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Review

A Perspective on Mammalian Caspases as Positive and Negative Regulators of Inflammation

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Members of the caspase family of cysteine proteases coordinate the morphological and biochemical events that typify apoptosis. However, neutralization of caspase activity in mammals fails to block death in response to most proapoptotic stimuli. This is because many cell death triggers provoke mitochondrial dysfunction upstream of caspase activation as a consequence of BAX/BAK channel opening. Although genetic or pharmacological inactivation of caspases fails to block cell death in most instances, it does convert the phenotype from apoptosis to necrosis. This has important implications for how the immune system responds to such cells, as necrotic cells provoke inflammation whereas apoptotic cells typically do not. Here, we propose an alternative perspective on apoptosis-associated caspase function by suggesting that these proteases are activated, not to kill, but to extinguish the proinflammatory properties of dying cells. This perspective unifies the mammalian caspase family as either positive or negative regulators of inflammation.

Introduction

Apoptosis is a mode of programmed cell death that is used to dispose of aged, superfluous, and injured cells with the minimum of disturbance to neighboring cells (Green, 2010). Apoptosis complements mitosis as a means of regulating cell numbers in multicellular organisms and for this reason is under molecular control by a dedicated set of enzymes and their regulators—the "cell death machinery." Members of a family of cysteine proteases, the caspases, become activated during apoptosis and coordinate the events that take place to ensure swift recognition and removal of apoptotic cells (Riedl and Salvesen, 2007; Taylor et al., 2008). However, although it is well established that caspases are required for the appearance of the major biochemical and morphological "hallmarks" of apoptosis, it is now clear that in many situations caspases are not required for terminating cell viability in mammals (Chipuk and Green, 2005; Kroemer and Martin, 2005). Because the great majority of injurious stimuli that promote caspase activation do so by promoting permeabilization of the mitochondrial outer membrane (Green and Kroemer, 2004), the latter event is usually sufficient to ensure cell death irrespective of whether caspases are activated downstream or not. Despite this, the caspases activated during apoptosis are still typically viewed as "death effectors," but much evidence now points toward a more complex role for these proteases as regulators of the inflammatory potential of apoptotic cells rather than as arbiters of cell fate.

An Alternative Perspective on Apoptosis-Associated Caspase Function

Here we propose that the primary function of apoptosis-associated caspase activation in mammals is the avoidance of proinflammatory engagement of the immune system and its attendant detrimental consequences—primarily autoimmunity—by cells undergoing programmed elimination. There is a growing body of evidence to argue that the caspase-dependent alterations to the cell that typically occur during apoptosis not only ensure recognition and uptake of apoptotic cells by phagocytes, but also switch on an anti-inflammatory program in the engulfing cell. Furthermore, there is also much evidence that apoptotic cells, even those undergoing secondary necrosis, can actively antagonize and override concurrent proinflammatory signals delivered to phagocytes that have engulfed an apoptotic cell (reviewed in Birge and Ucker, 2008).

In this review, we will discuss evidence to support the idea that caspases activated during apoptosis function to extinguish or dampen the proinflammatory properties of dying cells through directly inactivating and coordinating the sequestration of numerous potentially proinflammatory molecules, collectively called danger-associated molecular patterns (DAMPS) or alarmins, that reside within (Matzinger, 1994; Kono and Rock, 2008). Where death occurs without caspase activation, we propose that this results in a failure to sequester and inactivate endogenous DAMPs and may lead to local activation of macrophages and dendritic cells (DCs), the key antigen-presenting cell of the immune system. The latter event is particularly dangerous because, apart from unnecessary engagement of the immune system where a pathogenic threat is not present, DC activation can "license" these cells to present self-antigens to T cells (reviewed in Kono and Rock, 2008). This scenario is particularly undesirable from an immunological standpoint, as this can lead to a loss of tolerance toward "self" that is essential to safeguard against potentially catastrophic autoimmune responses. Caspase activation during cell death can thus be interpreted as a means of conveying information to the immune system concerning the qualitative nature of the cell death event (i.e., whether planned or pathogen induced), rather than simply a means to terminate cell viability. We suggest that apoptosis-associated caspase activation serves primarily to coordinate the silent

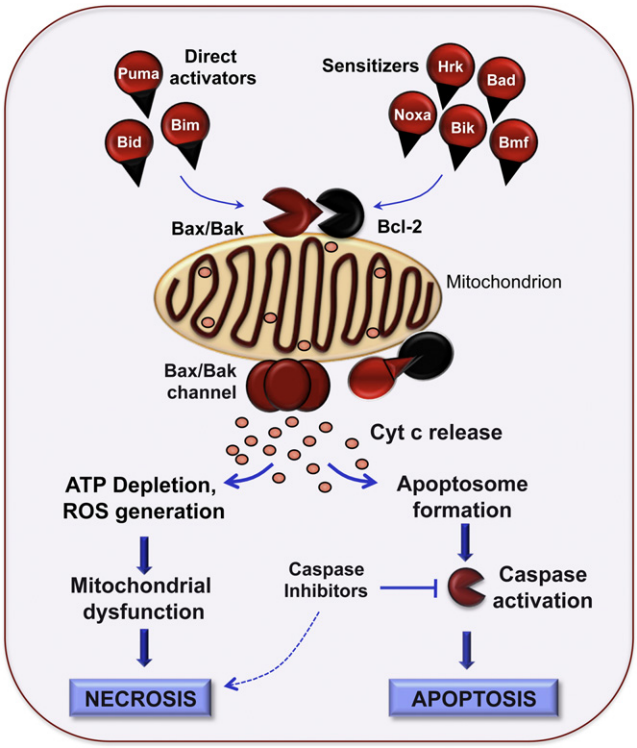

Figure 1. BAX/BAK-Induced MOMP Is Sufficient to Kill
BH3-only proteins act as specific sensors for various apoptotic stimuli and promote assembly of BAX/BAK oligomomers within the mitochondrial outer membrane, leading to cytochrome *c* release, apoptosome formation, caspase activation, and apoptosis. Importantly, caspase inhibition downstream of cytochrome *c* release does not protect against cell death as ATP depletion, reactive oxygen species generation, and subsequent mitochondrial dysfunction, brought about by release of mitochondrial intermembrane space proteins, leads to death by necrosis.

removal of dying cells by actively repressing the mechanisms normally used by the immune system to recognize and respond to inappropriate, nonprogrammed, cell deaths. From this perspective, apoptosis-associated caspase activation serves a predominantly anti-inflammatory role, as opposed to a cell death-inducing one.

The Classical View: Caspases as Regulators of Death or Inflammation

Mammals possess multiple caspases and these have traditionally been split into two major subgroups based upon sequence homology and function. The "inflammatory" caspases, which belong to the caspase-1-related subset, are activated in cells of the innate immune system, such as macrophages and DCs, in response to infection as well as noxious agents that trigger necrosis (Creagh et al., 2003; Schroder and Tschopp, 2010). Caspase-1 subfamily members (i.e., caspase-1, caspase-4, and caspase-5) have been implicated as regulators of inflammation through processing and activating two related cytokines, IL-1β and IL-18, which have diverse effects and act to initiate and amplify immune responses to infectious agents (Creagh et al., 2003). In addition, the murine caspase-4 ortholog, caspase-11, has recently been found to be important for caspase-1-dependent IL-1β and IL-18 production in response to a subset of inflammatory triggers (Kayagaki et al., 2011). However, the precise role of caspase-4 and caspase-5 in inflammation remains to be further clarified.

The "apoptotic" caspases, which belong to the caspase-3-related subgroup, are activated during apoptosis and are widely considered to participate in the execution of the cell (Taylor et al., 2008). To date, over 600 substrates for the cell death-related caspases have been identified (Lüthi and Martin, 2007). However, the vast majority of these substrates, with some notable exceptions, have not been linked with any specific feature of apoptosis (Taylor et al., 2008). Furthermore, the failure to cleave particular caspase substrates rarely, if ever, permits a cell to survive the events that precede caspase activation. One possibility is that these enzymes are engaging in a policy of redundancy; targeting numerous vital molecules to ensure that cell death is guaranteed. If this is so, it is impressive for the scale of overkill employed. Alternatively, it is also plausible that many of the proteins that are cleaved during apoptosis are either postmortem events or "innocent bystander" cleavage events that have no significance for the process and take place when cell viability has already effectively been terminated. Either way, given the sheer number of proteins that are cleaved during apoptosis, it seems highly unlikely that the majority of these proteins are targeted solely for the purpose of terminating cell viability. Moreover, because the events that lead to activation of the major "executioner" caspases irreversibly disrupt mitochondrial function (Green and Kroemer, 2004), an event that is sufficient to ensure that most cells will die, it seems implausible that caspases become activated merely to compound an already fatal blow. Before we outline why caspases are dispensable for cell death, it is necessary to take a brief look at how caspases become activated during apoptosis.

The MOMP Problem: Caspase Activity Is Dispensable for Cell Death within the Intrinsic Pathway to Apoptosis

A major route to caspase activation and apoptosis results from cellular stresses—such as cytokine deprivation, heat shock, and DNA damage, all of which provoke mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c* and other mitochondrial constituents into the cytosol (Figure 1). This has been dubbed the intrinsic or mitochondrial pathway to caspase activation and is employed by numerous proapoptotic stimuli (Green and Kroemer, 2004; Youle and Strasser, 2008). For simplicity, we will focus predominantly on the intrinsic pathway to caspase activation, but we will also refer to the second major route to caspase activation, the extrinsic or death receptor pathway, toward the end of the review.

MOMP is achieved through opening of a mitochondrial membrane channel comprised of the related proteins, BAX and BAK, as a consequence of transcriptional upregulation or post-translational modification of one or more members of the "BH3-only" protein family (Figure 1). BH3-only proteins are activated in response to diverse triggers of apoptosis, the details of which are outside of the scope of this review but have been well documented elsewhere (Youle and Strasser, 2008). Activated BH3-only proteins either directly or indirectly promote conformational changes within BAX and BAK that provoke their

oligomerization within the outer mitochondrial membrane. The latter event results in the formation of a pore or channel that permits the escape of numerous mitochondrial intermembrane space proteins into the cytosol (Green and Kroemer, 2004). The most notable of these is cytochrome *c*, as this protein acts as a cofactor for the assembly of a caspase-activating complex within the cytosol that has been dubbed "the apoptosome."

Binding of cytochrome *c* to Apaf-1 triggers its oligomerization into a wheel-like structure and permits recruitment, homodimerization, and activation of caspase-9 within the Apaf-1 apoptosome (Riedl and Salvesen, 2007). In turn, the apoptosome activates caspase-3 and caspase-7, setting off a chain of caspase activation events downstream and unleashing a torrent of protease activity within the cell (reviewed in Taylor et al., 2008).

Notwithstanding the dramatic activation of caspases upon efflux of cytochrome *c* into the cytosol, MOMP itself heralds the swift demise of the majority of cells in which this occurs. This is because, in addition to cytochrome *c*, numerous mitochondrial intermembrane space proteins exit mitochondria upon opening of the BAX/BAK channel, leading to a rapid decline in ATP synthesis as well as the generation of reactive oxygen, all of which swiftly compromise numerous cellular functions irrespective of the activation of caspases downstream (Green and Kroemer, 2004; Kroemer and Martin, 2005). Because of the essentially irreversible nature of MOMP, this is a key checkpoint in apoptosis and is heavily policed by a complex web of proteins that belong to the Bcl-2 family (Figure 1). Antiapoptotic Bcl-2 proteins bind to BAX/BAK, as well as activated BH3-only proteins (Figure 1), thereby preventing assembly of the BAX/BAK channel and consequently blocking MOMP and cytochrome *c* release (Taylor et al., 2008; Youle and Strasser, 2008). Consequently, it is the opening of the BAX/BAK channel, rather than caspase activation, which represents the point of no return for a cell death stimulus that engages the intrinsic pathway.

Caspase Activity Dictates the Switch between Apoptosis and Necrosis

Although caspases are often thought of as the direct effectors of cell death during apoptosis, this view is inconsistent with numerous observations where caspase activity has been pharmacologically inhibited or blocked through genetic inactivation. In mammals, caspase inhibition does not prevent cell death in response to stimuli that engage the intrinsic pathway to apoptosis (Marsden et al., 2002; Ekert et al., 2004; Chipuk and Green, 2005; Kroemer and Martin, 2005). Inhibition of caspase activation downstream of MOMP merely delays, but does not block, cell death. This explains why prosurvival members of the mammalian Bcl-2 family regulate MOMP rather than caspase activation to block apoptosis (Figure 1).

While caspase activation is dispensible for cell death once MOMP has occurred, there is a crucial difference in the outcome if apoptosis-associated caspase activity is inhibited. The failure to activate caspases dramatically changes the phenotype of cell death, converting it from an apoptotic to a necrotic one (reviewed in Chipuk and Green, 2005; Kroemer and Martin, 2005). This difference in outcome has very significant implications for how the immune system responds to such cells, as we shall discuss below.

Because MOMP is sufficient to ensure cell death for the majority of cells, this leads us to ask what purpose caspase activation serves during apoptosis? We suggest that the primary role of caspase activation is the sequestration and inactivation of cellular constituents, which—if permitted to leak out of the cell—could activate the immune system and promote potentially dangerous and unnecessary inflammatory responses. In support of this view, there is much evidence that phagocytes recognize and respond to necrotic and apoptotic cells in fundamentally different ways, even when apoptotic cells have entered secondary necrosis and are leaking their cellular contents (Birge and Ucker, 2008).

Sensing Danger: The Immune System Can Be Activated by Cell Death

The ability of the sentinel cells of the immune system (e.g., macrophages, DCs, and mast cells) to sense molecules released from dead cells makes a great deal of sense in biological terms (Matzinger, 1994). Our immune systems have evolved to protect us from infectious agents by attacking and killing these upon entry into the body. The simplest way to do this is to equip sentinel cells of the innate immune system with a battery of receptors that can detect molecules that are unique to foreign organisms. Indeed, this is an important feature of innate immunity, and macrophages, DCs, and other cells of the innate immune system bristle with an array of Toll-like receptors (TLRs) that are capable of detecting a wide variety of pathogen-associated molecular patterns (PAMPs). The consequences of a PAMP binding to its corresponding receptor are swift and lead to aggressive macrophage or DC activation followed by the triggering of immune functions that are directed at killing the infectious agent and presenting associated antigens to cells of the adaptive immune system (Iwasaki and Medzhitov, 2004).

Because it is not practical to have an endless array of TLRs capable of recognizing all possible PAMPs, infectious agents could well arise that may evade detection. Because of this threat, the immune system has also evolved its own array of endogenous danger-associated molecular patterns (DAMPs) that are released in response to sterile injury or infection that is associated with necrotic cell death (Matzinger, 1994; Kono and Rock, 2008). DAMPs can thus be viewed as surrogate markers for infection that enable a host to mount an effective immune response even in the absence of direct detection of a PAMP (Figure 2). DAMPs bind to receptors, such as members of the IL-1 receptor family, which have very similar intracellular signaling domains as TLRs and instigate responses from macrophages or DCs almost identical to TLR engagement. Indeed, some DAMPs have been reported to directly bind to TLRs, although this is somewhat controversial (reviewed in Kono and Rock, 2008). Thus, cells of the innate immune system can be activated through encounter with either PAMPs or DAMPs, with similar consequences. But how does infection or injury trigger DAMP release?

DAMPs are normally sequestered within healthy cells and only become released upon rupture of cells (i.e., necrosis), whereupon such molecules spill out into the extracellular space and trigger immune activation (Matzinger, 1994; Kono and Rock,

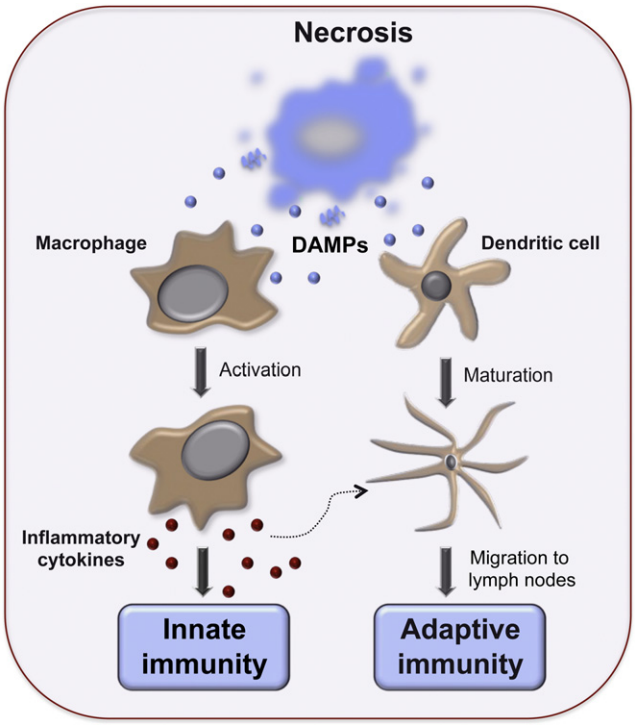

Figure 2. Necrotic Cell Death Promotes Inflammation
The release of endogenous danger-associated molecular patterns (DAMPs) from necrotic cells can activate various immune cell subsets. DAMPs activate macrophages to secrete inflammatory cytokines that have broad stimulatory effects on the immune system. Activation of DCs by DAMPs promotes DC maturation and subsequent migration to lymph nodes where mature DCs can initiate adaptive immunity by presenting antigens to antigen-specific T cells.

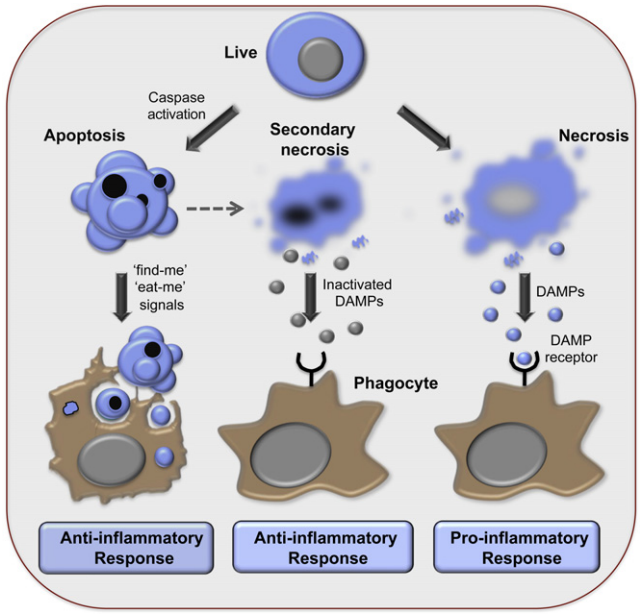

Figure 3. Apoptotic and Secondarily Necrotic Cells Are Anti-inflammatory
Caspase activation inactivates endogenous DAMPs and induces the production of "find-me" and "eat-me" signals to coordinate the swift removal of apoptotic cells by phagocytes and simultaneously suppress immune responses. Unlike cells that have entered necrosis directly, apoptotic cells that have entered secondary necrosis carry inactivated DAMPs and are also anti-inflammatory. In contrast, necrotic cells exhibit potent proinflammatory properties due to release of active DAMPs.

2008). Therefore, if an infectious agent provokes necrosis, this will trigger the release of DAMPs, which will then activate the immune system. It is highly appropriate that the presence of necrotic cells can instigate immune responses, as cell rupture is typically only caused by severe departures from normal physiology. Thus, necrotic cell death can betray the activities of viral infection (many viruses cause cell lysis during production of new virions), the activities of bacterial toxins and other severely cytotoxic molecules. Moreover, if necrosis is not a direct consequence of pathogen activities, necrosis of barrier tissues—due to compression injuries or burns, for example—is likely to lead to infection. Thus, our immune systems are "hardwired" to become activated in response to the detection of PAMPs or DAMPs.

Whereas necrotic cells typically provoke inflammation, apoptotic cells generally do not (Voll et al., 1997; Fadok et al., 1998; Lucas et al., 2003). Furthermore, several laboratories have independently reported that apoptotic cells are also capable of profoundly attenuating responses to PAMPs delivered in parallel (Voll et al., 1997; Lucas et al., 2003; Serhan and Savill, 2005). Indeed, apoptotic cells that have entered secondary necrosis and are leaking their cellular contents also retain this anti-inflammatory state, in contrast with cells that have entered necrosis directly (Cocco and Ucker, 2001; Birge and Ucker, 2008). This is curious and suggests that profound alterations to cellular composition occur during apoptosis to quell the activity of DAMPs within such that, even if these are inadvertently released, their proinflammatory activity is blunted.

So, how do DAMPs become inactivated during apoptosis? Because caspase activation is a fundamental difference between apoptosis and necrosis, caspases are prime suspects as the major effectors of the conversion of cells from a proinflammatory to a noninflammatory or actively anti-inflammatory state. We suggest that a major role of apoptosis-associated caspase activity is to quell the proinflammatory properties of apoptotic cells through inactivation (both directly and indirectly) and sequestration of potentially proinflammatory molecules (DAMPs) residing within (Figure 3). Before we examine the evidence in support of this idea, we will first consider other apoptosis-associated events that may limit the exposure of DAMPs during this process.

Apoptosis: A Calming Influence on the Immune System

Billions of cells die naturally on a daily basis as a consequence of homeostatic tissue turnover and the vast majority of these cell deaths occur via apoptosis (Green, 2010). This poses a significant challenge for the immune system in terms of discriminating between natural or programmed, as opposed to nonprogrammed or necrotic, cell deaths. Because of the capacity of intracellular DAMPs to provoke immune responses (Figure 2), it seems obvious that cells of the immune system need to be able to discriminate between cell deaths that are programmed

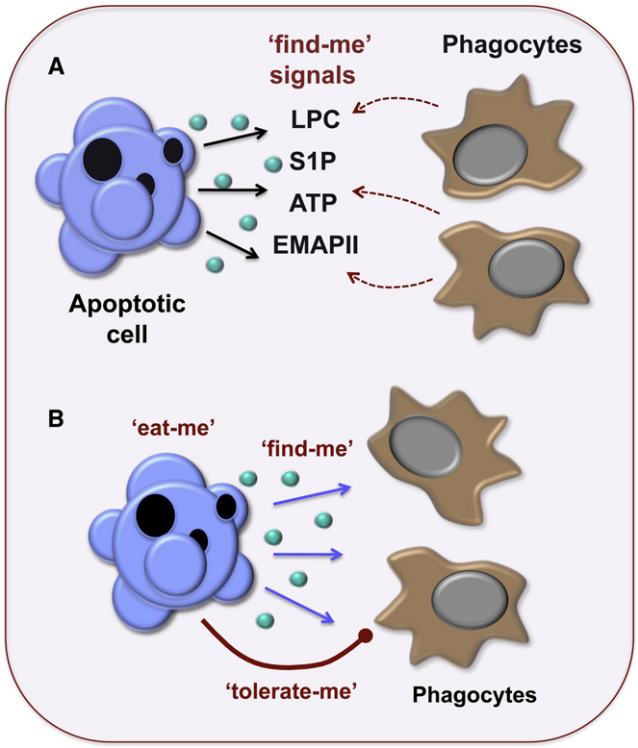

Figure 4. Apoptotic Cells Release "Find-Me" Signals to Attract Phagocytes

(A) Apoptotic cells release "find-me" signals such as ATP/UTP, sphingosine-1-phosphate (S1P), and lysophosphatidylcholine (LPC) that attract the attention of professional phagocytes.

(B) Apoptotic cells generate "find-me," "eat-me," and "tolerate-me" signals that actively downregulate immune responses and qualify this type of cell death as harmless.

from those that are not. Because of the scale of ongoing apoptosis in vivo, a failure to discriminate effectively between apoptotic and necrotic cells could result in inappropriate immune activation.

Because of the role of DAMPs as instigators of immune responses, a strong case can be made for the idea that when cells die by apoptosis, an important overarching objective is to prevent the release of cellular constituents that could provoke unnecessary immune responses. The simplest way of achieving this objective is to ensure that cells undergoing apoptosis are swiftly recognized and removed from a tissue before such cells have an opportunity to leak their contents. Indeed, this is probably what happens to the majority of apoptotic cells and this is ensured through caspase-dependent alterations to the plasma membrane (called "eat-me" signals) that trigger recognition of such cells by resident tissue macrophages, as well as nonprofessional phagocytes (Savill and Fadok, 2000). The molecules that have been implicated as triggers for phagocyte recognition and removal of apoptotic cells include phosphatidylserine (PS), which becomes externalized on the surface of apoptotic cells in a caspase-dependent manner (Martin et al., 1995). However, it is clear that PS exposure alone is insufficient to trigger uptake of apoptotic cells by phagocytes and that additional molecules are likely to appear in association with PS to qualify a cell as apoptotic (Segawa et al., 2011). Although these other ligands have yet to be identified, it is clear that caspase-dependent exposure of "eat-me" signals undoubtedly plays a key role in helping to ensure that apoptotic cells, in most cases, do not linger in tissues for long enough to permit escape of DAMPs into the extracellular space (Savill and Fadok, 2000). Moreover, it is becoming increasingly clear that this clearance process is not left up to chance encounters with neighboring phagocytes, as recent studies suggest that factors which actively elicit the attentions of phagocytes are released from apoptotic cells, once again in a caspase-dependent manner (Gregory and Pound, 2011; Ravichandran, 2011). Such factors have been dubbed "find-me" signals.

"Find-Me" Signals: Caspases Elicit Macrophage Recruitment to Apoptotic Cells

While nonprofessional phagocytes may be capable of engulfing neighboring cells that have undergone apoptosis, there is much evidence that this is also carried out by resident tissue macrophages and DCs. Because these cells typically represent a relatively small fraction of the cellular composition of most tissues, this poses the question of how apoptotic cells are discovered before they undergo secondary necrosis and awaken the full force of the immune system. Evidence is now accumulating to suggest that cells undergoing apoptosis signal their impending demise through the release of one or more soluble factors that act as chemoattractants for phagocytes (Gregory and Pound, 2011; Ravichandran, 2011). As mentioned above, such factors have been dubbed "find-me" signals and there is increasing evidence that apoptotic cells use such factors to guide phagocytes to their location to ensure a swift burial (Figure 4A).

Caspase-dependent release of lysophatidylcholine (LPC) was one of the first molecules to be implicated as a "find-me" signal that is released during apoptosis (Lauber et al., 2003). LPC production by apoptotic cells appears to be instigated as a consequence of caspase-3-dependent cleavage of calcium-independent phospholipase A2, resulting in the hydrolysis of membrane phosphatidylcholine to produce LPC (Lauber et al., 2003). LPC can act as a chemoattractant for monocytic cells and macrophages and may help to guide phagocytes to the dying cell. However, evidence that LPC is an important "find me" signal in vivo is still lacking. Endothelial monocyte-activating polypeptide II (EMAPII) is another molecule that has been implicated as a chemotactic factor released from apoptotic cells (Knies et al., 1998). EMAPII undergoes caspase-dependent proteolysis during apoptosis and the C-terminal fragment of this molecule can act as a trigger for monocyte attraction (Knies et al., 1998).

Recent studies from Ravichandran and colleagues have also implicated efflux of ATP and UTP as "find-me" signals for apoptotic cells (Elliott et al., 2009; Chekeni et al., 2010). Although ATP efflux is also associated with necrosis, the magnitude of release during apoptosis (~2% of the total cellular pool of this nucleotide) appears to be much lower than that seen during necrosis (Elliott et al., 2009). ATP/UTP efflux during apoptosis has been shown to be caspase-dependent as a consequence of proteolysis of the membrane channel pannexin-1 (Chekeni

et al., 2010). ATP efflux from apoptotic cells promotes selective recruitment of monocytes, but apparently not neutrophils, which could be due to two factors. On the one hand, low concentrations of ATP have been suggested to be anti-inflammatory, and data from Gregory and colleagues suggest that apoptotic cells may also release anti-inflammatory factors, such as lactoferrin, to suppress neutrophil chemotaxis and possibly their activation (Bournazou et al., 2009). Thus, although ATP release has been identified as a trigger of strong immune reactions, it is possible that release of more modest amounts of this nucleotide may have the opposite outcome during apoptosis.

Another possible "find-me" signal that been reported to be released in a caspase-dependent fashion during apoptosis is the sphingolipid, sphingosine-1-phosphate (S1P), which is generated through hydrolysis of ceramide (Gude et al., 2008). Two S1P kinases (SphK1 and SphK2) have been implicated in the generation of S1P, one of which (SphK2) undergoes caspase-dependent cleavage during apoptosis (Weigert et al., 2010). Proteolysis of SphK2 has been suggested to facilitate its release into the extracellular space where it can generate S1P and trigger chemotaxis of phagocytes toward apoptotic cells. However, an important caveat is that the concentrations of S1P released by apoptotic cells appear to be significantly lower than those required to trigger robust macrophage chemotaxis (Gude et al., 2008). Therefore the relative importance of S1P as a "find-me" signal for apoptotic cells awaits further clarification.

Fractalkine, a germinal center B cell associated chemokine, has also been proposed to serve as a "find-me" signal and is released from cells in a caspase-dependent fashion, although this chemokine is probably not cleaved directly by caspases (Truman et al., 2008). Furthermore, because fractalkine appears to be expressed predominantly by B cells, it is unlikely to represent a general "find-me" signal. This raises the somewhat unpalatable prospect that particular cell types may utilize specific "find-me" signals that are not shared by other cell types. What is perhaps more likely is that there are "find-me" signals common to all cells, as well as additional signals that may be used by particular tissues.

As the preceding discussion illustrates, there is evidence to argue that caspases are instrumental in the generation of both "find-me" and "eat-me" signals that coordinate the swift discovery, recognition and removal of apoptotic cells (Figure 4B).

"Tolerate-Me" Signals: Apoptotic Cells Are Actively Anti-inflammatory

Rapid clearance of apoptotic cells minimizes the probability that such cells will persist in tissues for long enough to undergo secondary necrosis and release DAMPs to awaken the immune system. Notwithstanding this, some studies indicate that apoptotic cells undergo a transformation that actively discourages inflammatory responses upon encountering phagocytes. This suggests that apoptotic cells are not merely passively noninflammatory, but are capable of actively inducing an anti-inflammatory state in macrophages and DCs that encounter such cells (Voll et al., 1997; Fadok et al., 1998; Stuart et al., 2002). How this is achieved is still a matter of debate, but a consistent observation is that direct contact with apoptotic cells triggers the production of the anti-inflammatory cytokine, TGF-β, as well as other anti-inflammatory mediators by the ingesting phagocyte (Voll et al., 1997; Fadok et al., 1998; Lucas et al., 2006). Ucker and colleagues have also reported that direct cell-cell contact between the phagocyte and the apoptotic cell is sufficient to confer this anti-inflammatory state, without recourse to soluble factors (reviewed in Birge and Ucker, 2008). Moreover, late stage apoptotic cells that have begun to leak their contents, and even cellular fractions derived from apoptotic cells, also retain their anti-inflammatory properties (Birge and Ucker, 2008), once again suggesting that alterations to their composition have occurred that have inactivated endogenous DAMPs within.

Collectively, these studies suggest that factors associated with, or released from, apoptotic cells may actively reprogram macrophages to an anti-inflammatory or "wound healing" phenotype. Alterations to the composition of apoptotic cells that renders their contents anti-inflammatory during apoptosis could thus be viewed as "tolerate-me" signals (Figure 4B). The induction of an anti-inflammatory program within a DC that has recently ingested an apoptotic cell (and is therefore loaded with self-antigens) would prevent simultaneous encounters with PAMPs from activating the DC and instigating an immune response against self-antigens. Therefore, the anti-inflammatory properties of apoptotic cells may be crucial for maintaining self-tolerance (Figure 3 and Figure 4B). In addition, the generation of "tolerate-me" signals would also act as a safeguard against situations where apoptotic cells may not always be removed prior to cell leakage. However, the cell-associated factors that confer anti-inflammatory properties upon apoptotic cells have yet to be convincingly identified.

In addition to the anti-inflammatory properties of apoptotic cells there is also accumulating evidence that caspase activation may also directly or indirectly inactivate DAMPs within apoptotic cells. It is also possible that inactive forms of DAMPs could exert anti-inflammatory roles as discussed above. Some of these DAMP-inactivating mechanisms will now be considered.

Switching Off the Alarm System: Caspases Coordinate the Inactivation of DAMPs

The full spectrum of DAMPs released by necrotic cells that are capable of engaging the innate immune system still await clarification, but current evidence suggests that these include cytokines such as IL-1α and IL-33, as well as other nonclassical cytokines and immunostimulatory molecules, such as HMGB1, uric acid, ATP, certain heat-shock proteins, single-stranded RNA, and genomic DNA (reviewed in Kono and Rock, 2008). Additional DAMPs almost certainly await discovery, as this is an active area of investigation at present. DAMPs all share in common the property that they represent "hidden self," as these molecules are not normally found in the extracellular space, thus their presence in this compartment is indicative of a severe departure from normality (Matzinger, 1994; Kono and Rock, 2008). Apart from limiting damage to neighboring cells by avoiding release of cellular contents, one of the major benefits of apoptosis may be to prevent the unmasking of "hidden self" or the conversion of DAMPs into harmless forms, thereby preventing unwanted immune responses (Figure 3 and Figure 5).

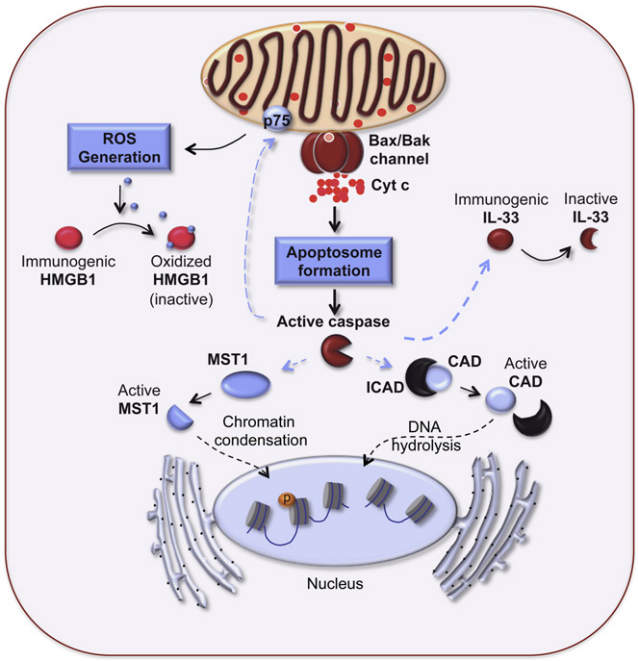

Figure 5. Caspases Coordinate the Inactivation of DAMPs during Apoptosis

Caspase activity, downstream of MOMP, directly and indirectly leads to the inactivation and/or sequestration of multiple endogenous DAMPs such as IL-33, genomic DNA, ATP, and HMGB1.

Recent studies suggest that genomic DNA is an important DAMP, capable of initiating DC maturation and the initiation of immune responses to coadministered antigens (Marichal et al., 2011; Ishii et al., 2001). It has long been known that aluminum salts (Alum) can provoke strong immune reactions to proteins, or protein fragments, that on their own elicit little or no immunity. Indeed, this property is widely exploited in the process of vaccination, to boost immune responses to molecules that would otherwise be ignored by the immune system. However, the molecular basis for the ability of Alum to trigger immune reactions to coadministered antigens has been debated. Recent studies suggest that Alum simply triggers necrosis of cells at the site of administration, leading to release of genomic DNA that acts as a DAMP to activate the immune system (Marichal et al., 2011). Indeed, administration of genomic DNA alone is sufficient to replicate many of the immunostimulatory effects of Alum (Marichal et al., 2011). Strikingly, hydrolysis of DNA with endonucleases strongly attenuated its immune activating properties, as well as those of Alum (Marichal et al., 2011). This may have particular relevance for apoptosis, as a curious feature of this mode of cell death is that genomic DNA undergoes extensive hydrolysis to small ~200 base pair fragments due to the actions of a caspase-activated DNAase (CAD/DFF) that becomes activated during this mode of cell death (Enari et al., 1998). Indeed, this was one of the earliest molecular characteristics of apoptotic cells to be reported but necrotic cells do not manifest any such chromatin hydrolysis (Kroemer and Martin, 2005). It has long been puzzling why extensive DNA fragmentation occurs during apoptosis. However, in light of the recent discoveries suggesting that short DNA fragments are much less effective immune activators than their high-molecular-weight equivalents, it seems plausible to suggest that caspases instigate DNA hydrolysis during apoptosis to dampen the immune activating properties of genomic DNA. Direct evidence in support of this proposal awaits further investigation; however, it is noteworthy that CAD$^{-/-}$ mice develop an autoinflammatory condition on a *DNAaseII*$^{-/-}$ background (Kawane et al., 2003). This appears to be due to incompletely digested DNA within apoptotic cells (due to CAD deficiency), persisting in macrophages (due to DNAase II deficiency) and leading to the activation of a pathway for sensing cytoplasmic DNA fragments, the RIG-I/IRF-3 pathway (Okabe et al., 2009).

Additional evidence in support of the idea that caspases associated with apoptosis suppress responses to endogenous DAMPs, which again may take the form of cytosolic genomic DNA, comes from a series of intriguing studies by Wallach and colleagues (Kovalenko et al., 2009; Rajput et al., 2011). These studies demonstrate that caspase-8 plays an important role in suppressing activation of the RIG-I/IRF-3 pathway, which is invoked in response to cytoplasmic DNA and RNA. During cornification of the skin, the nuclei of terminally differentiating keratinocytes break down and this might be sufficient to trigger activation of the RIG-I/IRG-3 pathway. However, recruitment of caspase-8 to the RIG-I complex results in proteolytic inactivation of RIPK1, a key signaling component of this complex, thereby attenuating expression of IRF-3-inducible genes which include the interferons and other inflammatory factors (Rajput et al., 2011). Conditional deletion of *CASP-8* in the skin leads to a spontaneous inflammatory disease due to excessive RIG-I-dependent IRF-3 activation, quite possibly in response to endogenous cytoplasmic DNA that is produced during keratinocye cornification (Kovalenko et al., 2009).

IL-33, a recently discovered cytokine and member of the IL-1 family, appears to be a bona fide alarmin and is released as a full-length protein during necrosis, but undergoes caspase-dependent proteolysis during apoptosis (Lüthi et al., 2009; Cayrol and Girard, 2009). Although an initial report suggested that IL-33 was activated through caspase-1-dependent proteolysis, several laboratories have comprehensively demonstrated that this cytokine is inactivated by caspases-3/-7-dependent proteolysis during apoptosis (Lüthi et al., 2009; Cayrol and Girard, 2009).

HMGB1 is another molecule that has been repeatedly implicated as a DAMP and represents another proinflammatory molecule whose activity, as well as availability for release, is differentially regulated in a caspase-dependent manner. This chromatin-binding molecule is loosely bound to DNA in healthy cells and is readily released during necrosis (Scaffidi et al., 2002). However, upon condensation of chromatin during apoptosis—a process that is caspase-dependent through protelysis of the Mst1 kinase (reviewed in Taylor et al., 2008)—HMGB1 becomes much more tightly associated to chromatin and its release is dramatically attenuated (Scaffidi et al., 2002). Thus, caspase-dependent chromatin condensation decreases the mobility of HMGB1 during apoptosis, thereby minimizing the potential for escape into the extracellular space to awaken the innate immune system. Furthermore, caspases have also

been implicated in the direct inactivation of HMGB1 through proteoysis of the mitochondrial caspase substrate p75NDUF (Kazama et al., 2008). The later event triggers a burst of reactive oxygen, leading to oxidation of a critical cysteine residue on HMGB1 that abolishes its DAMP activity (Kazama et al., 2008).

Studies also suggest that extracellular ATP has DAMP activity at high concentrations (Idzko et al., 2007). However, ATP production during apoptosis is sharply compromised in a caspase-dependent manner through proteolysis of the mitochondrial electron transport component p75NDUF (Ricci et al., 2004). Cellular ATP concentrations decline precipituously during apoptosis and this can be attenuated through blocking caspase-mediated proteolysis of mitochondrial proteins such as p75NDUF (Ricci et al., 2004). Thus, caspases may directly contribute to reducing the availability of this nucleotide for release if cell clearance is delayed.

Thus there is mounting evidence that caspases directly and indirectly coordinate the inactivation of several important DAMPs during apoptosis (Figure 5), although much work remains to be done to explore how other DAMPs may be affected during this process.

Challenges to the Model

Several observations, at first sight, are difficult to reconcile with the idea that apoptosis-associated caspase activation plays an anti-inflammatory role in mammals, rather than a strictly death-inducing one. Although the majority of Apaf-1-null mice die at birth, a small percentage of these mice do survive to adulthood on the C57BL/6 background without any apparent spontaneous inflammatory phenotype, or indeed any obvious tissue abnormalities (Honarpour et al., 2000). Note that these observations are problematic both for the idea that caspases are required for cell death, as well as for the idea that cell death-associated caspase activation suppresses inflammation. However, one reason for the lack of any obvious inflammatory phenotype in Apaf-1-null animals may be that, in the absence of Apaf-1, caspase activation may still occur via an alternative pathway to effector caspase activation, which remains to be defined. Indeed, Nagata and colleagues have recently shown that death induced by staurosporine treatment, a stimulus that would normally promote caspase activation via the Apaf-1/caspase-9 pathway, is associated with caspase-3 activation in *APAF1*$^{-/-}$ cells (Nagasaka et al., 2010), suggesting the existence of a compensatory route to caspase-3 activation in these animals.

Another possibility is that inflammation due to deficiencies in effector caspase activation only occurs in situations where large numbers of cells undergo apoptosis en masse—during infection or due to pathological injury involving death of large numbers of cells for example. Thus, stochastic rates of cell death under physiological conditions may not result in the release of sufficient quantities of DAMPs to overwhelm natural anti-inflammatory defenses, with caspase-dependent anti-inflammatory mechanisms becoming critical only when large numbers of cells die simultaneously. Therefore, the failure of Apaf-1-null animals to manifest spontaneous inflammation may relate to the threshold of DAMP release that is required to initiate inflammation. To address this possibility, it will be interesting to explore whether Apaf-1-null animals exhibit inflammatory phenotypes upon challenge with cytotoxic drugs or pathogens that provoke robust amounts of cell death within a restricted time window.

The recently described phenomenon of "immunogenic cell death" also appears to be at odds with the view that apoptosis is an immunologically silent mode of cell death (Obeid et al., 2007). Kroemer and colleagues have reported that certain drugs with the potential to induce apoptosis, predominantly anthracyclins, promote immune reactions in vivo that can result in the efficient clearance of tumors (Obeid et al., 2007). The latter observation challenges the idea that a key outcome of apoptotic cell death is the avoidance of immune activation. However, it is important to note that proapoptotic drugs capable of triggering immunogenic cell death were found to be in the minority when compared with numerous other proapoptotic stimuli in this model (Obeid et al., 2007). Second, it is possible that certain proapoptotic stimuli may override an anti-inflammatory outcome because these stimuli are inherently proinflammatory. For example, TNF is a major proinflammatory stimulus that is also capable of inducing apoptosis. Thus, cells dying in response to TNF treatment may also simultaneously produce and secrete proinflammatory cytokines, thereby negating the anti-inflammatory effects of "apoptotic" caspase activation. A similar argument can be applied to Fas/CD95 and TRAIL, which are also capable of triggering the production of proinflammatory cytokines, as well as apoptosis (Leverkus et al., 2003; Farley et al., 2006; Altemeier et al., 2007). Thus, it is plausible that triggers of immunogenic cell death may also promote the production of proinflammatory cytokines concurrently with apoptosis. Third, although anthracyclins may induce apoptosis in vitro, it is also possible that these agents induce a significant amount of necrosis in vivo, thereby releasing DAMPs and instigating inflammation that acts as a driver for tumor clearance. Therefore, the precise nature of the proapoptotic stimulus, in terms of its ability to instigate the production of proinflammatory cytokines, may be an important factor in determining whether apoptosis is immunologically silent or not.

Caspase Activation Also Plays an Anti-inflammatory Role within the Extrinsic Pathway

Much of the preceding discussion has focused upon caspase activation downstream of MOMP within the intrinsic pathway. However, a second major route to caspase activation and apoptosis involves members of the TNF receptor family, which includes TNF itself, Fas/CD95, and TRAIL, among others (Wallach et al., 1999). In this pathway, engagement of the latter receptors with their cognate ligands can recruit caspase-8 and caspase-10 to the cytoplasmic tails of these receptors, via adaptor proteins, and result in the activation of downstream effector caspases. Propagation of caspase activation within the extrinsic pathway occurs either through proteolysis and activation of the BH3-only protein, Bid, which leads to MOMP and/or through direct proteolytic processing and activation of downstream effector caspase-3 and caspase-7 (reviewed in Taylor et al., 2008). Unlike within the intrinsic pathway, neutralization of caspase activation within the extrinsic pathway does block apoptosis, which appears to contradict the idea that caspases play an anti-inflammatory role. However, what is frequently overlooked is that TNF, Fas, and TRAIL are potent

proinflammatory molecules that are capable of triggering cytokine and chemokine production from diverse cell types, through recruitment of RIPK1 as well as other signaling kinases to their receptor complexes (Leverkus et al., 2003; Farley et al., 2006; Altemeier et al., 2007). Inhibition of caspase activation in the latter contexts can enhance death receptor-induced production of proinflammatory cytokines through blocking cell death (Farley et al., 2006; Altemeier et al., 2007), which argues that caspase activation also plays an anti-inflammatory role in these situations.

Furthermore, similar to the role of caspase-8 as a negative regulator of RIG-I/IRF-3-dependent inflammation, as discussed earlier, a raft of recent papers have implicated caspase-8, as well as its regulators FADD and FLIP, as negative regulators of TNF-driven necrosis and inflammation (reviewed by Green et al., 2011). Collectively, these studies suggest that caspase-8 plays a role as an inhibitor of excessive RIPK1 and RIPK3 activation, possibly through direct proteolysis of RIPK1 itself and/or the RIPK1 deubiquitinating enzyme, CYLD (Green et al., 2011). Normally, caspase-8-mediated proteolysis of CYLD and/or RIPK1 restrains the activity of this kinase, keeping its activity within a desirable range. However, in the absence of caspase-8 (or its regulators FADD/FLIP), RIPK1 becomes deubiquitinated, due to CYLD stabilization, which leads to excessive RIPK1-driven RIPK3 activation. Deregulated RIPK3 activation promotes necrosis, also called necroptosis in this context, which is associated with considerable inflammation. This is another good example of caspase-dependent inhibition of molecules, RIPK1 and RIPK3, which have the potential to promote a mode of cell death that leads to robust immune activation. This pathway may act as a failsafe for the detection of viruses and other infectious agents that may inhibit caspase activity.

Evolutionary Conservation of an Immune-Regulating Role for Caspases

We have confined the preceding discussion to mammalian caspases, because it is in these organisms where MOMP rather than caspase activation appears to be the commitment point for cell death within the intrinsic pathway, although this may also apply to other vertebrates. Thus, the anti-inflammatory role of cell death-associated caspases proposed here may be a relatively recent adaptation, with caspases playing a predominantly cell killing-related role in more primitive organisms, such as nematodes. This could be due to the lower risk of autoimmune reactions in less complex and short-lived multicellular organisms that lack adaptive immune systems. However, it is worth noting that cell death-related caspases provide protection against bacterial and viral infections in the worm (Aballay and Ausubel, 2001; Liu et al., 2006) and the fly (Leulier et al., 2000). Therefore, the role of caspases as positive and negative regulators of immune reactions could well have an ancient origin.

Conclusions

As the preceding discussion illustrates, there is now much evidence to argue that a major function of caspase activation during apoptosis is to ensure swift recognition and engulfment of dying cells by phagocytes to prevent cell rupture and avoid release of proinflammatory DAMPs that could activate the immune system. Moreover, caspases are also capable of actively disabling DAMPs, such as IL-33, HMGB1, and genomic DNA, as we have discussed above, to minimize the proinflammatory potential of apoptotic cells. Viewed in this light, caspases activated during apoptosis can be interpreted to function primarily as anti-inflammatory enzymes, rather than cell killing enzymes, serving to avoid the potentially proinflammatory consequences of cell death. This leads to a new perspective concerning the role of the mammalian caspase family, with certain members of this family (the caspase-1-related branch) acting as proinflammatory proteases, and others (the caspase-9/caspase-3-related branch) acting as anti-inflammatory proteases. Thus, the majority of members of the mammalian caspase family can be construed to act as either positive or negative regulators of inflammation. This interpretation is a significant refinement of the existing model, which regards some caspases as cell death effectors and others as regulators of inflammation.

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