immediately following the formation of the phagosome upon dynamin-dependent membrane scission and is marked by multiple biochemical changes at the phagosome membrane. These alterations to the phagosomal membrane and the cargo contained within it are facilitated by vesicular trafficking events to and from the phagosome. Control of these processes is governed by the Rab GTPase family of proteins (Fig. 3a), which alternate between a GTP-bound (active) and a GDP-bound (inactive) state [104] Following activation, Rab proteins associate with effector molecules, the intracellular

functions of which include motor-driven trafficking, vesicle fusion, and signaling that can promote 'Rab conversion,' and the activation of other downstream Rab GTPase family members[105].

Early phagosomes acquire RAB5, which organizes endocytic trafficking and phagosome biogenesis during maturation[106]. RAB5 is critical to phagosome development as abrogation of its function leads to defects in maturation, preventing the formation of late phagosomes[107]. The precise mechanisms that initiate the early recruitment of RAB5 to the phagosome are not well understood[106]. RAB5 stimulates the fusion of nascent phagosomes with early endosomes (also known as sorting endosomes), and recruits and activates multiple effector proteins, including early endosomal antigen 1 (EEA1), and the vacuolar fusion proteins MON1A and MON1B, a process which has been reviewed elsewhere[108,109].

The class III PI3 kinase VPS34 is recruited to RAB5-positive early phagosomes, where it catalyzes the formation of the signaling lipid phosphatidylinositol 3-phosphate (PI3P) from phosphatidylinositol[110]. Both VPS34 and PI3P are required for the optimal progression of phagosome maturation. The catalytic activity and targeting of VPS34 to the membrane of the early phagosome is enhanced by the serine/threonine kinase VPS15 [108,110]. VPS15 forms a complex with VPS34, and also binds directly to active RAB5[111]. The function of VPS34 is considered further below, in our consideration of LC3-associated phagocytosis.

Early phagosomes transition to late phagosomes, which are defined by their acquisition of distinct biochemical markers, including the small GTPase RAB7, and the concomitant loss of early markers such as RAB5. Late phagosomes are more acidic than early phagosomes due to increased proton pumping into the phagosome lumen that is mediated by the multimeric protein complex vacuolar ATPase (V-ATPase), which translocates H^+ across endosomal and phagosomal membranes (Fig. 3a)[112,113].

RAB7 and its effectors are vital for the successful maturation of the phagosome, as inhibition of RAB7 activation blocks phagosome–lysosome fusion and results in failure to acidify the phagosome[108,114]. Two RAB7 effector proteins, RAB7-interacting lysosomal protein (RILP) and oxysterol-binding protein–related protein 1 (ORPL1), accumulate on late endosomes, phagosomes, and lysosomes[114]. Through their direct association with the molecular motor dynein–dynactin, these proteins coordinate microtubule-dependent vesicular trafficking of RAB7-positive compartments[114]. RILP, dynein, intact microtubules, and lysosome-associated membrane protein 1 (LAMP1) and LAMP2 are required for the fusion of late phagosomes with the lysosomal compartment[115]. LAMP-1 and LAMP-2 are heavily glycosylated membrane proteins that preserve lysosomal membrane integrity and are essential for facilitating phagosome-lysosome fusion through interaction with RAB7 [115].

The direct fusion of the mature phagosome with a lysosome is coordinated in part by the formation of a Ca^{2+}-dependent SNARE complex **[G]**, composed of VAMP7 and syntaxin 7 (Fig. 3a)[116,117]. Both syntaxin 7 and VAMP7 are recruited to phagosomes, and abrogation of syntaxin 7 function inhibits phagosome–lysosome fusion[118]. After fusion, the newly formed phagolysosome **[G]** can be defined by its high acidity (pH 4.5–5.0) and the presence of active cathepsins **[G]**. Acidification, which itself promotes degradation of the internalized

cell corpse, is also required for the activation of lysosomal acid hydrolases. These hydrolytic enzymes mediate the potent destructive capacity of the lysosome in concert with other effectors, including oxidants, cationic peptides, and lipases (Fig. 3a)[119].

Modifications to phagosome maturation

LC3-associated phagocytosis.—The phagosome can be modified to either facilitate or impair its maturation and thus influence degradation of phagosome contents following its fusion with lysosomes. One such modification occurs during LC3-associated phagocytosis (LAP) [G] (Fig. 3b). In LAP, a subset of proteins required for canonical autophagy, in combination with regulators unique to LAP, enable the conjugation of the microtubule-associated protein 1A/1B light chain 3 (LC3) family [G] to the phagosome membrane upon phagosome sealing (Fig. 3b)[120-124]. LAP is dependent on the protein Rubicon and reactive oxygen species (ROS) generated by the nicotamide adenine dinucleotide phosphate oxidases (NADPH oxidase-2, also known as NOX2, in macrophages), both of which are completely dispensable for canonical autophagy[121]. A subset of the autophagy proteins (ATGs) are also required for LAP (Fig. 3b). The roles of autophagy-related proteins in phagocytosis have been reviewed elsewhere[123,124].

LAP is engaged in response to certain phagocytic cargo, including dying cells[123], although the precise signaling events that trigger this process remain obscure. VPS34 is recruited to the phagosome as part of a complex that contains Rubicon, Beclin-1, UVRAG, and VPS15, all of which appear to be required for PI3K activity of the LAP complex. The generation of PI3P on the phagosome membrane recruits the LC3-conjugation machinery, and abrogation of LC3 lipidation at the membrane impairs phagosome maturation and lysosome-mediated degradation (Fig. 3B)[125,126]. In autophagy, LC3 family proteins promote autophagosome–lysosome fusion as such fusion is impeded by the depletion of LC3[127]. The RAB7 effector protein FYCO1 interacts with LC3 and PI3P and facilitates autophagosome–lysosome fusion[128]. Similarly, LC3 on the phagosome promotes phagosome–lysosome fusion to form the phagolysosome. A Rubicon homolog, PLEKHM1, interacts with both RAB7 and LC3 and putatively facilitates phagosomal and endosomal trafficking[129,130]. These processes, which are involved in lysosomal targeting and fusion, are likely to be common to autophagy and phagocytosis. To date, it is not believed that the ability to conjugate LC3 to the phagosome directly affects the functional capacity of the lysosome; the impaired maturation and degradation observed in LAP-deficient cells and animals has not been associated with a direct lysosomal defect[125,126].

Liver X receptors.—Other pathways with roles in efferocytosis include the liver X receptors (LXR s) and the peroxisome proliferator-activated receptors (PPARs), nuclear receptor families that regulate genes involved in lipid metabolism and transport[131-134]. Upon engulfing a dying cell, a phagocyte can effectively double its lipid content, requiring an appropriate transcriptional response that is mediated by these nuclear receptors. LXR deficiency in macrophages results in their inability to clear apoptotic thymocytes, but does not alter engulfment of inert beads or other substrates[135]. Likewise, mice that are deficient in LXRs accumulate dead cells in multiple tissues, due to a failure in efferocytosis in vivo[135]. Pharmacologic LXR agonists improve efferocytosis and clearance by stimulating the

expression of receptors, including MerTK, both in cell culture and in vivo[136]. In addition, engagement of LXRs during efferocytosis induces the expression of the cholesterol transporter, ABCA1, which effluxes excess cholesterol[137], and genes involved in lipid oxidation by mitochondria, permitting efficient catabolism of the excess lipids[137,138].

Similar to LXRs, the PPAR family affects efferocytosis. Expression of PPARγ occurs in response to engulfment of apoptotic cells and remains elevated until efferocytosis resolves[139]. In concert with activation of LXRs, PPARγ activation has been shown to enhance efferocytosis by macrophages[135]. Indeed, inhibition of PPARγ using a pharmaceutical antagonist resulted in impaired efferocytosis[139].

Resolution of phagocytosis

Once the phagosome has fused with lysosomes and its cargo has been degraded, a resolution phase restores homeostasis within the phagocyte, allowing further phagocytosis. Since dying cells are not the only phagocytic cargo, the outcome of phagocytosis can vary dependent upon what cargo is internalized. In the context of efferocytosis, some of the components of the cell corpse can be recaptured and recycled for use by the phagocytic cell following lysosomal degradation. Sugars, amino acids, lipids and nucleotides are recycled to replenish cellular stores and can potentially be used as building blocks and an energy source by the phagocytic cell[123]. The internalization and degradation of cargo can also influence the activation of signaling pathways; for example, DNA that escapes degradation can activate the DNA sensing cGAS-STING pathway and lead to the production of type I interferons (IFN), as discussed above. These events can lead to the loss of immune tolerance to the apoptotic corpse and can potentially result in autoimmunity[125].

Rapid maturation and generation of the phagolysosome and efficient degradation of the cell corpse is facilitated by LAP (as described above) and promotes the production of anti-inflammatory cytokines including IL-10, a process that promotes immune silence (Fig. 3B). In contrast, disruption of LAP decreases phagosome maturation, leading to activation of inflammatory signaling pathways and the production of pro-inflammatory mediators including IL-1β and IL-6 (Fig. 3B) [121].. The exact process leading to this switch in cytokine production in the absence of LAP has not been well defined. Furthermore, the failure to efficiently degrade the cell corpse in LAP-deficient phagocytes may result in leakage of phagosome contents, such as DNA, into the cytosol, which in turn induces type I interferon production via sensing by STING[140]. STING-dependent IFN production by tumor associated macrophages in LAP-deficient mice promoted T cell-mediated anti-cancer immunity in several cancer models[140].

Following efferocytosis, the phagocyte must restore the functions of cytoskeletal components, such as actin and microtubules, to ensure that its phagocytic capacity is maximized for further phagocytic events. This restoration of function has not been well-studied compared to the upstream processes of phagocytosis. However, some details can be inferred from studies evaluating effectors such as RAB27A in the events leading to phagocytosis. RAB27A negatively impacts phagocytosis by prolonging the actin-coating of nascent phagosomes, leading to impaired transition to the phagosome sealing stage[141]. Thus, RAB27A and similar effectors could be critical for the restoration of actin and other

cytoskeletal networks following completion of phagocytosis. However, it is clear that the restoration of the cytoskeleton following phagocytosis is an ATP-dependent process and likely requires multiple components[142]. An additional aspect of recovery is the recycling or continued expression of membrane receptors to recognize subsequent cargo. Impaired recycling results in decreased membrane expression of receptors such as TREM2 and TLR4, which recognize a variety of amyloids and pathogen products respectively[126,143], and may also decrease the membrane expression of receptors that recognize dying cells. This decrease in available surface receptors may impair the endocytic and phagocytic capacity of the phagocytic cell, suggesting that these restorative events are critical for ensuring the phagocyte can continue recognizing and clearing phagocytic cargo.

Efferocytosis and disease

The fact that so much redundancy in the recognition of dying cells is built into efferocytosis highlights its importance in pathophysiology. Indeed, many autoimmune and inflammatory pathologies are associated with defects in this process, with uncleared or improperly cleared cellular corpses resulting in inflammation, exacerbated tissue damage, and organ dysfunction. However, defects in other aspects of the efferocytotic machinery can result in increased protection against some pathologies.

Systemic pathology

The most common autoimmune disorder associated with defective efferocytosis is systemic lupus erythematosus (SLE), a chronic, systemic autoimmune pathology that affects multiple organ systems, such as the lung, kidneys, skin, and central nervous system (CNS). Although it is rare to observe uncleared dead cells when examining healthy tissue under the microscope, dead cells can often be observed in the blood, skin, and lymph nodes of patients with SLE, and disease severity is strongly correlated with defective efferocytosis in vitro and accumulation of dead cells in vivo [144,145]. Patients with SLE also display elevated levels of circulating autoantigens, such as extracellular DNA, which bind autoantibodies to form immune complexes that accumulate or are deposited in the glomerular and vessel walls of the kidney[146,147].

Defects at nearly every point of the efferocytosis pathway are implicated in the pathogenesis of SLE (Table 2). Mice with genetic deletions that abrogate components of find-me signaling pathways (for example, production of S1P), eat-me signaling pathways (for example, TIM4, MFG-E8, Protein S, MerTK, and C1q), and in components required for the processing of engulfed dead cells (LXRs, PPARs, ABCA1, and Rubicon) all display a progressive SLE-like disease (see Table 2 for references).

Neurodegenerative diseases

The central nervous system (CNS) contains phagocytic cells called microglia. These resident phagocytes are phenotypically similar to macrophages and clear dead cells and cellular debris in the CNS[148]. However, other cells of the CNS, such as oligodendrocytes, astrocytes, and neuronal progenitors can also act as mediators of efferocytosis. Like all organ systems, the CNS requires efficient efferocytosis for homeostasis, but efferocytosis is also

fundamental for the reorganization of neuronal circuits and initiating the regenerative response after injury[149]. Consequently, multiple neurodegenerative pathologies are associated with defects in efferocytosis (Table 2), including Multiple Sclerosis (MS), Alzheimer's disease, Parkinson's disease, and Huntington's disease[150,151]. Defects at multiple steps of efferocytosis can result in MS, including defects in Pannexin-1 find-me signaling, MerTK eat-me signaling, and LXRα and LXRβ, which are engaged upon processing of the corpse. Decreased expression of the bridging molecule MFG-E8 has also been observed in a mouse model of Alzheimer's disease, and expression of a dominant negative mutant of MFG-E8 in microglia cells decreased engulfment of apoptotic neurons[152,153].

Retinal degeneration

Retinal pigment epithelial (RPE) cells are the primary phagocytes in the eye, and shed photoreceptor outer segments (POS) are their primary phagocytic cargo. Although RPE are post-mitotic cells that must function throughout an organism's lifetime, POS are shed daily in concert with circadian rhythms, and defects in this process can result in macular degeneration and vision loss. The LAP machinery is required to process POS and to ensure proper chromophore **[G]** regeneration in RPE cells [154,155]. Similarly, defects in MerTK and integrins in RPE cells result in retinal degeneration[156]. Modulation of efferocytosis could be leveraged as a potential therapy for some eye diseases; for example, activation of LXRα and LXRβ signaling can alleviate inflammation associated with autoimmune uveitis[157].

Pulmonary disorders

The lung and associated airway structures are laden with both professional phagocytes (alveolar macrophages and dendritic cells) and non-professional phagocytes (airway epithelial cells). These phagocytic cells primarily clear apoptotic neutrophils, which are rapidly recruited to the lung during damage. Neutrophils are short-lived and their clearance is central to the resolution of inflammation[158].

Allergic airway inflammation is associated with an influx of neutrophils and eosinophils to the airway lumen. Deletion of genes encoding protein components of the efferocytotic machinery, such as MerTK or Rac1, in mouse macrophages results in airway hyperresponsiveness (AHR) and fibrosis , caused by unresolved allergic airway inflammation [159]. Other pulmonary disorders, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), are also characterized by the recruitment of neutrophils and other inflammatory cells to the airways, and alveolar macrophages from patients with CF and COPD display decreased phagocytic capacity for apoptotic cells[160,161].

Atherosclerosis

The heart and vasculature contain professional phagocytes, such as monocytes, macrophages, and dendritic cells, which engulf metabolically-derived oxidized lipids that then induce apoptosis in these phagocytes. These apoptotic but lipid-full cells, known as foam cells, must then be cleared via efferocytosis[162,163]. Failure to effectively clear dead cells in general is a major factor in the progression of atherosclerosis. Mature atherosclerotic plaques contain uncleared foam cells, and it has been suggested that defective efferocytosis

contributes to plaque expansion, plaque rupture, acute coronary syndromes and stroke[164-166].

Proper processing of engulfed dead cells requires lipid sensing and cholesterol efflux (see above), and mice lacking receptors for dead cells (e.g. MFG-E8[167]) also display increased atherosclerosis. As discussed above, uptake of apoptotic cells by phagocytes stimulates cholesterol efflux, primarily though upregulation of the cholesterol efflux regulatory protein ABCA1[168,169]. Decreased expression of ABCA1 promotes inflammation and atherosclerotic lesions, and overexpression of ABCA1 can improve disease progression and decrease inflammation[170,171]. Synthetic agonists of LXRs or PPARs improve the efferocytotic capacity of phagocytic cells and have been used successfully to treat patients with atherosclerosis. HMG-CoA reductase inhibitors, commonly known as statins, lower cholesterol and inflammation and also inhibit the action of RhoA, a negative regulator of engulfment that is highly expressed in atherosclerotic lesions[172]. In addition, LysM-Cre Atg5[flox/flox] mice, in which myeloid cells lack the autophagy protein Atg5, are deficient for canonical autophagy and LAP, and display more atherosclerotic lesions than wild-type animals in murine models of the disease[173,174]. It is therefore possible that effective pharmacologic treatments of atherosclerosis function, at least in part, by improving efferocytosis.

Liver pathologies

The liver neutralizes exogenous threats that reach this organ via the blood and has been referred to as a "graveyard" for dying lymphocytes, as apoptotic immune cells often traffic via the blood to the liver for their removal. Although much of the required efferocytosis is carried out by liver-resident macrophages called Kupffer cells, other liver cells, including hepatocytes, endothelial cells, and stellate cells can mediate efferocytosis[175].

Defective efferocytosis has been implicated in a variety of liver pathologies, such as autoimmune hepatitis, fatty liver disease, and primary biliary cholangitis (Table 2). Molecules such as TIM4 and GAS6 are critical for the resolution of hepatic ischemia reperfusion injury[176], suggesting dead cell clearance is essential for regulating hepatic inflammation.

Pathology of diabetes

Normal turnover of pancreatic islet β-cells is crucial for maintaining glucose homeostasis, but the excessive loss of β-cells or β-cell dysfunction results in diabetes mellitus (DM). The clearance of apoptotic islet β-cells is critical for maintaining immune tolerance, and type 1 DM (T1DM) is an autoimmune disease wherein T cells destroy the insulin-producing β-cells in the pancreas. While the etiology of T1DM is not fully understood, it is possible that defects in efferocytosis result in an accumulation of apoptotic β-cells, inflammation, and the generation of autoantibodies and autoreactive T cells that mediate T1DM[177].

Phagocytes from non-obese diabetic (NOD) mice, a polygenic model for spontaneous T1DM, display a marked defect in efferocytosis in vitro, as well as high levels of autoantibodies in vivo[178]. Similarly, mice lacking MFG-E8 exhibit increased insulin sensitivity compared with their wild-type counterparts[179]. A common complication in

patients with diabetes is compromised wound healing; phagocytes isolated from diabetic wounds have been shown to be defective in the clearance of apoptotic neutrophils from the wound site, increased inflammation, and delayed wound healing, suggesting efferocytosis is a general feature of diabetes[180,181].

Pathology of the gastrointestinal system

Intestinal epithelial cells (IECs) are continuously renewed to ensure that the intestine maintains optimal barrier and absorptive functions. Due to the high turnover of cells in this tissue, efferocytosis is constantly required to prevent unwanted inflammation. Although IECs themselves are capable of engulfing and clearing apoptotic cells, dendritic cells and macrophages are the primary phagocytes in the small intestinal lamina propria[6]. Defects in the efferocytotic machinery caused by ablation of the phosphatidylserine receptor CD300f is associated with inflammatory bowel syndrome and colitis, as well as the progression of colon carcinoma[73].

Impaired wound healing and regeneration

The skin experiences a high level of turnover of epithelial cells that are shed or cleared by macrophages, dendritic cells, and Langerhans cells **[G]** to promote an immune-silent response. Wound healing requires that apoptotic neutrophils be efficiently cleared, and mice deficient for C1q or CD36 display delayed wound healing and increased inflammation at the wound site[182].

Efferocytosis of apoptotic cells is fundamentally important for normal wound healing. Prevention of efferocytosis by blockade of phosphatidylserine recognition or ablation of MerTK impaired tissue repair in murine models of lung and intestinal damage[183]. Furthermore, in *Drosophila melanogaster,* the engulfment of apoptotic cells is required to 'license' the repair machinery of macrophages for wound healing[184].

Leukocytes are largely absent from skeletal muscle, but injury induces necrotic death and the release of DAMPs to quickly recruit immune cells to facilitate debris clearance. Inflammatory cytokines also promote the proliferation of muscle stem cells known as satellite cells. RAGE-deficient mice display delayed regeneration after acute injury of skeletal muscles due to decreased macrophage infiltration. Further, satellite cells can also engulf and clear dying cells, and RAGE expression is increased in satellite cells upon damage. In the absence of RAGE, damaged skeletal muscle displayed delayed differentiation of activated satellite cells[185].

Upon ischemia-reperfusion injury in the heart, efferocytosis of dead cells drives fatty acid oxidation and increased electron transport in the mitochondria, which inhibit inflammation[186]. Animals in which macrophages have a genetic defect in electron transport display inflammation and reduced wound repair upon heart injury.

Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease characterized by increased circulating autoantibodies, which results in progressive joint destruction. Studies have highlighted a

prominent role for an immune suppression by efferocytosis in the prevention of arthritic pathologies. Specifically, MerTK-deficient mice develop more severe collagen-induced arthritis, inflammation, and increased secondary necrosis than wild type mice as a consequence of defective efferocytosis[101]. Mice deficient in both DNAse-II and the type I IFN receptor, IFNAR (while DNAse-II deficiency is lethal, this is prevented by co-ablation of IFNAR, as discussed above) are unable to properly process engulfed cellular cargo and develop spontaneous polyarthritis with increased inflammation reminiscent of human rheumatoid arthritis[187]. Finally, LXR agonists, which mimic aspects of efferocytotic signaling, have therapeutic benefits in mouse models of inflammatory arthritis[188].

Reduced male fertility

During spermatogenesis, vast numbers of germ cells are generated with the majority undergoing apoptosis and clearance. Sertoli cells are large phagocytes that line the seminiferous tubules of the testes and are responsible for clearing apoptotic cells as well as providing support during germ cell differentiation. ELMO and MerTK are both required for Sertoli cell-mediated efferocytosis, and ablation of the genes encoding either of these molecules results in testicular pathology and reduced fertility[99,189]. Overexpression of BAI1 can rescue the fertility defects, but not the vision defects found in MerTK-knockout mice, suggesting that the phosphatidylserine receptor function of BAI1 can function in place of MerTK in Sertoli cells but not in retinal pigment epithelial cells.

Beneficial effects

In some cases, defects in efferocytosis are associated with beneficial effects. Mice lacking GAS6 show delayed graft versus host disease **[G]** (GVHD) compared with their wild-type counterparts[190]. Ablation of the LAP machinery (Rubicon, Beclin-1, UVRAG, ATG5, and ATG7) in myeloid cells display increased anti-tumor immunity[140]. Similarly, anti-tumor immunity is enhanced in mice lacking Tim4[123], or animals treated with annexin V to block recognition of phosphatidylserine on dead cells[191,192]. In melanoma, interfering with efferocytic clearance may be directly beneficial, as pharmacological inhibition of MerTK can block the growth of melanoma both in culture and in human melanoma xenograft models[193]. Finally, genetic ablation of CD36 protected animals from ischemia-reperfusion injury of the lung, although whether this relates to efferocytosis is unclear[194].

Conclusions and Perspectives

Efferocytosis is governed by a plethora of factors, including unique membrane lipids and lipid reorganization, multiple effector proteins that serve functions such as recognition, the activation of phagocytosis and, ultimately, degradation of the cell corpse. As discussed, the multistep process from cell death to cell clearance is a delicate process with multiple points of redundancy, indicating the importance of efferocytosis in development, homeostasis, and pathophysiology.

The homeostatic function of nearly every major physiological system is dependent, at least in part, on the ability of phagocytes to clear cellular debris. Dysfunction of efferocytosis leads to severe disease and activation of efferocytosis, in the context of clearing apoptotic

cells and he engagement of the LAP machinery, promotes immune silence through the production of anti-inflammatory signals. As current evidence suggests that efferocytosis and LAP engagement are beneficial in many settings, promoting an inflammatory response by targeting LAP and efferocytosis may prove beneficial in the treatment of various malignancies. The generation of targeted therapeutics against components of these pathways may facilitate the treatment of cancer, including checkpoint blockade, by increasing the immunogenicity of apoptotic tumor cells. As the study of efferocytosis and associated molecular pathways is an expanding field, new insights into the activation and regulation in response to dying cells is likely to lead to the discovery of new treatment paradigms for pathologies including cancer, autoimmunity, neurodegeneration, and beyond.

Acknowledgements

Glossary

Efferocytosis

Engulfment and clearance of dead and dying cells, usually (although not exclusively) by myeloid cells such as macrophages.

Entosis

A process by which a living cell invades or is engulfed by another. This process differs from the clearance of apoptotic cells by phagocytosis as the internalized cell is still alive.

Inflammasome

Any molecular complex capable of activating caspase-1.

Immune Tolerance

Lack or inhibition of an adaptive immune response to specific antigens.

Flippase

A membrane protein that transports phosphatidylserine from the outer leaflet to the inner leaflet of the plasma membrane.

Scramblase

A membrane protein that, upon activation, equilibrates ("scrambles") the distribution of phospholipids between the inner and outer leaflets of the plasma membrane.

MLKL

Mixed lineage kinase-like protein, MLKL is the executioner in necroptosis, disrupting the plasma membrane following its phosphorylation.

Gasdermins

Proteins that, upon activation, creates pores in the plasma membrane; Gasdermin D and Gasdermin E function in pyroptosis.

Dynamin

A member of the protein GTPase family, mainly involved in the scission of newly-formed vesicles from a membrane and their fusion with another membrane.

Guanine nucleotide exchange factors (GEFs)
Enzymes that activate monomeric GTPases including Ras, Rac, and Rho. GEFs stimulate the release of guanosine diphosphate (GDP) to allow binding of guanosine triphosphate (GTP).

SNARE complex
SNAP receptor complex involved in vesicle membrane fusion to other membranes.

Phagolysosome
The intracellular vesicle that results from the fusion of the phagosome with lysosomes.

Cathepsins
A class of proteases mostly present in lysosomes.

LC3-Associated Phagocytosis (LAP)
A non-apoptotic function of several proteins of the autophagy pathway, resulting in lipidation of LC3 family proteins on the phagosome membrane, enhancing fusion of the phagosome with lysosomes.

Microtubule-associated protein 1A/1B light chain 3 (LC3) family
The ATG8 protein family, including Microtubule-associated proteins 1A/1B light chain 3 (LC3) proteins, Gamma-aminobutyric acid receptor-associated protein (GABARAP), GEC-1, and GATE-16.

Langerhans Cell
A specialized dendritic cell residing in the epidermis of the skin.

Graft versus host disease
Pathology resulting from the introduction and action of allogeneic T lymphocytes, which can occur following transplant of bone marrow from which T cells have not been removed.

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BOX 1:

Modes of programmed cell death

Different modes of cell death have unique activating stimuli and present different signaling moieties to the phagocyte, leading to efferocytosis and a variety of distinct physiological outcomes (see the figure).

Apoptosis

Apoptosis can be triggered by the activation of a mitochondrial pathway by cellular stress (intrinsic apoptosis) or through the activation of death receptors at the cell surface (extrinsic apoptosis). The Bcl-2 proteins regulate intrinsic apoptosis; anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and BFL-1) prevent uncontrolled apoptotic initiation, whereas pro-apoptotic Bcl-2 proteins (Bak, Bax and Bok) trigger mitochondrial outer membrane permeabilization (MOMP). Mitochondrial intermembrane proteins SMAC, Omi, and cytochrome c are released into the cytosol following MOMP. Cytochrome c activates apoptotic protease activating factor-1 (APAF-1), which in turn activates the serine protease caspase-9; active caspase-9 activates the executioner caspases, caspase-3 and caspase-7, which contribute to the archetypal features of apoptotic cells by cleaving cellular proteins[246]. Death receptors known to mediate extrinsic apoptosis include the tumor necrosis family members, including TNFR1, the Fas receptor (CD95) and the TRAIL receptors. Receptor ligation promotes recruitment of adaptor proteins, including FADD, which bind and activate caspase-8 by oligomerization. Caspase-8 cleaves and activates the executioner caspases, which can be inhibited by X-linked inhibitor of apoptosis (XIAP). Caspase-8 also cleaves the BCL-2 family protein BID, activating it to induce MOMP[247] and releasing SMAC and Omi (as above). These proteins antagonize the function of XIAP, permitting executioner caspase activation and apoptosis.

Necroptosis

Necroptosis is a regulated form of necrosis that is also activated by extrinsic apoptotic receptors. Necroptosis is initiated through the activation of RIPK1, which binds and activates RIPK3 following autophosphorylation. RIPK3-mediated phosphorylation of the mixed-lineage kinase domain-like pseudokinase (MLKL) promotes its oligomerization and insertion into the plasma membrane, forming a membrane-disrupting pore, leading to death[248]. This process is inhibited by the activation of caspase-8, together with its apoptotic inhibitor c-FLIP$_L$, and the proteolytic activity of this complex cleaves RIPK1 to prevent necroptosis without engaging apoptosis[249].

RIPK3 and necroptosis can also be activated independently of RIPK1 by Toll-like receptors that engage the adapter protein TRIF, or by the activation of the viral sensor ZBP1[250,251].

Pyroptosis

During bacterial infection, inflammatory caspases (namely caspase 4, caspase 5 and/or caspase 11) directly recognize lipopolysaccharide on intracellular, Gram-negative bacteria and trigger pyroptosis[252-255]. Alternatively, the inflammatory caspase, caspase-1,

is activated by multiprotein inflammasomes. Inflammasomes generally contain a pattern-recognition receptor (PRR) that senses PAMPs or DAMPs, caspase-1, and the bridging molecule apoptosis-associated speck-like protein containing a CARD (ASC). The inflammasomes activate caspase-1 by oligomerization. Caspase-mediated cleavage of the pore-forming protein Gasdermin-D disengages its autoinhibitory domain, leading to insertion of the Gasdermin-D pore into the plasma membrane and subsequent plasma membrane permeabilization[255]. Active caspase-1 also proteolytically-processes the pro-inflammatory cytokines IL-1β and IL-18 into their active forms, which are subsequently released from pyroptotic cells. Pyroptosis clears pathogens by destroying their replicative niche and engaging the inflammatory response. Although beneficial under most circumstances, uncontrolled pyroptosis can lead to organ failure, sepsis, and death.

Ferroptosis

Free cytosolic iron can propagate lipid peroxidation via the Fenton reaction, leading to reactive intermediates that promote ferroptosis, which is characterized by membrane rupture as a result of high levels of lipid peroxides. Glutathione peroxidase 4 (GPX4) functions to reduce lipid peroxides in cellular membranes; GPX4 inhibition or a reduction in the levels of the GPX4 co-factor glutathione can therefore result in uncontrolled lipid peroxidation of poly-unsaturated fatty acids and ferroptosis[256,257]. Under pathological scenarios ferroptosis often accompanies other cell death routines; however, inhibition of apoptosis or necroptosis is not sufficient to inhibit ferroptosis.
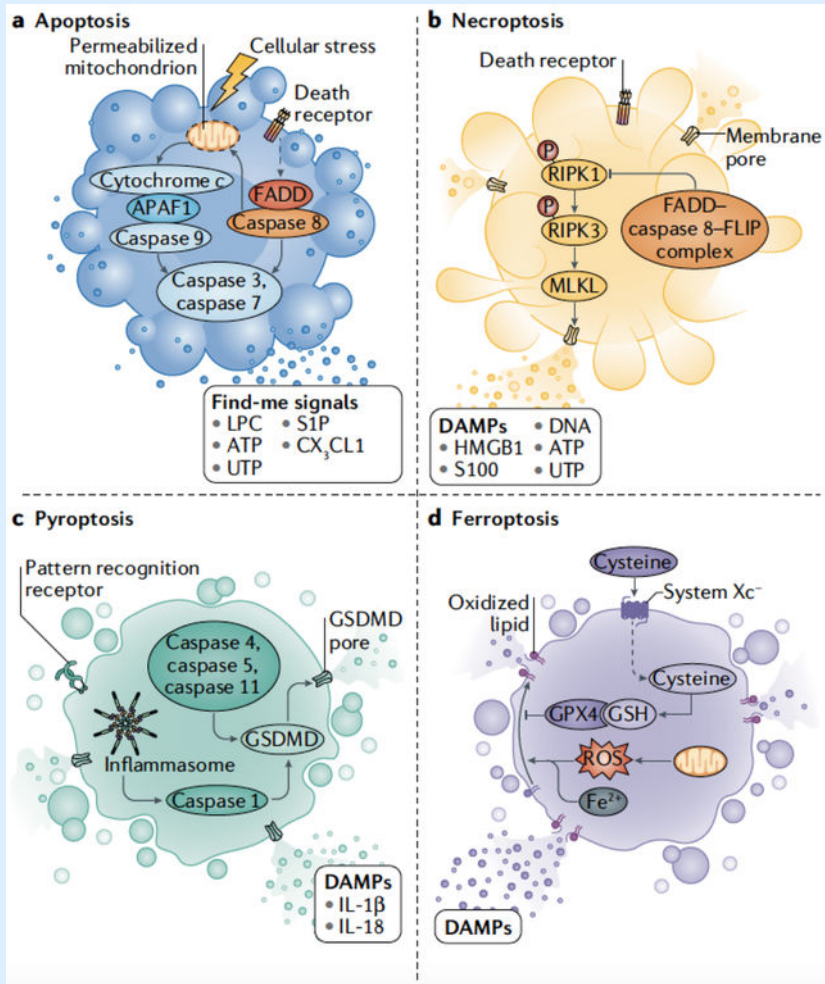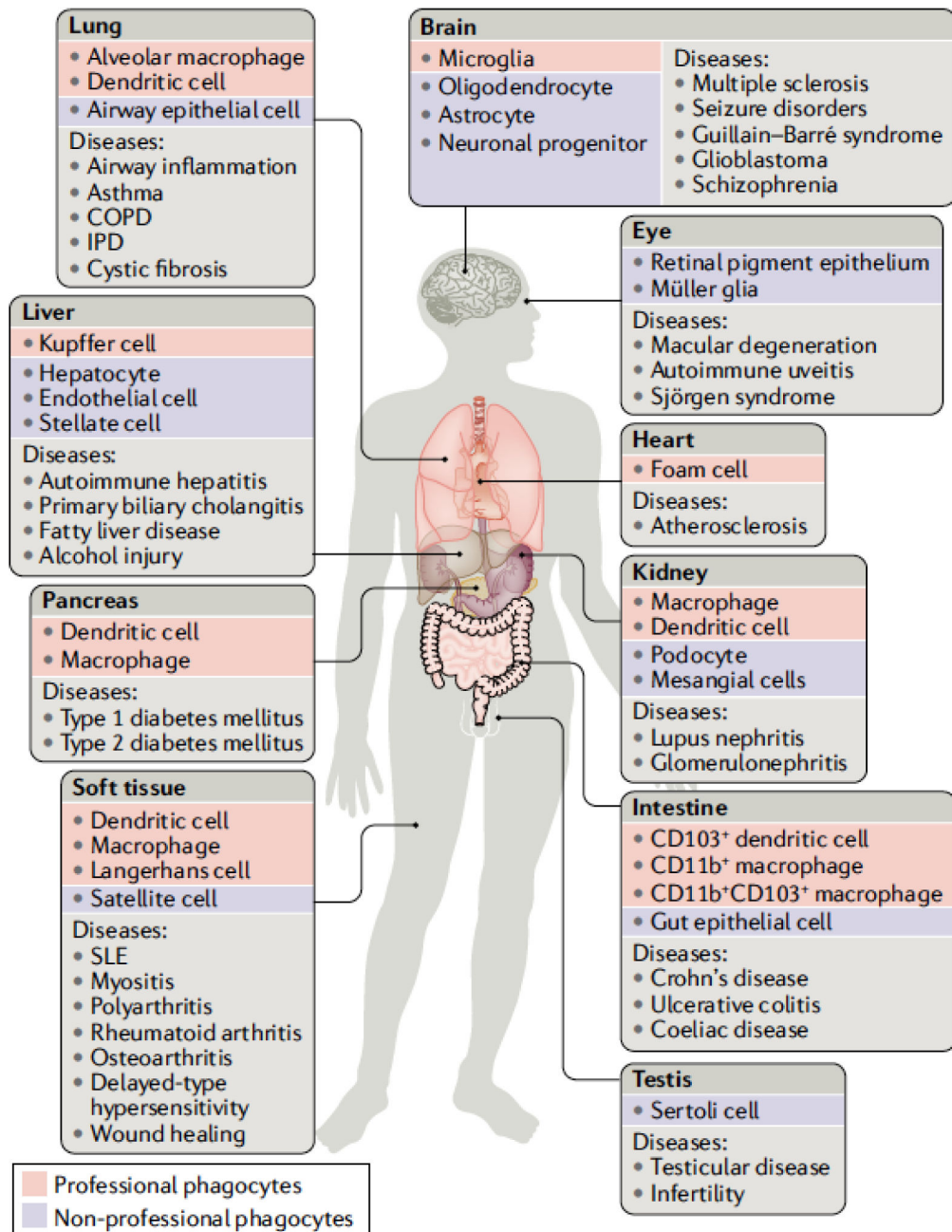
Box 1 Figure.

Figure 1. Efferocytosis is critical for tissue homeostasis.
Efferocytosis can be carried out by professional phagocytes (red boxes), such as macrophages and dendritic cells, or to a lesser extent by non-professional phagocytes (blue boxes) such as epithelial cells. Disruption of normal efferocytosis can contribute to the development of a wide range of pathologies (light grey boxes) across a variety of tissues. (dark grey boxes). COPD, chronic obstructive pulmonary disease; IPD, idiopathic pulmonary disease; SLE, systemic lupus erythematosus.
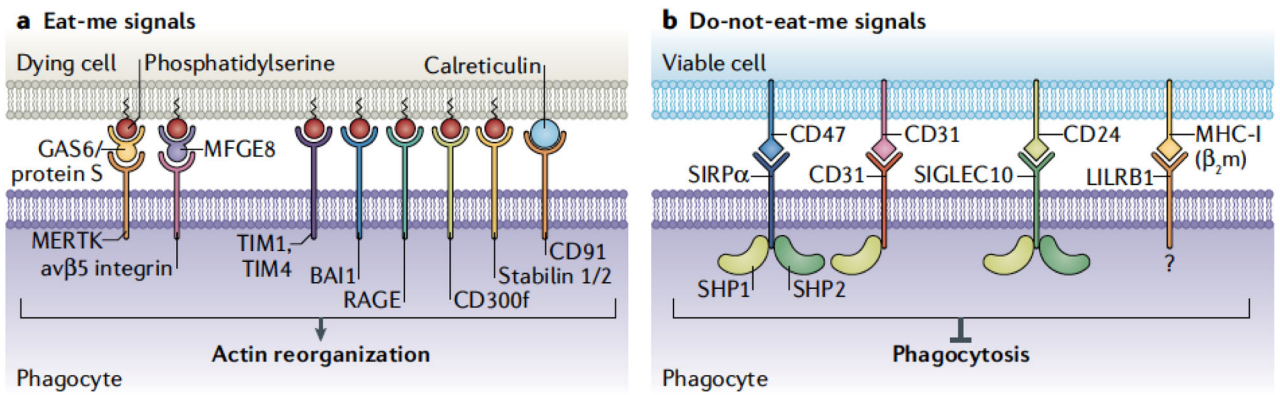
Figure 2. Cell surface signals regulating efferocytosis.
Cells display proteins and lipids to communicate with phagocytes. These protein signals can be "eat-me" signals or "don't eat me" signals, and interact with phagocytes via receptors on the phagocyte cell membrane. A) Dying cells expose a variety of signals on their surface that serve as "eat-me" signals for surveying phagocytes. A primary signal exposed on dying cells is the externalization of phosphatidylserine (PS). PS can interact with a variety of receptors on the surface of the phagocyte including TIM1, TIM4, BAI1, RAGE, the CD300 family, and the Stabilin proteins. Additionally, signaling can occur through receptors including MerTK, via adaptors including Gas6 and protein S, or through avβ5 integrin via MFGE-8. Calreticulin expressed on the surface of the dying cell can act as an eat-me signal through binding to CD91 expressed on the phagocyte outer membrane. B) Healthy cells decorate their plasma membranes with "don't-eat-me" signals that protect them from phagocytic targeting. Don't-eat-me signals include CD47 which is recognized by SIRPα on the phagocyte surface, and CD31, which homodimerizes with CD31 on phagocytes. Similarly, CD24 on viable cells engages Siglec-10 on phagocytes to inhibit engulfment. Signaling in each case occurs through SHP1/2 and suppression of pathways required for phagocytosis, including actin remodeling. Class I MHC (MHC-I) can signal through LILRB1 to mitigate phagocytic activation, although the signaling cascade is not fully delineated. In combination, these signals regulate the initiation of efferocytosis.
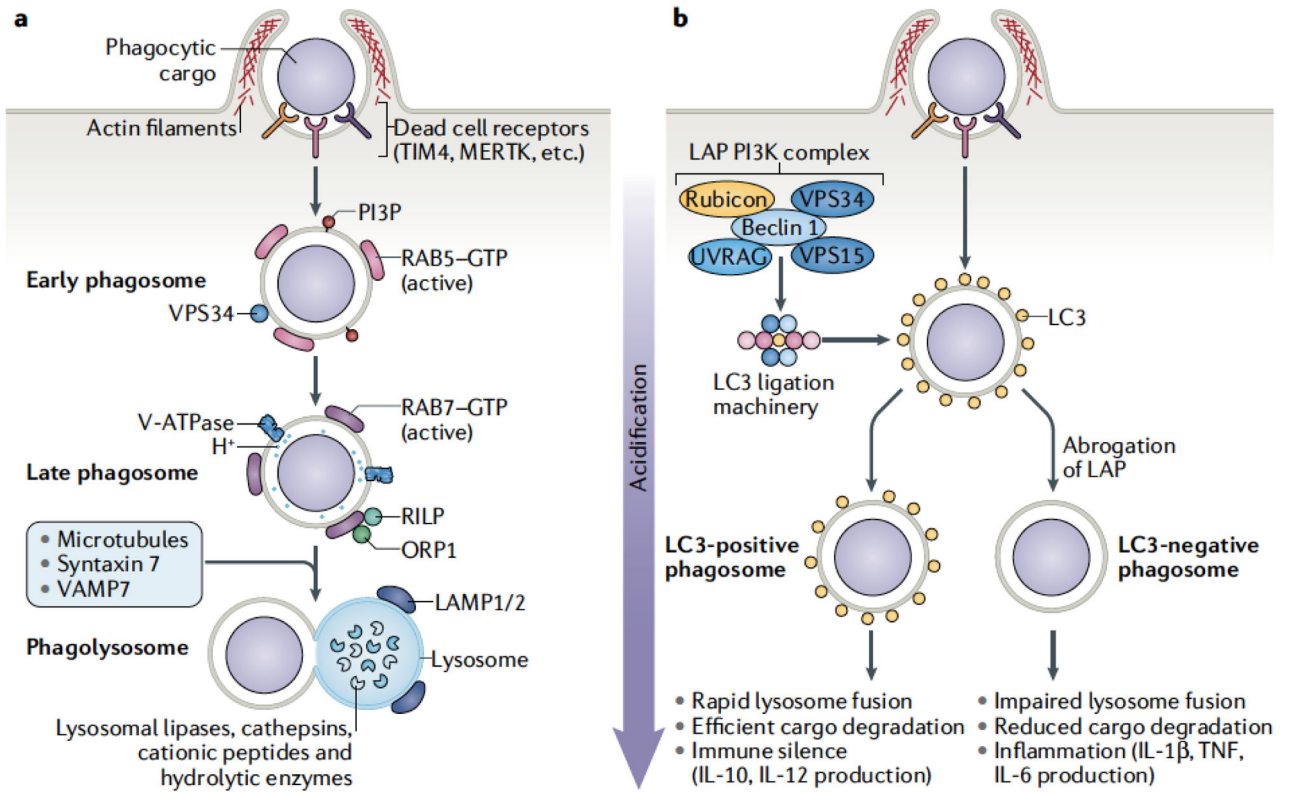
Figure 3. Phagocyte processing of a dying cell.

Upon engulfment by a phagocyte, a dying cell progresses through a canonical program leading to the degradation of the dead cell by the lysosome. Additional modifying pathways can be engaged that alter the degradative process and subsequent downstream immune signaling pathways. A) Efferocytosis of dying cells is a multistep process, involving initiation of phagocytosis, maturation of the phagosome, degradation of phagolysosome contents, and the resultant downstream effects on physiological outcomes. B) The process of LC3-associated phagocytosis (LAP) is characterized by recruitment of LC3 to the phagosome membrane. Following engagement of phagocytosis, a PI3-kinase complex containing Rubicon, UVRAG, Beclin1, VPS34, and VPS15, is assembled at the phagophore. Assembly of this PI3-kinase complex leads to the recruitment and activation of the LC3 ligation machinery. Ligation of LC3 to the phagosome lipid membrane promotes rapid maturation and lysosomal fusion. This expeditious process leads to efficient clearance of the dead cell and immune silence. If LAP is abrogated, there is a delay in phagosome maturation and lysosomal fusion. The impaired clearance of the dying cell leads to inflammatory immune activation through processes that are, to date, not fully understood.

Table 1.

Damage-associated molecular patterns released from necrotic cells

Data from refs[207,208,209,210,211,212,213,214,215,216]. BAI1, brain-specific angiogenesis inhibitor 1; cGAS, cyclic GMP–AMP synthase; C1qR, C1q receptor; HMGB1, high-mobility group protein B1; IL-1R, IL-1 receptor; IL-18R, IL-18 receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RAGE, receptor for advanced glycosylation end products; TIM, T cell immunoglobulin mucin receptor; TLR, Toll-like receptor.

Origin	Molecule	Receptor
Intracellular Pathogens	PAMPs	PRR (cell surface, endosome, cytosol)
Nucleus	HMGB1	TLR2, TLR4, RAGE
	Histones	TLR2, TLR4, TLR9
Nucleus and mitochondria	DNA	TLR9, cGAS, AIM2, ZBP1
	RNA	TLR3, TLR7, TLR8
Cytosol	ATP, UTP	P2Y2R
	Uric acid crystals	NLRP3
	S100	RAGE
	IL-1β, IL-18	IL-1R, IL-18R
Inflammasome	IL-1	IL-1R
	IL-33	ST2
Cell surface	Phosphatidylserine exposure	TIM1, TIM4, BAI1, Stabilin 2, CD300f, RAGE, MerTK, Axl, Integrin
	Calreticulin	CD91
Endoplasmic Reticulum	C1q	C1qR
	ICAM3	CD14

Footnotes: Citations for Table 1. [195-204]

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Table 2.

Diseases associated with aberrations in efferocytosis pathways

ABCA1, ATP-binding cassette transporter 1; BAI1, brain-specific angiogenesis inhibitor 1; C1qa, complement C1q subcomponent subunit A; EAE, experimental autoimmune encephalomyelitis; ELMO1, engulfment and cell motility protein 1; GVHD, graft-versus-host disease; IBD, inflammatory bowel disease; LRP1, LDL receptor-related protein 1; LXR, liver X receptor; MFGE8, milk fat globule-EGF factor 8; MS, multiple sclerosis; PPAR, peroxisome proliferator-activated receptor; RAGE, receptor for advanced glycosylation end products; SLE, systemic lupus erythematosus; TIM4, T cell immunoglobulin mucin receptor 4.

Signal	Molecule	Pathology Associated	Ref.
Systemic			
Find-me	G2A	Autoimmunity	205
	Sphingosine-1-phosphate	SLE	206
Eat-me	TIM4, MerTK	SLE	159,207,208
	RAGE	Sepsis	102,209
	CD300f	Autoimmunity	210
	Integrins	Autoimmunity	211
	MFG-E8	SLE; Autoimmunity	212,213
	Gas6	GVHD	190,214
	Protein S	Autoimmunity	215
	SCARF1	Autoimmunity	216
	C1qa	SLE	217
Processing	LXRα/β	SLE; autoimmunity	132
	PPAR	SLE	132
	ABCA1	SLE	218
	Rubicon, Beclin-1, VPS34, ATG5, ATG7, ATG16	SLE, anti-cancer immunity	125,140
Central Nervous System			
Find-me	Pannexin-1	MS/EAE	219
Eat-me	BAI1	Learning and memory	220
	TREM2	Alzheimer's disease	126
	MerTK	MS/EAE; Parkinson's	221
	Gas6	Toxin-induced demyelination	222
	C1qa	Epilepsy	223
Engulfment	GULP	Schizophrenia	224
Processing	LXRα/β	MS/EAE	132
	Rubicon	Alzheimer's disease	126
Eye			
Eat-me	MerTK	Retinal degeneration	221
Processing	LXRα/β	Autoimmune uveitis	211
Liver			

Signal	Molecule	Pathology Associated	Ref.
Eat-me	TIM4	Ischemia reperfusion injury	225
	Gas6	Ischemia reperfusion injury	226
Pancreas			
Eat-me	MFG-E8	Type 1 diabetes mellitus	167
Testes			
Eat-me	MerTK	Reduced fertility	221
Engulfment	ELMO1	Testicular pathology	227
Lung			
Find-me	P2Y2	Allergic airway inflammation	228
Eat-me	RAGE	Fibrosis; Allergic airway inflammation	102
	MerTK	Allergic airway inflammation	221
	CD36	Lung injury	194
Engulfment	RAC1	Allergic airway inflammation	229
Cardiovascular system			
Find-me	Pannexin-1	Hypertension	230
	CX3CR1	Atherosclerosis	231
Eat-me	BAI1	Dyslipidemia	137
	MFG-E8	Atherosclerosis	167
	CD36	Diet-induced obesity	232
	LRP1	Atherosclerosis	233
	C1qa	Atherosclerosis	234
	SLC2A1	Atherosclerosis	235
Processing	DOCK180	Cardiovascular pathologies	236
	LXRα/β	Atherosclerosis	237
	PPAR	Atherosclerosis	238
	DRP1	Atherosclerosis	239
Kidney			
Eat-me	Stabilin-1, Stabilin-2	Glomerular fibrosis	240
	Gas6	Nephritis	214
Engulfment	ELMO1	Diabetic nephropathy	227
Gastrointestinal system			
Find-me	G2A	IBD/Colitis	241
Eat-me	CD300f	IBD/Colitis	210
	MerTK	Colon cancer	221
	Integrins	IBD/Colitis	242
	MFG-E8	IBD/Colitis	167
	RAC1	IBD/Colitis	243
Skin, Muscle, Mucosal Tissue and Joints			

Signal	Molecule	Pathology Associated	Ref.
Eat-me	RAGE	Muscle regeneration	[244]
	C1qa	Wound healing	[182]
	MerTK	Arthritis	[101]
	Gas6	Melanoma	[245]
	Integrins	Carcinoma	[242]
	CD36	Wound healing	[194]
Processing	DNase II	Polyarthritis	[187]
Engulfment	RAC1	Arthritis	[243]