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Death-Defining Immune Responses After Apoptosis

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Abstract

Apoptosis is a programmed form of cell death whereby characteristic internal cellular dismantling is accompanied by the preservation of plasma membrane integrity. Maintaining this order during apoptosis prevents the release of cellular contents and ensures a noninflammatory death. Here, we consider examples of apoptosis in different contexts and discuss how the same form of cell death could have different immunological consequences. Multiple parameters such as cell death as a result of microbial infection, the nature of the inflammatory microenvironment, the type of responding phagocytic cells and the genetic background of the host organism all differentially influence the immunological consequences of apoptosis.

Keywords

Apoptosis; immunology; infection; innate and adaptive immunity; phagocytosis; tolerance

Introduction

It is estimated that over one million cells die every second throughout the human body (1). Aside from natural cell turnover, cell death can also occur as a result of infection with viruses and bacteria, presumably as a primitive method of limiting the replication and systemic spread of infectious pathogens (2).

Dead cells are cleared by the phagocytic sentinels of the innate immune system including macrophages (MΦ), monocytes (Mo) and dendritic cells (DC), which are also responsible for determining whether the internalized cargo poses any threat to the host. Indeed, these phagocytic cells are capable of discriminating between self and a variety of nonself-components *via* several germline-encoded pattern-recognition receptors (PRRs). These receptors are capable of signaling activation of the innate immune system by detecting both conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) as well as products generated as a result of cell death, known as damage-associated molecular patterns (DAMPs). Depending on the types of PRRs engaged, MΦ, Mo and DC

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undergo distinct activation and differentiation profiles that effectively orchestrate the appropriate innate and adaptive immune response. PRR engagement in response to cell death could lead to either suppressive or protective responses depending on the type and context of cell death encountered.

Recognition of microbial components such as lipopolysaccharides (LPS), peptidoglycans and flagellin are invariably associated with the transcriptional initiation of various immune response genes (3). With respect to the recognition of dying cells or components thereof, the classic dogma is that innate recognition of apoptotic cells results in the generation of a tolerogenic milieu, whereas DAMPs released during necrotic cell death initiate an inflammatory immune response. However, recent findings unraveling the mechanisms of apoptosis necessitate a revision of the manner in which cell death pathways are linked to tolerance and immunity (4). While the type of cell death plays a critical role in dictating the nature of the ensuing immune response, the context within which cells die is also important for proper conditioning of the immune response (2,5–7). Here we describe the intracellular mechanisms that lead to apoptosis including the extrinsic and intrinsic pathways. We delineate how apoptosis during infection can shape a suppressive, autoreactive or protective immune response.

Defining Cell Death

The first classification of mammalian cell death was formulated in 1972 by Kerr et al who used the term “apoptosis” to describe a mechanism of controlled cell deletion (8). These observations then led Schweichel and Merker to characterize three forms of cell death based on unique morphological changes to the cell (9), which are now referred to as apoptosis, autophagic cell death and necrosis (4). Today, rather than characterize cell death via morphological assessment that could lead to misinterpretations among investigators, the Nomenclature Committee on Cell Death urges researchers to follow a series of guidelines based on molecular signaling pathways involved during each death process, as well as a set of measurable biochemical features to correctly identify the type of cell death (4). In this review, we shall focus on apoptosis.

Apoptosis

The primary purpose for apoptosis is to dispose of unwanted cells in a controlled manner (8). In doing so, dying cells undergo a well-organized and coordinated internal dismantling in an effort to minimize damage to neighboring cells and prevent tissue stress (2). One way this can be achieved is through the release of immunosuppressive cytokines including IL-10 and TGF- β from both apoptotic cells and phagocytic cells responding to apoptosis (2). Phagocytic cells sense and clear apoptotic cell corpses via a sequence of “find me” and “eat me” signals expressed by dying cells (10). Examples of “find me” signals and the corresponding receptors on phagocytic cells directing chemotaxis include the G protein coupled receptor G2A, as well as sphingosine-1-phosphate (S1P) and the S1P-receptor 1 (10). “Eat me” signals on the apoptotic cell surface, such as phosphatidylserine (PtdSer), can then “directly” or “indirectly” trigger phagocytosis. For “direct” triggering, the T cell immunoglobulin and mucin domain (TIM) family of phagocytic receptors are required

whereas “indirect” triggering is achieved via $\alpha_v\beta_{3/5}$ integrins that bind the MΦ secreted product known as milk fat globule-EGF factor 8 (MFG-E8) in complex with PtdSer to enhance corpse clearance (10). These “find me” and “eat me” signals as well as the corresponding receptors have recently been reviewed by Hochreiter-Hufford and Ravichandran (10).

Not only do the release of the aforementioned cytokines and detection of “find me”/“eat me” signals condition the surrounding microenvironment, they also direct differentiation of regulatory T cells (Tregs), which supplement the suppressive nature of the apoptotic milieu (2). Moreover, the membrane of a dying cell is preserved during apoptosis concealing many potentially immunogenic DAMPs which if released could alert surrounding cells, including phagocytic cells, by triggering both surface and cytosolic PRR. These PRR include C-type lectin receptors, nucleotide-binding domain and leucine-rich repeat containing receptors, retinoic acid inducible gene I-like receptors (RLR) and Toll-like receptors (TLRs) (3,11). However, although many DAMPs may be concealed during steady-state apoptosis thus preventing inflammation, PAMP detection by PRR in the context of infection-induced apoptosis is vital for the successful elicitation of protective immunity and preservation of the host (12).

Essential for the execution of apoptosis and the internal dismantling process are the family of cysteine-aspartic acid proteases known as caspases, including the “initiators” (caspase 2, 8, 9 and 10) and “executioners” (caspase 3, 6 and 7) (13). Although not all caspases are involved in cell death, pan-inhibition using N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) during treatment with death-inducing stimuli will result in failure to induce apoptosis (4). To achieve activation of initiator and executioner caspases during cell death, cells must proceed through either the extrinsic or intrinsic apoptosis pathways.

Extrinsic apoptosis is initiated through ligation of the trimetric transmembrane receptors belonging to the tumor necrosis factor receptor (TNFR) superfamily of which TNFR1, Fas and TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) are the most extensively studied (4). . Whereas ligation of TNFR1 can instruct cellular survival, apoptosis and necroptosis (recently reviewed by Kaczmarek et al (14)), signaling through Fas and TRAIL-R1 is a potent inducer of extrinsic apoptosis (4). In brief, binding of Fas and TRAIL-R1 with the cognate ligands FasL and TRAIL, respectively, results in a conformational change to the cytoplasmic tails of the trimeric receptor complex, thus exposing the death domains (DDs). These DDs can then bind the adaptor molecule known as Fas-associated death domain (FADD) protein via a similar DD that can then recruit the initiator procaspase 8, and finally several isoforms of the cellular FADD-like IL-1 β -converting enzyme inhibitor protein (c-FLIP) family that are crucial for determining the fate of the cell (15). Interestingly, depending on the concentration of these isoforms including long, short and Raji variants (L/S/R), c-FLIP can induce both apoptotic and anti-apoptotic signaling (16). Although it was previously suggested that c-FLIP_S and c-FLIP_R blocked apoptosis by inhibiting procaspase 8 activation, the role of c-FLIP_L remained unclear and debated. To address this, Fricker et al used mathematical models of Fas signaling among other assays to demonstrate that the pro- or anti-apoptotic signaling induced by c-FLIP_L was dependent on (i) the strength of stimulus

through Fas, (ii) the concentration of c-FLIP_L and (iii) the presence of either c-FLIP_S or c-FLIP_R (17). In addition, these data also support an expanded role for c-FLIP_{S/R} as modulators of Fas signaling rather than simply inhibitors of procaspase 8 cleavage. Therefore, in the context of a death-inducing signal, the c-FLIP isoforms collectively, together with FADD and procaspase 8, form the death-inducing signaling complex, which facilitates the autocatalytic cleavage of procaspase 8 into the active form (15). Depending on the cell type, apoptosis can then proceed through the caspase 8-mediated cleavage of executioner caspases or through mitochondrial outer membrane permeabilization (MOMP), which requires further action from the B cell lymphoma 2 (BCL-2) family of proteins to be discussed below. Following caspase 8-mediated activation of executioner caspase 3, several substrate targets are cleaved including Golgi stacking proteins for Golgi apparatus fragmentation, nuclear lamins for nuclear membrane permeabilization, inhibitor of caspase activated DNase (ICAD) that releases CAD and facilitates DNA degradation, and finally proteins permitting PtdSer exposure on the membrane surface serving as “eat me” signals for phagocytes (10,13). Taken together, the culmination of these events leads to the demise and clearance of the cell without the induction of inflammation (Figure 1).

Unlike extrinsic apoptosis that relies on death receptor ligation to generate cell death, intrinsic apoptosis results from a variety of stress stimuli including ionizing radiation, DNA damage, oxidative stress, hypoxia, hyperthermia, endoplasmic reticulum stress from disruption of calcium stores or accumulation of unfolded proteins, and, importantly for this review, microbial infection, which all lead to MOMP and the destruction of mitochondria (4). If possible, the stressed cell will first attempt to correct the damage or alleviate the stress via repair mechanisms that are, in part, monitored by the ratio of pro- and anti-apoptotic proteins of the BCL-2 family (18). However, in the event that the cell cannot be salvaged, the Bcl-2 homology domain 3 (BH3) subgroup of the BCL-2 family is transcriptionally up-regulated including BH3 interacting domain death agonist (BID), NOXA and PUMA, which skew the BCL-2 family of proteins in favor of apoptosis. This is accomplished by alleviating the suppression of *pro-apoptotic* BCL-2 proteins including Bcl2-associated X protein (BAX) and Bcl2-homologous antagonist killer (BAK) by the *antiapoptotic* BCL-2 and BCL-extra-large (BCL-XL) proteins (18). The pro-apoptotic proteins BAX and BAK then form pores in mitochondrial membrane resulting in MOMP and the loss of mitochondrial membrane potential (18). This permits the release of apoptotic molecules such as apoptosis-inducing factor (AIF), endonuclease G (ENDOG), second mitochondria-derived activator of caspases (SMAC) and cytochrome-c from the inner membrane space of the mitochondria (19). AIF and ENDOG relocate to the nucleus to cause DNA fragmentation whereas SMAC and cytochrome-c are important for further caspase activation by inhibiting a family of ubiquitin ligases known as inhibitors of apoptosis (IAP), including cellular (c)IAP1 and 2 (19). These ubiquitin ligase proteins (recently reviewed in (20)) are defined by the conserved baculoviral IAP repeats (BIR) and, aside from preventing apoptosis, have critical roles in directing a variety of cellular signaling processes including cytokinesis, proliferation and differentiation. Moreover, IAPs have also been shown to modulate immune signaling through PRRs by regulating the ubiquitination status of several proteins required for activation of the transcription factor NF- κ B as well as those needed to initiate the mitogen-activated protein kinase signaling cascade for proinflammatory responses (20). To promote

cell survival, IAP bind to the caspase IAP binding motif (IBM) via the BIR domain, which inhibits the catalytic activity of the caspase. Likewise, the function of IAP can be blocked via a similar IBM domain on SMAC and SMAC mimetics, which are currently under clinical trials for anti-tumor therapy (21). In addition, cytochrome-c, together with apoptotic protease-activating factor-1 and homodimers of initiator caspase 9, form the apoptosome that is responsible for procaspase 3 cleavage and further destruction of the cell (13) (Figure 1).

Apoptosis, namely the intrinsic pathway of apoptosis, is also a critical determinant for both the progression and the severity of disease during pathogenic infection. Indeed, both bacterial and viral pathogens can compromise the apoptotic machinery required to elicit suppressive or inflammatory responses, which can greatly dictate the desired immune outcome. Depending on the type of pathogen, gaining entry into a host cell can be accomplished by adhesion/invasion using machinery to inject disruptive effector molecules into host targets (22) or by endocytosis, including pinocytosis and phagocytosis (23), which alter cellular function. One example considered here is the apoptosis of intestinal mucosal epithelial cells during infection with pathogenic enteric bacteria, such as enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (24,25), as well as the rodent pathogen *Citrobacter rodentium* (26). These bacteria possess virulence factors such as pore-forming toxins, adhesins and components of a type III secretion system (T3SS), all encoded by the locus of enterocyte effacement (24). The T3SS mediates firm adhesion of the bacteria to the host cell and injection of various bacterial effectors directly into the host cell cytosol to disrupt cellular functions and survival. One of these effectors is the EPEC secreted protein F (EspF) that translocates to the mitochondria to initiate MOMP and subsequent apoptosis (25,27). Conversely, viruses such as the enterovirus coxsackie virus B4 (CB4) enter host cells via macropinocytosis, a mode of nonspecific uptake of surrounding fluids (23), and can lead to autoimmunity and type 1 diabetes (T1D). In addition, severe infection from lymphocytic choriomeningitis virus (LCMV), which utilizes a noncoated pit entry method of endocytosis, has been shown to induce extensive apoptosis of erythrocytes. To further limit tissue destruction by an aggressive immune response, DC engulf these apoptotic erythrocytes in a process known as hemophagocytosis and release IL-10 to help regulate the severity of the infection (28). Taken together, apoptosis coupled with either bacterial or viral infection clearly plays an influential role in dictating how the immune response is regulated during pathogenic insult.

Consequences of Apoptosis Associated With Infection and Inflammation

MΦ, Mo and DC have the ability to internalize microbes and host apoptotic cells through a process of phagocytosis characterized by the formation of membrane-delimited compartments, known as phagosomes (recently reviewed in (29)). Phagocytic cargo can potentially serve as a source of antigens for the MHC-II and MHC-I (cross-presentation) pathways. The engagement of PRR during phagocytosis “instructs” phagocytes or the antigen presenting cells (APCs) about their phagosomal content, and modulates the kinetics and outcome of phagosome maturation accordingly (30,31). Engagement of TLRs during phagocytosis serves to alert the cells to the microbial nature of their cargo and initiates an inducible rate of phagocytosis characterized by enhanced fusion of phagosomes with

lysosomes and optimal MHC-II presentation, all within a T cell costimulatory context (30). In the absence of TLR signaling or during the phagocytosis of apoptotic cells *in vitro*, phagocytic DC remain immature, phagosome maturation is delayed (30) and antigens derived from apoptotic cells do not efficiently generate TCR ligands (31). This was demonstrated by analyzing T cell proliferation after *in vitro* incubation of DC that had phagocytosed apoptotic cells expressing Ea as a model antigen, with 1H3.1 CD4 T cells expressing the corresponding TCR. In contrast, when apoptotic cells were previously incubated with the TLR4 ligand LPS to mimic apoptosis associated with infection, the simultaneous presence of TLR ligands allowed phagosomes containing cellular antigens to fully mature, generate peptides loaded onto MHC-II molecules, induce the expression of co-stimulatory molecules and activate antigen-specific CD4 T cells (Figure 2). While presentation of microbial antigens derived from infected apoptotic cells may still be favored in this case, we have proposed that the presence of apoptotic cell derived peptides within a TLR signaling phagosome could lead to their potential presentation by MHC-II within an inflammatory context. This mechanism may precipitate the activation of autoreactive T cells in genetically predisposed individuals during microbial infections associated with significant tissue damage and death of infected cells (32).

Breaking tolerance and inducing autoimmunity

Evidence for activation of autoreactive CD4 T cells after tissue damage has been provided from studies with mouse models T1D (33). T1D is an autoimmune disease characterized by the destruction of pancreatic β islet cells responsible for insulin production (34). Epidemiological studies have shown correlations between infection with the pancreatic β cell tropic virus CB4 and the development of T1D (32). In a mouse model of T1D, it has also been demonstrated that CB4 infection of BDC2.5 TCR transgenic (Tg) mice, harboring CD4 T cells specific for a nonidentified pancreatic antigen, precipitated the development of T1D. Rapid-onset diabetes in CB4 infected BDC2.5 mice correlated with the activation of a preexisting population of autoreactive diabetogenic TCR Tg memory T cells, which resided within the pancreas (35) (Table 1). CB4 infection induces both damage to β islet cells and a highly inflammatory state, both that could potentially activate the autoreactive BDC2.5 Tg T cells. However, only damage to β islet cells upon treatment with the agent streptozotocin (STZ), and not high levels of type I interferon (IFN-I) triggered by injection of the viral dsRNA mimic poly (I:C), induced a rise in insulinitis (35). Notably, APCs purified from STZ-treated BDC2.5 Tg mice had acquired and processed cellular antigen from dying β islet cells as evidenced by their ability to induce proliferation of BDC2.5 CD4 T cells *in vitro* and transfer diabetes to naïve recipient Tg mice. These results demonstrated that the preexisting population of resting autoreactive memory T cells in BDC2.5 Tg mice could be activated *in vivo* upon release of pancreatic cell antigens during tissue damage (35). Presumably, a similar population of memory T cells specific to pancreatic antigens could exist in prediabetic patients having been triggered by pancreas tropic infections, and poised to cause disease upon infectious or environmental β islet cell damage.

Whereas autoreactive T cells with high affinity to self-antigen are efficiently deleted from the T cell repertoire via central and peripheral mechanisms of tolerance (32), T cells with low affinity to self-antigens are spared and could potentially precipitate autoimmunity (36)

(Table 1). However, this could only occur under circumstances where the self-antigen is presented in the context of a microbial infection. This was demonstrated experimentally by crossing RIP-mOVA mice, where a membrane-bound form of ovalbumin (OVA) is expressed as a self-antigen specifically in β islet cells, to V β 5 Tg mice carrying the V β used by OVA-specific OT-I CD8 T cells (36). In progeny mice, pairing of the Tg V β 5 with the endogenously rearranged TCR α genes created a polyclonal autoreactive CD8 T cell population with a range of TCR avidities to K^b-OVA. While the high-avidity autoreactive T cells were deleted in these mice, the lower avidity ones remained, activation of which was then monitored. Neither systemic inflammation nor antigen-specific help from CD4 T cells was capable of activating these low-avidity autoreactive CD8 T cells. Activation of these T cells could only be achieved upon infection with a *Listeria monocytogenes* recombinant bacterium expressing the self-antigen OVA (LM-OVA).

Breaking tolerance to self via the presentation of cellular self-antigen during inflammation is also dependent on the subset of phagocytic cells that engulf apoptotic cell cargo. Resident peritoneal M Φ express both TIM-4 and the enzyme 12/15-lipoxygenase (12/15-LO), which oxygenates free and phospholipid-bound polyunsaturated fatty acids (37). By studying the phagocytosis of apoptotic cells injected into the peritoneal cavity of mice undergoing thioglycollate-induced peritonitis, the authors observed that apoptotic cell clearance was confined to resident M Φ that expressed high levels of 12/15-LO and TIM-4, and not to the 12/15-LO-negative Ly6C^{hi} inflammatory Mo in those exudates. During peritonitis, 12/15-LO generated phospholipid oxidation products were enriched on the plasma membrane of resident M Φ , where they sequestered and bound distinct soluble factors such as MFG-E8, competing these ligands away from the Ly6C^{high} inflammatory Mo that utilize them to phagocytose apoptotic cells (37). This mechanism is important to avoid autoimmunity since uptake of apoptotic cells by inflammatory Mo in 12/15-LO-deficient (*Alox15*^{-/-}) mice, where diversion of apoptotic cell cargo to 12/15-LO⁺ resident M Φ is absent, led to the MHC-II presentation of apoptotic cell derived antigens in an inflammatory and T cell co-stimulatory context (37) (Table 1; Figure 2). The result was a break in self-tolerance as evidenced by the appearance of anti-nuclear and other autoantibodies in the serum of *Alox15*^{-/-} mice followed by the development of glomerulonephritis. Subjecting *Alox15*^{-/-} mice to sterile peritonitis induced by intraperitoneal injection of pristane, an experimental murine model of lupus, triggered the production of higher levels of autoantibodies and an exacerbated-form glomerulonephritis when compared to their WT counterparts (37). These studies were notable because they revealed two important findings. First, despite a rampant inflammatory response, the phagocytosis of apoptotic cells can be relegated to a dedicated subset of mononuclear phagocytes, the tissue-resident M Φ in this case, diverting phagocytic cargo carrying self-antigens away from the inflammatory Mo. Second, the type of mononuclear phagocyte conducting apoptotic cell clearance can profoundly impact the decision to induce tolerance versus immunity to self-antigens (Table 1).

Altered CD4 T cell differentiation fates depending on the nature of the apoptotic cell recognized

Phagocytosis of apoptotic cells and Treg differentiation—Phagocytic cells possess the ability to induce the differentiation of naïve CD4 T cells into either effector or

regulatory T cells (5,38). FOXP3⁺ Tregs are characterized by the expression of the transcription factor FOXP3 and by their suppressive function and contribution to immune tolerance (39). They can be divided into two major populations: thymus derived natural FOXP3⁺ Treg and extrathymically derived (also induced or adaptive) FOXP3⁺ Treg that are generated after antigen encounter in the periphery (39). Recently, the notion of tissue-resident Treg has also been introduced (40).

Studies have reported plasticity in FOXP3 expression. Some Treg during inflammatory conditions may lose FOXP3 expression and acquire an effector phenotype with pathological consequences (41). This is consistent with the finding that FOXP3 expression is essential for Treg suppressor function (42). However, these findings are controversial because of the use of *in vitro* re-stimulations and different mouse models to fate-map FOXP3 expression (41,42). Nevertheless, there is an agreement that the majority of Tregs are stable, especially thymic-derived T cells that express high level of FOXP3.

Early *in vitro* studies have shown that phagocytosis of apoptotic neutrophils by MΦs inhibited the production of pro-inflammatory cytokines while inducing the secretion of anti-inflammatory mediators like TGF-β1 (43), a key factor needed for the differentiation and maintenance of FOXP3⁺ Tregs *in vitro* and *in vivo* (42,44) (Figure 2). Accordingly, immune tolerance is generated after injection of CD3-specific mAb, which induces apoptosis of T cells and has been used as a treatment for transplant rejection (45). Apoptotic cell cargo were engulfed by MΦ and DC and stimulated TGF-β1 production, which allowed the conversion of FOXP3⁻ into FOXP3⁺ CD4 T cells, showing that phagocytosis of apoptotic cells is a physiological stimulus for Treg differentiation *in vivo* (45).

A promising therapeutic application of the immunosuppressive capacity of apoptotic cells is the infusion of donor apoptotic cells to enhance engraftment of bone marrow (BM) cells and prevent graft rejection (46–48). The advantage of this protocol is that it enhances cell engraftment and also prevents graft-versus-host disease (GvHD) without the need for additional treatments that can cause excessive immunosuppression (48). This approach was first used in engraftment models of murine BM transplantation (49) (Table 1). In these studies, apoptotic cells from a healthy donor were injected into sub-lethally irradiated recipients that had received a small number of allogeneic BM cells. Interestingly, investigators found a considerable improvement with the engraftment independently of (1) the origin of the apoptotic cells and (2) the cause of cell death (i.e. UV irradiation or anti-Fas-induced cell death), suggesting that apoptotic leukocytes could facilitate allogeneic BM engraftment (49). This treatment also prevented humoral allo-immunization against a BM graft in mice, in a TGF-β-dependent manner (50). TGF-β was also shown to induce the expansion of protective FOXP3⁺ CD4⁺ CD25⁺ cells during allogeneic BM transplantation (51). Interestingly, this protection was mediated by MΦ since treatment with clodronate-loaded liposomes, which deplete Mo/MΦ, prevented generation of Tregs. In parallel, DC depletion using CD11c-DTR mice, in which CD11c⁺ cells express the diphtheria toxin (DT) receptor and are eliminated by DT treatment, had no effect (51).

In both rat and mouse models of heart transplantation, transfusion of apoptotic splenocytes from the donor strain prior to transplant prolonged survival of heart allografts. In mice, the

protective effect was mostly mediated by phagocytic CD8 α^+ DC (52). To achieve long-term (>100 days) allograft survival, the authors combined allogeneic splenocyte infusion with a single dose of anti-CTLA-4/ CD154 mAb injection. This treatment induced the generation of IL-10 and TGF- β producing Tregs, which were responsible for indefinite cardiac allograft survival. In addition to M Φ , Mo and DC, plasmacytoid DC might also have a role as an effector population that can induce tolerance after infusion of apoptotic cells. Indeed, these cells have been shown to internalize and present alloantigens in a model of vascular cardiac transplantation upon a tolerizing injection of splenocytes and anti-CD40L (53).

T cell apoptosis in transplantation—Many immunosuppressive therapies that improve the outcome of allogeneic hematopoietic stem cell or solid organ transplantation rely in part on the apoptosis of alloreactive T cells, which mediate graft rejection (54,55). Pioneer studies explored the behavior of T cells after co-stimulatory blockade or inhibition of IL-2-mediated signal transduction by the immunosuppressive drug rapamycin (56,57). Wells et al generated transgenic mice where T cells overexpressed the anti-apoptotic protein BCL-XL and were resistant to both passive or active cell death (57). These mice were resistant to the induction of transplantation tolerance, thus identifying apoptosis as the mechanism responsible for permanent engraftment of vascularized cardiac allografts. Li et al corroborated these observations, additionally demonstrating that combined co-stimulatory blockade and rapamycin therapy could enhance T cell apoptosis and facilitate skin graft tolerance (56).

Several reports have analyzed the effects of selective elimination of host-reactive donor T cells by inducing cell death via cross-linking of the Fas receptor (see most recent works (58–60)) or targeted delivery of a chimeric IL-2/ caspase-3 molecule (61). Other approaches make use of immuno-toxins and magnetic beads to target specific molecules such as CD25 on allogeneic T cells. Beneficial effects in various settings of transplantation have also relied on the induction of cell death using extracorporeal photopheresis whereby donor lymphocyte infusions or the mononuclear cell fraction of leukapheresis products is treated with photosensitizing drugs such as 8-methoxyp-soralen or TH9402 and exposed to ultraviolet light. These treatments photodeplete alloreactive T cells and induce leukocyte apoptosis prior to infusion into the patient (discussed in (55) and (46)).

Phagocytosis of infected apoptotic cells and T helper 17 cell differentiation—Compared to these studies, when phagocytosis of apoptotic cells is combined with a TLR stimulus, the concomitant ligation of TLRs during phagocytosis of infected apoptotic cells constitutes a scenario where IL-6 and TGF- β are induced together (6) (Table 1). BM derived DC that phagocytosed infected apoptotic cells in vitro secreted more IL-23 and TGF- β than DC treated with free LPS, and importantly, only the TGF- β made in response to infected or uninfected apoptotic cells was biologically active. The cytokine milieu created in response to infected apoptotic cells was shown to be conducive for inducing the differentiation of naïve CD4 T cells into T helper (Th)17 cells, which secreted IL-17 and IL-22, and expressed the Th17 lineage-specific transcription factor ROR γ t. Conversely and consistent with the studies above, phagocytosis of apoptotic cells instructed naïve CD4 T cell differentiation into FOXP3 $^+$ Tregs.

The ability to instruct CD4 T cell differentiation into Th17 cells by DC that had received dual signals from apoptotic cells and microbial components was concordant with studies *in vivo* where mice were orogastrically infected with *C. rodentium*, which was known for its ability to induce apoptosis of intestinal epithelial cells and to trigger Th17 responses (25,26,62–64). Infection of mice with a mutant strain of *C. rodentium* that was incapable of inducing apoptosis (EspF) led to marked reduction in the characteristic Th17 response within the intestinal lamina propria (6). This was despite similar colonization, shedding in stool, and colonic hyperplasia. Similarly intraperitoneal injection with QVD-OPH, a pan caspase inhibitor that blocks apoptosis, blunted the Th17 response induced by WT *C. rodentium* (6) (Figure 2). These findings illustrate how the immunosuppressive nature of apoptotic cell clearance can coexist with the necessarily inflammatory nature of infection, and assign an important role to phagocytosis of apoptotic cells, in combination with TLR engagement, in the induction of Th17 cell differentiation.

Cell death induces regulatory mechanisms to control excessive immune responses

The anti-inflammatory nature of apoptotic cell clearance also induces regulatory mechanisms to avoid excessive responses to infection and tissue injury. One example is the role of hemophagocytosis, investigated during virus infection (28). This process could be induced in mice by injection of a high dose of the TLR ligands unmethylated CpG DNA and poly(I:C) or by infection with the LCMV variant clone 13 (C13), which achieves high levels of viral nucleic acids in infected hosts. IFN-I production by these stimuli induced the exposure of PtdSer on erythrocytes, and the expression of TIM-1, TIM-4, $\alpha_v\beta_3$ integrins and MFG-E8 on inflammatory monocyte-derived DC (Mo-DC). Injecting neutralizing antibodies against TIM-1, TIM-4 and $\alpha_v\beta_3$ blocked hemophagocytosis by Mo-DC, which was also dependent on IFN-I as it was impaired in mice deficient for IFN-I receptor. Interestingly, Mo-DC were implicated as the major cellular source of IL-10 at the early phase (24 h) of infection, and antibody-mediated neutralization of IL-10 blunted TGF- β 1 production in response to hemophagocytosis. The physiological significance of hemophagocytosis during an anti-viral immune response was demonstrated by impairing hemophagocytosis, blocking PtdSer receptors, neutralizing IL-10 activity or using CD11c-cre Il10^{fl/fl} mice, which lack IL-10 expression specifically in CD11c⁺ DC. In all of these cases, excessive virus-specific cytotoxic lymphocyte activity, liver damage and mortality were observed after LCMV C13 infection as compared to control mice. Therefore, the IFN-I response to viral nucleic acids initiates a regulatory program of hemophagocytosis by inducing the expression of “eat-me” signals on erythrocytes targeting them for specific clearance by Mo-DC. The resultant secretion of anti-inflammatory cytokines such as IL-10 and TGF- β 1 modulates the immune response in order to ameliorate tissue damage (Figure 2; Table 1). Apoptosis during infection here thus serves to balance an aggressive host defense response with protection of tissues from inflammatory pathology.

Finally, recent observations in a novel mouse model, where expression of a specific antigen was constitutive in the thymus but inducible in the periphery, raise the possibility that protective Tregs could be activated under certain circumstances where self-antigens become exposed (65) (Table 1). Transgenic mice expressing a membrane-bound form of OVA driven by a tetracycline response element were crossed to transgenic mice expressing the

tetracycline transactivator under control of the skin-specific keratin 5 promoter to generate K5/TGO mice. OVA expression in the skin was tightly regulated such that activation of adoptively transferred OVA-specific DO11.10 CD4 T cells was only observed upon doxycycline treatment to induce OVA expression. Constitutive OVA expression in the thymus led to modest deletion of OVA-specific DO11.10 T cells and an increase in OVA-specific Tregs when K5/TGO mice were further crossed with DO11.10 TCR Tg mice. Analysis of progeny K5/TGO/DO11 mice after doxycycline treatment showed marked infiltration of the skin with DO11 T cells that produced IL-17 and IFN- γ despite the presence of large numbers of OVA-specific Tregs. The resultant skin inflammation resolved spontaneously 20–30 days post-induction of antigen expression, and notably OVA-specific Tregs mediated this resolution. In fact, OVA-specific FOXP3⁺ Tregs were activated and expanded soon after inducing OVA expression, differentiating into potent suppressor cells. These Tregs persisted in the mouse despite extinguishing OVA expression and could mediate a form of protective “regulatory memory” against severe skin disease upon re-induction of OVA expression. Although the exact signals mediating Tregs activation in this case remain unclear, perhaps apoptosis of keratinocytes during effector T cell infiltration and inflammation of the skin constitutes one such signal that serves to control and ultimately resolve the inflammation.

Conclusion

An increasing body of knowledge regarding both the mechanisms of apoptosis and its role in the context of infection has not only helped redefine our view of apoptosis (4) but also enhanced our understanding of the delicate balance between tolerance and protective immunity. Based on the wide range of cell death programs (4), which may or may not be further coupled to a variety of PAMP and DAMP signals, it is conceivable that there exists a distinct immunological response for each mechanism of cell death encountered by phagocytic cells. As we continue to explore how homeostasis is maintained in the host, it will be important to examine these processes in the context of local cell death as these signaling pathways may provide future targets for therapeutic intervention.

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Abbreviations

12/15-LO	12/15-lipoxygenase
AIF	apoptosis-inducing factor
APC	antigen presenting cell
BAK	Bcl2-homologous antagonist killer
BAX	Bcl2-associated X protein

BCL	B cell lymphoma
BCL-2	B cell lymphoma 2
BCL-XL	BCL-extra-large
BH3	Bcl-2 homology domain 3
BID	BH3 interacting domain death agonist
BIR	baculoviral IAP repeats
BM	bone marrow
C13	clone 13
CB4	coxsackie virus B4
c-FLIP	cellular FADD-like IL-1 β converting enzyme inhibitor protein
DAMPs	damage-associated molecular patterns
DC	dendritic cells
DD	death domain
DT	diphtheria toxin
ENDO G	endonuclease G
EPEC	enteropathogenic Escherichia coli
EspF	EPEC secreted protein F
FADD	Fas-associated death domain
FasL	Fas ligand
GvHD	graft-versus-host disease
IAP	inhibitors of apoptosis
IBM	IAP binding motif
ICAD	inhibitor of caspase activated DNase
IFN-I	type I interferon
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharides
MΦ	macrophages
MFG-E8	milk fat globule-EGF factor 8
Mo	monocytes
Mo-DC	monocyte-derived DC
MOMP	mitochondrial outer membrane permeabilization
OVA	ovalbumin

PAMPs	pathogen associated molecular patterns
PRRs	pattern-recognition receptors
PtdSer	phosphatidylserine
SMAC	second mitochondria-derived activator of caspases
STZ	streptozotocin
T1D	type 1 diabetes
T3SS	type III secretion system
Tg	transgenic
Th	T helper
TIM	T cell immunoglobulin and mucin domain
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell

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Figure 1. Extrinsic and intrinsic apoptosis signaling pathways

Caspase activation by *extrinsic* apoptosis requires binding of the trimeric TNF family of death receptors by their cognate ligands such as TRAIL and FasL. Binding results in a conformational change within the cytoplasmic tails of these receptors and exposes DD sequences. FADD is then bound via similar DD sequences, which permit the recruitment of initiator procaspase 8 and several isoforms of the c-FLIP. Collectively, these proteins form the DISC, which facilitates the autocatalytic cleavage of procaspase 8 into the active form of caspase 8. Depending on the cell type, caspase 8 can then cleave and activate procaspase 3

that targets several proteins including ICAD, which in turn liberates CAD to trigger DNA degradation and apoptosis. Conversely, caspase 8 can also initiate MOMP by a pathway that overlaps intrinsic apoptosis and requires the BCL-2 family of pro- and anti-apoptotic proteins as well as the BH3-only subgroup. Active caspase 8 can then cleave BID into tBID and skew the balance of pro- and anti-apoptotic BCL-2 proteins. In doing so, *anti-apoptotic* proteins including BCL-2 and BCL-XL are sequestered away from the *pro-apoptotic* proteins BAX and BAK, which together form pores in the mitochondria resulting in MOMP and apoptosis. Similarly, during *intrinsic* apoptosis, induction of an internal/external cellular stress results in the transcriptional up-regulation of BH3-only proteins that function like tBID. Following the BAX/BAK-induced MOMP, apoptotic molecules are released from the mitochondria including AIF, ENDOG, SMAC and cytochrome-c. AIF and ENDOG relocate to the nucleus and cause DNA fragmentation, whereas SMAC and cytochrome-c inhibit the family of pro-survival ubiquitin ligases known as cIAP. Finally, cytochrome-c, APAF1 and homodimers of procaspase 9 form the apoptosome. The apoptosome cleaves and activates several executioner caspases to commit the cell to apoptosis. It also induces surface expression of “eat me” signals such as PtdSer to initiate phagocytic clearance. TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FasL, Fas ligand; DD, death domains; FADD, Fas-associated death domain; c-FLIP, cellular FADD-like IL-1 β -converting enzyme inhibitor protein; DISC, death-inducing signaling complex; ICAD, inhibitor of caspase activated DNAase; CAD, caspase activated DNAase; MOMP, mitochondrial outer membrane permeabilization; BCL-2, B cell lymphoma 2; BH3, Bcl2-homology domain 3; BID, BH3 interacting domain death agonist; tBID, truncated BID; BCL-XL, BCL-extra-large; BAX, Bcl2-associated X protein; BAK, Bcl2-homologous antagonist killer; AIF, apoptosis-inducing factor; ENDOG, endonuclease G; SMAC, second mitochondria-derived activator of caspases; cIAP, cellular inhibitors of apoptosis; APAF1, apoptotic protease-activating factor-1; PtdSer, phosphatidylserine.

Figure 2. Immune consequences of phagocytosis of apoptotic cells

Phagocytosis of two different types of apoptotic cells is depicted. Uninfected (left and center panel, blue cells and red erythroid cells) and infected apoptotic cells (blue containing gray microbes, right panel).

Left panel: Apoptosis can occur during sterile tissue damage. Immature DC or other phagocytes migrate to the site of cell death where phagocytosis takes place via interactions between PtdSer exposed on the apoptotic cell membranes and TIM-4, $\alpha_v\beta_{3/5}$ integrins and MFG-E8 expressed by phagocytic cells. The same phenomenon can be observed during infection with LCMV C13, which induces high levels of systemic type I interferon (IFN-I) leading to the expression of PtdSer on erythroid cells as well as phagocytic receptors and bridging molecules by Mo-DC. Direct or indirect binding of these molecules to PtdSer leads to erythroid cell phagocytosis by Mo-DC in a process called hemophagocytosis. In the absence of TLR signaling, phagosomes containing apoptotic cells fuse with lysosomes but undergo terminal degradation without antigen presentation.

Engulfment of apoptotic cells stimulates the release of anti-inflammatory cytokines such as TGF- β and IL-10, which temper inflammation during anti-viral responses. Center panel: During inflammatory peritonitis, resident M Φ are the dominant mononuclear phagocytes that clear apoptotic cell cargo and maintain tolerance. M Φ expressions of the enzyme 12/15-LO that generates oxidized phosphatidylethanolamine products (Ox-PE) on the M Φ plasma membrane. Ox-PE then sequester distinct soluble receptors such MFG-E8 away from the

$\alpha_v\beta_{3/5}$ integrins on the surface of inflammatory Mo robbing them of the ability to conduct apoptotic cell phagocytosis. Phagocytosis also induces the secretion of anti-inflammatory cytokines like TGF- β and IL-10 by resident M Φ . Right panel: While the recognition of PtdSer on apoptotic cells induces TGF- β secretion, microbial components from the infecting pathogen engage TLRs and stimulate the secretion of inflammatory cytokines like IL-6 and IL-23. Concomitant secretion of TGF- β , IL-6 and IL-23 induces differentiation of naïve CD4 T cells into Th17 cells. The maturation of phagosomes carrying infected apoptotic cells is also subject to TLR control. Because the phagosome contains both microbial and apoptotic cell derived proteins, TLR signaling is expected to tailor these phagosomes for optimal processing and presentation of both microbial and cellular antigens. Presentation of cellular antigens derived from infected apoptotic cells could have the potential to activate autoreactive T cells, which may have escaped thymic deletion. Activation in this inflammatory and T cell co-stimulatory context could lead to autoreactivity and may precipitate autoimmune disease in a genetically susceptible host. DC, dendritic cells; PtdSer, phosphatidylserine; MFG-E8, milk fat globule-EGF factor 8; LCMV, lymphocytic choriomeningitis virus; C13, clone 13; TIM-4, T cell immunoglobulin and mucin domain; LCMV, lymphocytic choriomeningitis virus; IFN-I, type I interferon; Mo-DC, inflammatory monocytes-derived DC; M Φ , macrophages; 12/15-LO, 12/15-lipoxygenase; TLRs, Toll-like receptors; pMHC, peptide-major histocompatibility complex.

Table 1

Consequences of apoptosis associated with infection and inflammation

Model	Molecular trigger/disease	Findings	Refs.
Breaking tolerance	CB4/T1D	Tissue destruction results in expansion of auto reactive memory T cells	(35,37)
Breaking tolerance	LM-OVA/T1D	Infection results in expansion of low-avidity self-specific effector T cells	(38)
Maintaining tolerance	Thioglycollate/peritonitis	Maintenance of tolerance requires apoptotic cell uptake by specific mononuclear phagocytes	(39)
Maintaining tolerance	Pristane/glomerulonephritis and lupus	Type of mononuclear phagocyte engulfing apoptotic cells can impact tolerance versus immunity	(39)
Inducing tolerance	Allograft/GvHD	Infusion of apoptotic donor cells induces tolerance to allograft	(50)
Inducing tolerance	Alloreactive T cells/GvHD	Improved graft tolerance by inducing apoptosis in alloreactive T cells	(54–61)
Driving CD4 T cell differentiation	<i>C. rodentium</i> /colitis	Combination of apoptosis and infection promotes Th17 differentiation	(6)
Regulatory mechanism	LCMV/hepatitis	Hemophagocytosis ameliorates excessive tissue damage during inflammation	(28)
Regulatory mechanism	OVA/dermatitis	Excessive exposure to self-antigen results in the generation of memory Tregs	(53)

GvHD, graft-versus-host disease; LCMV, lymphocytic choriomeningitis virus; LM-OVA, *Listeria monocytogenes* recombinant bacterium expressing the model antigen ovalbumin; T1D, type 1 diabetes; Th, T helper; Tregs, regulatory T cells.