Published in final edited form as:

Immunol Rev. 2010 July ; 236: 95–109. doi:10.1111/j.1600-065X.2010.00919.x.

The complex interplay between autophagy, apoptosis, and necrotic signals promotes T-cell homeostasis

Craig M. Walsh¹ and Aimee L. Edinger²

¹Institute for Immunology and the Department of Molecular Biology & Biochemistry,

²Department of Developmental and Cell Biology, University of California, Irvine. Irvine, CA, USA

Summary

Intense research efforts over the last two decades have focused on establishing the significance of apoptotic signaling in adaptive immunity. Without a doubt, caspase-dependent apoptosis plays vital roles in many immune processes, including lymphocyte development, positive and negative selection, homeostasis, and self-tolerance. Cell biologists have developed new insights regarding cell death, establishing that other modes of cell death exist, such as programmed necrosis and type-II/autophagic cell death. Additionally, immunologists have identified a number of immunological processes that are highly dependent upon cellular autophagy, including antigen presentation, lymphocyte development and function, pathogen recognition and destruction, and inflammatory regulation. In this review, we provide detailed mechanistic descriptions of cellular autophagy and programmed necrosis induced in response to death receptor ligation, including methods to identify them, and compare and contrast these processes with apoptosis. The crosstalk between these three processes is emphasized as newly formulated evidence suggests that this interplay is vital for efficient T-cell clonal expansion. This new evidence indicates that in addition to apoptosis, autophagy and programmed necrosis play significant roles in the termination T-cell-dependent immune responses.

Keywords

autophagy; T cells; apoptosis; programmed necrosis; tolerance; homeostasis

Introduction

As emphasized in many pages of this compendium of reviews regarding the termination of adaptive immune responses, apoptosis has long been known to orchestrate not only the development of self-tolerant lymphocytes but also the maintenance of peripheral lymphoid tolerance and homeostasis. However, it is becoming clear that apoptotic cell death and signaling pathways are not as simple as they first appeared. Recent studies have illuminated a previously unappreciated relationship between apoptosis, necrosis, and autophagy in activated peripheral T cells. A better understanding of the role metabolic reprogramming plays in cellular growth, studies uncovering the machinery of autophagy, and the identification of key signaling proteins regulating programmed necrosis are now becoming highly relevant to understanding how adaptive immune responses are terminated. Herein, we discuss recent progress in cell death and autophagy research and address the vital role that these alternative forms of death may play in the regulation of adaptive immunity.

Autophagy is commonly observed in both apoptotic and necrotic cells. Unlike apoptosis or necrosis, autophagy is likely an unfamiliar term to many readers, as its role in the immune response has only recently been elucidated. An introduction to autophagy therefore precedes a discussion of its specific role in T-cell proliferation and programmed cell death. The term autophagy comes from Greek roots: *auto* 'self' and *phagy* 'to eat'. The phrase 'macroautophagy, hereafter referred to as autophagy' is so commonly employed because there are at least three different forms of self-digestion: (i) (macro)autophagy is the enclosure of cytoplasm, cytoplasmic protein aggregates, or whole organelles in a double membrane-bound structure that is destined to be degraded in the lysosome (Fig. 1); (ii) microautophagy is the capture of cytoplasmic contents in small vesicles that bud directly into the lysosome; and (iii) chaperone-mediated autophagy does not involve vesicle formation, but instead, proteins containing a target sequence are translocated across the lysosomal membrane (reviewed in 1). It is macroautophagy, hereafter referred to as autophagy, that has been identified as playing a key role in the adaptive immune system.

Autophagy has distinct roles in different cellular contexts. Constitutive, or basal, autophagy is responsible for the turnover of old or damaged organelles. This 'housekeeping' level of autophagy maintains cellular homeostasis. For example, as the principle site of reactive oxygen species (ROS) generation, mitochondria are particularly susceptible to oxidative damage. Damaged mitochondria generate even more ROS than fully functional organelles (2-4). Turnover of defective mitochondria through autophagy thus plays an important role in reducing oxidative stress. Ribosomes, endoplasmic reticulum (ER), peroxisomes, and protein aggregates are all eliminated via autophagy (5). In the thymus, the constitutive degradation of cellular components through autophagy provides an excellent source of self-antigens. Thymic epithelial cells have a high basal rate of autophagy, and blocking autophagy in these cells interferes with the development of T-cell tolerance (6). There may be an as yet unidentified signal (e.g. lipid or protein oxidation) that targets old or damaged organelles for autophagic degradation, as it would be bioenergetically inefficient to catabolize fully functional useful organelles.

Autophagy can also be induced in response to cellular stress. Nutrient limitation and adenosine triphosphate (ATP) depletion are potent inducers of autophagy. Autocatabolism protects bioenergetically stressed cells by supplying nutrients from internal sources to support essential cellular processes until extracellular nutrients once again become available. Autophagy induced by bioenergetic stress is generally considered to be a non-selective, bulk degradative process. Autophagy can also be induced as a part of a cellular differentiation program that helps cells switch to a new phenotype. Autophagy promotes the clearance of mitochondria from reticulocytes during their maturation (7-9). Differentiation into adipocytes also requires autophagy (10, 11). As discussed later in this review, autophagy is also required during T-cell development and following activation (12, 13). Autophagy can also aid in the clearance of intracellular pathogens (reviewed in 14). In all of these cases, autophagy is a productive, homeostatic cellular response.

There is no evidence that inducing cell death is a physiologic function of autophagy in normal cells. When apoptosis is blocked, however, autophagy certainly can kill cells. When evaluating the role autophagy plays in a dying cell, it is critical to recognize that a cell filled with autophagic vacuoles may be dying because it is filled with autophagic vacuoles (Type II autophagic cell death) or in spite of the fact that it is filled with autophagic vacuoles. In the latter case, the homeostatic response of autophagy may be insufficient to compensate for the initiating stress. Homeostatic autophagy often precedes apoptosis, and when apoptosis is blocked, the failure to restore homeostasis leads to necrosis instead. By contrast, in Type II cell death, blocking autophagy protects from cell death. Making the distinction between helpful and harmful autophagy in a given cellular context is key to understanding the

function of autophagy. The ability to reduce the level of essential autophagy proteins through genetic ablation and RNA interference (RNAi) has simplified this process and dramatically enhanced our understanding of the interplay between death and autophagy in the adaptive immune system.

The nuts and bolts of autophagy

There are three stages of autophagy: (i) the initiation phase when the 'isolation membrane' begins to form around the cytoplasmic contents to be degraded, (ii) expansion of the crescent-shaped isolation membrane and eventual closure of the growing autophagosome into a double-membrane bound vesicle as a result of the fusion of the two tips of the crescent-shaped isolation membrane, and (iii) autophagosome maturation that culminates in degradation in lysosomes (Fig. 1). The basic molecular machinery that controls these stages is briefly described below and summarized in Table 1. For additional details, readers are referred to several excellent recent reviews (15, 16).

Initiation/nucleation

Two different multi-protein complexes are required to trigger autophagy by initiating formation of the isolation membrane. A complex containing the serine/threonine kinase Ulk1 is at the top of the autophagy induction cascade (17). Mammals have two Atg1 orthologs, Ulk1 and Ulk2, both capable of triggering autophagy (18). Deciphering the relative importance of these homologs in the process will require additional studies. The Ulk kinase forms a large multiprotein complex that is localized to the isolation membrane and plays an essential role in autophagosome formation (17). How this complex initiates autophagy remains unclear, but it plays a role in recruiting downstream autophagy proteins. The core components of the second autophagy initiation complex are Beclin1, the class III phosphatidylinositol 3-kinase (PI3K) Vps34, and p150 (19). Deletion of any of these proteins or inhibiting Vps34 kinase activity with 3-methyladenine (3-MA) or other PI3K inhibitors blocks autophagosome formation. This core PI3K complex is activated or inhibited through associations with other proteins, including Bcl-2 (20-23) (see below). Phosphatidylinositol 3-phosphate (PtdIns3P) generation may recruit other autophagy proteins to the isolation membrane (24).

Elongation and closure

Two ubiquitin-like conjugation systems are required for autophagosome elongation and closure (reviewed in 25). In both systems, Atg7 functions similarly to the E1 enzyme that activates ubiquitin prior to its conjugation to lysine residues by E2 and E3 enzymes. Two ubiquitin-like molecules, Atg12 and LC3, are conjugated to different partners. Atg12 is constitutively conjugated to Atg5 by Atg7 working with the E2-like enzyme Atg10. This conjugation is most likely irreversible. The Atg5-Atg12 conjugate binds Atg16L, which is specifically localized to isolation membranes. When associated with Atg16L, Atg5-Atg12 is thought to act like an E3 enzyme promoting LC3 lipidation and correct LC3 localization (26, 27).

Prior to conjugation, the second ubiquitin-like molecule, LC3 undergoes proteolytic processing catalyzed by the cysteine protease Atg4B (28). Atg4B removes the C-terminus of LC3 exposing a glycine residue required for the conjugation reaction (this processing event explains why LC3 is always tagged with fluorescent proteins on the N-terminus). The proteolytically processed form of LC3 is known as LC3-I. When autophagy is induced, the C-terminal glycine of LC3-I is conjugated to the lipid phosphatidyl ethanolamine (PE) by the E2-like protein Atg3 working in concert with Atg7 (29, 30). Conjugation to PE increases LC3's hydrophobicity and its mobility in SDS-PAGE (sodium dodecyl sulfate

polyacrylamide gel electrophoresis); LC3-II is not a proteolytically processed form of LC3-I. LC3-II is present on the outer surface of the growing isolation membrane and is retained on autophagosomes throughout the maturation process (Fig. 1). Thus, LC3-II is commonly used as a specific marker for autophagosomes. Prior to the fusion of autophagosomes and lysosomes, Atg4B cleaves the PE moiety from LC3-II, releasing LC3 into the cytoplasm where it is presumably recycled (28) (Fig. 1). The LC3-II on the inner face of the autophagosome is not accessible to Atg4B, however, and is degraded with the autophagosome. As a result, LC3 levels generally decline in response to a prolonged autophagic stimulus. Functionally, LC3-II is important for the elongation and closing off autophagosomes and is able to promote hemifusion of membranes *in vitro* (24, 30, 31). LC3-II may also serve as a scaffold supporting the expansion of the autophagosome membrane and participate in cargo selection (25, 32).

Degradation

Thus far, all proteins required for this stage of autophagy function in endocytic trafficking generally and are not autophagy specific. Proteins that play a role in lysosomal fusion, acidification, and lysosomal proteases are all required for degradation of autophagosomes.

The commonly used phrase ‘autophagic flux’ refers to the execution of all three stages of autophagy: initiation, expansion and closure, and degradation (7). If degradation is blocked, autophagosomes accumulate even in the absence of autophagy induction. Discriminating between increased autophagic flux and decreased autophagosome degradation is essential to understand the role of autophagy plays in a particular cellular process. In some instances, encapsulation of cellular contents in autophagosomes may fulfill the function of autophagy. In most cases, however, degradation of autophagosomes is required for autophagy to fully serve its homeostatic functions.

Monitoring autophagy

For seminal reviews of appropriate methods to study autophagy, interested readers are referred to previously published articles (7, 33). Some frequently employed techniques include LC3 localization, LC3 conjugation, and electron microscopy. For LC3 localization, LC3-I is present in the cytoplasm. Upon conversion to LC3-II, the protein becomes more hydrophilic and membrane-associated (Fig. 1). Thus, the induction of autophagy is marked by a shift in the subcellular distribution of LC3 from diffuse in the cytosol to punctate ‘dots’ representing autophagosomes. GFP-LC3 is commonly used in this assay. For LC3 conjugation, LC3’s electrophoretic mobility increases upon conjugation to PE, and thus LC3-II can be quantified by Western blotting. By itself, an increase in LC3-II levels does not indicate whether autophagic flux is increased or degradation is blocked. Autophagy occurs constitutively at a basal rate. Because some LC3-II is turned over with the autophagosome (Fig. 1), inhibiting lysosomal degradation will result in LC3-II accumulation even in the absence of autophagy induction. It is often inferred that autophagic flux has been increased when adding lysosomal inhibitors further increases the levels of LC3-II detected in a Western blot beyond the level detected with the autophagic stimulus alone. The reasoning is that if LC3 levels are increased because degradation is blocked, inhibiting lysosomal proteolysis should not increase LC3-II levels any further. For electron microscopy, autophagosomes are readily detected in electron micrographs. The double membrane around autophagosomes is not always obvious but they contain cytoplasmic contents and organelles at various stages of degradation. While commonly performed, evaluation of autophagy using electron micrographs is prone to misinterpretation if not conducted by an experienced observer and considered in the context of other assays.

Signaling inputs regulating autophagy relevant to the adaptive immune system

Given the key functions of autophagy in maintaining cellular homeostasis and the lethal consequences of excessive autophagy, it is not surprising that multiple signaling inputs regulate this process. Several regulatory inputs are particularly relevant in T cells: mTOR, ROS, loss of mitochondrial membrane potential, and Bcl-2/Bcl-X_L.

Mammalian target of rapamycin (mTOR) is found in two complexes in cells, the rapamycin-sensitive mTORC1 and the rapamycin-insensitive mTORC2 (reviewed in 34, 35). mTORC1 is activated by TCR signaling and cytokines and is inactivated by low amino acid levels and by bioenergetic stress. The mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) is also activated by TCR and cytokine signals and increases mTORC1 activity by inactivating upstream negative regulators. mTORC1 suppresses autophagy induction by inhibiting the kinase activity of Ulk1 (17). The release of Ulk1 from mTORC1 inhibition under starvation conditions stimulates the initiation of autophagy (36, 37). Intriguingly, Ulk1 may also negatively regulate TORC1 (38, 39). mTORC1 activation can also increase autophagy by increasing Hif1 α (40). For a comprehensive review of the role of TOR in autophagy regulation, interested readers are referred to Neufeld (41).

Many studies have identified ROS as an autophagy trigger. Inhibiting electron transport chain complexes I or II leads to ROS generation that stimulates autophagy (42). Treating cells with H₂O₂ or inhibiting superoxide dismutase-mediated ROS detoxification also induces autophagy (43). ROS generated in response to selenite treatment induces autophagy in cancer cells that contributes to their demise (44). In all of these circumstances where ROS levels are increased non-physiologically, autophagy induction hastens cell death. ROS generated in response to physiological insults, such as nutrient deprivation, triggers homeostatic autophagy that protects cells from death (45-47). Autophagy helps to reduce ROS production by decreasing mitochondrial mass and thus ROS generation. While ROS increase autophagy, ROS actually decrease the activity of Atg4B (47). It has been suggested that inhibiting Atg4B-mediated de-conjugation of LC3 and PE stimulates autophagy. It is likely that other autophagy proteins are also regulated directly or indirectly by ROS. For example, oxidative stress activates PARP-1 which can trigger autophagy by depleting ATP levels, which will lead to mTOR inhibition and the induction of autophagy (48).

A decrease in mitochondrial membrane potential precedes mitochondrial outer membrane permeabilization during apoptosis. Multiple studies have shown that depolarized mitochondria are more likely to be eliminated by autophagy (49-52). Reductions in mitochondrial membrane potential could be an autophagy trigger in either an apoptotic or necrotic setting.

Binding of ER-associated (but not mitochondrial) Bcl-2 to Beclin1 prevents its association with Vps34, thereby inhibiting autophagy at the initiation stage (53). Bcl-X_L also inhibits autophagy by associating with Beclin1 (53-55). The negative regulation of autophagy by Bcl-2 can be disrupted by BH3-domain proteins, JNK1-mediated phosphorylation of Bcl-2 (56), or phosphorylation of Beclin1 by DAPK (57). TCR signaling could increase autophagy by activating JNK. Inhibitor of NF- κ B (I κ B) kinase has recently been shown to promote autophagy in an NF- κ B-independent, JNK1 dependent manner (58) that could involve the release of Bcl-2 from Beclin1.

Autophagy supports proliferation and survival during the transition between naive and effector T cell

Given the signaling networks that are known to regulate autophagy, its role in mammalian T cells is particularly intriguing. As outlined above, autophagy is a catabolic response, and it was somewhat counter-intuitive that it would be intrinsically linked to T-cell activation and proliferation (59). Activation of CD4⁺ T cells cultured under optimal *in vitro* conditions leads to the induction of autophagy (60). He and colleagues (12) established for the first time that autophagy plays an essential role in both activated T cells through the use of fetal liver reconstitution experiments using Atg5^{-/-} T cells. Atg5 is essential for autophagosome formation (61) (Fig. 1). Atg5^{-/-} T cells undergo inefficient clonal expansion marked by profound levels of cell death. Atg5-deficient T cells are also present at greatly reduced levels in the periphery compared to wildtype T cells, suggesting a developmental defect or a reduced reconstitution capacity. These studies clearly establish a positive, homeostatic role for the catabolic process of autophagy in rapidly proliferating, highly anabolic T cells.

As we have previously speculated (59), autophagy may be critically important in activated T cells for several reasons. First, TCR stimulation leads to an oxidative burst that may produce massive levels of toxic ROS. T-cell activation leads to the opening of CRAC channels and subsequent entry of millimolar levels of calcium (62). This calcium stimulates oxidative phosphorylation at early timepoints after T cell stimulation, leading to high-level production of ROS (63-66). While these ROS may be beneficial for lowering T-cell activation thresholds through inhibition of phosphatase activity (67-70), these oxygen radicals also have the potential to induce significant organelle damage through lipid peroxidation and other damaging effects that may promote T-cell death (71). Thus, autophagy, or more precisely, mitophagy, may be used to limit the number of mitochondria producing ROS and to remove damaged mitochondria that would further amplify ROS production.

This hypothesis was tested by Pua *et al.* (12, 72) using mice conditionally deficient for a second required autophagy protein, Atg7 (Fig. 1, Table 1). T-cell-specific deletion of the Atg7 gene resulted in defective proliferation as observed using Atg5^{-/-} fetal liver transplants (12, 72). Interestingly, naive T cells recovered from these mice had high levels of ROS and failed to reduce mitochondrial content normally during the developmental transition from thymocytes to mature T cells. In parallel studies, Swat and colleagues (13) identified a similar increase in mitochondrial mass in T cells lacking Atg5 and noted enhanced biosynthesis of mitochondria-specific genes in T cells lacking the capacity to induce autophagy. These findings are consistent with the hypothesis that mitophagy has an important role during T-cell development and activation. T cells become less dependent on mitochondria for energy as they switch to glycolytic metabolism following their activation. Thus, the failure to remove damaged mitochondria could lead to the release of mitochondrial proteins that promote apoptosis (e.g. cytochrome C) and unnecessary mitochondria might lead to excessive ROS production that could trigger apoptotic or necrotic cell death.

Autophagy researchers have recently made advances that could facilitate further testing of the role of selective mitophagy in T-cell development and proliferation. Recent work in yeast has identified Atg32 as the protein that targets mitochondria for selective autophagic degradation (73, 74). Atg32 does not have an obvious homolog in higher eukaryotes, but Nix (also known as BNIP3L) has been proposed as a functional ortholog of Atg32 in mammalian cells. Nix is broadly expressed and dramatically upregulated during terminal erythroid differentiation when mitophagy clears reticulocytes of mitochondria as a part of their maturation into red blood cells (75). Like yeast Atg32 (73, 74), Nix is not required for starvation-induced autophagy (8, 76). Recently, Nix has been found to recruit LC3 to mitochondria causing their targeting to autophagosomes, similar to how p62 functions in the

clearance of ubiquitinated protein aggregates (32, 77). $Nix^{-/-}$ reticulocytes have a more profound defect in mitochondrial clearance than $Atg7^{-/-}$ reticulocytes (9). This result suggests that Nix can promote mitochondrial clearance through conventional and alternative autophagy pathways (78). It remains to be determined whether Nix plays a role in mitophagy in T cells.

Another potential explanation for the profound requirement for autophagy during the transition between naive and effector stages in T cells is the accepted function of autophagy in nutrient-limited cells. T cells require enormous amounts of energy and biosynthetic intermediates to drive the rapid clonal expansion necessary to eliminate pathogenic infections. Consistently, it has been estimated that T cells (especially $CD8^{+}$ cells) divide at maximal rates for mammalian cells, with doubling times that roughly equate to once every 4-6 h (79). Such rapid proliferation clearly places significant demands on the cell's bioenergetic and biosynthetic pathways (64), demands that may be alleviated to some extent through autophagy. Related to this, the transition from dependence on oxidative phosphorylation to aerobic glycolysis that is observed following the activation of naive T cells (80-82) may somehow address biosynthetic needs. Nutrient transporter proteins are expressed at very low levels in naive cells and are dramatically upregulated in the first two days after activation. Autophagy may be important in the early stages of T-cell activation, when extracellular nutrient access is limited but metabolic reprogramming to support proliferation has already begun. The degree to which catabolized organelles are used for the biosynthesis required for efficient clonal expansion is presently unclear. Autophagy is clearly a key mechanism by which amino acids are recycled, and glutamine in particular plays a critical role in biosynthetic reactions supporting rapid T-cell proliferation (64). Finally, it is possible that autophagy is required for efficient transitions through cell cycle stages. We have observed that blockade of cell cycle at the G1/S and G2/M stages of cell cycle using hydroxyurea and nocodazole, respectively, leads to enhanced recovery of LC3-II (authors' unpublished observations). This result suggests that transitions through distinct cell cycle stages may enhance autophagic flux, possibly as a means to accomplish cytoplasmic remodeling during these stages.

Whatever the function of autophagy, understanding the signaling that leads to the genesis of autophagosomes within activated T cells is an important future challenge. TCR signaling leads to high levels of mTOR and S6 kinase (S6K) activity in effector T cells if they are to avoid becoming anergic or differentiating into regulatory T cells (83-86). As described above, mTOR inhibits autophagy (87), and conversely, the mTOR-dependent signaling intermediate S6K is inhibited by activated Ulk1/Atg1 (38, 39, 88). Thus, a major question facing immunologists is how autophagy is induced when active mTOR signaling is present within T cells. ROS may have mTOR independent stimulatory effects on autophagy. Changes in the balance of Bcl-2 family members or their phosphorylation state may also be critically involved. Understanding the interplay between autophagy and proliferation in T cells will have far-reaching implications for other fields, especially cancer and developmental biology.

Death receptor signaling and T-cell homeostasis: roles for apoptosis, necrosis, and autophagy

The contribution of death-receptor induced apoptotic-signaling intermediates to T cell homeostasis

Death receptors (DRs) are subset of the TNF-receptor family that contain cytoplasmic death domains, many of which are essential for immune homeostasis and tolerance (89). These death domains promote the recruitment of adapter molecules such as FADD, TRADD, and

RIPK1 (90-93), all of which bear death domains for homotypic oligomerization with DRs. Upon DR ligation, these adapters are involved in the assembly of complexes that promote either inflammatory and survival signals, primarily involving the activation of the transcription factor NF- κ B, or apoptotic signals delivered through caspase-8 (94). Additionally, a non-catalytic paralog of caspase-8 called c-FLIP (95) is also recruited to caspase-8 containing death-induced signaling complexes (DISCs) (96) and serves to promote or inhibit DISC activity depending on the context (89).

While mice bearing mutations in individual or multiple DRs have been noted to develop notable hematopoietic deficiencies, attempts to address the involvement of apoptotic signaling by DRs in immune homeostasis and tolerance have been thwarted by the embryonic lethality of germline deletion of the DISC components FADD, caspase-8, and c-FLIP (97-100), findings that suggest a potentially non-apoptotic role for these DISC proteins. T cells lacking FADD specifically were first developed by Winoto and colleagues (98), who made use of Rag1-deficient blastocyst chimerism to assess the role(s) of FADD in T cells. We and two other groups (101-103) used an alternative approach in which a mutant form of FADD lacking a caspase-8 recruitment domain (FADD^{dd}) was ectopically expressed in T cells. Paradoxically, whereas loss of FADD function blocked DR-induced apoptosis in thymocytes, it did not establish the function of DR-induced apoptosis in T-cell homeostasis. Instead, these mutant mice suggested that FADD was essential for T-cell clonal expansion. Similarly enigmatic results using T cells conditionally lacking caspase-8 or c-FLIP established that these DISC proteins are also essential for T-cell clonal expansion (104-105). Research by Lenardo and colleagues (106) led to the observation that loss of caspase-8 function in humans also leads to striking T-cell defects. Taken together, these studies demonstrate that caspase-8, FADD, and c-FLIP are required for T-cell expansion following antigenic stimulation. However, it should be noted that the contributions that DISC-mediated apoptosis provide to effector T-cell death *in vivo* remain poorly understood. As we learn more about the various functions of these proteins in T cells, it will then be possible to establish the relevance of DR-induced apoptosis to T-cell homeostasis and tolerance.

RIP kinases, necroptosis, and autophagy

Although early reports proposed a relatively simple apoptotic signaling cascade downstream of death receptors (107), it is now clear that caspase-8 induced apoptosis is not the only path to DR-induced cell death (Fig. 2). As is often exclaimed, ‘the devil is in the details’, this can be no more apt than for DR signaling. Perhaps one of the earliest examples of this was provided by Nagata’s group (108), when it was observed that artificially multimerized mutation of FADD induced a caspase-independent form of cell death. Another early example of cells failing to follow this simple DR-signaling paradigm was provided by Vandenabeele and colleagues (109), when it was found that L929 cells underwent a caspase-independent form of cell death in response to TNF α when pre-incubated with the pan-caspase inhibitor zVAD-FMK. Subsequent work by Jürg Tschopp and coworkers (110) revealed an alternative, caspase-independent form of cell death activated by DR ligation that depends on the kinase activity of RIPK1. While initially perplexing, these results demonstrated the existence of an alternative DR-induced cell death pathway revealed in cells lacking the capacity to activate caspase-dependent apoptosis (94).

Many groups have now established that disabling caspase-8 dependent apoptotic cell death often leads to a form of cell death that has many hallmarks of cellular necrosis (111). Termed variously ‘programmed necrosis’, ‘necroptosis’, or ‘caspase-independent cell death’, this form of death is readily observed when caspase activity is blocked, but its relevance to cellular physiology is unclear. Does this death process exist as a parallel or independent pathway to apoptosis, and is it truly programmed? While these questions

remain to be fully explored, it is notable that inhibitors of RIPK1 activity, a key intermediate in DR-induced necroptosis, prevent myocardial infarction, cerebral ischemia, or acute organ failure (112–115). Indeed, perhaps the one unifying mechanistic feature of cell death by programmed necrosis is the involvement of RIPK1 activity. In addition to RIPK1 involvement, programmed necrosis has also been found to involve the generation of high levels of ROS (116) induced by RIPK1. As well, the translocation of AIF, a phylogenetically ancient mitochondrial flavoprotein, from mitochondria to nuclei has been shown to be involved in the induction of programmed necrosis (117). Considering the release of cytochrome C and its activation of caspase-9 in the Apaf-1-containing ‘apoptosome’ is essential for intrinsic apoptosis resulting from MOMP (118), this pathway to activate programmed necrosis is mechanistically reminiscent. The mitochondrial release of AIF is then thought to participate in the subsequent activation of PARP1 within the nucleus, the latter leading to significant depletion of cellular metabolites in the process of chromatin condensation (111). In summary, if apoptosis does not kill the cell, RIPK1-dependent programmed necrosis will.

The central role of RIPK1 in orchestrating programmed necrosis is rather paradoxical, since it is also required for cell survival following DR stimulation through activation of NF- κ B signaling (119–120). RIPK1 itself is the target of proteolytic inactivation by caspase-8, a feature originally thought to limit pro-survival signaling following DR ligation (121). However, it is likely that caspase-8 dependent cleavage of RIPK1 prevents the elaboration of a programmed necrotic response (112). Thus, one can consider caspase-dependent apoptosis and programmed necrosis to be cross inhibitory toward one another. DR-induced activation of RIPK1 leads to induction of NF- κ B, and anti-apoptotic signaling via the upregulation of factors such as c-FLIP and anti-apoptotic Bcl-2 family members (122). Concurrent caspase-dependent death (at least that induced through caspase-8) cleaves and inactivates RIPK1 (and likely other proteins), thereby blocking programmed necrosis. When caspase-8 fails to limit RIPK1 activity, excessive autophagy leading to necrotic death ensues. Although the complete basis for and significance of this cross inhibition remains to be determined, it is tempting to speculate that it is preferable to the host that an individual cell dies through apoptosis, but dying by necrosis is better than failing to die at all. Cells that die through caspase-dependent apoptosis promote immunological tolerance through the release of oxidized high-mobility group box 1 (HMGB1) protein (123). While beyond the scope of this treatise, death by necrosis vs. apoptosis leads to very distinct immunological outcomes, with necrosis thought to be antigenic and apoptosis generally tolerogenic (124).

Although originally suggested to be important for DR-induced programmed necrosis (110), Yuan and colleagues have profoundly implicated the kinase activity of RIPK1 in promoting both programmed necrosis and autophagy. In a search to define inhibitors of programmed necrosis, Degterev *et al.* (112) identified a compound called Necrostatin-1 (Nec-1) that blocks this form of death. Further characterization of Nec-1 demonstrated that this chemical compound specifically inhibits the catalytic activity of RIPK1, thereby preventing the elaboration of necroptotic signaling in a variety of contexts (125). In this work, it was also found that Nec-1 blocked the induction of autophagy induced in FADD-deficient Jurkat cells (126) treated with DR ligands. Curiously, while Nec-1 was capable of blocking death in these cells, the Vps34 and autophagy inhibitor 3-MA failed to rescue TNF α -treated FADD-deficient Jurkat cells (112). These findings reveal that necroptosis and autophagy may be linked processes, likely through RIPK1. However, the dominance of one pathway over another and how this controls the overall survival of cells remain to be established.

With significant interest in defining the programmed necrosis pathway downstream of RIPK1 during programmed necrosis, Hitomi *et al.* (127) made use of an siRNA screen to identify genes required for TNF α -induced programmed necrosis. Of a variety of targets

identified, CYLD was found to be vital to TNF-induced programmed necrosis. This is of significant interest, since CYLD is known to be recruited to TNF-R1 and to RIPK1 (128). Hitomi *et al.* (127) also identified glutathione peroxidase (gpx4) and glutathione S-transferases (gsta3 and gsto2), which are involved in the generation of free glutathione, potentially connecting the high ROS observed during programmed necrosis with RIPK1 signaling. Possibly consistent with previous work by Kroemer and coworkers (129), PARP-2 was also found to serve as a core component in the programmed necrosis pathway. Thus, this work links RIPK1 activation to programmed necrosis through ROS generation and ATP depletion, two key autophagy triggers.

RIPK3 is also a core component in TNF α -induced programmed necrosis (130), associating with RIPK1 to develop a complex termed the 'necrosome' that is vital to the induction of programmed necrosis (131). Recent results demonstrate that RIPK3 is recruited to the RIPK1-containing necrosome via its RIPK1-dependent phosphorylation (132-134). Blockade of RIPK3 signaling by RNA interference or ectopic expression of a dominant negative, kinase dead form of RIPK3 blocked TNF α -induced necroptosis and prevented the associated increase in ROS. While RIPK3-deficient mice display no obvious defects in DR-induced apoptosis, NF- κ B activation, or T-cell function (135), RIPK3-deficient mice demonstrated an impaired ability to control vaccinia virus infection, with a reduced level of tissue necrosis and inflammation (134). These findings suggest that programmed necrosis may serve beneficial roles *in vivo* by promoting desirable inflammatory responses during pathogenic challenges.

Han and coworkers (132) also identified an intriguing connection between the necrosome and cellular metabolism that helps explain the connection between RIPK1-dependent necroptosis and autophagy. Seven metabolic enzymes, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), fructose-1,6-bisphosphatase 2 (FBP2), fumarate hydratase (FH), glycosyltransferase 25 domain-containing 1 (GLT25D1), and isocitrate dehydrogenase 1 (IDH1), were identified in association with RIPK3 using a mass spectrometric approach. Three of these (PYGL, GLUL, and GLUD1) were found to directly bind to RIPK3, as assessed through co-immunoprecipitation assays, and RIPK1 activity was found to influence their activities. Thus, RIPK3 activity promotes enhanced oxidative phosphorylation leading to heightened production of ROS that could be involved in the activation of autophagy. However, the means by which the RIPK1/RIPK-containing necrosome influences autophagic flux and why the enhancement of cellular metabolism leads to the induction of programmed necrosis remain fundamental questions that bear further investigation.

Programmed necrosis and autophagy in T cells lacking FADD or caspase-8 activity As described earlier, T cells lacking FADD, caspase-8, or c-FLIP activity enigmatically fail to undergo efficient clonal expansion upon antigenic challenge or mitogen treatment. This has led to speculation that such T cells lack the capacity to respond to TCR proximal signals. For example, it was suggested that FADD^{dd}-expressing T cells display defective calcium mobilization following TCR crosslinking (136), a finding disputed by Strasser and colleagues (137). In human T cells bearing an inactivating mutation of caspase-8, the marked T-cell proliferative defects have been associated with impaired NF- κ B nuclear translocation (106, 138). Curiously, mouse T cells expressing FADD^{dd} or lacking caspase-8 or c-FLIP do not display overt defects in NF- κ B activity (137, 139-141), suggesting that there may be some differences in mouse and human T cells with respect to the involvement of DISC proteins in TCR signaling. Nonetheless, T cells deficient in FADD or caspase-8 had unanticipated proliferative defects.

The potential involvement of DISC proteins in negatively regulating autophagy was first proposed by Lenardo and colleagues (142), who demonstrated that zVAD-FMK treatment or siRNA knockdown of caspase-8 led to enhanced autophagy. This autophagy was non-homeostatic and associated with non-apoptotic cell death, e.g. type II cell death; blockade of autophagic signaling rescued these cells. Subsequent studies revealed a role for FADD in modulating autophagy through its physical association with Atg5 (143). TRAIL-induced apoptosis in normal epithelial cells was shown to involve a FADD-dependent pathway that induces both apoptosis and autophagy, a pathway lost during the immortalization of tumor cells (144). Prompted by earlier work demonstrating a selective defect in S6K signaling (84), we observed enhanced levels of autophagy in FADD^Δ-expressing and caspase-8 deficient T cells, and in FADD-deficient murine embryonic fibroblasts, as assessed by LC3 processing, LC3-GFP punctae, and morphologic analyses using transmission electron microscopy (145). Curiously, the hyper-autophagic phenotypes observed in FADD^Δ-expressing and caspase-8-deficient T cells were only observed following antigenic stimulation; naive T cells lacking DISC function demonstrated normal basal autophagy, demonstrating that this hyperautophagy required T-cell activation.

As described earlier, autophagy is induced during T-cell activation and is required for T-cell proliferation (12). Thus, our results imply that FADD and caspase-8 are required to maintain autophagy within homeostatic limits in T cells, perhaps through inactivation of RIPK1 and RIPK3 activity, both of which serve as proteolytic targets of caspase-8 (121-130). In addition, blockade of autophagy through 3-MA or through retrovirally enforced expression of dominant negative Vps34 or shRNA to Atg7 revealed that autophagy is not only activated in FADD^Δ expressing and caspase-8-deficient T cells during mitogenic activation but also promotes the death of these cells (146). While these results might implicate type II death, we also observed that blockade of RIPK1 with Nec-1 rescued proliferation of FADD^Δ-expressing and caspase-8-deficient T cells. Studies by Ch'en *et al.* with caspase-8 deficient cells demonstrated an essential role of RIPK1 in the premature death of these cells through similar use of Nec-1, and by knocking down RIPK1 with retrovirally-enforced expression of shRNA to RIPK1. Clearly, the death observed in cells lacking FADD or caspase-8 activity occurs through programmed necrosis as a result of excessive autophagy.

We have proposed that autophagy itself may promote the assembly of a DISC-like complex that includes caspase-8, RIPK1, and FADD in association with Atg5, Atg12 and Atg16 recruited to pre-autophagosomal structures (146) (Fig. 2). In this manner, T cells may control the outcome of autophagic signaling. At low levels of autophagy normally encountered during T-cell activation, anti-apoptotic factors such as c-FLIP and Bcl-xL may be sufficient to prevent the full induction of apoptosis in response to the generation of this signaling complex. At high levels of autophagy, sufficient signal will be generated to overcome this inhibition triggering caspase-dependent apoptosis (144) and preventing the induction of an unnecessary inflammatory response. When caspase-8 activity is blocked through mutation or by viral inhibitors of DISC signaling (147), apoptosis does not occur, and unchecked autophagy leads to programmed necrosis. While reminiscent, this is distinct from the case of DR-induced programmed necrosis, where autophagy accompanies, but does not induce necrosis (112). Indeed, it is likely that the issue of DISC assembly itself is crucial. In the case of autophagosome-induced DISC activation, necrotic death and autophagy would be blocked by interference with autophagy inhibitors or with Nec-1, since RIPK1 acts in a feedforward manner to promote autophagy and programmed necrosis. In the case of DR-induced DISC assembly, autophagy is not responsible for DISC assembly, and thus is not required for induction of programmed necrosis. Given this, it is likely that the downstream mediators of programmed necrosis are likely to be shared in these two DISC pathways. Thus, we speculate that blockade of RIPK3 signaling will likely rescue FADD^Δ-

expressing and caspase-8-deficient T cells in a manner similar to what has been observed for RIPK1 inhibition (140–145).

The role of c-FLIP in the control of autophagic signaling remains to be determined in the context of activated T cells. One plausible scenario is that c-FLIP may participate with the aforementioned FADD:Atg5 complexes to modulate caspase-8 activity. In this manner, c-FLIP isoforms may either promote caspase-8 activation (148), or limit its activity to prevent apoptosis (149). Although we failed to detect c-FLIP association with other DISC proteins recruited to Atg5 (unpublished data), definitive proof of this concept requires careful analyses of c-FLIP-deficient T cells. Recent work has revealed an alternative: c-FLIP is recruited to autophagosomes independent of FADD and Atg5. Instead, c-FLIP is recruited by its association with Atg3; ectopic expression of c-FLIP leads to blockade of autophagy through binding to Atg3, thus preventing its association with LC3 (150). Whether this independently regulates autophagy, or instead provides a source of c-FLIP to regulate autophagosome-bound caspase-8, remains to be established.

Conclusion

Although studies regarding apoptotic regulation of immunity have existed for roughly two decades, it is becoming clear that both autophagy and programmed necrosis are also vital to innate and adaptive immunity. Autophagy has shown itself to serve the immune system in many ways, including antigen presentation, antimicrobial activity, lymphocyte development, and most importantly for this compendium of reviews, lymphoid homeostasis. It is likely that future studies will reveal other important functions of this basic cell biological process in immunity. While less well characterized, programmed necrosis is likely to also hold important keys to unlocking the most perplexing issues related to immune function, lymphoid development, and homeostasis.

There are many questions that remain to be addressed in regard to the crosstalk between autophagy and programmed necrosis that takes place within proliferating T cells. Of these, a major unanswered question is why T cells are so sensitive to this crosstalk; other cell types (e.g. fibroblasts, epithelial cells) also possess this crosstalk, but T cells [and perhaps B cells (151–152)] are acutely sensitive to the lack of DISC function. Perhaps this crosstalk may explain the basis for the early embryonic lethality observed in FADD-, caspase-8-, and c-FLIP germline-deficient mice (94). If true, comparison of the affected embryonic cell types with T cells may help to uncover the basis for this sensitivity. Additionally, it remains unclear how T cells may possess significant caspase-8 activity during their proliferative response without succumbing to death during clonal expansion (153–154). Perhaps the answer to this question may hold the key to understanding what controls the sensitivity to DR-induced apoptosis as T cells switch from naive to effector stages. Most importantly, a major unanswered question yet to be resolved is the relevance of DR-induced apoptosis to immune homeostasis and tolerance, an issue we wished to investigate so long ago. Armed with knowledge about the various signaling paradigms in which DISC proteins operate, we believe the answer to this fundamental question will be obtained.

Acknowledgments

The authors acknowledge the following funding agencies for supporting our work: the National Institutes of Health (CMW, AI50506, AI63419), the Arthritis National Research Foundation (CMW), the National Multiple Sclerosis Society (CMW), the Juvenile Diabetes Research Foundation (CMW) and the Gabrielle's Angel Foundation (ALE).

References

1. Dice JF. Chaperone-mediated autophagy. *Autophagy*. 2007; 3:295–299. [PubMed: 17404494]

2. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med*. 2000; 192:1001–1014. [PubMed: 11015441]
3. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet*. 2005; 39:359–407. [PubMed: 16285865]
4. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003; 552:335–344. [PubMed: 14561818]
5. Williams A, et al. Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. *Curr Top Dev Biol*. 2006; 76:89–101. [PubMed: 17118264]
6. Nedjic J, Aichinger M, Emmerich J, Mizushima N, Klein L. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature*. 2008; 455:396–400. [PubMed: 18701890]
7. Klionsky DJ, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy*. 2008; 4:151–175. [PubMed: 18188003]
8. Sandoval H, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature*. 2008; 454:232–235. [PubMed: 18454133]
9. Zhang J, et al. Mitochondrial clearance is regulated by Atg7-dependent and -independent mechanisms during reticulocyte maturation. *Blood*. 2009; 114:157–164. [PubMed: 19417210]
10. Zhang Y, Goldman S, Baerga R, Zhao Y, Komatsu M, Jin S. Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *Proc Natl Acad Sci USA*. 2009; 106:19860–19865. [PubMed: 19910529]
11. Baerga R, Zhang Y, Chen PH, Goldman S, Jin S. Targeted deletion of autophagy-related 5 (atg5) impairs adipogenesis in a cellular model and in mice. *Autophagy*. 2009; 5:1118–1130. [PubMed: 19844159]
12. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med*. 2007; 204:25–31. [PubMed: 17190837]
13. Stephenson LM, et al. Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes. *Autophagy*. 2009; 5:625–635. [PubMed: 19276668]
14. Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. *Cell Host Microbe*. 2009; 5:527–549. [PubMed: 19527881]
15. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008; 451:1069–1075. [PubMed: 18305538]
16. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. *Curr Top Microbiol Immunol*. 2009; 335:1–32. [PubMed: 19802558]
17. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. *Curr Opin Cell Biol*. 2010; 22:132–139. [PubMed: 20056399]
18. Kundu M, et al. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood*. 2008; 112:1493–1502. [PubMed: 18539900]
19. He C, Levine B. The Beclin 1 interactome. *Curr Opin Cell Biol*. 2010; 22:140–149. [PubMed: 20097051]
20. Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA*. 2008; 105:19211–19216. [PubMed: 19050071]
21. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell*. 2008; 19:5360–5372. [PubMed: 18843052]
22. Matsunaga K, et al. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol*. 2009; 11:385–396. [PubMed: 19270696]
23. Zhong Y, et al. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol*. 2009; 11:468–476. [PubMed: 19270693]

24. van der Vaart A, Mari M, Reggiori F. A picky eater: exploring the mechanisms of selective autophagy in human pathologies. *Traffic*. 2008; 9:281–289. [PubMed: 17988219]
25. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. ‘Protein modifications: beyond the usual suspects’ review series. *EMBO Rep*. 2008; 9:859–864. [PubMed: 18704115]
26. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell*. 2008; 19:2092–2100. [PubMed: 18321988]
27. Hanada T, et al. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem*. 2007; 282:37298–37302. [PubMed: 17986448]
28. Kirisako T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol*. 2000; 151:263–276. [PubMed: 11038174]
29. Ichimura Y, et al. A ubiquitin-like system mediates protein lipidation. *Nature*. 2000; 408:488–492. [PubMed: 11100732]
30. Sou YS, et al. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol Biol Cell*. 2008; 19:4762–4775. [PubMed: 18768753]
31. Nakatogawa H, Ichimura Y, Ohsumi Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell*. 2007; 130:165–178. [PubMed: 17632063]
32. Pankiv S, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. 2007; 282:24131–24145. [PubMed: 17580304]
33. Mizushima N. Physiological functions of autophagy. *Curr Top Microbiol Immunol*. 2009; 335:71–84. [PubMed: 19802560]
34. Huang J, Manning BD. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem J*. 2008; 412:179–190. [PubMed: 18466115]
35. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006; 124:471–484. [PubMed: 16469695]
36. Hosokawa N, et al. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell*. 2009; 20:1981–1991. [PubMed: 19211835]
37. Jung CH, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell*. 2009; 20:1992–2003. [PubMed: 19225151]
38. Lee SB, et al. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. *EMBO Rep*. 2007; 8:360–365. [PubMed: 17347671]
39. Scott RC, Juhasz G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr Biol*. 2007; 17:1–11. [PubMed: 17208179]
40. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev*. 2010; 20:51–56. [PubMed: 19942427]
41. Neufeld TP. TOR-dependent control of autophagy: biting the hand that feeds. *Curr Opin Cell Biol*. 2010; 22:157–168. [PubMed: 20006481]
42. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *J Cell Sci*. 2007; 120:4155–4166. [PubMed: 18032788]
43. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ*. 2008; 15:171–182. [PubMed: 17917680]
44. Kim EH, et al. Sodium selenite induces superoxide-mediated mitochondrial damage and subsequent autophagic cell death in malignant glioma cells. *Cancer Res*. 2007; 67:6314–6324. [PubMed: 17616690]
45. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. *Cell Death Differ*. 2009; 16:1040–1052. [PubMed: 19407826]
46. Bensaad K, Cheung EC, Vousden KH. Modulation of intracellular ROS levels by TIGAR controls autophagy. *Embo J*. 2009; 28:3015–3026. [PubMed: 19713938]

47. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J.* 2007; 26:1749–1760. [PubMed: 17347651]
48. Huang Q, Wu YT, Tan HL, Ong CN, Shen HM. A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. *Cell Death Differ.* 2009; 16:264–277. [PubMed: 18974775]
49. Twig G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J.* 2008; 27:433–446. [PubMed: 18200046]
50. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol.* 2008; 183:795–803. [PubMed: 19029340]
51. Vives-Bauza C, et al. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc Natl Acad Sci USA.* 107:378–383.
52. Geisler S, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol.* 2010; 12:119–131. [PubMed: 20098416]
53. Pattingre S, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell.* 2005; 122:927–939. [PubMed: 16179260]
54. Maiuri MC, et al. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J.* 2007; 26:2527–2539. [PubMed: 17446862]
55. Oberstein A, Jeffrey PD, Shi Y. Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J Biol Chem.* 2007; 282:13123–13132. [PubMed: 17337444]
56. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol Cell.* 2008; 30:678–688. [PubMed: 18570871]
57. Zalckvar E, Berissi H, Eisenstein M, Kimchi A. Phosphorylation of Beclin 1 by DAP-kinase promotes autophagy by weakening its interactions with Bcl-2 and Bcl-XL. *Autophagy.* 2009; 5:720–722. [PubMed: 19395874]
58. Criollo A, et al. The IKK complex contributes to the induction of autophagy. *EMBO J.* 29:619–631. [PubMed: 19959994]
59. Bell BD, Walsh CM. Coordinate regulation of autophagy and apoptosis in T cells by death effectors: FADD or foundation. *Autophagy.* 2009; 5:238–240. [PubMed: 19077534]
60. Li C, et al. Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. *J Immunol.* 2006; 177:5163–5168. [PubMed: 17015701]
61. Mizushima N, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol.* 2001; 152:657–668. [PubMed: 11266458]
62. Cahalan MD, Zhang SL, Yeromin AV, Ohlsen K, Roos J, Stauderman KA. Molecular basis of the CRAC channel. *Cell Calcium.* 2007; 42:133–144. [PubMed: 17482674]
63. Krauss S, Brand MD, Buttgerit F. Signaling takes a breath--new quantitative perspectives on bioenergetics and signal transduction. *Immunity.* 2001; 15:497–502. [PubMed: 11672532]
64. Jones RG, Thompson CB. Revving the engine: signal transduction fuels T cell activation. *Immunity.* 2007; 27:173–178. [PubMed: 17723208]
65. Jones RG, et al. The proapoptotic factors Bax and Bak regulate T Cell proliferation through control of endoplasmic reticulum Ca(2+) homeostasis. *Immunity.* 2007; 27:268–280. [PubMed: 17692540]
66. Chaudhri G, Clark IA, Hunt NH, Cowden WB, Ceredig R. Effect of antioxidants on primary alloantigen-induced T cell activation and proliferation. *J Immunol.* 1986; 137:2646–2652. [PubMed: 2944959]
67. Rolli V, et al. Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol Cell.* 2002; 10:1057–1069. [PubMed: 12453414]
68. Reyes BM, Danese S, Sans M, Fiocchi C, Levine AD. Redox equilibrium in mucosal T cells tunes the intestinal TCR signaling threshold. *J Immunol.* 2005; 175:2158–2166. [PubMed: 16081782]
69. Capasso M, et al. HVCN1 modulates BCR signal strength via regulation of BCR-dependent generation of reactive oxygen species. *Nat Immunol.* 2010; 11:265–272. [PubMed: 20139987]

70. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. 2005; 120:649–661. [PubMed: 15766528]
71. Hildeman DA, et al. Reactive oxygen species regulate activation-induced T cell apoptosis. *Immunity*. 1999; 10:735–744. [PubMed: 10403648]
72. Pua HH, Guo J, Komatsu M, He YW. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. *J Immunol*. 2009; 182:4046–4055. [PubMed: 19299702]
73. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell*. 2009; 17:98–109. [PubMed: 19619495]
74. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev Cell*. 2009; 17:87–97. [PubMed: 19619494]
75. Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death Differ*. 2009; 16:939–946. [PubMed: 19229244]
76. Schweers RL, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci USA*. 2007; 104:19500–19505. [PubMed: 18048346]
77. Novak I, et al. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep*. 11:45–51. [PubMed: 20010802]
78. Nishida Y, et al. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature*. 2009; 461:654–658. [PubMed: 19794493]
79. van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol*. 2001; 2:423–429. [PubMed: 11323696]
80. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol*. 2001; 21:5899–5912. [PubMed: 11486029]
81. Frauwirth KA, et al. The CD28 signaling pathway regulates glucose metabolism. *Immunity*. 2002; 16:769–777. [PubMed: 12121659]
82. Jones RG, et al. CD28-dependent activation of protein kinase B/Akt blocks Fas-mediated apoptosis by preventing death-inducing signaling complex assembly. *J Exp Med*. 2002; 196:335–348. [PubMed: 12163562]
83. Braylan R, Diamond L, Powell M, Harty-Golder B. Percentage of cells in the S phase of the cell cycle in human lymphoma determined by flow cytometry. *Cytometry*. 1980; 1:171–174. [PubMed: 7021101]
84. Arechiga AF, et al. A Fas-Associated Death Domain Protein/Caspase-8-Signaling Axis Promotes S-Phase Entry and Maintains S6 Kinase Activity in T Cells Responding to IL-2. *J Immunol*. 2007; 179:5291–5300. [PubMed: 17911615]
85. Delgoffe GM, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*. 2009; 30:832–844. [PubMed: 19538929]
86. Delgoffe GM, Powell JD. mTOR: taking cues from the immune microenvironment. *Immunology*. 2009; 127:459–465. [PubMed: 19604300]
87. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol*. 2000; 150:1507–1513. [PubMed: 10995454]
88. Klionsky DJ, Meijer AJ, Codogno P. Autophagy and p70S6 kinase. *Autophagy*. 2005; 1:59–60. discussion 60–61. [PubMed: 16874035]
89. Siegel RM. Caspases at the crossroads of immune-cell life and death. *Nat Rev Immunol*. 2006; 6:308–317. [PubMed: 16557262]
90. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*. 1995; 81:505–512. [PubMed: 7538907]
91. Boldin M, Varfolomeev E, Pancer Z, Mett I, Camonis J, Wallach D. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J Biol Chem*. 1995; 270:7795–7798. [PubMed: 7536190]

92. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*. 1995; 81:495–504. [PubMed: 7758105]
93. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*. 1995; 81:513–523. [PubMed: 7538908]
94. Walsh CM, Luhrs KA, Arechiga AF. The “fuzzy logic” of the death-inducing signaling complex in lymphocytes. *J Clin Immunol*. 2003; 23:333–353. [PubMed: 14601642]
95. Irmeler M, et al. Inhibition of death receptor signals by cellular FLIP. *Nature*. 1997; 388:190–195. [PubMed: 9217161]
96. Kischkel F, et al. Cytotoxicity-dependent APO-1 Fas/CD95 -associated proteins form a death-inducing signaling complex DISC with the receptor. *EMBO J*. 1995; 14:5579–5588. [PubMed: 8521815]
97. Yeh WC, et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science*. 1998; 279:1954–1958. [PubMed: 9506948]
98. Zhang J, Cado D, Chen A, Kabra N, Winoto A. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature*. 1998; 392:296–300. [PubMed: 9521326]
99. Varfolomeev EE, et al. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity*. 1998; 9:267–276. [PubMed: 9729047]
100. Yeh WC, et al. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity*. 2000; 12:633–642. [PubMed: 10894163]
101. Newton K, Harris A, Bath M, Smith K, Strasser A. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J*. 1998; 17:706–718. [PubMed: 9450996]
102. Walsh C, Wen B, Chinnaiyan A, O'Rourke K, Dixit V, Hedrick S. A role for FADD in T cell activation and development. *Immunity*. 1998; 8:439–449. [PubMed: 9586634]
103. Zornig M, Hueber AO, Evan G. p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. *Curr Biol*. 1998; 8:467–470. [PubMed: 9550704]
104. Salmena L, et al. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev*. 2003; 17:883–895. [PubMed: 12654726]
105. Chau H, et al. Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *J Exp Med*. 2005; 202:405–413. [PubMed: 16043518]
106. Su H, et al. Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science*. 2005; 307:1465–1468. [PubMed: 15746428]
107. Ashkenazi A, Dixit V. Death receptors: signaling and modulation. *Science*. 1998; 281:1305–1308. [PubMed: 9721089]
108. Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, Nagata S. Caspase-independent cell killing by Fas-associated protein with death domain. *J Cell Biol*. 1998; 143:1353–1360. [PubMed: 9832562]
109. Vercammen D, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med*. 1998; 187:1477–1485. [PubMed: 9565639]
110. Holler N, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000; 1:489–495. [PubMed: 11101870]
111. Penninger JM, Kroemer G. Mitochondria, AIF and caspases--rivaling for cell death execution. *Nat Cell Biol*. 2003; 5:97–99. [PubMed: 12563272]
112. Degterev A, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol*. 2005; 1:112–119. [PubMed: 16408008]
113. Lim SY, Davidson SM, Mocanu MM, Yellon DM, Smith CC. The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovasc Drugs Ther*. 2007; 21:467–469. [PubMed: 17965927]

114. Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM. Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc Drugs Ther.* 2007; 21:227–233. [PubMed: 17665295]
115. You Z, et al. Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. *J Cereb Blood Flow Metab.* 2008; 28:1564–1573. [PubMed: 18493258]
116. Lin Y, et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem.* 2004; 279:10822–10828. [PubMed: 14701813]
117. Cande C, Cecconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? *J Cell Sci.* 2002; 115:4727–4734. [PubMed: 12432061]
118. Li P, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 1997; 91:479–489. [PubMed: 9390557]
119. Ting A, Pimentel-Muinos F, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J.* 1996; 15:6189–6196. [PubMed: 8947041]
120. Pimentel-Muinos FX, Seed B. Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity.* 1999; 11:783–793. [PubMed: 10626900]
121. Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* 1999; 13:2514–2526. [PubMed: 10521396]
122. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* 2001; 11:372–377. [PubMed: 11514191]
123. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity.* 2008; 29:21–32. [PubMed: 18631454]
124. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol.* 2009; 9:353–363. [PubMed: 19365408]
125. Degterev A, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol.* 2008; 4:313–321. [PubMed: 18408713]
126. Juo P, et al. FADD is required for multiple signaling events downstream of the receptor Fas. *Cell Growth Differ.* 1999; 10:797–804. [PubMed: 10616904]
127. Hitomi J, et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell.* 2008; 135:1311–1323. [PubMed: 19109899]
128. Wright A, et al. Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. *Dev Cell.* 2007; 13:705–716. [PubMed: 17981138]
129. Cande C, et al. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie.* 2002; 84:215–222. [PubMed: 12022952]
130. Feng S, et al. Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. *Cell Signal.* 2007; 19:2056–2067. [PubMed: 17644308]
131. Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. *Cell.* 2009; 138:229–232. [PubMed: 19632174]
132. Zhang DW, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science.* 2009; 325:332–336. [PubMed: 19498109]
133. He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell.* 2009; 137:1100–1111. [PubMed: 19524512]
134. Cho YS, et al. PPhosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell.* 2009; 137:1112–1123. [PubMed: 19524513]
135. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol.* 2004; 24:1464–1469. [PubMed: 14749364]

136. Hueber AO, Zornig M, Bernard AM, Chautan M, Evan G. A dominant negative Fas-associated death domain protein mutant inhibits proliferation and leads to impaired calcium mobilization in both T-cells and fibroblasts. *J Biol Chem.* 2000; 275:10453–10462. [PubMed: 10744735]
137. Newton K, Kurts C, Harris AW, Strasser A. Effects of a dominant interfering mutant of FADD on signal transduction in activated T cells. *Curr Biol.* 2001; 11:273–276. [PubMed: 11250157]
138. Kataoka T, et al. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr Biol.* 2000; 10:640–648. [PubMed: 10837247]
139. Arechiga AF, et al. Cutting edge: FADD is not required for antigen receptor-mediated NF-kB activation. *J Immunol.* 2005; 175:7800–7804. [PubMed: 16339514]
140. Ch'en IL, et al. Antigen-mediated T cell expansion regulated by parallel pathways of death. *Proc Natl Acad Sci USA.* 2008; 105:17463–17468. [PubMed: 18981423]
141. Zhang N, He YW. An essential role for c-FLIP in the efficient development of mature T lymphocytes. *J Exp Med.* 2005; 202:395–404. [PubMed: 16043517]
142. Yu L, et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by Caspase-8. *Science.* 2004; 304:1500–1502. [PubMed: 15131264]
143. Pyo JO, et al. Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem.* 2005; 280:20722–20729. [PubMed: 15778222]
144. Thorburn J, et al. Selective inactivation of a Fas-associated death domain protein (FADD)-dependent apoptosis and autophagy pathway in immortal epithelial cells. *Mol Biol Cell.* 2005; 16:1189–1199. [PubMed: 15635090]
145. Bell BD, et al. FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proc Natl Acad Sci USA.* 2008; 105:16677–16682. [PubMed: 18946037]
146. Bell BD, Walsh CM. Coordinate regulation of autophagy and apoptosis in T cells by death effectors: FADD or foundation. *Autophagy.* 2009; 5:238–240. [PubMed: 19077534]
147. Wu Z, et al. Viral FLIP impairs survival of activated T cells and generation of CD8+ T cell memory. *J Immunol.* 2004; 172:6313–6323. [PubMed: 15128821]
148. Dohrman A, Russell JQ, Cuenin S, Fortner K, Tschopp J, Budd RC. Cellular FLIP Long Form Augments Caspase Activity and Death of T Cells through Heterodimerization with and Activation of Caspase-8. *J Immunol.* 2005; 175:311–318. [PubMed: 15972663]
149. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem.* 2001; 276:20633–20640. [PubMed: 11279218]
150. Lee JS, et al. FLIP-mediated autophagy regulation in cell death control. *Nat Cell Biol.* 2009; 11:1355–1362. [PubMed: 19838173]
151. Beisner DR, Ch'en IL, Kolla RV, Hoffmann A, Hedrick SM. Cutting edge: innate immunity conferred by B cells is regulated by caspase-8. *J Immunol.* 2005; 175:3469–3473. [PubMed: 16148088]
152. Imtiyaz HZ, et al. The Fas-associated death domain protein is required in apoptosis and TLR-induced proliferative responses in B cells. *J Immunol.* 2006; 176:6852–6861. [PubMed: 16709845]
153. Alam A, Cohen LY, Aouad S, Sekaly RP. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med.* 1999; 190:1879–1890. [PubMed: 10601362]
154. Kennedy NJ, Kataoka T, Tschopp J, Budd RC. Caspase activation is required for T cell proliferation. *J Exp Med.* 1999; 190:1891–1896. [PubMed: 10601363]
155. Liang CC, Wang C, Peng X, Gan B, Guan JL. Neural-specific deletion of FIP200 leads to cerebellar degeneration caused by increased neuronal death and axon degeneration. *J Biol Chem.* 2010; 285:3499–3509. [PubMed: 19940130]
156. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci USA.* 2003; 100:15077–15082. [PubMed: 14657337]
157. Komatsu M, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol.* 2005; 169:425–434. [PubMed: 15866887]

158. Kuma A, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004; 432:1032–1036. [PubMed: 15525940]
159. Cadwell K, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature*. 2008; 456:259–263. [PubMed: 18849966]
160. Cadwell K, Patel KK, Komatsu M, Virgin HWt, Stappenbeck TS. A common role for Atg16L1, Atg5 and Atg7 in small intestinal Paneth cells and Crohn disease. *Autophagy*. 2009; 5:250–252. [PubMed: 19139628]
161. Cann GM, et al. Developmental expression of LC3alpha and beta: absence of fibronectin or autophagy phenotype in LC3beta knockout mice. *Dev Dyn*. 2008; 237:187–195. [PubMed: 18069693]
162. Sou YS, Tanida I, Komatsu M, Ueno T, Kominami E. Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. *J Biol Chem*. 2006; 281:3017–3024. [PubMed: 16303767]
163. Komatsu M, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell*. 2007; 131:1149–1163. [PubMed: 18083104]

Fig. 1. Summary of autophagic degradation

Autophagy is initiated by signal transduction events that lead to the activation to two multiprotein complexes containing either the Ulk1 protein kinase or the lipid kinase Vps34 (1). The activation of these complexes leads to the formation of the crescent-shaped isolation membrane that encompasses cytoplasm, organelles, and protein aggregates in a double membrane-bound autophagosome during the expansion and closure stage (2). Expansion and closure is regulated by two ubiquitin-like conjugation systems, one that forms Atg5-Atg12 and one that forms LC3-PE (known as LC3-II). The isolation membrane is studded with LC3-II, shown in green. p62 recruits ubiquitinated protein aggregates in the cytosol to the growing autophagosome, most likely as a result of its ability to simultaneously bind both ubiquitin and LC3-II. Prior to entry into the degradative pathway, Atg4B cleaves LC3 from PE allowing LC3 to be recycled. LC3-II on the inner membrane of the autophagosome is not accessible to Atg4B and is degraded with the autophagosome. At this point, autophagosomes enter the normal pathway for endocytic degradation (3). When autophagosomes fuse with the multivesicular body (MVB), their outer membrane becomes part of the limiting membrane of the MVB and the inner membrane-bound autophagosome is released into the compartment. Following MVBs fusion with or mature into a lysosome, the contents of the autophagosome are degraded. At least some of degradation products are exported from the lysosome into the cytosol where they are re-used/recycled.

Fig. 2. Model for the regulation of apoptosis and programmed necrosis induced by death receptor ligation or generation of autophagosomes

(A) Induction of caspase-dependent apoptosis by DR ligation or by hyper-induction of autophagy. In upper part of figure, ligation of DRs leads to assembly of the DISC. This results in the induction of apoptosis via caspase-8 dependent cleavage of effector caspases, or via Bid cleavage (not shown). In a similar manner, assembly of a DISC on isolation membranes/PAS (pre-autophagosomal structures) promotes caspase-8 activation, and subsequent caspase-dependent apoptosis in a similar fashion as for the DR/DISC. The threshold for apoptotic cell death is set by anti-apoptotic factors such as c-FLIP and Bcl-2. RIPK1 and RIPK3 activity is limited by caspase-8 dependent cleavage, limiting induction of autophagy and necrosis. (B) Induction of autophagy and necrosis with DISC assembly in the absence of caspase-8 or FADD activity. In this case, failure to activate caspase-8 activity leads to generation of a stable complex between RIPK1 and RIPK3. Instead of inducing caspase-dependent apoptosis, RIPK1 and RIPK3 promote programmed necrosis and autophagy, likely through the generation of ROS. Note that in this scenario, induction of necrosis by the DR/DISC will not be affected by autophagic blockade, whereas necrosis induced by the PAS/DISC is blocked by autophagic inhibition.

Table 1

The molecular machinery of autophagy.

Protein	Stage	Function	Autophagy specific?	Genetic ablation?
Ulk1/2	Induction	Serine threonine kinase, complexes with FIP200 and Atg13	?	(18)
FIP200	Induction	Complexes with Ulk1/2 and Atg13; also known as RB1CC1	No	(155)
Atg13	Induction	Recruits Ulk-FIP200-Atg13 complex to isolation membrane?	Probably	No
Beclin1	Induction	Forms complex with Vps34 and p150	?	(156)
Vps34	Induction	Catalytic subunit of class III PI3-kinase that complexes with Beclin1; PtdIns3P likely recruits proteins to isolation membrane	No	No
p150	Induction	Regulatory subunit of class III PI3-kinase (Vps34) that complexes with Beclin1; orthologous to yeast Vps15	No	No
Atg7	Maturation	E1-like enzyme that promotes the formation of Atg5-Atg12 and LC3-PE	Probably	(13,157)
Atg10	Maturation	E2-like enzyme that works with Atg7 to promote the formation of Atg5-Atg12	Probably	No
Atg5	Maturation	Conjugated to Atg12; Atg5/Atg12 complexed with Atg16L promotes LC3 lipidation and correct localization	Probably	(13,158)
Atg12	Maturation	Conjugated to Atg5; Atg5/Atg12 complexed with Atg16L promotes LC3 lipidation and correct localization	Probably	No
Atg16L	Maturation	Found on isolation membranes; complexes with Atg5/Atg12	Probably	(159,160)
LC3	Maturation	Pro-form is processed by Atg4B to become LC3-I; LC3-I is conjugated to PE by Atg7 working with Atg3 generating LC3-II; LC3-II promotes elongation and closing off of autophagosomes	Probably	(161,162)
Atg3	Maturation	E2-like enzyme that works with Atg7 to promote the formation of LC3-II	Probably	(30)
Atg4B	Maturation and Degradation	Atg4B processes pro-LC3 to LC3-I by cleaving C-terminus; Atg4B also recycles LC3 by cleaving PE from LC3-II	Probably	No
UVRAG	Degradation	Promotes fusion with lysosomes	No	No
p62	Autophagosomal targeting	Binds ubiquitin and LC3 homologs and recruits protein aggregates to autophagosomes, also called SQSTM1	No	(163)