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How dying cells alert the immune system to danger

Hajime Kono, M.D. Ph.D and Kenneth L. Rock, M.D.

Department of Pathology University of Massachusetts Medical School 55 Lake Avenue North
Worcester, MA 01655

Abstract

When a cell dies *in vivo* the event does not go unnoticed. The host has evolved mechanisms to detect the death of cells and rapidly investigate the nature of their demise. If cell death is a result of natural causes, that is, it is part of normal physiological processes, then there is little threat to the organism. In this situation, little else is done other than removing the corpse. However, if cells have died as the consequence of some violence or disease, then both defence and repair mechanisms are mobilized. The importance of this process to host defence and disease pathogenesis has only been appreciated relatively recently. This article will review our current knowledge of these processes.

The immune system was recognized in ancient times and rediscovered by Jenner and Pasteur based on its ability to confer protection upon repeat exposure to a pathogen. Through subsequent studies by Von Behring and others it rapidly became apparent that the immune system had the potential to respond not only to whole microorganisms, but to virtually any molecule that was “foreign” to the host. However, whereas injection of such molecules would often provoke a robust immune response, this did not invariably occur. Immunization protocols improved in the 1920s with the discovery by Ramon and Glenny of immunostimulatory molecules (adjuvants [G]) that could boost immune responses to co-administered antigens. Adjuvants were typically of microbial origin and became widely used to promote the effectiveness of immunizations. In the 1960s, Dresser showed that a foreign protein when highly purified would only elicit an immune response if it was admixed with a microbial adjuvant¹. Injected by itself, the antigen not only failed to elicit immunity but actually induced a state of tolerance². However, the significance of these observations was not well appreciated and adjuvants remained one of those things that everyone used because they were part of standard operating procedures.

In 1989 Janeway put these empirical observations into a conceptual framework³ (Fig. 1). He proposed that the immune system did not respond to all foreign antigens but only to those that are potentially associated with infection. The underlying idea here was that the immune system evolved to protect organisms against microorganisms and this discrimination between infectious versus noninfectious antigens focused defences on real threats rather than innocuous situations.

At this time, it was already recognized that in order to stimulate T cell responses, antigens had to first be acquired and presented on MHC molecules of an antigen presenting cell (APC). Moreover, it was further known that APCs also provided additional costimulatory signals necessary to activate T cells. Janeway incorporated these principles into his model (Fig. 1). He postulated that the discrimination between infectious and noninfectious non-self molecules was made by the APCs of the innate immune system through receptors that would recognize pathogen-associated molecular patterns (PAMPs) made by microorganisms that were

molecularly distinct from those made by mammals. PAMPs are naturally associated with infections and the active ingredients used in many adjuvants. Upon recognition of such molecules, the APCs were stimulated to express all of the signals needed to activate naïve T cells. This idea presaged the discovery of Toll-like receptors (TLRs) and other microbial sensors ^{4, 5}.

We now know that Janeway's model is largely correct. However, it could not explain all immune responses. In this article we will discuss the evidence supporting the concept that the immune system has also evolved mechanisms to sense primary or secondary necrotic cell death (which we will henceforth refer to as simply necrotic cell death) and respond to it with innate and adaptive immune responses. We will highlight both what's known and as yet unknown about the mechanisms underlying these processes and how they may contribute to health and disease.

The Danger hypothesis

Janeway's hypothesis did not explain why robust T-cell immunity was generated to tissue transplants, some tumours and also in autoimmune diseases because these were all situations in which there were often no obvious microbial components (although in some situations microbes may help initiate these responses). Another possible exception was certain viral infections, because all viral molecules are synthesized by the host and don't contain obvious unique features (although it is now clear that there are molecular features of some viruses that are recognized by TLRs and other sensors like helicases ⁶).

Considering these exceptions led Matzinger to propose the danger hypothesis in 1994 (Fig. 1) ⁷. She postulated that the adaptive immune system evolved to respond not to infection *per se* but to non-physiological cell death, damage or stress. According to this idea, abnormal cell death is a potential threat to the organism whether it is caused by an infection or other pathological process and could be a universal sign of danger. This could explain how adaptive immune responses were stimulated by both infectious and noninfectious agents, such as tumours and transplanted tissues, all situations in which necrotic cell death would be occurring. In the danger model, dying cells were postulated to release endogenous adjuvants that, using similar nomenclature to PAMPs, have been called damage-associated molecular patterns (DAMPs); for simplicity we will use this terminology even though it is not clear whether endogenous danger signals really have distinctive molecular patterns as do PAMPs, although some have been proposed ⁸. Although the term DAMP was originally introduced to cover damage-associated-molecular patterns in all living organisms⁸, we will use it here primarily to refer to danger signals of non-microbial origin. Like PAMPs, DAMPs have been proposed to activate local APCs to become stimulatory to the adaptive immune system.

The danger hypothesis was proposed entirely on theoretical grounds and at the time of its conception there was little experimental evidence to support its central tenets. It was known that cells by themselves, including dead ones, could be immunogenic ⁹, but whereas such findings were consistent with the model, there was no direct evidence that cellular immunogens actually contained adjuvant activity. However, it was subsequently shown that when dead cells were admixed with an antigen and injected into animals, they augmented antigen-specific CD4⁺ and CD8⁺ T-cell responses ^{10 11}. This was formal proof that dead cells contained endogenous adjuvants that promoted T-cell immunity. It was also found that dead cells could stimulate dendritic cells (DCs) to mature into immunostimulatory cells in culture ¹⁰ and promote the migration of mature DCs into draining lymph nodes in animals ¹².

When healthy cells were treated with protein synthesis inhibitors, their endogenous adjuvant activity was not reduced ¹¹. Moreover, when healthy cells were killed instantaneously by

freeze-thawing, they provided adjuvant activity^{10 11}. Therefore, some or maybe even a majority of DAMPs come from preexisting molecules.

If DAMPs are preexisting in cells, then how does the innate immune system distinguish dead cells from live ones? The key event upon necrotic cell death is thought to be the release of DAMPs from intracellular stores, which we will refer to as the hidden self model (Fig. 2). In support of this notion, cytosol obtained from healthy cells has adjuvant activity, indicating that DAMPs are present intracellularly¹¹. It isn't yet clear whether there are also membrane-associated DAMPs, but nuclei have little adjuvant activity¹¹. In living cells, these intracellular molecules are normally sequestered from the innate immune system by the plasma membrane. However, when cells undergo necrosis they lose the integrity of their plasma membrane and release their intracellular contents, including the cytosolic DAMPs, into the extracellular milieu. This can also occur when apoptotic cells aren't rapidly cleared (Fig. 2). Thus, the release of DAMPs into the extracellular environment serves as a sign of death to the innate immune system.

Danger and disease

The impetus for proposing the danger hypothesis was the observation that strong immune responses were generated to transplanted tissue, to tumours and in autoimmunity, all in the apparent absence of microbial infection. However, what is the actual evidence that cell death and/or specific DAMPs are involved in these responses? There are now an increasing number of examples in which cell death has been linked to the initiation of these immune responses. In the case of autoimmunity, a developmentally programmed death of pancreatic β -islet cells precedes the initiation of autoimmune diabetes in the Non-Obese Diabetic (NOD) mouse [G]¹³. Preventing this death by treating NOD mice with a caspase inhibitor blocked the subsequent development of autoimmunity¹⁴. Similarly, when β -islet cell death is induced, autoimmune diabetes can be triggered¹⁵. Such effects are not just limited to autoimmune diabetes. Thus, injury to one eye can initiate an autoimmune response that destroys the contralateral eye and ischemic necrosis of the heart can lead to an autoimmune pericarditis^{16 17}. In the case of tumours, injection of dead cancer cells can stimulate T-cell responses⁹. Moreover, killing tumour cells with chemotherapeutic agents *in vivo* can also lead to increased T-cell immunity¹⁸. There is less direct evidence for this phenomenon in transplants, but it is clear that the more ischemic damage there is in an organ before it is transplanted, the more rapidly it is rejected¹⁹. The concept that cell death stimulates immune responses has often raised the question as to why cell injury and its attendant release of cell proteins and adjuvants does not lead to autoimmunity more frequently; that it does not is in fact not surprising (see Box 1).

Although the above observations are consistent with the danger hypothesis, the observation that cell death helps to stimulate immune responses does not in itself prove that this is occurring through the release of DAMPs (as opposed e.g. to the exposure of cellular antigens). However, there is limited evidence that the depletion of one DAMP (uric acid, discussed below) can reduce the activation of T cells to an autoantigen²⁰, some cancers²¹ and a transplanted cell line²⁰. However, the loss of this DAMP did completely inhibit these responses and for responses to some other transplanted tumours or tissues the loss had no effect (A. Hearn, Y. Shi, D. Greiner and KLR, unpublished). These incomplete effects are presumably due to additional DAMPs that were not eliminated, although it cannot be ruled out that DAMPs have only a minor role in these responses. To determine the full contribution of danger signals to immune responses, it will be necessary to elucidate all of these molecules and then test the effect of eliminating them.

DAMPs that function as adjuvants

In this Review we restrict our discussion of DAMPs to host molecules that are not classical cytokines. Before reviewing specific DAMPs, it is important to discuss the criteria for establishing a candidate molecule as a bona fide DAMP. First, a DAMP should be active as a highly purified molecule. Second, it is important to show that its biological activity is not due to contamination with microbial molecules (PAMPs). Such contaminants are easy to introduce, may co-purify by binding tightly to host molecules, and many have similar immunostimulatory activities. Caution is especially warranted if the putative DAMP is found to work through receptors for PAMPs, such as TLRs. Third, the DAMP should be active at concentrations that are actually present in pathophysiological situations. Fourth, selectively eliminating or inactivating the DAMP (for example, with antibodies, specific enzymes, targeted mutations or [define]RNAi[G]) should ideally inhibit the biological activity of dead cells in *in vitro* and *in vivo* assays. Of course if the assay system is reading out several different DAMPs with redundant activities, then this final criterion may be difficult to establish. As discussed below, these criteria have largely been met for only a few DAMPs identified so far.

Heat-shock proteins

One of the first sets of endogenous cellular molecules that were described to have potential adjuvant properties were heat-shock proteins (HSPs). Injection of HSP-peptide complexes into animals was shown to promote immune responses to the bound antigenic peptides²². This effect was due in part to the ability of HSPs to target the associated peptides into APCs²². However, in addition, the HSPs seemed to provide an adjuvant activity as they could augment immune responses when admixed with antigens and injected into mice²³. Moreover, purified HSPs have been shown to stimulate DCs to mature *ex vivo*²⁴ and to also stimulate these APCs to migrate to lymphoid organs *in vivo*²⁵. However, whether these immunostimulatory activities are due to the HSPs themselves is controversial. A number of studies have suggested that the immunostimulatory activities of HSPs are due to contaminating microbial products, such as lipopolysaccharide (LPS)²⁶, or other bioactive molecules (eg., concanavalin A) introduced during the purification procedure. Experiments have not tested the effect of depleting HSPs on the adjuvant activity of dead cells; this is not a trivial experiment given the number of different HSPs that might have to be eliminated. Therefore, the importance of HSPs as DAMPs is not yet clear.

Uric acid

A second cellular molecule with adjuvant activity is uric acid. This was identified in a study that purified from cells adjuvant activities for CD8+ T cell responses²⁷. The active form of this molecule is thought to be monosodium urate (MSU) microcrystals that form when uric acid is released into the sodium-rich extracellular fluids. Thus, this is an example in which a DAMP becomes active by both release from an intracellular pool and a change in its structure. Monosodium urate crystals were shown to stimulate DCs *in vitro* and have adjuvant activity *in vivo*. Importantly, the adjuvant activity of cells was reduced by enzymatic depletion of uric acid, indicating that it is a major DAMP, at least in some cells²⁷²⁰. However, uric-acid depletion did not eliminate all endogenous adjuvant activity from dead cells and cytosol was found to contain other activity²⁷²⁰, indicating that there must be other important DAMPs.

HMGB1

The high mobility group box 1 protein (HMGB1) also has adjuvant activity²⁸. HMGB1 is an intracellular DNA-binding protein that stabilizes nucleosomes, has a role in bending DNA, and regulates transcription. Certain viable leukocytes, such as DCs, can be stimulated to release HMGB1 by a nonclassical secretion mechanism and once in the extracellular fluids this molecule functions as a cytokine²⁸. Because HMGB1 was identified as an inflammatory

DAMP released from necrotic cells ²⁹ (see below), it was tested for adjuvant activity. It was found that when recombinant HMGB1 was added to immunizations with soluble antigen it boosted antibody responses and with cellular antigens it induced protection against tumour challenge ³⁰. In addition, supernatants from necrotic fibroblasts provided adjuvant activity when added to a tumour immunization. When this necrotic supernatant came from HMGB1-deficient cells or was neutralized with specific antibody its adjuvant activity was reduced by about 50%, indicating that HMGB1 is an endogenous adjuvant, but not the only one in these cells ³⁰. HMGB1 was also shown to play roles in the tumor immunity developed by chemotherapy- and radiotherapy-induced cell death by selective inhibition with neutralizing antibodies or RNAi ³¹. Consistent with its adjuvant activity *in vivo*, HMGB1 was found to stimulate cultured DCs to mature³⁰. These data indicate that HMGB1 contributes to the endogenous adjuvant activity of at least some cells.

Genomic dsDNA

Yet another molecule shown to have endogenous adjuvant activity is double-stranded (ds) genomic DNA. Mouse dsDNA stimulated cultured DCs to mature, although it did not induce cytokine secretion ³². This stimulatory activity was perhaps not surprising because unmethylated CpG-rich DNA is known to stimulate dendritic and other cells through TLR9, and such sequences are present in mammalian DNA, albeit at low frequency ³³. However, mouse genomic DNA still stimulated DCs after being methylated and was inactivated by denaturation into single strands, suggesting that its activity was not entirely due to TLR9 recognition of CpG sequences ³². When purified dsDNA was mixed with an antigen and injected into mice it functioned as an adjuvant and boosted both antibody and CD8+ T-cell responses ³². How much of the adjuvant activity from dead cells is provided by dsDNA is not clear because depletion experiments have not been performed. However, in other studies when subcellular fractions were tested for adjuvant activity for CD8+ T cells *in vivo*, cytosol was active but purified nuclei were not ¹¹.

Collectively, the above studies show that cells contain multiple DAMPs. There remain a number of important unresolved questions. How many DAMPs exist? There are almost certainly additional ones to be discovered because other endogenous adjuvant activities have been detected in cell fractions that seem to be distinct from the DAMPs identified so far. (Y. Shi, HK and KLR unpublished). Which DAMPs are the most important ones and does this vary among different cell types or pathophysiological conditions? Finally, do all DAMPs elicit the same biological responses or do different ones have specialized functions? So, the complexity of the repertoire of DAMPs is at present unclear.

Cell death, DAMPs and inflammation

When cells die *in vivo*, the innate immune system not only alerts the adaptive immune system to potential danger, but also induces an inflammatory response ³⁴. In this process mediators are generated that act on the local vasculature in ways that cause arterioles to dilate and venules to leak fluid and recruit leukocytes from the blood into the tissue (Fig. 3). The net effect of these processes is to increase blood flow and deliver protein-rich plasma and leukocytes to the site of injury. Grossly, this causes the signs and symptoms of redness, heat and swelling that are the hallmarks of an inflammatory response. The inflammatory response to cell death is robust and is so stereotypical that on pathological examination the time of tissue injury can be established based on how far this response has progressed.

The inflammatory response to necrotic cell death was recognized long before the danger hypothesis was conceived. Although this response was not part of the danger hypothesis, we believe that it serves a similar function and is best viewed as a component of the same overall process. So, as discussed above, necrotic cell death is potentially dangerous and in response

to this threat the innate immune system responds both directly with inflammation and also by calling in reinforcements from the adaptive immune system, such as preexisting antibodies and complement (Fig. 3). While this is potentially useful for defence and repair, the host pays a price for marshalling the inflammatory response and it is one that can be quite costly (Box 2).

The basic underlying mechanisms by which dead cells trigger inflammation and adaptive immune responses are very similar. Thus both responses can be stimulated by DAMPs that are preexisting and sequestered in the interior of cells³⁵ (HK, unpublished data). Moreover, there is evidence that at least some of the DAMPs that stimulate inflammation are the same ones that provide adjuvant activity, as will be discussed further below (Table I). In fact, this overlapping activity could reflect a mechanistic link between inflammation and adjuvant effects. However, it is presently unknown whether all of the proinflammatory DAMPs are ones with adjuvant activity and whether inflammation is always necessary for adjuvant activity. To answer these unresolved questions it will be necessary to elucidate and characterize the various DAMPs.

There has been some progress recently in identifying proinflammatory DAMPs. They can be broadly subdivided into two categories based on their origin and mechanism of action (Fig 3). In one class, the DAMPs are bioactive mediators of cellular origin that directly stimulate cells of the innate immune system. These structures are recognized by known or as yet unknown receptors. In the other class, molecules released from dead cells work more indirectly by generating DAMPs from other extracellular molecules (some may also work by activating extracellular mediators such as complement, which many would not consider to be DAMPs). The actual bioactive mediator in these cases comes from extracellular components. We will consider each class of these DAMPs separately. Again, we will restrict our discussion of DAMPs to host molecules that are not classical cytokines.

Intracellular DAMPs with proinflammatory activity

HMGB1, which was discussed for its adjuvant properties above, was first identified as a DAMP based on its role in inducing inflammation²⁹. HMGB1 released from necrotic cells was shown to stimulate monocytes *in vitro* to produce proinflammatory mediators³⁶ and injection of purified HMGB1 into mice stimulated an acute inflammatory response³⁷. However, it presently is unclear whether all of these effects are due to HMGB1 itself or an associated molecule. This is because HMGB1 binds tightly to certain molecules of microbial or cellular origin and in some experiments highly purified HMGB1 had little proinflammatory activity³⁸. It also isn't clear to what extent HMGB1 from dead cells contributes to the inflammatory response. After toxic or ischemic damage in the liver, treatment with HMGB1-specific antibodies reduces the inflammation that ensues, but only partially²⁹. Although this implicates a role for HMGB1 in these responses, it is uncertain whether the source of HMGB1 is from injured cells and/or from living leukocytes which secrete HMGB1 after activation. However in a more direct test of this issue, injection of dead HMGB1-negative fibroblasts stimulated inflammation as well as HMGB1-sufficient cells³⁵. Therefore, HMGB1 is not the only, or maybe even the major, proinflammatory DAMP released from dying cells.

Another DAMP with adjuvant activity that may also have a role in stimulating inflammation to dead cells is uric acid. As discussed above, cells contain very high levels of uric acid that is released after necrotic cell death into the sodium-rich extracellular fluids and converted to MSU. It has long been known that when MSU crystals form spontaneously in patients with hyperuricaemia they cause the acute inflammation of gout. Moreover, injection of pure MSU into animals or humans causes inflammation³⁹. Therefore, the uric acid released from dead cells might contribute to initiating the ensuing inflammatory response.

Two other sets of molecules that have been shown to have proinflammatory activity both *in vitro* and *in vivo* are galectins and thioredoxin. Galectins are cytosolic lectins that can bind a number of cell-surface receptors including CD2 and CD3 which can stimulate leukocytes⁴⁰. They are expressed primarily in certain leukocytes and endothelium and therefore their potential contribution would be limited to these cells. Thioredoxin is a ubiquitous intracellular enzyme with antioxidant activity⁴¹. Both galectins^{42–44} and thioredoxin^{45, 46} have been shown to stimulate various leukocytes and endothelium *ex vivo* and when injected *in vivo* to stimulate inflammation. To what extent these molecules actually contribute to the death-induced inflammatory response, if at all, is unknown.

There are a number of other intracellular molecules that have been reported to stimulate the production of proinflammatory cytokines from leukocytes primarily in *in vitro* experiments and therefore are potential candidates for contributing to death-induced inflammation. These include HSPs^{47, 48} 4950, S100 proteins^{51, 52}, nucleosomes^{53, 54}, purines such as ATP and adenosine^{55, 56}, antimicrobial peptides^{57, 58} 59 and N-formylated mitochondrial peptides⁶⁰. The contribution of these molecules to death-induced inflammation *in vivo* is also unknown.

Extracellular DAMPs and other mediators.

Non-muscle myosin heavy chains (type IIA and C) are released from dead cells and are not themselves proinflammatory. However, normal animals have circulating natural IgM antibodies [G]specific for these cellular proteins that bind the released myosin molecules⁶¹. The resulting immune complexes activate the complement cascade, leading to the generation of the proinflammatory fragments C3a, C4a and C5a. That this process is important to death-induced inflammation, in at least some settings, is suggested from a number of findings. Immunodeficient mice that lack antibody show reduced inflammation to ischemia-reperfusion injury of the bowel⁶², skeletal muscle⁶³ and heart⁶⁴. Injection of a monoclonal myosin-heavy-chain-specific antibody into these animals restores the inflammatory response after injury⁶⁵. Moreover, injection of a myosin peptide into normal (antibody-sufficient) mice to block their natural myosin-specific antibodies inhibits the inflammatory response to ischemia-reperfusion injury⁶¹. Therefore, the non-muscle myosin functions to trigger an extracellular inflammatory pathway.

There are a number of molecules that are exposed or released upon necrotic cell death that trigger the generation of extracellular proinflammatory molecules (Table I). These include cellular molecules that directly activate the complement cascade^{66, 67} or cleave extracellular matrix molecules such as collagen⁶⁸ 69, fibrillar protein⁷⁰, hyaluronic acid⁷¹ and heparan sulfate⁷² into proinflammatory fragments. Cellular molecules may also trigger the clotting, fibrinolytic and kinin cascades which generate other proinflammatory mediators⁷³. The cellular molecules that are responsible for these effects are not known but are presumably various hydrolases and proteases. The extent to which these cellular triggers and the extracellular DAMPs they help generate contribute to the inflammatory response to dead cells is not clear.

Receptors for DAMPs

Much more is known about how the innate immune system senses microbial PAMPs than how it recognizes DAMPs. In the case of extracellular PAMPs, one of the major sensors is TLRs^{74, 75}. As PAMPs and DAMPs both have adjuvant and inflammatory properties one possibility is that they actually work through the same receptors⁷⁴. A potential role for TLRs has been explored so far primarily in inflammatory responses stimulated by dead cells and selected DAMPs (Table I).

Experiments injecting dead cells into TLR-deficient mice has shown that no single TLR examined to date is required for the inflammatory response, although TLR5 and TLR8 were not analyzed³⁵. Similarly mice lacking toll-like receptor adaptor molecule 2 (TICAM2), a signaling adaptor protein used by TLR3 and TLR4, displayed normal responses. By contrast, mice that were deficient in both TLR2 and TLR4 showed a decrease in inflammation, but the magnitude of this effect was quite modest³⁵. Death-induced neutrophilic inflammation was markedly decreased in mice lacking myeloid differentiation primary response gene 88 (MyD88), a signaling adaptor protein that is required downstream of all TLRs (except TLR3) as well as the IL-1 and IL-18 receptors. However, this effect was primarily due to a key role for the IL-1 receptor in the recruitment of neutrophils³⁵. Therefore, it seems that TLRs, and in particular TLR2 and TLR4, may have only a small role in the death-induced inflammatory response.

There are a number of purified DAMPs that have been reported to stimulate TLR2 and TLR4. These include HMGB1^{76,31}, uric acid⁷⁷, some HSPs^{78,79}, some defensins [G]⁸⁰, hyaluronic acid⁸¹, heparan sulfate⁸², and some fragments of extracellular matrix proteins⁷⁰. Whereas this data may well be correct, caution is warranted because TLRs can be stimulated by microbial contaminants that are easily introduced during the purification of molecules. Perhaps such contaminants explain why there is also conflicting data that some of these same DAMPs (including uric acid^{83,84}, HSPs⁸⁵ and HMGB1⁸⁶) do not stimulate responses through TLR2 or TLR4.

In addition to TLRs, some other receptors have been implicated in the actions of various DAMPs (Table I). RAGE (Official symbol: AGER (advanced glycosylation end product-specific receptor) binds HMGB1 and S100 proteins and has been reported to mediate the adjuvant⁸⁷ or inflammatory effects⁵¹ of these DAMPs. A number of molecules including CD91⁸⁸, CD14⁴⁸ and scavenger receptors⁸⁹ have been implicated in the action of HSPs. Galectins [G] have been reported to stimulate glycosylated surface receptors such as CD2⁴⁰. Integrins⁹⁰, chemokine receptors⁶⁹ and CD44⁹¹ have been reported as the targets of DAMPs from extracellular matrix components.

Clearly, much more work is needed to elucidate the receptors involved in sensing DAMPs. This is an important issue not only for understanding how death is perceived by the innate immune system, but also because the nature, diversity and distribution of these receptors will determine the nature of the pathophysiological responses stimulated by necrotic cell death; these receptors may also be molecular targets for therapeutic intervention.

Conclusions

Cell death is potentially dangerous to the host and therefore the immune system has evolved mechanisms to detect and respond to this process. It accomplishes this important task by having the innate immune system monitor tissues for cells that are disintegrating. Upon detecting the release of intracellular contents, an inflammatory response is rapidly mobilized. This provides initial defence and also attempts to clear and repair the damage. In parallel, DCs are stimulated to mature and stimulate adaptive immune responses if immunogenic antigens are present. Collectively, these responses help to protect the host and limit the injurious process, but can themselves also cause tissue damage and disease. There remain major unresolved issues in understanding these responses. The number of different danger signals, their molecular identity, and whether different ones cause different biological effects are all unresolved questions. Similarly the receptors and pathways that these sense the release of these signals need to be better elucidated. Once the molecular participants are identified it will be necessary to define their roles in both health and disease. Ultimately, it may be possible to use these

molecules to manipulate responses (e.g. as vaccine adjuvants) or to inhibit them to treat autoimmune or inflammatory diseases.

Box 1

Danger and autoimmunity

If necrotic cell death releases endogenous adjuvants that promote immune responses to cellular antigens then why doesn't autoimmune disease develop whenever there is cell injury? Indeed, the same question could be asked as to why microbial infections, which contain potent microbial adjuvants, don't generally also stimulate autoimmune disease to cellular antigens released from damaged cells. The reason may be that the host lacks autoreactive T cells that can respond, either because they were deleted from the T cell repertoire or are held in check by immunoregulatory mechanisms. In other words, adjuvants of either microbial or endogenous origin may be necessary, but they are not sufficient to trigger responses. It is true that occasionally cell death and infections may trigger the development of autoimmune disease, however this is the exception rather than the rule. Where it has been studied there is a strong multigenic component to several autoimmune diseases and while the underlying genetic defects are not fully understood at least some of the disease-associated genes are involved in immunoregulatory processes¹²².

Thus perhaps in individuals that are genetically predisposed to developing autoimmunity, microbial or endogenous adjuvants are sufficient to tip the balance and initiate disease.

Box 2

Sterile inflammation and disease

Whereas the inflammatory response to cell death may neutralize or contain the underlying injurious process and promote tissue repair, it can also cause its own set of problems. The leukocytes that are recruited, particularly neutrophils, leak proteases and other noxious agents that can damage the surrounding viable tissue. This is evident from the findings that the amount of damage that occurs in ischemic injury of the heart¹¹⁶, lung¹¹⁷ and skeletal muscle¹¹⁸ and in toxic insults to the liver¹¹⁹ or lung¹²⁰ was markedly reduced when neutrophils were acutely depleted with antibodies. Similar experiments have shown that neutrophil depletion can accelerate wound healing¹²¹. Moreover, blocking the signals that are required to generate acute neutrophilic inflammation to necrotic cell death (for example, the interleukin-1 receptor pathway) decreases the damage to drug-induced hepatocyte death³⁵. Therefore, the collateral damage that occurs in inflammation to sterile tissue injury can be quite significant. Although this may be a reasonable price to pay to contain a serious threat such as an infection, it is much more costly when the inflammatory defence mechanisms aren't useful to combat the underlying cause of cell death. Consequently, the sterile inflammatory response is thought to underlie the pathogenesis of a number of acute and chronic diseases in which cell death has occurred (for example, ischemia-reperfusion injury, chronic lung diseases). Given the importance of death-induced inflammation to host defence and disease pathogenesis, it is important to understand how necrotic cell death stimulates inflammation and to identify molecular targets for therapeutic intervention.

Online Summary

- A large number of different pathological processes can damage cells in ways that lead to the common end result of death. Therefore, when non-physiological cell death occurs *in vivo* it is a harbinger of a potentially dangerous situation developing in a host.

- The innate immune system has evolved mechanisms to identify potential danger by detecting abnormal cell death. This is accomplished by sensing the release of a subset of molecules (DAMPs) that are normally hidden in living cells or their local environment but are released or exposed when cell die and lose integrity of their plasma membrane.
- Upon detecting the presence of DAMPs the innate immune system initiates an acute inflammatory response that rapidly delivers soluble and cellular defenses to the site of damage. This response is a double-edged sword that can contain and repair the damage but can also damage normal tissues and in so doing cause disease.
- DAMPs also stimulate antigen presenting cells of the innate immune system to migrate to lymphoid tissues and become immunostimulatory for T cells. In this way the innate immune response alerts the adaptive immune system to potential danger in ways that help initiate responses to any immunogenic antigens at the site of damage. This may play an important role in initiating T cell responses to tumors, transplants and in autoimmunity.
- There has been recent progress in identifying some of what are probably a multitude of DAMPs. There remains much to be learned about these molecules, the cells and receptors that sense them, and the pathways they stimulate.

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About the authors

Hajime Kono

Hajime Kono is a postdoctoral fellow with Dr. Kenneth L. Rock at University of Massachusetts Medical School. He received his M.D. from University of Hokkaido, Sapporo, Japan and his Ph.D. from University of Tokyo School of Medicine, Tokyo, Japan. His research focuses on the mechanisms of cell death-induced inflammatory responses.

Kenneth L. Rock

Kenneth L. Rock studied in the laboratory of Drs. John Kappler and Pippa Marrack while earning his M.D. degree from the University of Rochester. He subsequently did post-doctoral training with Dr. Baruj Benacerraf at Harvard Medical School and a residency in Pathology at the Peter Bent Brigham Hospital. After 16 years on the faculty of Harvard Medical School he moved to his current position of Professor and Chairman of Pathology at University of Massachusetts Medical School. He has also founded two biotechnology companies. His laboratory works on elucidating the processes underlying immune surveillance by T cells including the mechanisms of antigen presentation and perception of immunological danger.

Glossary

Adjuvant, Adjuvant are immunostimulatory agents that enhance adaptive immune responses to co-administered antigens during vaccination.; primary and secondary necrotic cell death, Primary necrosis (oncosis) is a form of cell death that is characterized by vacuolization of cytoplasm and swelling of the mitochondria, nucleus and cytoplasmic that leads to rupture of the plasma membrane. Secondary necrosis is a process that occurs in apoptotic cells that are not cleared by phagocytes in which the integrity of plasma membrane is lost and the constituents of the cell are released.; NOD mouse, The NOD mouse spontaneously develops type 1 diabetes

mellitus due to autoreactive T cell mediated pancreatic beta cell destruction.; Natural IgM antibodies, Natural antibodies are ones that present in individuals without immunization (although they may be stimulated by host flora). They are predominantly of IgM isotype, have not undergone somatic mutations, and have low affinities but high cross-reactivities to many microbial pathogens and self antigens.; Defensins and cathelicidins, Defensins and cathelicidins are members of small antimicrobial polypeptides abundant in neutrophils and epithelial cells, and contribute to host defense by disrupting the cytoplasmic membrane of microorganisms.; Galectins, Galectins are lectins that bind wide arrays of glycoproteins or glycolipids with beta-galactoside and exert extracellular and intracellular functions including regulation of apoptosis, Ras signaling, cell adhesion and angiogenesis..

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Fig. 1. Stranger and danger models

Dendritic cells (DCs) are present in all tissues where they gather antigens from the local environment but are not in an immunostimulatory state. In Janeway's "stranger" model³ antigen presenting cells (later appreciated to be DC) were endowed with pattern recognition receptors that recognized the unique features of microbial molecules (pathogen-associated molecular patterns or PAMPs). When PAMPs were present, for example, from an infection or adjuvant, then DCs were stimulated to migrate to lymphoid tissues and present both antigen and costimulatory molecules to T cells. In Matzinger's danger model⁷, the key event controlling the initiation of an immune response was not infection, but the production of danger signals (DAMPs) from cells stressed, damaged and/or dying in the local tissue. These were postulated to act on DCs in ways that also caused them to migrate and present antigens to T cells in an immunostimulatory manner. It has been speculated that DAMPs might be produced in response to PAMPs and actually be the final mediator promoting immune responses in all situations, including infection. This may occur, however it is also possible, and in our view likely, that DAMPs and PAMPs can alert the immune system to a problem independently and possibly even in a synergistic manner.

Fig. 2. Discriminating between viable cells, necrosis and apoptosis

The hidden-self model proposes that innate immune cells have receptors that detect certain intracellular molecules, called damage-associated molecular patterns (DAMPs), that are normally hidden in the interior of cells and which are only revealed after necrosis. This model can explain why live cells, which contain preexisting danger signals, don't stimulate the innate immune system. Moreover, it can also explain why necrotic cells always stimulate the innate immune system, whereas apoptotic cells are only stimulatory in some situations⁹² but not others⁹³. This is because necrotic cells always lose membrane permeability and release their intracellular contents. By contrast, apoptotic cells initially maintain membrane integrity. If apoptotic cells are then rapidly cleared by phagocytes, then the dead cells don't release their intracellular DAMPs and the immune system is not stimulated. However, if apoptotic cells are not rapidly cleared, as may occur in a solid organ, then they undergo secondary necrosis and become permeable. The resulting release of DAMPs then stimulates the innate immune system. So, the event that communicates "danger" is not how the cell dies *per se*, but whether or not it eventually loses membrane integrity and releases its intracellular contents into the extracellular milieu. This concept of hidden signals being revealed may also apply to DAMPs of extracellular origin (e.g. when revealed through the action of enzymes released after cell damage) and even, as has been proposed, hidden portions of molecules⁶.

Figure 3. Cell death and inflammation

Necrotic cell death releases intracellular damage-associated molecular patterns (DAMPs) that are recognized by receptors on leukocytes and possibly other host cells, stimulating the generation of proinflammatory cytokines such as interleukin-1 (IL-1). Other molecules that are exposed or released from dead cells act on extracellular components to generate mediators (for example, complement fragments) or DAMPs (for example, fragments of extracellular matrix) that then trigger the production of proinflammatory cytokines from host cells. The proinflammatory mediators act on local vascular endothelium causing them to become 'leaky' and attract neutrophils and monocytes. Once in the tissue, these soluble and cellular defences attempt to neutralize or contain microorganisms or other injurious agents if present. They also clear dead cells and stimulate tissue repair.

Table I
Adjuvant and pro-inflammatory activity of intracellular and extracellular DAMPs

	Adjuvant activity	Proinflammatory activity	Potential Receptors	Refs
Intracellular DAMPs				
HMGB1	In vivo Adjuvant activity by purified molecule Adjuvant activity shown by selective depletion	In vivo Inflammation to liver injury blocked by neutralizing Ab Neutrophil recruitment by purified molecule	RAGE TLR2, -4*	28 29 30 31 36 37
	In vitro DC activation	In vitro Chemotaxis Cytokine induction		
Uric acid (MSU)	In vivo Adjuvant activity by injection of purified molecule and selective depletion	In vivo Gout induced by purified molecule Neutrophil recruitment by purified molecule	TLR2, -4* CD14	9 83 20 27 39 77 84
	In vitro DC activation	In vitro Cytokine induction		
Chromatin/ Nucleosome/DNA	In vivo DC maturation by purified molecule	In vivo Neutrophil recruitment by purified molecule	TLR9 (with BCR or Fc receptor)	32 54 94 95
	in vitro DC activation DC activation in forms of chromatin-IgG complex	in vitro Cytokine induction B cell activation in forms of chromatin-IgG complex		
HSPs	In vivo Tumor immunogenicity enhanced by overexpressed molecule or addition of purified molecule (HSP70) DC migration to LNs by purified molecule (gp96)	In vivo ND	CD14 (HSP70, HSP60) CD91 (HSP70, HSP90, gp96, calreticulin) Scavenger receptors (HSP70, gp96, calreticulin) TLR4 (HSP60) TLR2, -4 (HSP60, gp96) CD40 (HSP70)	23 24 25 48 49 50 79 96 97 98
	in vitro DC maturation (gp96 & hsp70), (gp96)	in vitro Cytokine induction (HSP60) (HSP70) (gp96, hsp70, hsp90)		
Adenosine and ATP	In vivo Exacerbation or Abrogation of bronchial asthma by purified molecule or specific inhibition, respectively	In vivo Exacerbation of nephritis by purified molecule	ATP: P1, P2X, P2Y receptors Adenosine: A ₁ , A _{2A} , A _{2B} , A ₃ receptors	55 56 99 100 101 102
	in vitro DC maturation	in vitro chemotaxis		
Galectins	In vivo ND	In vivo Monocyte recruitment by purified molecule	CD2 and others with beta-galactosidase	42 40 43 44
	In vitro DC maturation	In vitro Chemotaxis		
Thioredoxin	ND	In vivo Chemotaxis by purified molecule In vitro Chemotaxis	ND	45
S100 proteins	ND	In vivo Neutrophil recruitment by purified molecule	RAGE	51 52
		In vitro Chemotaxis and cytokine induction		
Cathelicidins	In vitro DC maturation DC activation in forms of LL37 - self DNA complex	In vitro Chemotaxis	FPRL-1	58 103 104 123
Defensins	In vivo Adjuvant activity by co-administration of	In vivo ND	CCR6 TLR4	59 80 103 105

	Adjuvant activity	Proinflammatory activity	Potential Receptors	Refs
	purified molecule			
	In vitro DC maturation	In vitro Chemotaxis		
N-formylated peptide	In vivo ND	In vivo Neutrophil recruitment by purified molecule	FPR, FPRL-1	60106107
	In vitro DC chemotaxis	In vitro Chemotaxis		
Extracellular DAMPs				
Hyaluronicacid	In vivo Inhibition of Langerhans cell maturation by blocking peptide Adjuvant activity by administration of purified molecule	In vivo Reduction of bleomycin induced lung inflammation by blocking peptide	CD44 [*] TLR2, -4	8191108109110
	In vitro DC maturation	In vitro Cytokine induction		
Heparan sulfate	In vitro DC maturation	In vitro Cytokine induction	TLR4	7282
Fibrinogen	In vitro DC maturation	In vitro Cytokine induction	Integrins TLR4	70· 90111
Collagen derived peptide	In vivo ND	In vivo Neutrophil recruitment by purified molecule	CXCR2	68· 69112
	In vitro DC maturation	in vitro chemotaxis		
Fibronectin	In vitro DC maturation	In vitro Chemotaxis	Integrins	113
Elastin derived peptide	In vivo ND	In vivo Abrogation of smoke-induced emphysema by specific Ab	Integrins	114
	In vitro ND	In vitro Chemotaxis		
Laminin	In vivo ND	In vivo Neutrophil recruitment by purified molecule	Integrins	115
	In vitro ND	In vitro Chemotaxis		

ND = not determined.

* Some papers show opposite results.