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Intertwined pathways of programmed cell death in immunity

Summary: Programmed cell death (PCD) occurs widely in species from every kingdom of life. It has been shown to be an integral aspect of development in multicellular organisms, and it is an essential component of the immune response to infectious agents. An analysis of the phylogenetic origin of PCD now shows that it evolved independently several times, and it is fundamental to basic cellular physiology. Undoubtedly, PCD pervades all life at every scale of analysis. These considerations provide a backdrop for understanding the complexity of intertwined, but independent, cell death programs that operate within the immune system. In particular, the contributions of apoptosis, autophagy, and necrosis in the resolution of an immune response are considered.

Keywords: T cells, apoptosis, autophagy, cell activation, signaling proteins

Convergent evolution of PCD in prokaryotes and eukaryotes

The selective advantage of programmed cell death (PCD) in multicellular organisms is easy to understand. There are many examples where disposing of cells would be, on balance, beneficial. An example is parasitism by infectious agents where cellular suicide is a ubiquitous form of immunity that denies intracellular parasites the opportunity to replicate (1). But PCD transcends immunity. It is clearly a part of the developmental program of development for all metazoans examined, being particularly prevalent in degenerating tissues (think, the resolution of webbed digits) (2) but present during all phases of generative development as well (3). Less obviously, PCD is highly developed in both single-cell prokaryotes and eukaryotes, illustrating the selective power of altruism at least in clonal organisms. Such selective power has given rise to multiple pathways of PCD in metazoans, and these pathways have been found to be highly intertwined—so much so that there can be difficulty in sorting out the proximal cause of death in certain examples of PCD. To emphasize the universality of PCD and better understand its origins, this review starts with a brief
survey of PCD phylogeny, and ends with a detailed analysis of PCD in T cells.

Prokaryotes

Although the selective advantage for PCD is not obvious for unicellular organisms, a cursory analysis of the current state of the field shows that both prokaryotic (4) and eukaryotic (5) unicellular organisms exhibit robust PCD. Thus far there does not appear to be an example of PCD in Archea, but that may be for lack of discovery. What can we learn from distantly related examples of PCD and how would this affect our understanding of the complexity of PCD in mammals?

In one example, *Bacillus subtilis* uses environmental cues or quorum sensing to act as a multicellular organism, differentiating into distinct subpopulations. Under conditions of nutrient limitation, a subpopulation delays sporulation and the SpoOA operon is activated (6). The SpoOA-active cells produce an adenosine triphosphate (ATP)-binding cassette (ABC) and an ABC transporter that pumps sporulation killing factor (Skf) from the cell. This example of toxin-anti-toxin resistance simultaneously confers resistance to Skf and targets SpoOA-inactive cells for lysis. In addition, SpoOA-active cells produce a signaling peptide (SdpC) that may repress sigma factor in SpoOA-inactive cells, rendering cells more sensitive to the effects of Skf. SpoOA-active cells utilize the released nutrients from dead cells, survive, and delay sporulation. As it is clearly beneficial to delay sporulation until absolutely necessary, this represents a selective advantage for a genetically identical population. The population of bacteria acts as a multicellular organism, digesting parts of itself to the selective advantage of its descendents. Although the mechanics of this system of PCD have no connection to eukaryotic cell death, it illustrates one of the concepts driving the selection of PCD, that is, selective resource utilization under conditions of stress. Clearly, this can convey an important advantage even in the prokaryotic kingdom. As an aside, cheaters, insensitive to the effects of Skf, SpoOA-active cells produce the released nutrients from dead cells, survive, and delay sporulation. As it is clearly beneficial to delay sporulation until absolutely necessary, this represents a selective advantage for a genetically identical population. The population of bacteria acts as a multicellular organism, digesting parts of itself to the selective advantage of its descendents. Although the mechanics of this system of PCD have no connection to eukaryotic cell death, it illustrates one of the concepts driving the selection of PCD, that is, selective resource utilization under conditions of stress. Clearly, this can convey an important advantage even in the prokaryotic kingdom. As an aside, cheaters, insensitive to the effects of Skf, would have an initial advantage as a minor proportion of a wildtype population but a disadvantage as they become too prevalent. We note that this is but one form of cell death in *B. subtilis* (7).

Bacteria have many forms of immunity to phage and plasmids, including one that has been termed ‘adaptive immunity’ (8, 9). Among their arsenal of defense mechanisms, bacteria use PCD as a form of immunity. The mazEF system is another toxin-anti-toxin ‘addiction module’ encoded within the chromosome of *E. coli* and other bacteria. MazF is a stable toxin that cleaves mRNA at a specific site, whereas MazE is a labile anti-

MazF. Thus, prevention of MazF-induced death requires continuous production of MazE. This system was shown to act as an altruistic form of immunity to prevent the spread of bacteriophage P1 (10). Addition of phage P1 to wildtype cultures did not prevent the bacteria from growing to stationary phase, whereas ΔmazEF cultures grew for a period of time but ultimately collapsed due to massive phage lysis. Other examples of PCD as a form of immunity in prokaryotes have been found, and we surmise that they abound in the prokaryotic world (11). Resource limitations and infectious agents are but two selective pressures sufficient to give rise to distinct pathways of PCD in prokayotes.

Unicellular eukaryotes

One of the most phylogenetically distant (from us) eukaryotes is *Dictyostelium discoideum*, a soil ameba that multiplies as a unicellular organism in nutrient abundance. In nutrient-deficient conditions, it undergoes cellular differentiation and morphogenesis to form a multicellular slug-like structure with viable spores and a stalk of vacuolated cells (5). These stalk cells apparently undergo a form of PCD with characteristics of apoptotic and non-apoptotic death (5). A genome analysis has shown that *Dictyostelium* may have two genes that are orthologous to the death pathway genes of multicellular eukaryotes. One encodes a paracaspase that possesses protease activity (12) and orthologous to Malt1 in mammals. Malt1 is involved in the signal cascade leading to nuclear factor κB (NFκB) activation. *Dictyostelium* also has an ortholog of Atg1, and as described for other organisms (13), studies showed that *Dictyostelium* Atg1 is required for macroautophagy (14). Nonetheless, neither Atg1 nor the single paracaspase Malt1 is needed for PCD in the stalk of the starved slug (15, 16).

*Dictyostelium* cells subjected to starvation and differentiation-inducing factor (DIF) undergo mitochondrial uncoupling, and this is sufficient to induce events associated with a cell death described as necrotic: perinuclear clustering of mitochondria, disintegration of the plasma membrane, and lysosomal rupture (17, 18). Unfortunately, no other molecular details have been described, and other than the paracaspase and Atg1 kinase mentioned above, no other death pathway orthologs have thus far been found in the *Dictyostelium* genome.

Viruses able to infect *Dictyostelium* have never been described, and to date, there is no evidence for PCD in ameba responding to the threat of infection by various pathogenic bacteria. Interestingly, there does appear to be an immune response in starved *Dictyostelium* (19). In the migrating slug, a small subset of cells develops into sentinel cells and circulates...
within the slug, engulfing bacteria and sequestering toxins. A TIR-domain containing protein, TirA, was found to be necessary for at least some of these functions. Whether sentinel cells also maintain and utilize PCD as a means of sequestering pathogenic bacteria is unknown. Regardless, this observation suggests that an ancient foraging mechanism gave rise to phagocytosis as a form of immunity. In addition, the very same bacterial virulence factors that inhibit phagocytosis in Dictyostelium inhibit phagocytosis by mouse macrophages (20), which suggests that bacterial phagocytosis by amebae may have provided the selective pressure for bacteria to evolve anti-phagocytic virulence factors. The world of bacterial pathogenesis might have been enabled by the step-wise selection of bacteria evermore resistant to amoebic foraging.

These studies on amebae suggest the possibility that autophagy is a fundamental process of eukaryotes predating metazoan evolution, as all eukaryotes examined to date possess highly conserved means of autophagic reutilization of cytoplasmic constituents (21, 22). However, at least in extant Dictyostelium, it is not the mechanism of PCD. Rather the most distant mechanism of PCD appears related to programmed necrosis with the caveat that until we have the mechanistic components of necrotic cell death in examples of pre-metazoan protists, we do not know that this morphologically defined process is evolutionarily related to programmed necrosis found in mammals (18).

Metazoans

PCD in metazoans is most commonly carried out via apoptosis; yet, we can deduce that apoptotic cell death as it exists today was not present prior to multicellular diversification. At least it has not been maintained in the protists examined thus far. The basic components of apoptosis such as Apaf-1 and Caspase-3-related caspases are not found in ameba, although they are found in Cnidaria (e. g. hydra), one of the most distantly related metazoan phyla (23). There is a strong conservation of Bak and Bax, caspase activation, and DNA fragmentation in sea anemone (phylum, Cnidaria) and sponges (phylum, Porifera), and so it would seem that the apoptotic machinery was present at the root of metazoan diversification. However, as reviewed recently, these orthologs often regulate apoptotic cell death in surprisingly diverse ways, e.g. CED-9 serves a different mechanistic role in C. elegans compared with Bcl-2 in mammals (24), albeit both are pro-survival. In addition to apoptosis, PCD may take the form of autophagy or necrosis. An analysis of these forms of death reveals that they are highly interrelated.

Diverse mechanisms of cell death in the immune system

In present day mammals, there are thought to be three types of PCD: Type I apoptosis, Type II autophagy, and Type III necrosis. The history and basis for these classifications have been comprehensively reviewed (22, 25–27). Although PCD has been extensively recorded and studied since the dawn of cell theory in the 19th century (28), we still do not have a clear understanding of the way in which each of these three forms of death contribute to many physiological processes. The next three sections constitute an attempt to understand how these types of cell death are intertwined and whether they are truly distinct.

Type I-apoptosis

The phenomenon of ‘chromatolysis’ was described in many tissues, and it included characteristics typical of Type I apoptosis, a relatively recent term applied to describe a particular morphological description of death (29, 30). It appears to be a dominant pathway of cell death, found naturally in virtually all pre- and post-developmental tissues and constitutes a default state that must be actively inhibited in most thriving cell types. Simple removal of cells from their cognate cellular interactions is sufficient to induce an apoptotic cascade leading to cell death (31–33). Apoptosis has now been clearly and extensively characterized in terms of the molecular events that occur and the way in which these molecular events result in cell death (26, 34–37). Most relevant here, apoptotic PCD constitutes an important aspect of host immunity, and this is illustrated by the many viruses and bacteria that have evolved apoptotic regulatory factors (38–44). In fact, pathogenic virulence factors provide a high-resolution reflection of pro- and anti-apoptotic pathways.

Although described in detail and reviewed many times by those with primacy in the field, the simple outline of intrinsic apoptosis involves mitochondrial outer membrane permeabilization (MOMP) by Bax and Bak (45, 46). Prosurvival effectors such as Bcl-2 or Bcl-XL serve to inhibit Bax and Bak, whereas proapoptotic factors, such as Bim and Puma, inhibit Bcl-2 and Bcl-XL. The exact details of these signaling events are still under active study, with experiments revealing a survival activity for Bcl-XL and Bcl-2 independent of their binding to Bax (47–49). The formation of MOMP allows the irreversible release of death effectors such as cytochrome c, apoptosis-inducing factor (AIF), Smac (Diablo), Omi HtrA2, and endonuclease G (50). Each has a different function, but cytochrome c causes the oligomerization of Apaf-1 and the activation of Caspase-9. From there, a proteolytic cascade of
effector caspases (Caspases-3, -6, -7) ensues, resulting in the orchestrated breakdown of the cell by way of DNA fragmentation and the membrane expression of signals for engulfment (51). A hallmark of apoptotic death is that it usually occurs without exciting an inflammatory response (52).

Alternatively, there exists an extrinsic pathway of apoptosis that involves death receptors (DRs), which in human beings include Fas [tumor necrosis factor receptor superfamily member 6 (TNFRSF6)], TNFRI (TNFRSF1A), DR3 (TNFRSF25), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1) or DR4 (TNFRSF10A), TRAILR2 or DR5 (TNFRSF10B), and DR6 (TNFRSF21) (53, 54). In addition, there are three decoy TRAIL-binding receptors: TRAILR3 or DcR1 (TNFRSF10C), DR6 (TNFRSF21), and osteoprotegerin (55). Ligation of Fas, DR4, or DR5 results in the direct binding of Fas-associated death domain protein (FADD) and the formation of the death-inducing signaling complex (DISC) that includes the initiator caspase, Caspase-8, and Caspase-10 (Caspase-10 is not found in mice) (56). Alternatively, the ligation of TNFR1 and DR3 bind TRADD and RIPK1 and activate NfκB and mitogen-associated protein kinase (MAPK) pathways, although depending on context, TRADD and RIPK1 can also bind FADD and form a DISC (53). Caspase-8 activation, in turn, activates effector Caspases-3, -6, and -7, thus achieving a similar end result as that of the intrinsic pathway. A central enigma in understanding the extrinsic pathway is that, in activated T and B cells, it appears to also behave as a survival mechanism, and this is the focus of later sections.

Type 2-autophagic degeneration

The second morphological category of cell death has been described as type 2 macroautophagy or simply autophagy, and in fact if this is a death pathway at all, its regulation is seemingly convoluted. It is characterized by the presence of abundant double-membraned vacuoles. Starvation, the loss of growth factors, protein aggregation, and other cellular stresses increase the normal basal level of autophagy. Autophagy in mammalian cells is further characterized by a punctate vesicular distribution of an ortholog of yeast autophagy protein, Atg8 (microtubule-associated protein-1 light chain-3, LC3) (57). Mechanistically, double membrane-bound autophagosomes deliver cytoplasmic constituents destined for degradation to the late endosomes and lysosomes (58). The process is directly related to autophagy in yeast, where cellular constituents are broken down and reutilized under conditions of resource deprivation. Its function is to allow starved cells to survive until resources are again plentiful (21). A similar pathway was shown to be operative in mammalian cells by examining the withdrawal of IL-3 from IL-3-addicted immortalized cells. In Bax−/−Bak−/− cells, removal of IL-3 resulted in an extended autophagic process ending with the expiration of size-diminished cells containing little cytoplasm. By contrast, without Atg5 or Atg7 in such cells, removal of IL-3 resulted in rapid, presumably non-apoptotic cell death (57). Clearly, in the absence of apoptosis, autophagy was able to rescue or at least delay cell death, whereas in the absence of both pathways, yet another mechanism of death was engaged.

The components of autophagy have been identified by the selection for at least 30 autophagy-related (Atg) mutants in Saccharomyces cerevisiae (59, 60). Many of these identified mutant genes have orthologs in eukaryotic species (58, 61). Autophagy can be initiated by two ubiquitin-conjugating systems based on the conjugation of the ubiquitin-like products Atg8 and Atg12 (62–64). Atg12 is activated by an E1-like enzyme, Atg7, transferred to an E2-like enzyme, Atg10, and coupled to Atg5 (65, 66). Atg8 (mammalian ortholog is LC3) is also activated by Atg7; it is transferred to the E2-like enzyme, Atg3, and conjugated to phosphatidylinositol-3-phosphate (becoming LC3II in mammalian cells) (63). Thus, Atg7 is essential for both ubiquitin-like conjugation systems. Its function was deciphered from experiments on Atg7-deficient mice, where no autophagy was reported, and mice died 1 day postpartum (67). Among the many phenotypes exhibited by these mice, adults with a liver-specific Atg7 deletion revealed a profound deficiency in the fasting-induced degradation of cytosolic proteins and organelles (67). Atg1 and Atg5 are also thought to be required for both autophagic pathways (68).

More recently, an alternative Atg5/Atg7-independent form of macroautophagy has been discovered in mouse embryonic fibroblasts subjected to the stress of etoposide. Similar to the results described above, autophagic vacuoles were detected in the brains, livers, and hearts of fetal Atg5−/− mice. It also operates in the autophagic elimination of organelles in developing erythrocytes, indicating that this alternative form of autophagy has a physiological role (69). As a targeted deletion of Atg7 is embryonic lethal, the alternative form of autophagy is not redundant, but one idea is that it is key to the type 2 autophagic death pathway.

Autophagy defined by morphology can be found in association with many examples of naturally occurring cell death throughout the eukaryotic kingdom (70, 71). As described above, it is also found in cells that have lost the ability to undergo apoptosis because of a genetic deficiency in Bax and Bak (57, 72). All such cells ultimately die without detectable DNA fragmentation, perhaps as a consequence of collapsed...
cell energetics. However, this is often only a correlation of morphologic appearance and progression to death, as the relationship between autophagy and cell death is still not well understood. Although there are examples in which autophagy appears to be directly causative of cell death, these examples are still rare (22, 73–77). In many instances in which autophagy is associated with cell death, viability is not rescued by prevention of autophagic pathways (78, 79). In other investigations, evidence was largely gathered by the use of an inhibitor of macroautophagy, 3-MA, and yet this drug has many substrates (80). Even knockdown of Beclin-1, a necessary component of autophagy, can have other effects, most importantly on apoptosis (81).

Several molecular connections between autophagy and apoptosis have been described, possibly indicating a contingency of one to the other. In one study, Atg5 was shown to interact with FADD (82), and the investigators proposed that Atg5 mediates both vacuole formation and death, with the death being dependent upon FADD-induced caspase activation (75). No connection was made to the downstream effector of FADD, Caspase-8, and the implication was that this constituted evidence of a novel FADD-dependent pathway to apoptosis and autophagy (83).

Bcl-2, known to be a potent inhibitor of Bak and Bax, also functions to regulate autophagy through a direct interaction with Beclin-1 (an Atg6 mammalian ortholog that associates with phosphoinositide 3-kinase and required for autophagosome formation) (81, 84, 85). The direct interaction was demonstrated in both yeast and mammalian cells. An analogous form of regulation was also found in the plant hypersensitive response (HR), a form of PCD induced in response to virus infection. In BECLIN-1-deficient Arabidopsis, HR spread to uninfected cells, whereas in wildtype plants, HR was limited to those cells infected by tobacco mosaic virus (86).

A third interaction between the apoptosis pathway and autophagy was shown to occur between Flice-like inhibitor protein (Flip) and Atg3. Cellular (or viral) Flips were shown to suppress autophagy by interfering with the reaction of Atg3 and LC3. Thus, Flip inhibits Caspase-8, preventing the extrinsic form of apoptosis, and also inhibits autophagy. These connections between apoptosis and autophagy make an analysis of the proximal cause of cell death difficult (22, 74, 75).

In T cells, the lack of autophagy results in increased cell death, especially under conditions of activation. As thymocytes mature and migrate to the secondary lymphoid organs, there normally occurs a loss of mitochondria. In Atg7-deficient T cells, this loss of mitochondria does not take place, and the autophagy-deficient T cells have increased reactive oxygen species as well as an imbalance in pro- and anti-apoptotic protein expression (87). The result is a decrease in T-cell survival. Activation of Atg7-deficient T cells results in substantially reduced survival compared with wildtype cells without a marked alteration in either the rate of cell cycle progression or the infection-induced kinetics of T-cell expansion and contraction (I. L. Ch’en and S. M. Hedrick, unpublished data). In addition, autophagy constitutes an important facet of immunity against bacteria and viruses (88, 89). It is vital to the elimination of group A Streptococcus, Mycobacterium tuberculosis, and Shigella flexneri (90–92). It is also activated and possibly protective in response to Sindbis virus (84) and Herpes viruses (93), and it is an essential part of antigen presentation in dendritic cells (94, 95).

Type 3 non-lysosomal cytoplasmic degradation, necrosis

In a highly cited review from 1990 (25), Clarke redefined Type 1 apoptosis and Type 2 autophagic degeneration, but he found no literature describing cell death precisely equivalent to the type 3 death originally described by Schweichel and Merker (96). Rather, he listed a Type 3b ‘cytoplasmic’ cell death that still differed from Types 1 and 2. In this form of cell death, mainly characterized in dying neurons, organelles such as the rough endoplasmic reticulum, the nuclear envelope, and the Golgi become dilated, and the cytoplasm becomes vacuolated (97–99). Defining characteristics of Type 3b cytoplasmic degeneration included a lack of autophagic vacuoles and nuclear condensation, the presence of dilated cytoplasmic organelles, and a loss of plasma membrane integrity (25, 100). None of these characteristics provides a definitive biochemical trait for necrosis, such as the activation of caspases or DNA fragmentation provides for apoptosis. Loss of membrane integrity is characteristic of all dying cells, with kinetics being the only feature distinguishing apoptotic from necrotic cells.

This form of cell death has since been explicitly reported in many examples of developmental PCD (101–106), and perhaps the most medically relevant example arises from ischemia-reperfusion events that result in the opening of the mitochondrial permeability transition pore (mPTP) (107). Components of the mPTP were thought to include the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), the peptidyl-prolyl cis-trans isomerase, cyclophilin D, and other molecules (108); however, only cyclophilin D has been verified by genetic ablation experiments (109). Mice deficient in the gene encoding cyclophilin D (Ppid) do not exhibit necrosis induced by ischemia-reperfusion.
injury (110–112). At the same time, cells from these mice remained sensitive to various mediators of apoptosis. One might be tempted to conclude that the requirement for cyclophilin D provides a unique characteristic of programmed necrotic cell death.

In addition to the loss of plasma membrane integrity and the formation of the mPTP, necrotic cell death has been characterized by the production of reactive oxygen species (ROS), ATP depletion, dysregulation of Ca2+ homeostasis, activation of proteases such as calpains and cathepsins, and lysosomal rupture (100). With all this information, what are the constituent components of necrotic cell death?

One of the most tractable examples of necrotic cell death comes from studies on cell lines that lack components of the DISC. L-929, Jurkat T lymphoma, and mouse embryonic fibroblasts undergo apoptosis when treated with ligands of the death receptors: Fas, TNFRI, or Trail-R. However, in the presence of the small molecule pan-caspase inhibitor zVAD-fmk or poxvirus-derived caspase inhibitor CrmA, Fas-ligand, TNF, or Trail-treated cells undergo a delayed form of death that morphologically resembles necrosis (113–116). Further work showed that a loss of FADD or Caspase-8 sensitized cells to necrotic death, and a necessary downstream effector of this death was Ripk1 (previously known as RIP1) (116). In a study to find small molecule inhibitors of necrotic death in the absence of apoptosis, one molecule designated Necrostatin-1 was found to inhibit TNF-mediated necrosis in the presence of zVAD-fmk. The investigators termed this form of programmed necrotic death necroptosis (78) and found the target of Necrostatin to be the kinase activity of Ripk1 (117). Indeed, Necrostatin was also found to inhibit the death associated with ischemia reperfusion, and so there would appear to be functional connection between Cyclophilin D-dependent necrosis and Necrostatin-sensitive necroptosis. Although autophagy was evident in cells undergoing TNF-mediated necroptosis, it could not be inhibited by knockdown inhibition of any of several autophagic components (78). A conclusion was that inhibition of the DISC under conditions of a death signal provided by TNF led to Ripk1-dependent necroptosis.

Apoptosis, autophagy, and necroptosis in the regulation of T-cell homeostasis

Natural T-cell expansion and contraction

Although PCD appears to be important in every organism and in most, if not all, physiological organ systems, it is most evident within the immune system. T cells exhibit dramatic expansion and contraction in response to many infectious agents, and in fact, T cells are almost certainly the most dynamic cell population in metazoans. By some estimates, the 50–200 precursor T cells specific for a particular viral epitope may expand 10 000–50 000-fold over the course of 7–8 days. In studies with lymphocytic choriomeningitis virus (LCMV), up to 90% of the total T-cell population present at the peak of an immune response are virus-specific (118, 119). Equally dramatically, after the peak of expansion, the population rapidly dies off with similar kinetics, leaving only a rear-guard of memory cells. Control of this dynamic contraction constitutes the major topic scrutinized in this issue of Immunological Reviews. A note of caution here, this rapid die-off and resolution is true for T-cell responses to viruses and bacteria that are neatly cleared by the immune system. Those viruses that utilize persistence as a mechanism of transmission, e.g., those that rely on venereal transmission, do not elicit the same type of tidy expansion and contraction of T cells. Rather, persistent viruses induce a T-cell response that is poorly resolved, resulting in exhausted and anergic effector T cells (120, 121).

With so much cell death occurring during the resolution of an immune response, what signals induce cell death, and which mechanisms of death are in evidence? During the contraction phase of the response, dying cells show hallmarks of apoptosis (122, 123). Most prominently, the die-off was diminished in the absence of the pro-apoptotic BH3-only Bcl-2 family members, Bim (124–126) or Puma (127, 128), or the ultimate MOMP mediators, Bax and Bak (129). However, the overexpression of Bcl-2 or Bcl-XL did not appear to affect the contraction of virus-specific CD8+ T cells (130). Moreover, the contraction phase of a virus-specific immune response was shown to be insensitive to caspase inhibitors (131). One hypothesis is that T cells in the contracting population lack sufficient growth factors and undergo apoptosis by default (132, 133), and yet in competition experiments, this does not appear to be the case (134). The mechanism and regulation underlying the contraction of CD8+ T cells is yet unresolved.

The participation of the extrinsic apoptosis pathway is also still being debated. The original contention was that lymphocyte contraction was Fas-dependent based upon the discovery of activation-induced cell death in T cells (135–138). However, Fas or TNFRI-deficient CD8+ T cells or even double-deficient T cells exhibited normal contraction following expansion induced by an acute virus infection (130, 139, 140). The deletion of both TNFR1 (death domain-containing) and TNFRII (non-death domain-containing) appeared to diminish or delay the contraction of LCMV-specific T cells,
although the double mutant did not maintain a high number of effector T cells (141). This observation indicates that TNF does play a role in the contraction phase of a T-cell response, but it is not the only mechanism and it may not be the primary mechanism of cell death.

Another facet of the extrinsic death pathway is that Fas- or FasL-deficient mice harbor an excess of T cells with the unusual phenotype of B220^+CD4^-CD8^+, and yet, a T-cell-specific deletion of Fas shows no such phenotype. The lymphoproliferative disease of Fas-mutant mice required Fas deletion in both lymphoid and non-lymphoid tissues, and the origin of this is still not entirely understood (142). Mice with a T-cell-specific Fas deletion exhibited severe lymphopenia and the accumulation of FasL on activated T cells.

Further investigation of Fas revealed that it is important for the elimination of antigen-presenting cells and chronically stimulated T cells, such as those activated by persistent viruses or autoimmune disease (143–145). These investigations were extended in experiments examining the dynamics of an immune response in Fas-deficient (lpr) mice with the additional deletion of Bim. In one analysis, the findings were consistent with those cited above: Bim, but not Fas, was found to be important in the clearance of T cells following an acute Herpes simplex virus-1 (HSV-1) infection, whereas the combination of a Fas mutation and Bim deletion was needed to see an abnormal increase in the number of antigen-specific cells in the spleen following a chronic murine γ-herpesvirus (MHV) infection (146). A second study on Fas- or Bim-deficient mice examined the much-studied acute virus response to LCMV-Armstrong and showed little change in the contraction of T cells in the spleen, but in lpr, Bim double deficient mice, T cells exhibited no contraction in the lymph nodes (147). One problem with all these studies is that they do not show whether the requirement for Fas and Bim is T-cell intrinsic or related to the other complicated aspects of immune regulation involving non-lymphoid or non-hematopoietic cells. Clarification might come from either adoptive transfer experiments or T-cell-specific deletions of Fas and Bim.

The extrinsic death pathway regulates survival: a wealth of experiments, a paucity of understanding

A difficulty in studying the role of the extrinsic death pathway in the immune system is that there are several DRs that can coordinate overlapping pathways, ultimately initiating an apoptotic cascade through the activation of Caspase-8. If there exists DR redundancy, perhaps mutants in common downstream effectors would exhibit a stronger phenotype. As such, several groups produced mice with a defect in FADD or Caspase-8 to study the dynamics of the ensuing immune response. Enigmatically, the observed phenotype was opposite to that expected. In mice with a defect in either FADD or Caspase-8, the expansion of T cells in vitro or in vivo was substantially diminished (148–156). Although early reports purported to provide evidence for a defect in cell cycle progression (157–159), our experiments showed that this was not the case. FADD- or Caspase-8 deficient T cells, optimally stimulated, progressed through exactly the same number of divisions as wildtype T cells. In addition, Caspase-8 deficient T cells took up BrdU both in vitro and in vivo at exactly the same rate as wild-type cells (154, 160). Rather, the reduced expansion of T cells was entirely due to an increased rate of cell death as measured by uptake of 7AAD or staining by Annexin V. In activated T cells, the DISC thus acts to promote cell survival!

The origin of this death sensitivity has also been controversial. One prominent study on both human and mouse T cells deficient for Caspase-8 purported to show that there was a deficiency in NFκB activation (161). In addition, deletion of Caspase-8 in B cells was likewise reported to result in an NFκB activation defect (155). This result could explain an increased rate of death, as NFκB regulates a number of genes important for cell survival. However, other comprehensive studies on T and B cells harboring a FADD or Caspase-8 deficiency clearly showed that no defect exists in the antigen or mitogen stimulation of NFκB activation, measuring five different parameters of NFκB activation including a gene array analysis of 107 known NFκB target genes (153, 156, 160, 162). In fact, the cause of cell death was found to be uncharacteristic of apoptosis and unrelated to the death mediated by genetic knockout of NFκB components. Caspase-8 or FADD-deficient T cells optimally stimulated with anti-CD3 and anti-CD28 showed little DNA fragmentation or caspase activation. By contrast, proliferating NFκB-defective T cells divided at a reduced rate with dying cells found actively fragmenting their DNA (160).

If not apoptosis, then what was the cause of cell death in Caspase-8 deficient stimulated T cells? One group proposed that the T cells lacking Caspase-8 die by autophagy (159), and this conclusion was based on the formation of autophagic vesicles and the presence of LC3-II in TCR-stimulated T cells with a FADD-dominant interfering transgene. In addition, these investigators found evidence for a complex consisting of Caspase-8, FADD, Atg5, 12, 16, and Ripk1. This is in line with a report on the nature of death found in cell lines treated with inhibitors of Caspase-8. Here, Caspase-8 inhibition was sufficient to cause autophagy that appeared to depend on Beclin-1, Atg7, and Ripk1 (163).
An alternative is that Caspase-8-deficient T cells die via necroptosis. Indeed, Caspase-8-deficient T cells stimulated to divide in culture were completely rescued by the addition of Necrostatin-1 or its active derivatives (159, 160). As the target of Necrostatin was found to be the kinase activity of Ripk1 (117), further work showed that a knockdown of Ripk1 also rescued Caspase-8-deficient T-cell expansion in response to antigen stimulation in vivo (160). Thus, in a manner not yet understood, FADD and Caspase-8, presumably in a complex, maintain Ripk1 in an inactive state. Although work has shown Caspase-8 able to cleave Ripk1 directly (164), T cells lacking Caspase-8 did not accumulate increased amounts of Ripk1. In fact, the amount of Ripk1 in Caspase-8-deficient T cells was reduced, although the specific activity was markedly increased (160). As Ripk1 may be also involved in the regulation of autophagy, the possibility exists that Caspase-8 deficient T cells increase autophagic activity and die as a result of autophagic or necroptotic mechanisms. The proximal cause of death has not been determined.

Apoptosis or necrosis: inhibition of Caspase-8 as a PAMP

These results are consistent with the notion that death by apoptosis, autophagy, and necrosis are highly interwoven, and perhaps the circuitry acts as a means of detecting viral infection. In an instance of infection, T cells would die rather than proliferate and potentially propagate the infection. The only time T cells would naturally lack Caspase-8 activity is if they were virally infected, as many viruses harbor anti-Caspase-8 virulence factors (44). In this sense, Caspase-8 inhibition is a pathogen-associated molecular pattern (PAMP). For example, cytomegalovirus (CMV) encodes a specific inhibitor of Caspase-8 known as vICA (165). This inhibition is key to virus replication, and yet if there is an alternate pathway, how does the virus prevent premature death? The answer may be that CMV also encodes the M45 product that suppresses Ripk1. Thus, as would be expected for Red Queen co-evolution (166), for each futile attempt by the host to counteract viral virulence factors, there is a selection-driven evolutionary response by the virus. Although we do not know the order in which vICA and M45 were evolved, a reasonable guess is that it was viral-host co-evolution that drove the link between the inhibition of Caspase-8 and Ripk1.

The role of Ripk1 in survival, apoptosis, or necroptosis

Ripk1 appears to hold a strong nodal position in the signaling network governing the fate of many cells including T and B lymphocytes. It can coordinate apoptosis through the assembly of a DISC, it can activate NFkB through the recruitment of a signaling complex that induces IKK, it forms a complex with components of the autophagy process, and it is required for necroptosis. The specifics of the latter signaling pathway are less well established. We know there are three Ripk-related molecules encoded separately, Ripk1, Ripk2, and Ripk3, and they are similar only within the amino-terminal kinase domain (167). Ripk2 is involved in Nod2-mediated NFkB regulation, both positive and negative (168, 169), but it does not appear to participate in the DISC pathway or the pathway regulating necroptosis. Ripk1 can enucleate two or more signaling complexes (170), at least one of which includes Ripk3 (171–173). These complexes propagate distinct signals and understanding these pathways is the key to understand the circuitry of PCD in many cells, including T cells.

DRSs and, most particularly, TNFRI can form two different signaling complexes. In response to TNF ligation, TNFRI can recruit TRADD, Traf2, and Ripk1 along with cIAP1 and cIAP2 to form what is referred to as Complex I (170, 174). In a Traf2 and cIAP1,2-dependent manner, Ripk1 is ubiquitinated, resulting in the recruitment of a complex consisting of Tak1 and Tak1-binding proteins as well as the IKKα/β/γ heteromeric combination. The result is phosphorylation of IkB followed by the activation of NFkB dimers (175, 176). Although not as well understood, there can also occur the activation of Jnk and p38 (177–179). This is opposed by the deubiquitinating enzyme, cylindromatosis (Cyld) or A20 (180–182). A20 can remove K63 ubiquitin from Ripk1 and replace it with K48 ubiquitin. With the reduction of K63 ubiquitination, Ripk1 may instead promote cell death (183, 184). Either TRADD or Ripk1 can recruit FADD and Caspase-8 to form the DISC-like complex II (174). This DISC may be held in check by cFlip but can be disrupted by K48 cFlip ubiquitination and degradation initiated by the E3 ligase, Itch (185). Above is but a brief summary of these interactions, and the reader may refer to more comprehensive reviews (167, 174, 184).

Most recently, three studies (171–173) further characterized necrotic cell death downstream of TNFRI signaling, showing that it requires the interaction of Ripk1 and Ripk3. Using the same type of model system in which TNF-stimulated cells were blocked for caspase activation, an siRNA knockdown screen found an essential role for Ripk3. The interaction of Ripk1 and Ripk3 was found to occur via a Rip homotypic interaction motif (RHIM), and this was required to mediate necroptosis. In one of the studies, this protein complex was found to interact with glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate...
dehydrogenase 1 (GLUD1) (171). The mechanism of death was proposed to be the activation of metabolic pathways leading to the overproduction of ROS (171). Ripk3-deficient mice were also found to be resistant to a model of pancreatitis disease progression. Strikingly, VSV infection revealed an important role for apoptosis and necrosis in viral immunity. VSV encodes the viral caspase inhibitor B13R, and VSV-infected cells were indeed resistant to DR-induced apoptosis. However, they become sensitized to TNF-induced necrosis (186). Wildtype T cells were found to exhibit activation-induced cell death induced by anti-CD3 antibody, whereas VSV-infected Ripk3−/− cells were significantly more resistant (173). In wildtype mice, VSV caused myeloid infiltration in visceral fat pads, and this was found to be absent in Ripk3−/− mice. The suggestion was that necrosis is an important aspect of virus-induced inflammation. There was detected a Ripk1-Ripk3 complex in the liver of wildtype VSV-infected mice, whereas the hepatic inflammation normally associated with VSV infection was not found in Ripk3−/− mice. This was associated with a dramatic increase in viral titers in Ripk3−/− mice. The conclusion is that necrosis and perhaps the inflammation associated with necrosis is an important part of innate viral immunity. Whether necrosis is also important for the resolution of an adaptive immune response awaits further experimentation.

The mechanisms of death operative in natural immune responses appear to be diverse. Apoptosis, autophagy, and necrosis may each play a role, depending on the strategies of the infectious agent. In addition, they are not mutually exclusive or even independent pathways. Some of the pivotal regulatory factors characterized for apoptosis or autophagy also appear to guide the induction of necrotic mechanisms of death. Despite at least a decade of elegant work probing the nature of T-cell dynamics, we believe that there is still much to be discovered.

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