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Armed Response: How Dying Cells Influence T-Cell Functions

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Summary

Immune responses during infection, injury, and cancer proceed in the presence of tissue injury and cell death. Consequently, the system must deal with its own dead cells while it determines the appropriate response to the invader. Since apoptotic cells are known to induce immune tolerance and necrotic cells can be potent stimulators of immunity, this decision becomes more complex. The key to understanding the immunologic choices made during cell death is to examine the mechanisms of tolerance induction by dying cells and then relate them to the mechanisms of immunity. Ideally, immunogenic cell death should be directed towards tumor cells and infected cells, whereas tolerogenic cell death should be associated with preventing unwanted immune responses to self. In this review, we discuss how the decision is made by focusing on the biochemical process of cell death and how its key components can influence both tolerance and immunity.

Keywords

tolerance; apoptosis; immunity; HMGB1; ROS; T cell; dendritic cell

Introduction

Among the first concepts that accompanied the descriptions of the vertebrate immune system in the early 1900s was the notion that this system must discriminate 'self' from 'non-self'. This principle was so pervasive that nearly half a century would pass before the recognition of acquired tolerance and autoimmunity, two findings that would change this core dogma from 'inherent' to 'learned' and thus set the stage for contemporary immunology. Acquired immune tolerance prevents the immune response from recognizing self, while the breakdown of these mechanisms can lead to autoimmunity. It then was anticipated that a defective immune system could be further 'trained' to correct such disease. Unfortunately, this has proven to be a greater challenge than first imagined.

One way to approach this issue is to ask how the immune system deals with its own dead and dying cells that expose self-antigens to the immune system. Every day many cells in the body die (including millions of lymphocytes) during normal tissue turnover, and in most cases, there appears to be little impact on the immune response. During infection however, cell death is accompanied by foreign antigens to which the immune response must react. Studies of cell death during infection have shown that dying cells can influence the outcome of an immune response to pathogens (1, 2). Some forms of cell death (e.g. apoptosis) are

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accompanied by the induction of tolerance, while other methods of death (e.g. necrosis) can promote immunity. In recent years, however, it has been recognized that this concept is oversimplified, and a number of factors related to cell death determine whether death is tolerogenic or immunogenic. In this review, we discuss how the immune system makes this distinction by focusing on the biochemical process of cell death and the consequences of the process for the immune response.

Tolerance and immunity to dying cells

One potent method for experimentally inducing peripheral tolerance involves the injection of apoptotic cells carrying an antigen (3–11). However, apoptotic cells are not always tolerogenic, as has been demonstrated for some tumors subjected to chemotherapy; in these systems, tumor cells killed by agents that induce apoptosis and activate caspases (e.g. chemotherapeutics, radiation) can effectively cross-prime the immune response, generating productive immunity (12, 13). These observations point to the idea that there must be multiple factors that determine if dying cells promote immunity or tolerance. Among these, dendritic cells (DCs) play central roles in the recognition of apoptotic cells and in the initiation and course of an immune response. Factors produced by the dying cells can influence the outcome of interaction with the DCs and can change the balance between tolerance and immunity. The most prominent examples are the exposure of phosphatidylserine on the outer leaflet of the plasma membrane of dying cells, the release of inflammatory cytokines [interleukin-10 (IL-10) or transforming growth factor β (TGFβ)] (14), and the immunogenic properties of end-stage degradation products [high mobility group box 1 (HMGB1), uric acid, heat shock proteins] (15, 16). The type of cell undergoing death, the activation (or stress) status of the cell, and even the location of the dying cells can be influential. In some experimental situations, the route of injection can determine the resultant immune response. These factors have been discussed in detail in a recent review (17) and are not be discussed further here. Examination of systemic tolerance induced by apoptotic cells produced during infection in the eye (discussed in more detail below) revealed many of the initial principles relating apoptotic cells to acquired tolerance (9). Our recent studies on the induction of immune tolerance induced by dying cells have used experimental systems such as those described in Fig. 1. We refer to this system throughout this review.

DCs in tolerance versus immunity

DCs are specialized antigen-presenting cells that initiate and direct T-cell immunity (18). These cells capture antigens as they reside in and traffic through non-lymphoid tissue in their immature form. When antigen is encountered in the presence of inflammatory stimuli, they ‘mature’ and take on an immunostimulatory function characterized by the upregulation of costimulatory molecules and an enhanced capacity to stimulate T-cell immunity. Studies have shown that in addition to stimulating immunity, DCs are important in the regulation of immunity and the induction of tolerance through the presentation of antigens associated with apoptotic cells to $CD8^+$ T cells. This has been termed cross presentation or cross tolerance (19–24).

Most DCs can tolerize or stimulate immunity depending on the conditions; however, evidence suggests that several DC subsets exist that perform different functions depending on their lineage or regional localization (18, 24). For example, splenic $CD8\alpha^+$ DCs are potent stimulators of tolerance, while $CD8\alpha^-$ DCs promote immunity (24, 25). While many studies on phagocytosis of apoptotic cells have used macrophages to examine the uptake and influence of apoptotic cells on immune function (10), there is compelling evidence that macrophages are incapable of cross presentation (26). However, the role of the macrophage

in tolerance induction should not be dismissed. In the absence of splenic marginal zone macrophages, apoptotic cells are not rapidly cleared, and immunity to their associated antigens is induced via the DCs (10). This is also highlighted by the importance of the spleen for tolerance in several systems (27, 28). Peripheral tolerance seems to depend on the ingestion of apoptotic cells by DCs, or at least contact between these entities; whether this results in tolerance or immunity is thought to depend on the maturation state of the DC, although there is evidence that mature DCs can induce tolerance (9, 29).

In our studies, the $CD8\alpha^+$ DC was found to be crucial for tolerance induction (7), and it is interesting that the same cells ($CD8\alpha^{high}$ $CD11c^{high}$) that cross prime $CD8^+$ cytotoxic T-lymphocytes (CTLs) also cross-prime regulatory T cells (24). This tolerogenic DC population is extremely effective in transducing a tolerogenic signal (7), as only 0.02–0.03% of the cells in the spleen are sufficient for the observed effects. Similarly, when DCs are derived from GM-CSF-stimulated bone marrow cultures, less than 1% of the DCs are $CD8\alpha^+$; however, removal of this population prevents tolerance induction following exposure to antigen-coupled apoptotic cells (9, 30). This is consistent with (similar) findings by den Haan *et al*. (25), who found only a small percentage of cells isolated from the spleen contained antigen for cross-priming of CTLs. It is also noteworthy that there seems to be a difference in antigen processing and presentation that is intrinsic to these DC subsets: $CD8\alpha^+$ DCs tend to process antigens predominantly for presentation by major histocompatibility complex (MHC) class I molecules, whereas $CD8a^-$ DCs present antigens on MHC class I and class II molecules (31). This observation suggests that the preferential induction of tolerance by $CD8\alpha^+$ DCs might induce diminished $CD4^+$ T-cell-mediated help but enhanced $CD8^+$ T-cell-mediated immune responses. This is directly relevant to the induction of T-cell immunity *in vivo* by apoptotic cells discussed in the next section. A number of cell surface markers have been described to distinguish $CD8\alpha^+$ and $CD8\alpha^-$DCs (24); however, other mechanisms that distinguish the DC populations by their ability to cross prime and/or cross tolerize remain elusive.

Necrotic cells prime $CD4^+$ T-cell help, apoptotic cells do not

Antigens that are associated with dying cells are engulfed by DCs and are then cross-presented on MHC class I molecules to $CD8^+$ T cells. However, the resulting immune response can be quite different, as necrotic cells can prime an immune response, while apoptotic cells are tolerogenic. To reconcile this difference, we examined the mechanisms of cross-presentation and cross-tolerance, as defined in other systems (32). Following antigen recognition by $CD8^+$ T cells and their development into CTLs, the long-term fate of these cells is determined by additional signals provided by activated $CD4^+$ T cells, such as the action of CD40-ligand on the DCs. Without these additional signals, acting to ‘license’ the DCs, the activated ‘helpless’ CTLs function as primary effector T cells but have a short lifespan (29, 32, 33) and die by activation-induced cell death following subsequent exposure to antigen. This activation-induced cell death is mediated by the expression of the death ligand TRAIL (TNF-related apoptosis-inducing ligand; also known as TNFSF10), which triggers apoptosis in helpless CTLs and other activated T cells (32).

The relationship between these observations and the induction of tolerance by apoptotic cells was revealed when we examined T-cell priming with apoptotic and necrotic cells (8). DCs that had encountered necrotic cells presented antigen to both $CD4^+$ and $CD8^+$ T cells, but those that engulfed apoptotic cells presented antigen to $CD8^+$ T cells but not $CD4^+$ T cells. The latter $CD8^+$ T cells produced TRAIL following re-exposure to the antigen, which inhibited the induction of a cell-mediated immune response (that is, they mediated tolerance). TRAIL-mediated suppression was directed toward the $CD4^+$ T cells responding to a subsequent antigen challenge and may be mediated by demise of the responding cells

(34, 35). The induction of tolerance could be overcome by activation of CD40 at the time of exposure to apoptotic cells (7), and therefore it is likely that T-cell help can prevent tolerance through such a mechanism. Consequently, exposure to apoptotic cells shifts the system from classical 'helped' CTL-mediated immune responses to those induced by tolerogenic 'helpless' CTLs that produce TRAIL following re-exposure to antigen. In support of this shift, TRAIL-deficient mice were resistant to the induction of tolerance mediated by the intravenous injection of apoptotic cells (8). Thus, encounter with apoptotic cells results in the induction of a $CD8^+$ Treg that mediates tolerance by producing cytotoxic TRAIL (see Fig. 2 for a summary of these findings). Interestingly, this tolerance is relatively short lived, lasting approximately 60 days (34), which may limit its potential for long term therapeutic purposes.

On a historical note, these results may relate to some of the earliest descriptions of $CD8^+$ Tregs, once termed T-suppressor cells (Ts cells) (36–39), that regulate immune responses through the secretion of a soluble factor that suppresses immunity by inhibiting T-cell function (called T-suppressor factor) (40, 41). Although it is not our intent to explain all of the properties ascribed to 'T-suppressor factor', it is interesting to speculate that at least some of the suppressor activity in the supernatants of $CD8^+$ Ts cells was TRAIL. Indeed, descriptions of such factors as composed of trimers of a 23 kDa monomer (42), is consistent with the electrophoretic behavior of TRAIL (43, 44). Thus, $CD8^+$ T cells that suppress immune responses may not belong to a unique T-cell subset with specialized function but may instead manifest a regulatory activity of otherwise normal cytotoxic $CD8^+$ T cells. Our data, showing a $CD8^+$ Treg in our system is consistent with a helpless CTL, support this idea (8). This may explain why it was previously difficult to identify unique cell surface markers that identified 'Ts' cells as a functional population [and one of the issues that fueled the 'suppressor cell' controversy (45)]. Perhaps our results also explain why Ts cells were not effectively cloned; they underwent TRAIL-dependent, activation-induced cell death upon restimulation.

The role of apoptotic cells in other settings of tolerance induction

Tolerance via antigen exposure in an immune privileged site

The connection between apoptosis of lymphoid cells and active immune regulation in an *in vivo* system was first described in the mid-1990s in studies on immune privilege in the eye (4, 46). The mechanisms of immune privilege have been discussed in detail elsewhere (47); however, it is useful to point out the properties that distinguish immune-privileged sites such as the eye from conventional ones. First, tissue transplanted to the eye enjoys a higher rate of success compared with other areas. There is extraordinary success of corneal transplants in humans, where high graft acceptance rates are observed without tissue matching or systemic immunosuppressive therapy (48). Second, the eye constitutively expresses CD95L, which induces apoptosis in invading $CD95^+$ lymphoid cells (49). CD95L, which is expressed throughout the retina and on the corneal epithelium and endothelium, is critical for controlling inflammation and is instrumental in the successful transplantation of the cornea. When CD95L is defective in the eye, as in *gld* mice, inflammation induced by virus (48, 49) and other agents (50) cannot be controlled, resulting in significant damage to the ocular tissues. Third, and most important for this discussion, the apoptosis of lymphoid cells by CD95L in the eye leads to systemic immune tolerance to associated antigen. Thus, what is most important for tolerance in this system is the invasion of the eye by inflammatory cells and their subsequent apoptosis (4). Injection of antigen without associated inflammation (and apoptosis) is not tolerogenic (4, 28).

The importance of inflammatory cell apoptosis to the induction of tolerance was demonstrated in experimental herpes simplex virus type 1 (HSV-1) infection of the eye.

When virus was injected into the anterior chamber (AC) of the murine eye, infiltrating inflammatory cells underwent apoptosis within 48 h (49), and tolerance [as measured by systemic delayed-type hypersensitivity (DTH)] to the viral antigens developed (4). When virus was injected into the AC of CD95- or CD95L-defective mice, HSV-1 infection did not result in the death of inflammatory cells, and tolerance did not occur. Importantly, there was massive uncontrolled inflammation in these infected eyes (49). Furthermore, if the eye (containing the dead cells) in wildtype mice was removed within the first 3 days of viral injection, tolerance was not established. These enucleated mice instead became immune to the virus, suggesting that sufficient viral antigen can leave the eye to induce immunity, but the eye must remain intact for a time so that tolerance induction by apoptotic cells can be established. This three day time frame may allow the dead cells within the eye to initiate the systemic tolerance response (4).

Using the experimental system in which TNP-coupled spleen cells (TNP-spl) were injected into the eye (similar to Fig. 1, AC instead of intravenous), we first realized that cell death had a significant role in tolerance induction (4). In this system, the genotypes of the injected cells and the recipients were readily controlled, and we found that the presence of functional CD95 on the injected cells and CD95L in the eye were essential for tolerance induction. When either was defective or absent, immune tolerance was not established. Surprisingly, while CD95L in the eye was required for tolerance, it was the process of apoptotic cell death that was the critical factor in tolerance induction. That is, CD95-defective *lpr* TNP-spl, which did not undergo apoptosis or induce tolerance when injected into the AC of mice, induced tolerance if they were made to undergo apoptotic cell death by irradiation or heat shock prior to injection. In contrast, if these cells were induced to undergo necrosis, e.g. by freeze–thaw, tolerance did not occur. Further, tolerance was not established if apoptosis was prevented by overexpression of BCL-xL. The implication from these studies with virus or TNP-spl was that the CD95L-induced dying cells influenced the immune outcome, overriding the induction of immunity and promoting tolerance. Also, while CD95-mediated apoptosis was critical, it was the mitochondrial pathway of apoptosis that was required for tolerance to develop, as indicated by the effect of BCL-xL. This idea was critical for understanding tolerance induction and laid the foundation for studies, described below, that established a critical role for caspase activation and mitochondrial destruction for the induction of tolerance by apoptotic cells (9).

We then undertook studies to define how the apoptosis of lymphoid cells in the eye could induce active systemic unresponsiveness. We felt that it was important to show how the dead cells in the eye reached the immune machinery in the spleen, as the spleen had been previously established as the ‘site of action’ for this form of tolerance (27, 28). We examined several ideas including the possibility that phagocytic cells present in the eye captured the apoptotic cells and delivered them to the spleen, as phagocytic cells in the eye have been proposed to capture antigen and deliver it to the spleen in a tolerogenic form (51). However, we were not able to demonstrate any transit of antigen-laden phagocytes from the eye to the spleen, nor were we able to show that the phagocytic cells within the eye even captured apoptotic cells or antigen. In fact, definitive proof that antigen was delivered systemically by cells residing in the eye has, to our knowledge, never been obtained. What we did observe, however, was that apoptotic cells were washed from the eye, directly entered the blood, and then lodged in the spleen. The apoptotic cells were subsequently captured by splenic DCs, which were responsible for initiating the tolerance response (52). This sequence of events is identical to what happens in tolerance induced by intravenous injection of antigen-coupled splenocytes (see below). Subsequently it was realized that the antigen-presenting cells resident in the eye are not inherently tolerogenic, but instead can act as potent inducers of immunity (53). Thus, their role in tolerance induction upon administration of antigen into the AC of the eye is doubtful. It is interesting to note that

there is no $CD4^+$ T-cell activation in this system and that tolerance induced by apoptotic cells following antigen injection in the eye is mediated by a TRAIL-producing $CD8^+$ T cell (T.S. Griffith and T.A. Ferguson, unpublished results).

Apoptosis and immune tolerance in sepsis

Sepsis, the leading cause of death in most intensive care units, induces a severe immunosuppression, making it difficult to control the primary infection and predisposing patients to secondary nosocomial infections (54, 55). This immune defect may be critical to the pathogenesis and subsequent mortality in sepsis (56). Because sepsis induces significant apoptosis in lymphoid and myeloid cells, and apoptotic cells promote tolerance (see above), we recently tested the idea that suppression of immunity in sepsis may be mediated by the tolerogenic properties of apoptotic cells (57). We immunized mice for DTH on various days following cecal ligation and puncture (CLP), which induces sepsis, and then tested their recall response. We found that for seven days following CLP, mice were unresponsive to antigen, but by day 10, normal responses were observed.

CLP induces massive apoptosis in lymphocytes that was blocked by transgenic expression of BCL-2 or Bim deficiency (58, 59), and such blockade preserved immunocompetence. Importantly, injection of apoptotic splenocytes into $Bim^{-/-}$ mice, again suppressed immunity following CLP. This observation suggests a causal link between apoptotic cells and immune suppression in sepsis. Interestingly, when TRAIL-deficient mice were subjected to CLP, apoptosis was not blocked, but the animals retained their DTH responses. So even though the apoptosis was not dependent on TRAIL, the immune suppression that resulted was mediated by this cytokine. Consistent with this finding (and with the tolerance studies discussed above), we found that TRAIL in this system is produced by a $CD8^+$ regulatory T cell (57). Thus, in this system, there is an important link between apoptotic cells and immune suppression during sepsis, and TRAIL-producing $CD8^+$ Treg cells are critical to the mechanism of this short-lived induction of immune unresponsiveness.

Apoptotic cells and tolerance following peripheral T-cell deletion

During an immune response, clones of reactive T cells rapidly proliferate, followed by a contraction phase. In lymphoid organs, this contraction is mediated by Bim-dependent as well as CD95-mediated apoptosis (60, 61). In tissues such as the skin, however, CD95L-CD95 interactions are essential to return the T-cell response to homeostasis (62).

Can such apoptosis during immune contraction promote tolerance in some settings? We recently tested this idea, using a system in which a relatively large number of T cells undergo peripheral deletion in a short period time following cognate antigen exposure (34, 35). Naive, T-cell receptor (TCR) transgenic $CD4^+$ T cells, responsive to ovalbumin (OVA), were adoptively transferred to syngeneic recipients, following which the OVA peptide was administered. This resulted in rapid deletion of the transgenic T cells from the spleen and lymph nodes, mediated by CD95L-CD95 interactions. Following deletion, these mice were then incapable of generating a DTH response to OVA. When these mice were given a second infusion of naive TCR T cells of the same specificity, the mice remained insensitive to OVA, and thus the unresponsiveness was actively maintained. In contrast, if the tolerant mice were seeded with $CD4^+$ TCR transgenic T cells of different antigen specificity, immunity to that antigen was produced following immunization. This antigen-specific tolerance was dependent on the number of T cells undergoing deletion. Similarly, tolerance was also induced by administration of irradiated TCR transgenic cells. Further, animals lacking TRAIL or TRAIL receptor maintained immune responsiveness, and thus, the process was similar to that seen in other tolerance systems, discussed above. Interestingly, the tolerance in this system appeared to be mediated by a cytotoxic $CD8^+$ T cell capable of

killing the TCR transgenic T cells, raising the possibility that the effect was targeted to TCR-derived peptides. Such 'clonotype-specific' regulation by $CD8^+$ T cells has been described in other systems (63). It is tempting to speculate that during the course of an immune response, sufficiently large number of apoptotic T cells bearing a particular TCR may promote a tolerance mechanism that directs subsequent responses to their own (and other specificities) in the recall response. This may be consistent with changes in specificities observed in the recall response to influenza, for example (64).

The influence of the process of apoptosis in dying cells on the DCs that engulf them

One distinction between apoptotic and necrotic cells is the release of damage-associated molecular patterns (DAMPs) from the latter (15). We reasoned that understanding the pathways of cell death and how these pathways impact DAMPs would give us insight into the mechanism of modulation of immunity by dying cells. A major distinction between apoptosis and other forms of cell death is the involvement of caspase proteases (65). These enzymes are activated by signaling pathways in the apoptotic cells and orchestrate apoptosis through their cleavage of specific substrates. Caspases are important for many of the cellular events associated with apoptosis, including DNA fragmentation, membrane blebbing, and fragmentation of the cell into membrane-bound apoptotic bodies (66). The mitochondrial pathway of apoptosis involves mitochondrial outer membrane permeablization (MOMP), followed by cytochrome c release and the activation of the executioner caspases 3 and 7. These caspases then attack the permeabilized mitochondria leading to destruction of the electron transport chain, the loss of ATP, and the production of ROS (67, 68). With these well characterized events in mind, we explored the role of this pathway in tolerance induction.

We based our studies on two observations concerning apoptosis that we previously made in our tolerance system (see above). First, blocking caspase activity during apoptosis converts a tolerogenic signal into an immunogenic one (7), and second, blocking MOMP by overexpression of Bcl-xL prevents tolerance induction by lymphocytes that are undergoing CD95-mediated apoptosis (4). These observations pointed to a pivotal role for both MOMP and caspases in altering a mitochondrial function that might be important for the tolerogenic effects of apoptotic cells on the immune system.

Ultraviolet (UV) irradiated wildtype or caspase-3,-7-double deficient mouse embryonic fibroblasts were cultured with hapten-modified DCs, which were then injected into mice, following which immunity versus tolerance was assessed (Fig. 1). These experiments showed that cells deficient in executioner caspase expression were immunogenic not tolerogenic. As one consequence of caspase activation is the production of ROS by the dying cells, we tested whether such ROS production might contribute to tolerance induction by apoptotic cells. We found that tolerance induction was converted to immunity if apoptotic cells were treated with a ROS scavenger or with a reducing agent [dithiothreitol (DTT)]. Conversely, necrotic cells, treated with an oxidizing agent [hydrogen peroxide (H_2O_2)] became tolerogenic. Together these studies suggested that caspase-dependent ROS production is critical to the tolerogenic nature of apoptotic cells.

Previous work demonstrated that during apoptosis activated caspases feed back on the permeabilized mitochondria, cleaving NADH dehydrogenase Fe-S protein-1 (p75 NDUFS1), the 75 kDa subunit of respiratory complex I; this results in a loss of ATP production and the generation of ROS (68). Mutation of a single amino acid in the caspase cleavage site of NDUFS1 delayed mitochondrial destruction and prevented ROS production during apoptosis. These observations established a molecular link between caspase-

dependent apoptosis and ROS production, and we asked if this link was involved in tolerance induction. UV-irradiated HeLa cells expressing wildtype NDUFS1, cultured with antigen-coupled DCs induced potent tolerance, as expected. In contrast, UV irradiated HeLa expressing the non-cleavable mutant of NDUFS1 failed to induce tolerance in this system and instead induced potent immunity. Thus, caspase-mediated cleavage of NDUFS1 in mitochondria that had undergone MOMP is required for tolerance induction, possibly as a consequence of ROS production.

Induction of immunity by UV irradiated HeLa expressing the non-cleavable mutant of NDUFS1 could be recapitulated by using supernatants from these cells in a co-culture with apoptotic cells and DCs. Supernatants from expressing wildtype NDUFS1 did not have this function. This finding suggested that cells without mitochondrial degradation were releasing something that might promote immunity by perhaps mimicking necrosis. One of the reported differences between apoptotic and necrotic cells is that necrotic cells can release high mobility group box 1 protein (HMGB1) (69). However, when the supernatants from apoptotic HeLa cells expressing the wildtype or mutant NDUFS1 were examined, HMGB1 was found to be released in similar quantities (9). Importantly, however, depletion of HMGB1 from the supernatant of the cells expressing the mutant protein abolished the ability to stimulate immunity, while HMGB1 in the supernatant of the wildtype apoptotic cell was non-functional. The importance of HMGB1 in this system was further confirmed as necrotic cells from HMGB1-de cient mice were tolerogenic rather than immunogenic, unless supplemented with exogenous HMGB1. Together, these results suggested that it was not the quantity but rather the quality of HMGB1 that determined the immunologic outcome.

One difference between apoptotic cells expressing wildtype versus mutant NDUFS1 was the production of ROS in the wildtype cells. HMGB1 acts as a sensor of oxidative stress in others system (70), and therefore, we prepared recombinant HMGB1 in which the cysteines (23, 45, and/or 106) were mutated to serines, making these residues resistant to oxidative conditions. Using these recombinant proteins, we found that the oxidation of Cys106 (and not Cys23 or Cys45) destroyed the ability of HMGB1 to function as an immunostimulatory molecule. Thus, the oxidative state of HMGB1 regulates the outcome of the immune response (9). These results are summarized in Fig. 3.

HMGB1 in immunity versus tolerance

HMGB1 is a DNA-binding protein historically known as a non-histone chromosome architectural protein that is conserved across species. HMGB1 stabilizes nucleosomes and allows bending of DNA to facilitate gene transcription and rearrangement (71). It contains 215 amino acids and has two homologous DNA-binding domains (called the A box and B box) and a C-terminal acidic region. HMGB1 is recognized as the prototypical DAMP, and the B-box region contains this activity in some systems (72, 73). It has been reported to be a potent activator of macrophages (74) and DCs (75), signaling via the receptor for advanced glycation end products (RAGE) (76), as well as TLR2, TLR4, and/or TLR9 (77, 78). It is thought to be a mediator of inflammation and to participate in a number of pathogenic processes including septic shock (79), acute lung injury (76), and arthritis (80). Recently the Cys106 in HMGB1 was identified as a key residue in its ability to activate proinflammatory activity in macrophages (e.g. TLR4 binding and TNFα production). Interestingly, mutation of Cys106 to Ser106 abolishes the ability of HMGB1 to interact with TLR4 (81).

The inflammatory function of extracellular HMGB1 has been challenged, as the recombinant HMGB1 (rHMGB1) from different sources elicited varying pro-inflammatory activity. For example, rHMGB1 obtained from prokaryotic sources (*E. coli*) can stimulate inflammation via TLR4 (82); however, when prepared from eukaryotic cells, it does not

necessarily display this function (83, 84). Thus, some effects of HMGB1 may be due its binding to contaminating bacterial molecules (83, 84). HMGB1 has also been found to bind numerous other proteins, including cytokines such as IL-1β, TNFα, and IFNγ (73, 85, 86), and free nucleic acids. The latter can generate signals through TLR9 and RAGE (87). Of course, rather than 'contamination', such binding by HMGB1 may function in a biological context, that is, bound cofactors may be directed by HMGB1 to produce its biological effects. This is consistent with our results; HMGB1 plus antigen, without the presence of dying cells, does not stimulate DC to promote immunity (Fig. 4). The required component from the dying cells is currently unknown. Thus, identifying the co-factors involved in HMGB1 function in any given setting may be critical to our understanding of the immunologic function of this protein.

The suggestion that HMGB1 is released during necrosis but not apoptosis (69) does not apply to all cell types and may be an artifact of overexpression systems. During apoptosis, fragmented nuclear DNA and associated proteins are released (88), and the binding of HMGB1 to DNA is increased during apoptosis, consistent with the idea that late-stage apoptotic cells can release both DNA and HMGB1 (89). Apoptotic tumor cells can release HMGB1, and this is dependent on caspase activation (9, 78, 89). Such dying cells are immunogenic by virtue of their release of HMGB1(13, 78, 89). It has been suggested that post-translational modifications (acetylation, phosphorylation) of HMGB1 might determine its immunostimulatory capacity; however, this seems to apply only to cells that actively secrete HMGB1 during inflammation (e.g. DCs, macrophage) (90, 91) and not cells undergoing programmed cell death or necrosis. We were unable to identify such modifications to HMGB1 in our systems.

Our results (discussed above) showed that the oxidation of HMGB1 is a major determinant in the outcome of an encounter between dying cells and the immune response. We found that during apoptosis HMGB1 is oxidized, preventing its function as a DAMP. In contrast, HMGB1 released from necrotic cells (non-oxidized) or HMGB1 released in the absence of caspase activation (also not oxidized) is capable of blocking tolerance by apoptotic cells through interaction with DCs. Interestingly, in the process of blocking tolerance, HMGB1 did not induce overt maturation of DCs or measureable proinflammatory cytokine production (9). In addition, HMGB1 alone did not stimulate immunogenic activity of DCs, while immunity was induced by addition of HMGB1 to apoptotic cells (Fig. 4). How HMGB1can exert its effects on tolerance and immunity without overt stimulatory effects remains unknown.

In our system, HMGB1 can exert its effects on DCs when used in the nanogram range (9). Many other studies (73, 81, 92) have used 10–100 times that concentration to stimulate proinflammatory cytokines and other functions. Perhaps we were unable to detect HMGB1 effects because we simply did not use enough of the protein; however, a recent paper (93) examining the role of CD24 in the regulation of HMGB1 function found little cytokine production from DCs in the absence of this cell surface protein. These authors showed that CD24 on DCs was a negative regulator of HMGB1 function and that CD24 deletion ($CD24^{-/-}$ mice) increased the production of cytokines by DCs. When wildtype ($CD24^{+/+}$) DCs were examined, proinflammatory cytokine induction by HMGB1 was minimal, consistent with our studies (9). Thus, HMGB1 may affect different pathways, depending on local levels of this protein. However, it should also be noted that increasing HMGB1 concentrations would amplify the influence of protein contamination with other factors from the source of the recombinant protein (LPS or other factors, see above).

HMGB1 has been implicated in a number of biological processes, including septic shock, tumor immunity, and arthritis (15, 76, 94). It is released by DCs, macrophages, and other

cells to promote inflammation and immunity. In sepsis and endotoxemia, for example, HMGB1 is thought to be a late mediator of the pathology observed. During these processes, HMGB1 is (rapidly) released, and neutralization of HMGB1 can block the lethal effects of LPS or cecal ligation and puncture (CLP) (95). The source of HMGB1 in these systems is thought to be the activated macrophage; however, it is well established that there is massive apoptosis and necrosis in these models (96). One possibility is that cell death contributes to the increased levels of HMGB1 identified in the blood, although this has not been tested. It is also not known if the redox state of HMGB1 has any influence on lethality in this model. The high level of oxidative conditions during these infections might suggest that HMGB1 is oxidized, and indeed, anti-oxidants can ameliorate lethality in some infections (97). It will be interesting to determine whether reduced HMGB1, which promotes immunity, would have beneficial effects in sepsis and endotoxemia.

Another unexplored area of HMGB1 function is the role of oxidative modification in the interaction of HMGB1 and its receptors. Perhaps oxidation/reduction may alter the binding of the protein to one of the known receptors (TLR2, TLR4, TLR9, or RAGE) (77). It is also possible that the redox state of HMGB1 can influence the binding to one of the co-factors discussed above. Indeed, a recent study suggested that HMGB1 binding to TLR4 required Cys106. When this cysteine was converted to serine (which prevents oxidation), binding to TLR4 was not observed (81). Is it possible that oxidation of HMGB1 at Cys106 may increase its binding to TLR4? Similarly, HMGB1 was identified as a CpG-ODN–binding protein and a cofactor for proinflammatory cytokine synthesis (87), and oxidation of HMGB1 alters its binding to DNA (98, 99). Thus, the costimulatory activity of HMGB1 and DNA binding may be regulated by oxidation of the protein.

One effect of apoptotic cells on DC function is to prevent activation of $CD4^+$ T-cell help for CTLs, as discussed above. However, in some cases, DCs that have engulfed apoptotic cells can drive $CD4^+$ T-cell differentiation toward the Th2 type via the production of IL-10 (3). We do not know if such Th2 cells can license DCs to promote $CD8^+$ T-cell immunity, but if not, such polarization may promote the generation of helpless $CD8^+$ T cells to suppress immune responses. In contrast, HMGB1 has been shown to direct $CD4^+$ T cells toward a Th1 phenotype (75), promoting DTH and ‘helped’ $CD8^+$ T cells. The role of oxidation on this function of HMGB1 has not been explored but would be consistent with our results.

Clearance of dying cells is important in preventing autoimmunity, as animals deficient in mechanisms responsible for phagocytosis of dying cells often become autoimmune (100–102). It has been suggested that secondary necrosis of apoptotic cells is responsible for this effect (10, 11). However, there is another possibility. Recent studies have shown that the uptake of dying cells by phagocytes engages a cholesterol clearance mechanism via activation of liver-X-receptor (LXR). This function of LXR may also be immunosuppressive, and indeed, autoimmunity in *lpr* mice was effectively prevented by treatment with an LXR agonist (103). It is an intriguing possibility that the redox state of dying cells influences the activation of LXR, thereby affecting the immune outcome. HMGB1 may act as the carrier for this signal.

Concluding remarks

Therapeutic application of the principles of immune tolerance induced by cell death requires a thorough understanding how dying cells impact the immune response. Translating this knowledge to infection, cancer, and autoimmunity requires a more complete understanding of how the immune system makes this important decision. Clearly the modulation of the immune response by dead and dying cells is complex, relying not only on the properties of the phagocytic cells but also the type of T-cell immunity that ensues. It is important to

realize, however, that dying cells are much more active participants in these decisions than that were originally thought. Clearance of dying cells is certainly important, but the biochemical events evolving in the death process prior to clearance also determine many of the subsequent events. Processes such as caspase activation, mitochondrial degradation, ROS production, and oxidative modification of DAMPS can tip the balance between tolerance and immunity. This complex interplay can maintain system homeostasis but perhaps be manipulated to treat disease processes.

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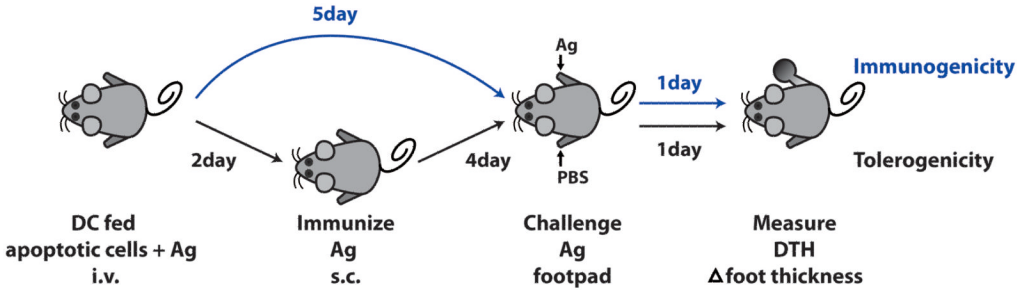

Fig. 1. Assays to measure the immunogenicity and tolerogenicity of dying cells

Immunogenicity test (indicated in blue): dendritic cells (DCs) fed apoptotic cells and antigen (Ag) are injected intravenously (i.v.) into syngeneic mice. Five days later, mice are injected with the Ag in the right footpad and PBS in the left footpad. Twenty-four hours later, DTH is measured by the difference in thickness between the right and left footpads. Positive DTH indicates priming of the immune response by the apoptotic cells fed to DCs. Tolerogenicity test (indicated in black): DCs fed apoptotic cells and Ag are injected i.v. into syngeneic mice. Two days later, mice are immunized with the Ag subcutaneously (s.c.). Four days following immunization, mice are injected with the Ag in the right footpad and PBS in the left footpad. Twenty-four hours later, DTH is measured by the difference in thickness between the right and left footpads. Negative DTH indicates tolerance induction by the apoptotic cells fed to DCs compared to mice that received only a subcutaneous immunization with antigen.

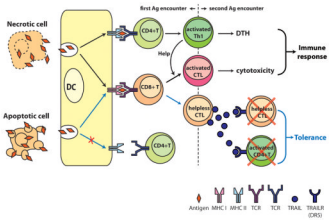

Fig. 2. Immune response and tolerance induction by dying cells

Antigens associated with dying cells are taken up by DCs. In the case of necrotic cells, the antigens are processed and presented on both MHC class II and MHC class I, leading to presented to both $CD4^+$ and $CD8^+$ T cells, respectively (first antigen encounter). Antigen encounter a second time results a delayed type hypersensitivity (DTH) mediated by $CD4^+$ T cells or cellular cytotoxicity mediated by $CD8^+$ cytotoxic lymphocytes (CTLs). However, antigens associated with apoptotic cells are presented via MHC class I only to $CD8^+$ T cells. The activated $CD8^+$ T cells then differentiate to CTLs without the benefit of $CD4^+$ T-cell help (first antigen encounter). The helpless CTL produces TRAIL upon second encounter of the antigen, depleting the helpless CTLs and activated $CD4^+$ T cells. This leads to immune tolerance.

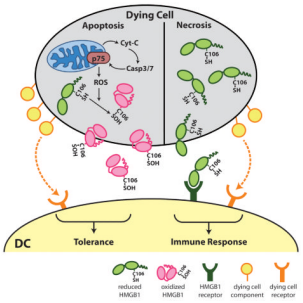

Fig. 3. Redox status determines the immunogenicity of HMGB1

Apoptotic and necrotic cells both release HMGB1. HMGB1 from necrotic cells is in a reduced form (−SH) and can stimulate an immune response when it interacts with the DC in the presence of apoptotic cells. Reduction of Cys106 (C106) is most critical. During apoptosis, cytochrome C release and the activation of executioner caspases 3 and 7 (casp3/7) leads to the cleavage of NDUFS1 (p75) and the release of ROS. ROS oxidize the Cys106 (C106) on HMGB1, preventing stimulation of the DC. Without active (reduced) HMGB1, apoptotic cells are free to interact with the DC and induce immune tolerance.

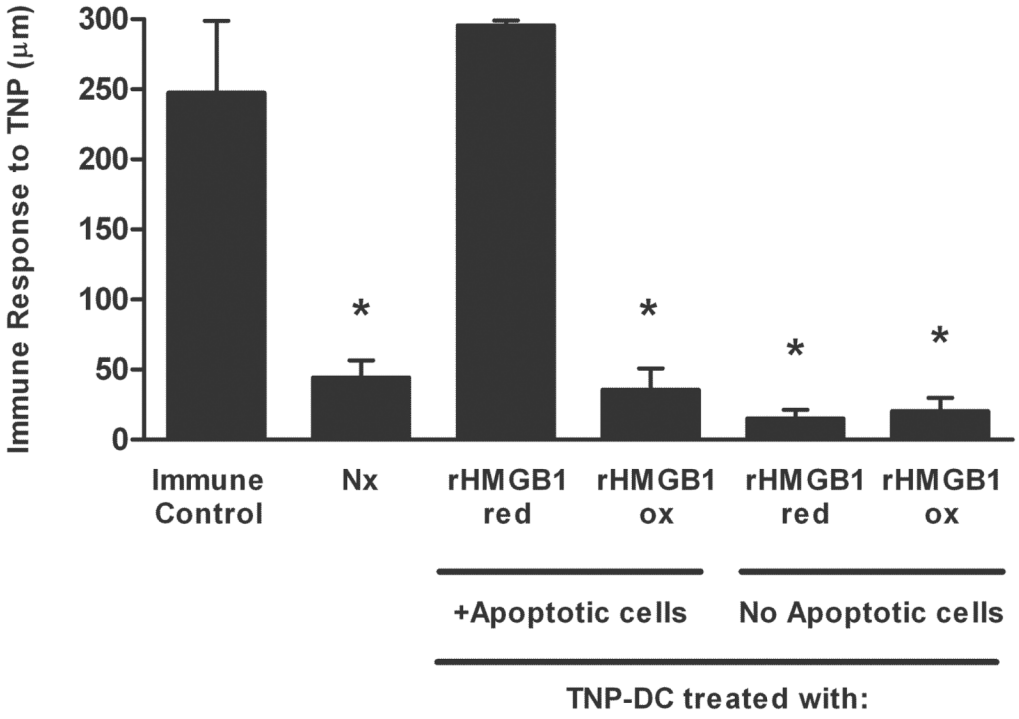

Fig. 4. HMGB1 does not stimulate immunity without apoptotic cells

Hapten-modified CD8α$^+$ dendritic cells (TNP-DCs) were cultured over night in the presence of H_20_2 oxidized (ox) or DTT reduced (red) rHMGB1 (250ng/ml) with or without apoptotic cells (γ-irradiated spleen cells), as described (9). TNP (TNP-DCs) were then injected i.v. into syngeneic mice (5×10^5/mouse). Five days later, mice were challenged with TNBS in the right footpad and PBS in the left footpad. Measurements (μm ± standard error) were taken 24 hours later and represent the difference between the right footpad (antigen challenge) and left footpad (PBS challenge). Immune control groups were injected with 0.1 ml 10 mM TNBS subcutaneously on the day of TNP-DC injection. Nx indicates no treatment of the TNP-DC. * denotes significantly different from the immune control ($p< .01$).