Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity

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The immunological consequences of apoptosis have been hotly debated. Apoptosis was originally described as a set of cellular morphological changes that occur in the absence of inflammation but the term has been redefined on the basis of a set of conserved molecular events that include the activation of caspases. Though the apoptosis occurring during normal development is immunologically bland or even tolerizing, the apoptotic death after viral infection or after the ligation of Fas can trigger powerful innate and adaptive immune responses. The molecular machinery at the nexus of apoptosis and inflammation includes caspase-1 – an activator of IL-1β and IL-18 – as well as the double-stranded-RNA-dependent protein kinase pathway and RNaseL pathway, which are key effectors of antiviral immunity. New proapoptotic vaccines induce immune responses that may be able to prevent or treat infectious disease and cancer.

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Abbreviations

CrmA	cytokine-response modifier A
DC	dendritic cell
dsRNA	double-stranded RNA
eIF-2α	eukaryotic translation initiation factor 2α
FasL	Fas ligand
ICE	IL-1β-converting enzyme
TGF-β	transforming growth factor β
TNFR	TNF receptor

Introduction

There is a great deal of death in the normal life of an organism and most of this death is apoptotic. During development, apoptotic death of cells results in the separation of the digits, the creation of the gyri in the brain and the formation of a variety of tubular structures, such as those in the digestive and respiratory systems. The death occurring during development is not inflammatory and hence from an immunological standpoint could be considered bland. Indeed, developmental apoptosis may even be immunologically tolerizing.

We will address three major questions in this review. First, is apoptotic death always bland? Second, what is the molecular basis of both the inflammation that accompanies the apoptotic death that occurs during a viral infection and of the apoptotic death that is induced by the interaction of Fas and its ligand? And finally, perhaps most importantly, can inflammatory signals produced during apoptotic death be used to enhance vaccine function?

Apoptosis is generally referred to in the literature without any modifiers, as if it were of a single type. Further, it is generally thought of as being an immunologically innocuous event that does not activate immune cells, such as dendritic cells (DCs) [1•–3•]. However, rather than being a single entity, apoptosis is complex, adaptable and flexible and the immunological effects of apoptosis vary depending on the circumstances in which it occurs. We argue here that apoptosis can be either bland or inflammatory depending on how it is initiated, in what cell type it occurs and whether or not particular co-factors, for example type I interferons, are present. In this short review, we hope to describe the molecular pathways that trigger inflammation and the activation of both innate and adaptive immunity. We will also explore how these immune responses might be useful in the develpoment of vaccines for infectious diseases and cancer.

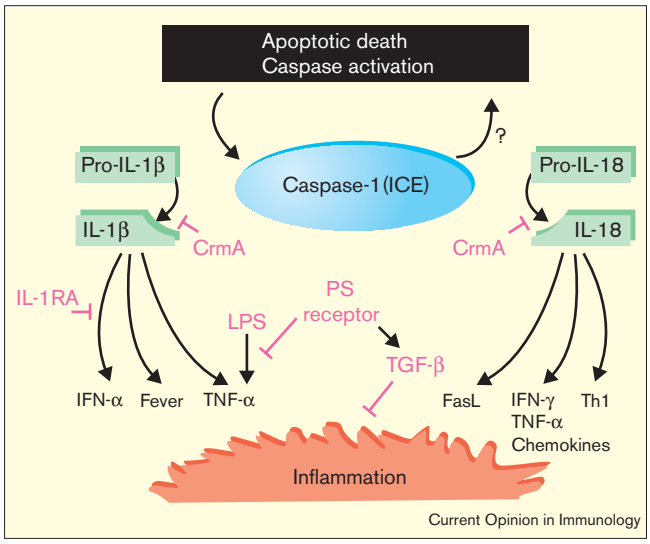
Redefining apoptosis

Though programmed cell death was initially described by Carl Vogt in 1842, the term apoptosis was coined in 1972 by Kerr *et al.* [4] and is derived from ancient Greek ('apo' means 'off', 'ptosis' means 'a falling' e.g. the way that leaves fall from a tree). The original descriptions of apoptosis focus on the characteristic morphological appearance of the dying cells: organelles appear shrunken and nuclear chromatin appears condensed and fragmented. Dense blebs develop and become apoptotic bodies that are phagocytosed by other cells. The lack of inflammation was another hallmark property of death by apoptosis [4,5].

Apoptosis was originally defined in contrast to necrosis, a process resulting from sudden and catastrophic cellular destruction, such as that caused by hypoxia, hyperthermia or physical injury. Necrotic cells have a have a loss of membrane integrity and the entire cell, as well as nucleus and mitochondria, swells. The chromatin initially clumps then disperses in a process known as karyolysis. Thus, cells dying by apoptosis have a very different appearance from those dying by necrosis. Furthermore, in sharp contrast to apoptosis, necrosis was described as "usually" being associated with inflammation [5].

A process of redefinition was initiated by Andrew Wyllie — one of the original authors of the paper [4] in which the term apoptosis was coined — when he observed [6] that the morphological changes that were originally described were "…closely associated with excision of nucleosome chains from nuclear chromatin, apparently through activation of an intracellular, but nonlysosomal, endonuclease…". Since these early studies, the molecular components of the apoptotic death machinery have been further defined. The premier genetic system for studying

Figure 1



Death and inflammation: how caspase-1 (ICE) activates IL-1β and IL-18 to induce innate and adaptive immunity and how this inflammation may be modulated. Apoptotic death induced by a variety of factors (such as ligation of Fas by its ligand) triggers a caspase cascade that can include ICE. Caspase-11 may be involved in the activation of ICE or may form a complex with it [16]. Activated ICE is then capable of cleaving the pro-forms of IL-1β and IL-18, which in turn trigger other proinflammatory cytokines. There is also evidence that activated ICE plays a role in the induction of apoptosis under some conditions (hence the question mark) [19••]. During poxviral infection, CrmA can block the activity of ICE. A number of other factors may regulate or even abrogate the activation of innate and adaptive immune responses: ICE, or its substrates IL-1β and IL-18, may not be expressed by the dying cell; IL-1-receptor antagonist (IL-1RA) may modulate or block the effects of IL-1β; and macrophages that take up apoptotic cells through the phosphatidylserine (PS) receptor do not produce TNF-α in response to lipopolysaccharide (LPS) but may produce the anti-inflammatory cytokine, TGF-β [34••].

the molecular machinery of apoptotic death is *Caenorhabditis elegans*, in which death genes and their protein products have been defined. In mammals, orthologs of these molecules have been identified and further characterized *in vitro* and *in vivo*.

The concept that has now become well accepted is that apoptosis involves a shared biochemical suicide program that exists in most cells and is turned on by a variety of normal developmental and pathogenic triggers, as well as by experimental manipulations. Though the original definition of apoptosis excluded inflammation, it has now become apparent that, in some cell types under certain circumstances, the generation of inflammatory signals can be an intrinsic component of the apoptotic machinery.

How members of the TNF-receptor family can induce death and inflammation

Our own interest in inflammatory apoptosis came from experiments in which we studied the expression of Fas ligand (FasL; also known as CD95L or APO-1L) by tumor cells [7,8,9•,10,11]. It was initially believed that Fas and its ligand mediated immune privilege and, furthermore, that FasL transfection could make transplantation easier and enable tumors to escape [12]. However, it was found that expression of FasL on β cells in the pancreas resulted in rapid rejection of islet cells accompanied by a massive inflammatory response. Tumors expressing FasL also are rapidly rejected (summarized in [9•]) and the sites of these regressing tumors coalesce into abscesses, thus linking the activity of a death receptor and an inflammatory immune response.

The way in which these events are linked with cellular death are becoming clearer. Initiation of apoptosis occurs when FasL — a member of the TNF family of proteins — interacts with its receptor, Fas. Not surprisingly, Fas is a member of the TNF receptor (TNFR) family of proteins that also includes a handful of other death receptors [13•]. The intracytoplasmic death-domains of these receptors activate a cascade of cysteine proteases — called caspases — via a set of adaptor molecules [13•,14,15].

There is ample evidence that, in some cell types, inflammatory signals can be an intrinsic part of this caspase cascade and this can activate innate and adaptive immune responses. One caspase at the nexus of death and inflammation is caspase-1, also known as IL-1β-converting enzyme (ICE) (Figure 1). Other ICE-like caspases that may be important for inflammation are caspase-4, -5, -11, -12 and -13 but their full activities remain incompletely elucidated [16–18]. ICE is rapidly activated by some apoptosis-inducing stimuli, including ligation of Fas by its ligand.

In addition to being activated during death, there is some evidence that ICE itself can be involved in the induction of apoptotic death. Indeed, ICE is a requisite component of the apoptotic death induced by *Shigella flexneri* in macrophages [19••]. However, ICE is not an essential component of developmental apoptosis: ICE-knockout mice develop normally. Its true function is as a mediator of the inflammatory events that can accompany apoptosis and ICE mediates this inflammation by activating IL-1β and IL-18.

In experiments using a variety of mouse tumors transfected with FasL, signaling through Fas has been directly linked to production of IL-1β [20]. Ligation of Fas is also known to induce IL-18 and — as a consequence — the inflammatory chemokine, IL-8 [21]. ICE-activated IL-1β augments DC maturation [22] and migration [23], and can activate NF-κB, triggering a variety of immune-activating cytokines and chemokines. IL-1β receptors can be found in one form or another on most cell types [24,25,26•]. In addition, IL-1β is the most potent known endogenous pyrogen [27•,28].

The cleavage of pro-IL-18 into active IL-18 by activated ICE is structurally similar to the cleavage of pro-IL-1β. Like IL-1β, only the mature form of IL-18 is bioactive and pro-IL-18 lacks a signal sequence. The external receptors

for IL-1β and IL-18 are different but the intracellular signaling pathways are identical [29]. Previously known as IFN-γ-inducing factor (IGIF), IL-18 induces NF-κB and has a number of other activities including the induction of FasL, TNF-α and chemokines, the augmentation of IFN-γ release after IL-12 stimulation and the stimulation of a Th1-type response [30–32].

It is known that Fas can induce apoptotic death through two different pathways but it is not known how these pathways differentially promote inflammation [33]. Inflammatory components of apoptosis may be regulated, in part, by controlling the expression of the 'pro' forms of IL-1 and IL-18, which are primarily produced in monocytes and macrophages. A receptor antagonist has been described for IL-1 that may counteract the inflammatory activities of IL-1β. Finally, potentially anti-inflammatory cytokines, including transforming growth factor β (TGF-β), are produced by macrophages upon the uptake of apoptotic cells via the phosphatidylserine receptor that may regulate or abrogate inflammatory signals [34••]. Regulatory events at the nexus of death and inflammation are likely to determine whether apoptosis is bland or inflammatory.

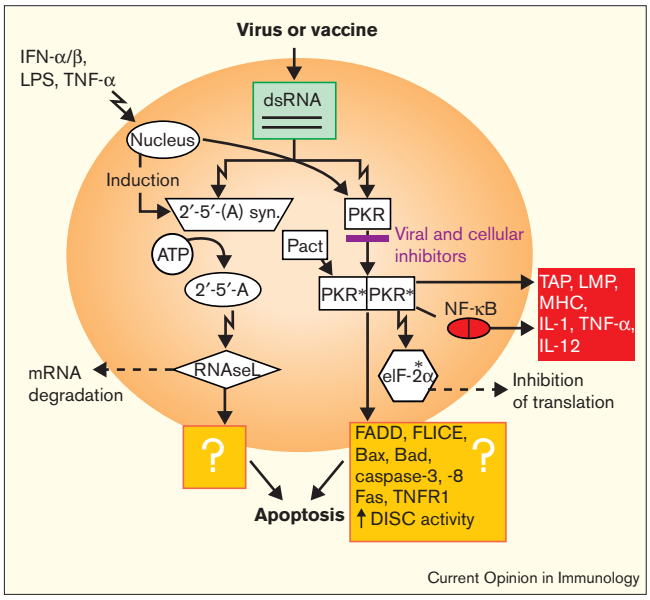
How double-stranded RNA induces both death and inflammation: the PKR and RnaseL pathways

Another example of inflammatory apoptosis is cell death associated with the recognition of double-stranded RNA (dsRNA). Many viruses, especially some RNA-based viruses, mediate the expression of dsRNA during transcription of overlapping RNA species or during viral replication. Higher organisms have evolved a number of redundant and complementary pathways for the recognition of dsRNA, including the dsRNA-dependent protein kinase (PKR) pathway and the 2′-5′-oligoadenylate-synthetase/RNaseL pathway. Activation of either of these pathways can induce apoptotic death. Though less is known about RnaseL pathway, the PKR and RNaseL systems can also induce robust innate and adaptive immunity (Figure 2).

The RNaseL pathway starts with the IFN-α/β-induced 2′,5′-oligoadenylate synthetases, a family of enzymes capable of linking ATP into oligomers of adenosine [35,36]. These 2′-5′-linked oligomers are capable of activating RNaseL, an enzyme named for its ability to degrade mRNA. The consequent inhibition of protein synthesis is reinforced by the activity of PKR, a serine/threonine kinase capable of dimerization and subsequent autophosphorylation in the presence of dsRNA [37,38].

The recent solution of the structure of human PKR provides insight into the mechanism of its activation by dsRNA [39]. Once PKR is phosphorylated (activated), it can phosphorylate the eukaryotic translation initiation factor 2α (eIF-2α). The phosphorylated eIF-2α can then be chemically linked with guanosine by an enzyme called guanosine exchange factor and thus be inactivated. When functional eIF-2α is phosphorylated, protein translation cannot efficiently occur [40].

Figure 2



How both apoptotic death and immunity may be triggered by dsRNA. The cellular 'death-inducing' events after exposure to dsRNA – following virus infection or vaccination – include the RNaseL and PKR pathways, which destroy mRNA and stop translation, respectively. Though the mechanisms are not fully known, apoptosis is induced in part by enhancing efficiency in the transmission of death signals through the adaptor, FADD, and enhanced activity of the death-induced signaling complex (DISC) [77,78]. Two proapoptotic members of the Bcl-2 family, Bad and Bax, are also induced. The PKR pathway can be activated (phosphorylated; this is indicated by the asterisks) by an endogenous gene product called Pact or inhibited by a variety of viral and cellular inhibitors that can act at a number of points in the pathway; these are summarized in [79]. Activated PKR also induces NF-κB and a variety of molecules that facilitate T cell recognition of infected targets (red box). LPS, lipopolysaccharide; syn., sythetase; TAP, transporter associated with antigen processing.

Thus, the PKR and RNaseL pathways shut-down translation and cause mRNA degradation. But the death observed after activation of these pathways is not merely due to the inhibition of host protein synthesis. The rapid death is mediated by a caspase cascade and thus is apoptotic. Our own experiments have demonstrated that replicon-containing nucleic-acid constructs (described in more detail below) that are known to mediate the production of dsRNA can induce caspase-dependent apoptotic death [41•]. Furthermore, phosphorylation of PKR has been linked to the elaboration of the death receptors, Fas and TNFR1 and to the activation of caspase-3 and -8 [42] (Figure 2).

In addition to its death-enhancing functions, phosphorylation of PKR also has immune-modulating effects mediated in part through its induction of NF-κB [43]. These include the induction of the expression of a number of molecules — including MHC molecules, the LMP-2 and -7 components of the proteasome, and the ATP-dependent transporters associated with antigen processing (TAPs) — that facilitate T cell recognition of targets. Thus, immune activation and

apoptotic death are both intrinsic components of the PKR and RNaseL pathways and form important links between cell death and immunity.

Viral blockade of apoptosis and inflammation

Viral infection of host cells triggers mechanisms that, if effective, can limit viral replication. As described above, cells respond to viral infection with apoptosis and the activation of innate and adaptive immunity. It has long been known that viruses encode proteins that counter these cellular defenses [44•,45–50] but the recent characterization of anti-apoptotic viral genes that double as anti-inflammatories sheds new light on how death and immunity are linked.

One example involves a viral protein called M11L. Myxoma viruses lacking M11L are highly attenuated. Despite their lack of virulence, lesions are characterized by vigorous inflammatory activity that far exceeds the inflammation associated with the wild-type virus [51,52]. In addition to its anti-inflammatory activity, M11L is also antiapoptotic at the mitochondrial checkpoint — downstream caspase-3 is not activated [53]. Interestingly, only the M11L-knockout virus induced apoptosis in monocytes, which are known to be capable of releasing activated IL-1β and IL-18 upon their death by apoptosis. Like another antiapoptotic viral protein, the poxviral serpin cytokine-response modifier A (CrmA) which inhibits ICE [54], observations with M11L provide a link between apoptosis and virally induced inflammation. These viral proteins may mimic the activity of natural inhibitors of ICE activity such as the serpin PI-9, whose downregulation in human vascular smooth muscle cells has recently been implicated in the inflammatory pathogenesis of atherosclerosis [53].

Inflammatory apoptosis compared with bland apoptosis in vaccine design

It may be possible to induce inflammatory apoptosis as a way of enhancing vaccine function. Others and we have focused on developing recombinant and synthetic cancer vaccines [55–60], especially those targeting normal 'self' antigens [61••,62–71]. We have previously reported on a new type of 'naked' nucleic-acid vaccine in which the gene encoding an antigen is inserted with an RNA replicase from an alphavirus (called a replicon) to form an RNA- or DNA-based immunogen [41•,72•,73,74]. The replicon mediates the copying of positive-stranded RNA to negative-stranded RNA and back again in the cytosol. Replicon-containing plasmids are 100–1000 times more efficient at eliciting immune responses than conventional DNA vaccines. Though initially designed as a way of enhancing antigen production *in vivo*, replicon-containing plasmids do not produce measurably more antigen that do conventional DNA vaccines. Instead, replicon-based nucleic-acid vaccines may be more effective at inducing immune responses because they employ qualitatively different mechanisms for immune activation: specifically, they induce caspase-dependent death in host cells [41•,42,43,44•,72•].

The apoptotic death mediated by these vaccines is a likely consequence of the requisite production of dsRNA intermediates that results from the activity of the replicon. dsRNA then can activate the PKR and 2′-5′-oligoadenylate synthetase pathways described above. Though these pathways may limit antigen production by mimicking viral infection [50], they also may activate the immune system. There are a variety of gene products known to modulate these pathways that may be useful in vaccine design (see Figure 2). It is not yet known how viral and cellular modulators of the PKR and 2′-5′-oligoadenylate-synthetase pathways will affect vaccine function.

The critical question from a vaccinologist's perspective is whether a particular dying cell contains signals that stimulate DC maturation. This activity may not be identical to factors known to induce 'inflammation' as described above. DC maturation signals can be obtained from supernatants of freeze-thawed 'necrotic' cells but these factors have not yet been fully characterized [1•,2•]. Apoptotic death of some cell types but not other types may induce the migration and maturation of DCs. Candidate signals that mediate these activities include chemokines such as CCR6 [75] and cytokines, for example IL-1β and type I interferons [22,23,76].

Conclusions

Apoptotic cell death can induce inflammation and promote the activation of an immune response. Apoptosis-associated immune effects are mediated by caspase-1 and other potentially inflammatory caspases, and by innate components of the antiviral machinery. The design of more effective vaccines for infectious diseases and cancer may be one of the first applications of a better understanding of the molecules at the nexus of apoptotic death, and innate and adaptive immunity.

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- • of special interest
- •• of outstanding interest

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