HMGB1 and Microparticles as Mediators of the Immune Response to Cell Death

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Abstract

In a wide variety of diseases, cell death represents both an outcome and an important step in pathogenesis. This duality occurs because cell death leads to the extracellular release of molecules and structures that can potentially induce the innate immune system. These mediators include the alarmins which are endogenous cellular constituents that exit activated or dying cells to stimulate toll-like receptors (TLRs) as well as non-TLR receptors. Of alarmins, the nonhistone protein HMGB1 is the prototype. Like DNA and RNA, HMGB1 can translocate from cells as they die. The activity of HMGB1 may reflect its interaction with other molecules such as LPS, DNA, and cytokines. In addition to alarmins, dead and dying cells can release subcellular organelles called microparticles that contain cytoplasmic and nuclear constituents, including DNA and RNA. These particles can impact on many cell types to induce inflammation. The release of HMGB1 and microparticles shows important similarities, occurring with cell death as well as stimulation of certain but not all TLRs. Furthermore, nitric oxide can induce the release of both. These observations suggest that the products of dead cells can serve as important mediators to drive immune responses and promote inflammation and autoreactivity. *Antioxid. Redox Signal.* 15, 2209–2219.

Introduction

DISEASE IS ALMOST ALWAYS A MATTER OF LIFE AND DEATH, with death representing a critical step in pathogenesis as well as a final outcome. In diseases across the spectrum of medicine, cell death and its consequences result from the interplay of extrinsic factors (e.g., infection, physical-chemical trauma, toxins) as well as intrinsic factors arising in the endangered tissue. These intrinsic factors act to counteract death or to trigger a protective host response. Among these downstream events, the stimulation of the innate immune system can modify pathogenesis and shift the balance of life and death at the level of the cell, tissue and whole organism.

In many respects, the response to cell death resembles the response to infection since both involve mediators arising directly or indirectly from an offending cell type (i.e., a bacterium or a dying cell). These mediators can stimulate innate immunity to promote host defense and induce a cascade of events leading to recovery and repair (4). If the response is excessive, however, tissue injury as well as severe systemic complications can ensue, leading to shock and organ dysfunction among other dire events.

In the response to infection, the foreign molecules inducing responses have been identified as PAMPs or pathogen associated molecular patterns. These molecules, which are het-

erogeneous in structure, can interact with a set of receptors termed pattern recognition receptors or PRRs. These receptors encompass the toll-like receptors (TLRs) as well as non-TLR receptors, including elements of the inflammasome. Together, stimulation of these receptors leads to the production of a broad array of conventional mediators including cytokines and nitric oxide (NO). PRRs are widely distributed among cells of the innate immune system with macrophages, dendritic cells, and B cells the main targets of PAMP stimulation (17, 30, 31).

In the response to cell death, those mediators that are produced by the dying cell itself are called DAMPs for damage or death associated molecular patterns. These molecules can also be designated as alarmins, signifying the alarm function of these molecules to stimulate innate immunity, prevent further damage, and initiate repair (21, 63). As currently defined, alarmins are intracellular molecules that are released from dead or dying cells to act as cytokines to induce immune responses or to act as chemokines to promote inflammation. Dead cells are a major source of alarmins, although these molecules can also be released by activated cells by nonconventional secretory pathways. Once outside the cell, alarmins can alert the immune system to cell injury and thereby trigger inflammation. As such, alarmins can play a key role in host defense as internal “danger” signals.

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Alarmins can be either large or small molecules. As a group, alarmins differ from conventional pro-inflammatory mediators such as prostanoids or cytokines because they are normal cellular constituents that serve essential functions, whether in the nucleus, cytoplasm, or mitochondria. In other terminology, alarmins are dual function molecules since, in addition to their ordinary structural or enzymatic activity, they are immune mediators once outside cells. For these molecules, location determines function, with death and activation driving forces of movement.

The death process is complicated and messy, however, with molecules (including alarmins) exiting dying and distressed cells, often leaving as ensembles or subcellular organelles called microparticles. Microparticles are small membrane-bound vesicles that are released from activated or dying cells (3, 13, 52). In the case of apoptosis, microparticles may correspond to blebs which are bubble-like structures that form on the membrane surface as apoptosis proceeds (10). While the function of blebs is not known, translocation events can fill the bleb with cytoplasmic as well as nuclear macromolecules, some of which have alarmin activity. In many instances, alarmin and microparticle release occurs concurrently and reflects similar inductive events.

Like alarmins, microparticles are immunologically very active and can subserve many of the same functions of alarmins; in addition, microparticles have powerful pro-coagulant activities that may contribute to their physiological function as well as a role in disease pathogenesis (3). At present, the term alarmin is used for molecules rather than structures. Until the terminology in this area is refined, alarmins and microparticles will be viewed separately, although at least some alarmins reside in particles and some of the activity of particles may result from their content of alarmins. This review will therefore focus on both nuclear alarmins and microparticles and advance a conceptual model to explain their activity in settings of cell death.

The Mechanisms of Cell Death

In the organism, cell death occurs in both physiological and pathological settings to produce remnants or debris differing in biological properties. In the simplest conceptualization, cell death can be dichotomized into apoptosis and necrosis.

Apoptosis is a form of regulated or programmed cell death that can occur in normal settings such as embryonic development or in abnormal settings such as sepsis or tissue infarction. In either setting, enzyme cascades systematically disassemble the cell, leading to the cleavage of proteins and nucleic acids, structural rearrangement of organelles, and volume shrinkage. Apoptosis requires energy and occurs over the course of hours (20, 35, 49).

In contrast to apoptosis, necrosis is almost always pathological and results from chemical or physical trauma. During necrosis, the cell loses its permeability, swells, and spills its contents. Given the triggering events (*e.g.*, toxin exposure, thermal injury), necrotic death can be very rapid although, for some inducing agents, the process can be much slower and follow a time-course similar to that of apoptosis. Necrosis does not require energy and, while macromolecular constituents can be denatured, they are not usually degraded or cleaved if the process involves a rapid traumatic event such as heat. With stimuli that induce a more gradual death process,

protein cleavage can occur, albeit mediated in a manner distinct from that occurring during apoptosis (20).

While apoptosis and necrosis can be distinguished morphologically and biochemically, these death forms may represent points along a spectrum where other factors (*e.g.*, oxygen supply) determine which pathway is operative. Apoptosis itself is likely heterogeneous, with caspase-dependent or independent subtypes identified on the basis of the effects of broad spectrum caspase inhibitors; since these agents may inhibit other intracellular pathways. However, categorizing forms of apoptosis from inhibitor studies can be problematic (35). Furthermore, depending on the cell type, the default mechanism for cell death may be either apoptosis or necrosis. Certain intracellular events can push the system in one direction or the other to set the cell on the path to demise. In some systems, the same stimulant (*e.g.*, anti-Fas) can induce either apoptosis or necrosis depending on the integrity of the cell death machinery and activity of enzymes and other proteins mediating apoptotic death (29, 59). These considerations are important in understanding the inter-relationship between cell activation, cell death, and alarmin release.

Among other features, apoptosis and necrosis may differ in their immune potential. In general, apoptotic cells are considered non-inflammatory or even anti-inflammatory while necrotic cells are considered pro-inflammatory (6, 43, 51). Furthermore, humoral and cellular systems can scavenge apoptotic cells to prevent their transition to secondary necrosis where their immune properties and release of internal molecules may change. Thus, as cells undergo apoptosis, they display membrane changes (*e.g.*, exposure of phosphatidylserine [PS] and other "eat me" signals) that promote phagocytosis. With rapid elimination of apoptotic cells, the spillage of internal molecules is reduced (41, 45, 60). In contrast, a cell dying by necrosis when subjected to major trauma that impairs permeability can instantly release its contents when it bursts. In the case of necrosis occurring in less disruptive circumstances, the release process has not been as well defined but could involve a distinct quantitative or qualitative pattern of release of internal constituents. Whether cells die by apoptosis or necrosis, the release of internal molecules can occur, thereby providing a supply of alarmins to drive immune responses.

The Mechanisms for Alarmin Generation

The products of dead and dying cells appear prominently in the blood and are an important part of the blood proteome. Such products include enzymes such as lactate dehydrogenase, a general marker of cell damage, or creatinine phosphokinase, a specific marker of damage of muscle or brain cells. Other proteins that populate the extracellular space include troponin, a marker of myocardial damage (14, 18, 62). While extensively utilized as biomarkers, these proteins have generally been viewed as inert byproducts of cellular injury rather than effector molecules themselves. In addition to proteins, both DNA and RNA, emanating from dead and dying cells, circulate in the blood, most likely released under the same circumstances as the proteins. Such molecules can constitute what could be called the blood "nucleome" (8, 54).

Of discoveries galvanizing interest in the alarmins, perhaps the most significant has been the delineation of the immune properties of HMGB1 (high mobility group box 1 protein).

HMGB1 is a nonhistone nuclear protein that plays an important role in nuclear architecture as well as transcriptional regulation. HMGB1 is 215 amino acids long and has two DNA binding domains (A box and B box) as well as a long C-terminal domain. Inside the nucleus, HMGB1 interacts with chromatin as well as transcription factors, showing preferential binding to DNA of certain conformations (e.g., bent DNA). Given its function, HMGB1 is less tightly bound to DNA than histones, allowing greater mobility inside the nucleus (15, 39).

The recognition of the immune properties of HMGB1 resulted from seminal experiments to identify late mediators of septic shock that could serve as targets for novel therapy. In septic shock, the role of cytokines such as TNF- α and IL-1 is well established in animal models, although efforts to target these cytokines for therapy in man have been generally unsuccessful. The failure of anti-cytokines in shock could result from many factors, although an important possibility concerns the kinetics of cytokine production. Thus, if cytokines are produced early or transiently in infection, anti-cytokines administered later in disease would be destined to fail. On the other hand, a mediator with more sustained production would afford a wider therapeutic window (57).

To identify such a mediator, Wang *et al.* stimulated macrophages in culture with LPS and analyzed the media to identify proteins with cytokine properties (61). The results of these studies were remarkable since the molecule identified was HMGB1. Subsequent studies demonstrated that HMGB1 levels are elevated in shock in animal models as well as humans. Furthermore, in *in vitro* experiments, purified or cloned HMGB1 stimulated a wide array of responses that resemble those induced by LPS as well as cytokines such as TNF- α . Importantly, HMGB1 appeared to be a valid target for therapy since, in shock models in mice, antibodies to HMGB1 reduced disease severity and prolonged survival (1, 15, 39, 61).

As these considerations indicate, HMGB1 fulfills criteria of an alarmin since it is an intracellular molecule with cytokine or chemokine activity. In another terminology, HMGB1 shows features of a DAMP or a damage (or death) associated molecular pattern by analogy to a PAMP or pathogen associated molecular pattern. Importantly, in this conceptualization, for HMGB1 to act as a cytokine, it has to exit the cell, a process which occurs in two distinct but related settings: cell activation and cell death. During the activation of macrophages, HMGB1 undergoes post-translational modifications, including acetylation and phosphorylation (7, 64). These modifications alter the charge of HMGB1 and its trafficking from the cytoplasm to the nucleus; in the cytoplasm, HMGB1 enters endolysosomes for eventual secretion. As a result of this translocation, the nuclear content of HMGB1 drops markedly. This translocation can result from activation by toll-like receptor (TLR) ligands, as well as cytokines such as type 1 and 2 interferon.

While original models conceptualized HMGB1 as an independently acting agent, more recent studies have indicated that the alarmin activity of this protein may reflect a partnership with other foreign or self molecules present in the extracellular milieu. Thus, for the classical alarmin activity, HMGB1 may need to bind to cytokines such as IL-1 or TNF- α as well as LPS, intensifying their pro-inflammatory activity. Similarly, HMGB1 can bind to DNA to create a more im-

munostimulatory complex to facilitate DNA entry into cells or to promote interaction with TLR and non-TLR internal sensors (5, 22, 48). In this conceptualization, the activity of HMGB1 may set the poise of the host response and act alone or in concert with other molecules (foreign or self) during the course of a response including the phase of healing and repair. In some instances (e.g., angiogenesis), HMGB1 may function in the absence of another molecule, although the ability of HMGB1 to interact with so many different structures can limit assessment of this possibility. Even if HMGB1 is introduced into a culture system or an animal as an isolated protein devoid of other molecules, it can pick up a companion to stimulate cells in tandem. While these issues require further exploration, the role of HMGB1 in inflammation is clear since blockade of its activity can attenuate inflammation in a variety of animal models including arthritis.

In our laboratory, we have explored the processes leading to HMGB1 release from stimulated macrophages, focusing on two main issues: the range of toll ligands that can stimulate release of HMGB1 and the role of other mediators in inducing HMGB1 translocation during macrophage activation. Thus, using murine macrophages such as RAW264.7 cells as well as bone-marrow derived macrophages, we showed that LPS, a ligand of TLR4, and poly (I:C), a ligand of TLR 3, can both induce HMGB1 translocation and release from macrophages stimulated *in vitro*. In contrast, stimulation of macrophages with a CpG oligonucleotide, a ligand of TLR9, failed to induce HMGB1 release despite induction of the expected cytokine response (26).

The failure of CpG DNA to induce HMGB1 translocation is notable since it suggests that macrophage activation *per se* may be insufficient for this process but rather that HMGB1 release reflects a particular pattern of activation. As shown in other studies, the downstream pathways elicited by ligands of TLR 3, 4, and 9 differ, with stimulation of TLR3 and TLR4, but not TLR9, activating the TRIF pathway. In contrast, TLR4 and TLR9 stimulation activate MyD88. These findings suggest that TRIF activation may be important in inducing the pathways that lead ultimately to HMGB1 translocation and release (26).

The differences in the macrophage responses induced by the various TLR ligands may be relevant to the *in vivo* effects of these agents. Thus, both LPS and poly I:C stimulation can lead to shock, with LPS treatment of mice often used as a model for sepsis. In contrast, the effects of CpG DNA administration to animals appear much more limited, with immunostimulatory oligonucleotides *in vivo* leading to cytokine production without the same systemic complications as LPS. The induction of shock by CpG DNA can be enhanced *in vivo* by prior treatment with galactosamine, which makes an animal dramatically sensitive to TNF- α (50). The use of this model has perhaps contributed to confusion about the activity of CpG DNA, placing it in the framework of other TLR agonists, although its ability to induce shock is actually limited. The correlation between release of HMGB1 and induction of shock is striking, focusing attention on the role of TRIF in these processes and the differences among TLR agonists in their effects on innate immunity.

In subsequent studies, we explored the effect of downstream mediators on HMGB1 release from macrophages. Thus, with stimulation of macrophages by LPS, nitric oxide (NO) can mediate the release of HMGB1 as shown by the

effects of blocking NO production with 1400W, a specific iNOS inhibitor. Furthermore, NO itself, generated *in vitro* from the NO donor NOC-18, can induce HMGB1 release directly in the absence of LPS. In contrast, for stimulation by poly (I:C), type 1 IFN plays a key role in the response. Thus, antibodies to IFN can block the HMGB1 release and IFN can induce HMGB1 release itself (27). The difference in the role of these mediators highlights the functional heterogeneity of TLR systems.

HMGB1 Release During Cell Death

Whereas cell activation leads to the release of modified HMGB1, dead cells can release this protein directly in a putatively unmodified form, with this process possibly causing the pro-inflammatory state of these cells (44, 46). For cells undergoing necrosis, especially when subjected to extreme death-inducing measures such as freeze-thawing or hypotonic lysis, release from the nucleus occurs readily. HMGB1 is not tightly adherent to chromatin and may simply diffuse away from the cell as permeability barriers break down (16). The kinetics and extent of this process likely reflect the type of necrosis occurring. Certainly, in published studies, freeze-thawing leads to very substantial HMGB1, although this intervention can be devastating to cells. In this regard, studies on the immune properties of necrotic cells have also used homogenized organ preparations. While these treatments certainly induce death, the resulting material may more closely resemble an extract than the remains of a dying cell.

For cells undergoing apoptosis, however, the HMGB1 release process may depend on the cell type, inducing stimulus as well as stage in the death process. Initial studies of the behavior of HMGB1 during apoptosis indicated that, with apoptosis, HMGB1 remains intracellular and increases its adherence to chromatin, showing decreased nuclear mobility as assessed biophysically. This result implies a modification in either the structure of HMGB1 or chromatin to enhance binding. Subsequent studies, as illustrated in Figure 1, have indicated that at least some cells release HMGB1 in late apoptosis, a stage sometimes termed secondary necrosis, when permeability barriers break down (2, 32).

Any comparison between HMGB1 release during necrosis and apoptosis is inherently complicated because of the differences in the mechanisms of these death forms, as well as uncertainty in the experimental systems used as models. Apoptosis can be induced by well-defined agents and has morphological and biochemical hallmarks, despite heterogeneity in mechanisms and differences in response of different cell systems in the effects of caspase inhibitors (35). In contrast, necrosis remains a vague entity that, to some investigators, is a final common pathway for a variety of death processes. Until the definition of necrosis matches that of apoptosis in its precision and rigor, caution is indicated in comparing release of HMGB1 and other alarmins during death to avoid the perhaps unwarranted conclusion that necrotic death is always equivalent to a cellular explosion in which all cell contents burst out.

These considerations pertain to the quantitative release of HMGB1. Post-translational modifications occurring during apoptosis (or necrosis) may affect the functional properties and alarmin activity of this protein. Thus, while HMGB1 release may occur during apoptosis, the protein may be inactive because of its oxidation state (2, 32, 58). Other modifications could also impact on its functional activity as well as its attachment to nuclear proteins as well as other molecules (e.g., DNA or cytokines) that may contribute to its activity. Assaying the activity of HMGB1 in media or cell extracts is complicated, however, because of the presence of other alarmins. It can be difficult therefore to characterize both the quantitative release of HMGB1 (and other alarmins) during death and the functional consequences.

Since HMGB1 release can occur during apoptosis, we investigated other mechanisms by which stimulation by TLR agonists can promote its translocation and release, seeking to reconcile the release process in these disparate settings. Both LPS and poly (I:C) can induce macrophage apoptosis, raising the possibility that HMGB1 release occurring during activation may result from apoptosis. To explore this possibility, we compared the magnitude of extracellular release of HMGB1 with the extent of apoptosis in *in vitro* systems with RAW264.7 cells stimulated with LPS, poly (I:C), or CpG DNA. In these experiments, the extent of HMGB1 release as

FIG. 1. Western blot analysis of high mobility group box 1 protein (HMGB1) release from apoptotic Jurkat cells. Jurkat cells were induced to undergo apoptosis (**A**) by treatment for the indicated times with staurosporine (STS), etoposide (Etop), or camptothecin (Camp). Apoptosis was inhibited by a 30-min pretreatment with 100 μ M Z-VAD-fmk. Necrosis (**B**) was induced by three cycles of freeze-thawing or heating cells at 56°C for 30 min. Supernatants were concentrated and run on SDS-PAGE gels and stained with an anti-HMGB1 antibody. Figure adapted with permission from Bell *et al* (2).

measured by Western blotting was correlated with the number of cells undergoing apoptosis. Interestingly, cells stimulated with CpG DNA neither released HMGB1 nor showed signs of apoptosis, providing further evidence for the differences in the response to TLR stimulation (28).

The simultaneous occurrence of HMGB1 release and apoptosis during LPS stimulation does not prove that the cells releasing HMGB1 are undergoing apoptosis or that apoptosis is inducing HMGB1 translocation. Rather, these events, while occurring in the same culture, may not be causally related, with distinct subpopulations of cells undergoing HMGB1 release, on one hand, and apoptosis, on the other hand. To elucidate the relationship between these processes, we tested the effects of caspase inhibitors on HMGB1 release in LPS stimulated cells. The results of these experiments were notable since the inhibition of caspase activity increased HMGB1 release (28). These results, while surprising, can nevertheless be explained by the effects of caspase inhibitors on LPS-stimulated cells. In this setting, inhibitors such as z-VAD can stabilize the MEF-2 transcription factor, leading to induction of NUR77, an "orphan" receptor implicated in cell death. With NUR77 induced, a non-caspase-mediated form of cell death follows stimulation by LPS with z-VAD (33, 34). These findings highlight the interplay of signaling events during activation and cell death and the occurrence of HMGB1 release during various forms of cell death. Furthermore, they are important in emphasizing the potential of at least certain forms of TLR stimulation to induce cell death.

In the setting of experimental shock, the expression of HMGB1 is prolonged and differs significantly from that of conventional cytokines such as TNF- α or IL-1 that have transient production. The long duration of HMGB1 expression is striking and may reflect the release of HMGB1 from dying as well as activated cells; in this setting, the sustained expression during shock may indicate ongoing cell death that accelerates as the shock state persists. Whatever the origin of HMGB1, however, the presence of this protein can intensify shock and contribute to immunological and vascular events that characterize this syndrome.

HMGB1 is not the only macromolecule shifting its location during death processes since, as part of apoptosis, there is extensive movement of nuclear molecules throughout the cell, including entrance into blebs for subsequent release as microparticles (9, 37, 47). Indeed, as shown in studies from our laboratory as well as others, DNA leaves cells during apoptosis both *in vitro* and *in vivo* and, in the extracellular milieu, displays a size distribution indicative of nuclease digestion into nucleosomes (12, 23, 24). The presence of extracellular DNA may be immunologically relevant since DNA can stimulate both TLR9 as well as non-TLR sensors following reuptake into cells. The stimulation of cells by DNA is likely potentiated by molecules such as HMGB1 and LL37 which bind DNA to form immunostimulatory complexes (36). While nuclear DNA itself may not stimulate responses, it can gain activity by complexing other molecules to form compound alarmins. On the other hand, mitochondrial DNA, which is also released during cell injury and death, has the potential to act by itself because its structure resembles that of bacterial DNA (65). Whether mitochondrial DNA emanating from dead and dying cells is physically associated with other cellular components is unknown, although the complex formation could also influence its immunological properties.

This process of DNA exteriorization occurs with apoptosis with the greatest levels *in vitro* observed during apoptosis or secondary necrosis. This DNA shows laddering, suggesting extracellular release following cleavage by enzymes such as CAD. The situation with necrosis is more complicated, with the extent of DNA release dependent on how death is induced. With extreme measures such as freeze-thawing, death is associated with an immediate and dramatic release of DNA. This DNA may have a short half-life since it can be degraded rapidly by either cellular nucleases exposed during the trauma of death or nucleases in the sera. With less violent death, the release of DNA may be more gradual and levels more sustained.

In *in vitro* situations, the presence of macrophages can influence the extent of DNA release. Using a co-culture model, we showed that macrophage cell lines such as RAW264.7 or J774 can influence the release of DNA from dying Jurkat cells although the outcome differs with apoptotic and necrotic cells as well as the ratio of dead cells and phagocytes (11). Thus, with apoptotic cells, phagocytes can reduce the extent of DNA release whereas with necrotic cells (induced by heat or ethanol), the phagocytes can increase the release of DNA. These results suggest two important elements in the interaction of dead cells with macrophages: the difference in the nature of the interaction with apoptotic and necrotic cells with phagocytes and the role of phagocytes in determining the extent of nuclear molecule release.

These findings are important in conceptualizing the dynamics of nuclear alarmin release during death and the potential basis of the immunological activity. Since the activity of HMGB1 can be influenced by bound DNA, the ultimate consequences of cell death *in vivo* may be determined by the formation of complexes of nuclear molecules that are subject to variable degrees of release and degradation. The findings further suggest the extent of alarmin immune activity generated in a culture (or blood or an effusion) will result not just in the presence of dying cells which are releasing nuclear molecules but rather will also depend on the presence of macrophages that can increase or decrease the components present. Furthermore, since complexes may be the operative form of nuclear molecules released during death, nucleases (or proteases) may modulate the level of activity observed by removing one or another component.

In Vivo Release of Nuclear Molecules

Studies *in vivo* support the concept that both immune cell activation and cell death can lead to release of nuclear molecules. Thus, levels of HMGB1 and DNA are elevated in settings such as shock, trauma, and sepsis, although few studies have addressed either their concomitant elevation in the same systems or presence as complexes. In experimental mice, DNA attains high blood levels following treatment with LPS but not CpG DNA; blood DNA levels also rise in mice with the induction of liver cell death by apoptosis (anti-Fas) or necrosis (carbon tetrachloride or acetaminophen); while the liver may be the main target organ for these interventions, other cells may die (23, 53). In these model systems, both apoptotic and necrotic cell death leads to high levels of circulating DNA that can be measured either by immunoassay for nucleosomes or direct measurement of DNA by a fluorometric dye called PicoGreen.

In another model system to track nuclear molecule translocation events, we infused apoptotic or necrotic cells into healthy mice by the intraperitoneal route (24). In these studies, we used Jurkat cells made apoptotic or necrotic *ex vivo* prior to administration to mice. Since Jurkat cells were derived from a male, we used female mice as recipients so that we could identify the source of any circulating DNA by PCR amplification of a Y-chromosome sequence; we also performed PCR amplification for the murine GAPDH sequence to assess whether this treatment leads to any DNA release from the recipient mice. For these experiments, we measured total DNA by PicoGreen.

Results of these experiments bolster observations from the *in vitro* models and show that infusion of either apoptotic or necrotic cells leads to a prompt rise in DNA in the blood, with a peak at about 5–6 hours. The DNA was derived from the infused Jurkat cells, although we could also detect DNA from the mouse. As in the case of the *in vitro* models, macrophages play an essential role in the generation of extracellular DNA. Thus, in mice depleted of macrophages by prior clodronate treatment, blood DNA did not occur despite the infusion of dead cells (24). On the other hand, in mice in which peritonitis was induced by prior treatment with agents such as thioglycollate, periodate, or peptone, the blood DNA response was modified (25). The effects were variable among the inducing agents, suggesting that inflammation may influence the generation of extracellular DNA from dead and dying cells in a way that may reflect factors in addition to the presence of peritoneal macrophages. Interestingly, these experiments suggested a correlation between the presence of DNA in the peritoneal fluid and the ability of infused cells to generate a blood DNA response.

In these experiments, we used female mice to allow tracking of the DNA from the dead (male) cell into the blood stream. In preliminary results that are surprising, we found that the same intervention that causes a blood DNA response in female mice does not affect male mice (40). Thus, with the infusion of dead cells in the male mice, the elevations of DNA are very limited (Fig. 2). Castration of female mice produces a situation similar to that of the male (*i.e.*, minimal elevations of DNA following dead cell administration).

We think that these findings are interesting in view of the marked male–female differences in the occurrence of autoimmune disease where females develop diseases such as systemic lupus erythematosus or Sjogren's syndrome five to ten times more commonly than males. In this regard, data on sepsis also indicate male–female differences in outcome with data suggesting that women may have a better outcome. To the extent that our findings on DNA can be extrapolated to other nuclear molecules such as HMGB1, we would suggest that sex differences in alarmin generation may impact on disease and likely reflect the manner in which products from dead cells are released into the extracellular milieu by the interaction with macrophages.

Together, these studies indicate that the release of nuclear molecules during death is not simply a matter of cell demise but rather can represent the end-product of a regulated pathway in which the extent of molecule release relates to both the mechanism of cell death (apoptosis vs. necrosis) as well as the interaction with neighboring phagocytic cells of which macrophages have a dominant influence. Furthermore, our findings suggest that other influences (*e.g.*, estrogen hor-

FIG. 2. The effect of sex on the generation of blood DNA from apoptotic cells. Jurkat cells (10^8 cells) were treated with etoposide *in vitro* to induce apoptosis and then administered by the intraperitoneal route to normal male and female BALB/c mice. Blood was obtained at various times subsequently and DNA levels were determined by fluorimetry using the PicoGreen dye. Values are expressed as mean ($\pm$ SD). Figure adapted with permission from Pisetsky and Jiang (40).

mones) can modulate the release of nuclear molecules most likely by their effects on phagocytic cell function. While we have assumed at least some similarities in the process of HMGB1 and DNA release, there are potential differences in these processes that can impact on the immunological role of these nuclear molecules. These differences may relate to the translocation and exteriorization of these molecules as well as their stability once they are outside cells. Further influencing these interactions are the effects of post-translational modifications of HMGB1 which could, in this context, influence the trafficking of this protein, its intrinsic activity as an alarmin as well as the interaction with other molecules such as DNA or cytokines.

Microparticles

Whereas studies on the release of nuclear molecules (*i.e.*, HMGB1 and DNA) with immunological activity have analyzed these molecules separately, a reductionist approach can easily ignore the realities of cell structure and the nature of the cytoplasmic and nuclear milieus. Rather than a solution in which molecules float freely, these compartments are organized matrices with multiple protein and other macromolecular components that organize and restrict the environment. During death, these compartments can rearrange, allowing trafficking and intracellular migration of these components which can facilitate release from cells to allow either new biological activity or facilitate clearance.

Of mechanisms for cellular reorganization, the formation of blebs is among the most dramatic, providing a hallmark for cells undergoing stress or death. This process, which is a frequent concomitant of apoptosis, leads to translocation of nuclear and cytoplasmic constituents into bubble-like extrusions on the cell surface (10). The function of blebbing is unknown but it could help sequester degraded or toxic cell products for safe export. Alternatively, like the release of alarmins, blebbing could represent a stage in the cellular demise process that creates immunostimulatory structures that can signal danger and activate innate immunity.

As currently defined, microparticles are small membrane-bound vesicles that, once released from cells, display pro-inflammatory and pro-thrombotic activities. These structures are generally 0.1–1.0 microns in diameter and contain an ensemble of cytoplasmic and nuclear constituents surrounded by a cell membrane. Since apoptosis is a common setting for particle generation, microparticles may correspond to blebs that have detached from cells, although other mechanisms may lead to particle release. Indeed, while blebs can entirely cover the surface of a cell, each cell releases only a few particles as death proceeds. It is possible therefore that, while blebbing and particle release both accompany apoptosis, they may be distinct processes. Particle release is also a feature of cell activation although, in this setting, the formation of blebs has not been extensively investigated (3).

Flow cytometry is the most common analytic approach for assessing the presence of particles in biological fluids. Since microparticles are small structures enclosed by a membrane, their assay can entail measurement either on the basis of size or display of cell surface markers. The assay of particles by flow cytometry can be problematic, however, since most cytometers cannot accurately detect structures in the size range of microparticles using ordinary parameters of light scattering. The smaller particles can simply not be detected since their size profile overlaps with noise. Detection by fluorescent agents to surface markers is also commonly used for particle measurement, although this approach is limited by the very small surface area of particles and uncertainties in the expression of various proteins on the surface. For example, while the presence of phosphatidylserine on the particle surface provides the basis for detection by annexin V binding, not all particles bind annexin V.

In the context of our work on nuclear alarmins, we were interested in the relationship between the release of particles and other nuclear molecules such as DNA, and the extent to which microparticles are a transport vehicle for alarmins. We therefore assessed the content of nuclear molecules in particles generated *in vitro* by apoptotic cells and determined parameters of particle release. For this purpose, we used some of the same systems we used to investigate HMGB1 and DNA release to determine whether microparticle release has similar mechanisms of extracellular generation.

This work is ongoing, although we can already draw certain important conclusions about the relationship between alarmin and particle release. Thus, we have shown that MPs are produced during *in vitro* apoptosis and are an important source of extracellular DNA and RNA (41). In these experiments, we demonstrated the presence of nucleic acid by immunochemical, biochemical, and flow cytometric assays. Particles contain DNA that shows laddering indicative of apoptotic cleavage; ribosomal RNA, including a degradation product that likely arises during apoptosis; mRNA species that can be amplified by PCR; and microRNA. Because free RNA molecules are very sensitive to nucleases, their presence in microparticles may provide a sanctuary in the extracellular space to allow transfer of genetic information or provide a source of nucleic acid to stimulate TLR and non-TLR sensors.

The content of nucleic acids provides the basis of a new flow cytometric assay which detects particles by the binding of SYTO13, a cell permeable dye that interacts with nucleic acids. Using this assay, we showed that flow cytometry with SYTO13 can detect approximately three times as many par-

FIG. 3. Comparison of flow cytometric measurement of microparticles by SYTO 13 staining and side scatter. HL-60, Jurkat, and MOLT-4 cells (10^7 cells) were treated with $1 \mu\text{M}$ staurosporine and supernatants harvested 24 h later for analysis. The MPs produced by these cells were detected in cell-free supernatants by fluorescence from SYTO 13 staining (shaded bars) or side scatter (clear bars) in unstained samples. MP counts by SYTO 13 detection or SSC detection were analyzed and were significantly different. $*=p < 0.0004$ in all cases. Figure adapted with permission from Ullal *et al* (55).

FIG. 4. Detection of microparticles by light scatter (SSC) and SYTO 13 staining. Cell-free supernatants containing MPs from Jurkat cells induced to undergo apoptosis by treatment with $1 \mu\text{M}$ staurosporine were analyzed by flow cytometry. The dot-plot compares MPs detected by SSC (black dots) with those detected by SYTO 13 staining (gray dots). Numbers in **bold face** denote percentage of MPs detected by SSC in each of the quadrants (Q1–Q4), and numbers in regular font indicate percentage of MPs detected by fluorescence. The populations of MPs detected by both SSC and fluorescence appear in quadrant Q2. Figure adapted with permission from Ullal *et al* (55).

ticles as does light scattering using preparations of particles from Jurkat cells undergoing apoptosis *in vitro* (Fig. 3; 55). This increased sensitivity results from the detection of particles too small for accurate measurement by light scattering (Fig. 4). As shown by the effects of DNase and RNase, SYTO13 binds to both DNA and RNA in particles, consistent with the content of both nucleic acids.

The phenotypic properties of particles generated *in vitro* indicate that these structures are dynamic and that they may undergo membrane changes following release from cells treated with agents to induce apoptosis. With particles harvested from the media of Jurkat cells treated with staurosporine, a protein kinase C inhibitor, we showed differences in the phenotype of particles released within 2 hours of treatment and those obtained in cultures treated for 18–24 hours. Whereas the particles from the longer duration cultures were positive for annexin V and propidium iodide (PI), particles from the shorter duration cultures had much lower levels of binding to both agents. Importantly, the early release

particles could mature with subsequent culture so that they bound both annexin V and propidium iodide (Fig. 5). The annexin V and PI negative or low particles were also present in untreated cultures, but their levels were very low (56).

These observations may be important in understanding the immune activities of MPs since surface membrane phosphatidylserine (PS) may be a signal for both clearance and immune activity. Indeed, the presence of PS on apoptotic cells may represent one of the molecules leading to the anti-inflammatory activity of this form of cell death; PS is also an “eat me” signal that may restrain immune activity from dying cells by promoting phagocytosis and therefore preventing any transition to necrosis and the potential release of alarmins. If this paradigm also operates with particles, the early release particle may have greater immune activity because of the absence of surface PS. As the particle persists in the extracellular space, its immune properties could diminish as surface PS expression rises. Together, these findings suggest that MPs are packets of potentially immunostimulatory molecules with surface marker expression that influence the overall activity of the structure.

As in the case of HMGB1 where both cell activation and cell death can lead to release, MPs can originate from activated cells as well as those that are dying. To understand particle release with activation, we used the RAW264.7 system to elucidate the effects of TLR stimulation on particle release (19). Using flow cytometry for MP detection, we showed that stimulation with either LPS or poly (I:C) leads to particle release where CpG DNA under the same conditions failed to elicit a comparable response (Fig. 6). Furthermore, we showed that the release of MPs from RAW264.7 cells resulted from the action of nitric oxide since 1400W, an iNOS inhibitor, blocked particle release elicited by LPS or poly (I:C) while an NO donor caused particle release (Fig. 7). In this system, unlike the results with HMGB1 release, we did not observe an effect of IFN on particle release.

FIG. 5. The phenotype of microparticles released into cultures of apoptotic cells. MPs released from staurosporine-treated Jurkat cells were isolated at the intervals of 0–2 h (dark gray peak), 2–4 h (solid line), 4–6 h (broken line), and 6–8 h (light gray peak) after treatment and assessed for propidium iodide, PI (top panel) and annexin V-binding (bottom panel). Figure adapted with permission from Ullal and Pisetky (56).

FIG. 6. Production of microparticles by RAW 264.7 cells in response to stimulation by LPS, poly (I:C), or CpG DNA. RAW 264.7 cells were treated with 0.05 (gray), 0.5 (stippled), 5 (black), or 50 (stripe) $\mu\text{g/ml}$ LPS, or 0.25 (gray), 2.5 (stippled), 25 (black), or 250 (stripe) ng/ml P(I:C), or 0.1 (gray), 1.0 (stippled), 10 (black), or 100 (stripe) μM CpG DNA oligonucleotide, or medium alone (white) for 24 h. MPs were measured with flow cytometry using a side scatter threshold. Figure adapted with permission from Gauley and Pisetky (19).

FIG. 7. The role of nitric oxide in microparticle production by RAW 264.7 cells. (A) RAW 264.7 cells were stimulated with 50 $\mu\text{g/ml}$ LPS or 0.25 $\mu\text{g/ml}$ poly(I:C) for 24 h following pre-incubation with (black bars) or without (white bars) the inducible nitric oxide synthase (iNOS) inhibitor, 1400W. (B) RAW 264.7 cells were treated with 0 (C), 200, 400, 600, 800, or 1000 μM of the nitric oxide donor, DPTA (dipropyleneetriamine NONOate) for 24 h. Microparticles were measured with side scatter using flow cytometry. * $p < 0.05$ control vs. treatment. Error bars represent SD of the mean. Figure adapted with permission from Gauley and Pisetsky (19).

Since particle release can occur with apoptosis and activation, we followed the format for the experiments on HMGB1 to assess whether caspase inhibition could affect particle release from cells treated with LPS or poly (I:C), both agents which can induce apoptosis under conditions of stimulation. The results of these studies resembled those with the HMGB1 system since caspase inhibition increased particle release, an effect that appears due most likely to a shift or perturbation of the death pathway that leads to necrosis and more extensive release of particles as well as other intracellular components, including alarmins.

While particles are much smaller than cells, they are nevertheless sufficiently large to incorporate both nuclear and cytoplasmic molecules, including DNA and RNA. Since particles can interact with cells to transfer both membrane and internal components to recipient cells, they are vehicles for immunostimulatory nucleic acids that can activate both TLR and non-TLR receptors as well as other signaling pathways.

Our studies show notable similarities between HMGB1, DNA, and particle release, although we have not yet measured all of these analytes in the same *in vitro* and *in vivo* systems. It is of interest therefore that HMGB1 released by enterocyte-like CaCo-2 cells stimulated with cytomix (a mixture of cytokines) can exist in both soluble as well as particulate forms (38). In this case, the particles display features of exosomes that are a particle type that originates from the multivesicular body; exosomes are smaller than microparticles and bear different proteins. While the localization of HMGB1 on particles in different systems requires future study, we would suggest that both the alarmins and microparticles are part of the same overall defense system that uses internal components to stimulate immune responses.

When distressed or dying, all cells can generate alarmins, discharging these products into the blood to activate the innate immune system both locally and systemically. For alarmins, the active moiety may be a complex, while for MPs the active moiety may be an organelle. Whether these systems are truly separate is a matter of straightforward molecular and cell biological experiments to define the disposition and location of these nuclear components in the extracellular milieu; importantly, such experiments will determine the extent to which molecules that are nominally soluble are in fact particulates. Future studies will define further the manner in which cells are destroyed during death and how the immune system recycles debris, as a very flexible and effective signaling system.

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Abbreviations Used

CAD = caspase activated DNase
CpG = cytosine guanosine dinucleotide
DAMP = damage (or death) associated molecular pattern
DNA = deoxyribonucleic acid
DNase = deoxyribonuclease
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
HMGB1 = high mobility group box 1 protein
IFN = interferon
IL-1 = interleukin 1
iNOS = inducible nitric oxide synthase
LPS = lipopolysaccharide
MyD88 = myeloid differentiation primary response gene 88
NO = nitric oxide
PAMP = pathogen associated molecular pattern
PCR = polymerase chain reaction
PI = propidium iodide
poly (I:C) = polyinosinic-polycytidylic acid
PRR = pattern recognition receptor
PS = phosphatidylserine
RNA = ribonucleic acid
RNase = ribonuclease
TLR = Toll-like receptor
TNF- α = tumor necrosis factor- α
TRIF = Toll/IL-1R domain containing adaptor-inducing IFN- β
Z-VAD = benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone