



Endogenous DAMPs, Category I: Constitutively Expressed, Native Molecules (Cat. I DAMPs)

12

12.1 Introduction

Endogenous DAMPs which are constitutively expressed in their native state are denoted as Cat. I DAMPs. Depending on their mode of emission, they comprise molecules passively released from necrotic cells, actively exposed at the surface of stressed cells, and actively secreted by stressed or dying cells. This category of danger signals includes molecules that are regarded as the “prototypical” or “historical” DAMPs which marked the start of the era of DAMPs; the HMGB1 and HSPs can be found among these molecules but also proteins of entirely different nature, structure, and function such as MHC Class I chain-related proteins.

Of note, some of the DAMPs described in this but also other subsequent chapters refer to so-called Hyppos in terms of proteins or lipids with hydrophobic surfaces. These hydrophobic molecules have been characterized by Seong and Matzinger already very early (in 2004!) as integral parts of endogenous DAMPs [1]. For example, as outlines in this anecdotal article, uric acid, HSPs, and hyaluronan (HA) polymers (held together by evenly spaced hyppos) have such hydrophobic binding sites. In the following, they will be described under the various subchapters concerned.

Together, the global role of the Cat. I DAMP in the initiation of various efferent innate immune pathways and adaptive immune responses justifies their description in the first chapter.

12.2 DAMPs Passively Released from Necrotic Cells (Class IA DAMPs)

12.2.1 Introductory Remarks

In case of ACD or RN as defined by a complete rupture of the plasma membrane, the whole content of the cell is passively released. The various intracellular native compounds including organelles and cell debris operate in the extracellular milieu

as constitutively expressed native DAMPs, denoted here as Class IA DAMPs (for ACD and RN, compare Part V, Chap. 17 and Sect. 19.3). Most of these DAMPs such as HMGB1 [2–4], HSPs [5–7], S100A8/A9 proteins [8, 9], and NAs [10, 11] can be directly sensed via binding to a variety of PRMs. These PRMs in terms of “classical” PRRs include TLRs, CLRs, NLRs, ALRs along with other DNA receptors such as cGAS, and RAGE (for informing articles, see Refs. [10, 12–21]). Many of these Class IA DAMPs signal danger to the surrounding PRMs-bearing cells such as leukocytes and phagocytes to trigger sterile inflammation and to PRM-bearing DCs to elicit adaptive immunity. In addition, some of these Class IA DAMPs were shown to activate sessile PRM-bearing cells of the innate immune system such as fibroblasts, myofibroblasts, and epithelial and vascular cells, thereby promoting repair and regeneration, for example, wound-healing processes following infectious/sterile injury-induced inflammation (for relevant articles, see Refs. [22–27]).

In the following, those DAMPs will be described which are predominantly or exclusively recognized by TLRs such as TLR4 and TLR2 (but also by RAGE) as well as NA receptor molecules such as RIG-I and cGAS. Historically, HSP72 plays a particular role as such a DAMP, because descriptions of endogenous agonists of TLRs up-regulated *in vivo* during sterile tissue injury started with this molecule [28]. Up to now, however, HMGB1 is the by far most investigated DAMP. Thus, these two molecules will be addressed in more detail.

12.2.2 The Prototype of DAMPs: High Mobility Group Box 1 (Subclass IA-1 DAMP)

12.2.2.1 General Remarks

The high mobility group (HMG) nuclear proteins were discovered in 1973 in an effort to better define the specific regulators of gene expression. Regarding their quick migration during electrophoresis, they were named after this property [29]. As a member of a subfamily of the HMG proteins, HMGB1 is both a nucleus-resident factor released following cell membrane rupture and—under certain conditions—a secreted (modified) protein (see also below, Sect. 14.3.3). As an intracellular nonhistone chromatin-associated protein, it operates as a DNA chaperone under homeostatic conditions by binding the DNA double helix transiently and bending it reversibly. As a DNA chaperone, the protein enhances the formation of nucleosomes; contributes to the assembly of site-specific DNA binding proteins to their cognate binding sites within chromatin, including transcription factors that underwind DNA upon binding; and is involved in transcription, replication, and DNA repair. Indeed, HMGB1 is an extremely mobile protein. The entire pool of HMGB1 roams the nucleus, resting on a specific DNA site for only fractions of a second. The transient binding to chromatin enables HMGB1 to perform its activities in transcription and other nuclear transactions [30]. The native HMGB1 is constitutively expressed in almost all cell types, and to operate as a DAMP, it is passively released following ACD or RN. Of note, in terms of an inducible DAMP, HMGB1 is actively

secreted in a processed/modified state during severe stress (for competent articles and reviews, see Refs. [31–34]).

Clearly, the history of the development of the molecule and current knowledge of its function are fascinating. During recent years, the number of investigations on HMGB1 is steadily growing. Justifiably, today, one can state that HMGB1 is apparently the best characterized DAMP. By utilizing comprehensive review articles as a guide (see Refs. [30, 31, 35–37]), a brief summary of the properties of this most relevant DAMP is depicted in the following.

12.2.2.2 The High Mobility Group Box Family of Proteins

The HMG proteins comprise three families: HMG-A, HMG-N, and HMG-box (HMGB) proteins. The HMGB proteins are by far the largest group, playing critical roles in recognition and maintenance of DNA in DNA-dependent cellular processes (reviewed in [38]). The HMGB family in mammals comprises the three evolutionarily highly conserved proteins HMGB1 (previously HMG1), HMGB2 (previously HMG2), and HMGB3 (previously HMG4 or HMG2b).

High mobility group box protein 1, an abundant 215 amino acid residue-containing protein present at variable levels in almost all mammalian tissues and cells, is highly conserved among various species. The molecule has a tripartite structure and consists of two homologous L-shaped DNA-binding domains (termed N-terminal HMG A box and central HMG B box) and a negatively charged (acidic) C-terminal tail. The tail interacts with the HMG boxes and may modulate their intermolecular interactions (for relevant articles, see [34, 39–42]) (Fig. 12.1). These separate structural motifs seem to function in different ways when isolated from HMGB1. Thus, several studies have identified the B-box domain as important for many of the pro-inflammatory properties of HMGB1 including cytokine release. In comparison, the A-box does not have the pro-inflammatory capabilities of the B-box and instead competes with HMGB1 for binding sites leading to mitigation of the inflammatory cascade [43].

12.2.2.3 Release of HMGB1 from Necrotic Cells Upon Sterile and Infectious Tissue Injury

Necrotic cells passively release HMGB1 and trigger sterile inflammation [44, 45]. As shown in early experiments performed by Scaffidi et al. [32], HMGB1 is released passively during cellular necrosis by almost all cells, which have a nucleus, and signals neighboring cells of ongoing damage. These early findings were convincingly confirmed by *in vitro* studies on Jurkat T cell leukemia cells treated to induce necrosis in terms of ACD by freeze-thawing, heat, hydrogen peroxide, or ethanol [46]. Among treatments tested, freeze-thawing produce the highest levels of extracellular HMGB1. Similar lines of studies are in support of these findings by showing an important role for oxidative stress mediated by hydrogen peroxide (H₂O₂) in inducing passive HMGB1 release from macrophages and monocytes in a time- and dose-dependent manner [47]. Indeed, there is now general agreement that the death modalities of ACD but also RN (i.e., necroptosis, pyroptosis, and NETosis, described

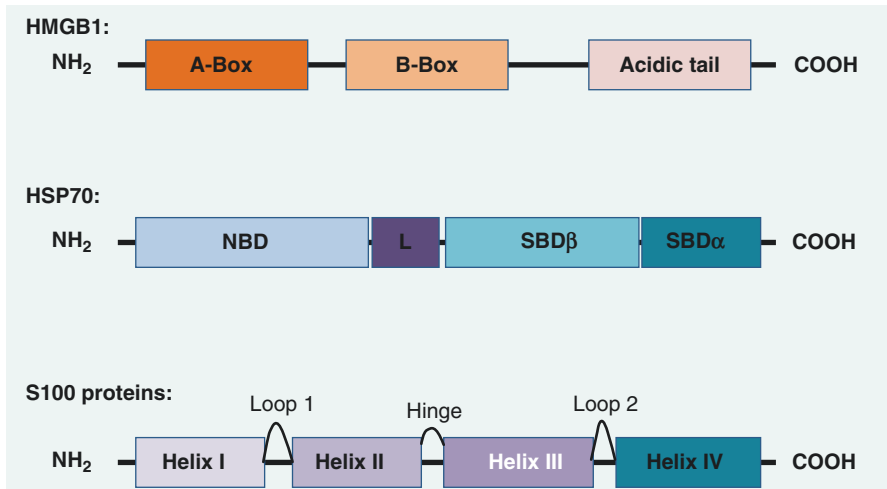


Fig. 12.1 Schematic diagram of the basic structure of HMGB1, HSP70, and S100 proteins. The molecule HMGB1 has a tripartite structure and consists of two homologous L-shaped DNA-binding domains (termed NH₂-terminal HMG A box and central HMG B box) and a negatively charged (acidic) COOP-terminal tail. The tail interacts with the HMG boxes and may modulate their intermolecular interactions. HSP70 consists of an NH₂-terminal nucleotide-binding domain, also termed ATP-binding domain, and a COOP-terminal protein substrate-binding domain that binds extended polypeptides. The latter domain is subdivided into a COOP-terminal α -helical subdomain providing a flexible lid of SBD β . The S100 proteins are calcium-binding proteins which are characterized by four helices (two helix-loop-helix segments), a central hinge region, and NH₂- and COOP-terminal variable domains. *COOP* carboxy terminus, *Hinge* hinge region, *HMGB1* high mobility group box I, *HSP70* heat shock protein70, *L* linker segment, *NH2* amino-terminus, *NBD* nucleotide-binding domain, *SBD* substrate-binding domain. Sources: Refs. [34, 39–42]

in Part V, Chap. 17 and Sect. 19.3) represent a huge source of the passive emission of this DAMP (reviewed in Refs. [48–51]).

Typical sterile tissue injuries causing passive release of HMGB1 from dying cells include ROS-mediated oxidative damage such as IRI [52, 53], traumatic lesions in patients [54–57], or chemically induced toxic liver injuries [58, 59] (reviewed by Lu et al. in Ref. [34]). As also competently examined by Lu et al. [34], HMGB1 can be passively released from cells infected by various viruses (such as West Nile, salmon anemia, dengue, and influenza viruses), indicating HMGB1 to operate as a critical mediator and amplifier of infectious tissue injury to elicit of virus-induced inflammatory diseases [60]. More recent data from studies in children suffering from enterovirus 71-induced *hand, foot, and mouth disease* suggest that HMGB1 is involved in the inflammatory pathogenesis of this disease and that the serum level of HMGB1 could be applied as a clinical indicator for the severity of the viral infection [61]. Another recent report presented evidence for a role of HMGB1 as a potential biomarker for severe viral hemorrhagic fevers [62].

12.2.2.4 The Binding Step: Recognition of HMGB1 Through Pattern Recognition Molecules

Innate immune functions of HMGB1 are mediated by its binding to receptors operating in the innate immune system, the PRRs/PRMs (see Part II). Several important receptors have been implicated in HMGB1 signalling either via direct binding of HMGB1 or its indirect binding in complex with other molecules.

Receptor for Advanced Glycation End Products

The first receptor described for HMGB1 is the RAGE, a multifunctional transmembrane protein of the immunoglobulin (Ig) superfamily [63] (see also Part II, Sect. 5.2.8). Signalling through RAGE results in activation of the transcription factors NF- κ B and MAPKs [64] (compare Part VI, Sect. 22.3.9). In particular, activation of the *extracellular signal-regulated protein kinase* (ERK) MAPK pathway is crucial in mediating cell migration, tumor proliferation and invasion, and expression of MMPs. Indeed, the HMGB1 \rightarrow RAGE axis is critically involved in the recruitment and migration of cells, directly by inducing expression of adhesion molecules, such as ICAM-1 and *vascular cell adhesion molecule-1* (VCAM-1) [65], or indirectly by causing secretion of chemokines, in particular CXCL12, which in turn forms a heterocomplex with HMGB1 [66] (for chemokines and adhesion molecules, see Part VI, Sects. 22.5.11 and 22.5.12).

Toll-Like Receptors

Besides RAGE, the Toll-like family of receptors has been demonstrated to be important in recognition of the DAMP HMGB1 associated with subsequent HMGB1-triggered signalling. Already in 2004, TLR4 and TLR2 were reported to be such candidates [67]: Murine macrophage cell lines transfected with dominant-negative constructs of TLR2 and TLR4 were found to show a decreased activation on stimulation with HMGB1. In particular, a decrease in NF- κ B-dependent reporter gene expression after transfection with dominant-negative constructs to TLR2, TLR4, or both, demonstrated that TLR2 and TLR4 are both involved in HMGB1-induced activation of NF- κ B [67]. Most crucial evidence in support of the notion that injury-induced HMGB1 is recognized by TLR4 came from the early seminal experiment performed by the Pittsburgh group [68]: TLR4-defective mice (C3H/HeJ mice) exhibited less damage in the hepatic postischemic reperfusion model than did wild-type mice (C3H/HeOuj mice). Anti-HMGB1 antibody failed to provide protection in C3H/HeJ mice but successfully reduced damage in C3H/Ouj mice. These Pittsburgh results demonstrated that HMGB1 is an early mediator of injury and inflammation in liver IRI and implicates TLR4 as one of the receptors that are involved in the process [68].

During the past decade, the contribution of the HMGB1 \rightarrow TLR4 axis to inflammation and immune regulation has been demonstrated in a wide range of experimental models, such as liver and lung damage, cancer, and epilepsy (reviewed by Venereau et al. [31]). In addition, other lines of studies revealed that HMGB1, when bound to nucleosomes, activates macrophages and DCs through TLR2 [69] and,

when complexed with CpG oligodeoxynucleotides (ODNs), binds to TLR9 to enhance cytokine production in pDCs [30, 70].

HMGB1 Promiscuously Binds Multiple MAMPs

Intriguingly, HMGB1 was observed to form complexes with almost all kinds of NAs as well as LPS, promoting sensing by their cognate receptors and increasing the robustness of inflammatory and immune responses to those agents. As a matter of fact, high-purity HMGB1 was even discussed to possess a limited pro-inflammatory activity on its own (for competent articles, see Refs. [70–73]). On the other hand, one may speculate that those MAMPs concerned may only acquire immunogenicity by complexing with HMGB1.

12.2.2.5 Redox Status-Dependent Functions of High Mobility Group Box 1

Once in the extracellular milieu, HMGB1 signals danger to the surrounding cells, triggers inflammation, and activates innate and adaptive immunity by interacting with multiple receptors mentioned above. Intriguingly, however, these various functions of HMGB1 vary depending on the redox state of this prototypical DAMP. Thus, the redox status of HMGB1 reportedly distinguishes its cytokine-inducing and chemokine activity. In fact, a large body of evidence has been published demonstrating that the redox state of cysteines modulates the binding of HMGB1 to its receptors and consequently its activities (reviewed by Venereau et al. [31]). The puzzle is based on three cysteines in positions 23, 45, and 106 possessed by HMGB1: C23 and C45 can form a disulfide bond, and C106 is unpaired. These cysteines are modified by redox reactions, causing three isoforms called *fully reduced HMGB1* for the all-thiol form, *disulfide HMGB1* for the partially oxidized one, and *sulfonyl HMGB1* for the terminally oxidized form [74] (Fig. 12.2). Fully reduced HMGB1 has sole chemokine activity by creating a complex with the chemokine CXCL12, which binds with increased affinity to its CXCR4 receptor to promote recruitment of inflammatory cells to sites of damaged tissues [66]. Conversely, the *myeloid differentiation factor 2* (MD-2), an extracellular adaptor of TLR4, binds specifically to disulfide HMGB1 but not to the other redox forms, thereby triggering the upregulation of chemokines and cytokines [75] (for MD-2, see Part VI, Sect. 22.3.3.1). Of note, interaction with MD-2 also requires the third cysteine, in the fully reduced form. Thus, the disulfide bond between C23 and C45 qualifies HMGB1 as a pro-inflammatory cytokine, whereas further cysteine oxidation to sulfonates abolishes both the chemoattractant and pro-inflammatory activities of HMGB1 [45]. Also, reduced HMGB1 protein promotes autophagy, whereas oxidized HMGB1 fosters apoptosis (for autophagy and apoptosis, compare Part V, Sects. 18.2 and 19.2). Further and for clinicians essential to know, a correlation between the presence of the disulfide HMGB1 and the onset of pathologies could be clinically shown, for example, concerning brain injury, liver damage, myositis, and juvenile idiopathic arthritis (reviewed in [31]). Remarkably, disulfide HMGB1, and not the reduced form, contributes to nociceptive signal transmission via activation of TLR4 [76] (for nociceptors sensing endogenous DAMPs, compare below, Chap. 15).

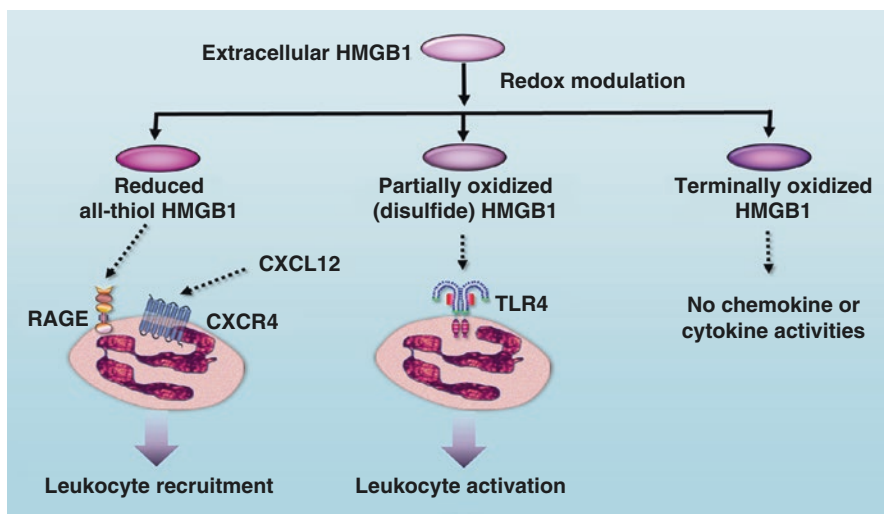


Fig. 12.2 The redox state of HMGB1 determines its different activities. The redox state of cysteines modulates the binding of HMGB1 to its receptors and consequently its activities. The puzzle is based on three cysteines possessed by HMGB1. These cysteines are modified by redox reactions, causing three isoforms called fully reduced HMGB1 for the all-thiol form, disulfide HMGB1 for the partially oxidized one, and sulfonyl HMGB1 for the terminally oxidized form. *CXCL* chemokine (C-X-C motif) ligand, *CXCR* C-X-C motif receptor, *HMGB1* high mobility group box1, *TLR4* toll-like receptor 4, *RAGE* receptor for advanced glycation end products. Sources: Refs. [45, 66, 74, 75]

Apart from redox status-mediated HMGB1 changes, many PTMs have been identified in altering HMGB proteins, including acetylation, phosphorylation, methylation, and oxidation [77] (for PTMs, see also Part VI, Sect. 24.3). These PTMs lead to different functional consequences, whereby acetylation obviously enables the molecule to be secreted. In fact, acetylation of Lys 75 (K73 in humans) in Sox 2 was shown to promote transport of the molecule from the nucleus to the cytoplasm, indicating that acetylation of HMG boxes might serve as a transport signal for this entire class of proteins [38, 78] (for secreted HMGB1 acting as an inducible DAMP, see below Sect. 14.3.3).

Together, at the time being, it is now essential and unavoidable to identify the redox state of HMGB1 as well as its potential modification in each specific condition and under given circumstances in vivo (for redox HMGB1, further reading is recommended in [79, 80]).

12.2.2.6 Concluding Remarks

As already mentioned above, HMGB1 is by far best investigated DAMP induced by tissue injury.

The proven generation of this molecule during all kinds of sterile tissue injury positions this molecule in the center of initiating steps leading to the development of acute and chronic inflammatory processes as well as adaptive immune responses

against bacterial and viral antigens, as well as autoantigens to promote autoimmune diseases and alloantigens to induce transplant rejection. The intense capability to operate as a DAMP may lie in the fact that it is recognized by three different receptors, TLR4, TLR2, and RAGE and, importantly, when complexed with NAs or other MAMPs and DAMPs, to be sensed by their cognate receptors.

12.2.3 Heat Shock Proteins (Subclass IA-1 DAMPs)

12.2.3.1 General Remark

All organisms respond to heat by synthesizing a group of stress proteins called heat shock proteins (HSPs). These highly conserved biomolecules exist ubiquitously throughout the evolutionary scale, from archaeobacteria to eubacteria, from plants to animals, and from animals to humans [81]. They are abundant across species in nearly all subcellular compartments. They are both constitutively and inducibly expressed. After a sudden rise in temperature of a few degrees, all cells immediately, but transiently, activate a small number of specific genes. Some of these encode HSPs. First observed in 1962, chromosomal “puffing” owing to the “unwrapping” of chromatin for gene activation occurred after the exposure of isolated salivary glands of the fruit fly, *Drosophila melanogaster*, to temperatures slightly above physiological levels [82]. By 1974, “puffing” was noted to be accompanied by high-level expression of a unique set of HSPs [83]. Investigations on *Drosophila* showed that each of four members of a small HSP family was induced during development in response to heat stress via cell-specific enhancers in the gene promoter regions [84]. Investigations into members of different species soon revealed that cells produce neosynthesized proteins when exposed not only to an abrupt increase in temperature but also to other diverse toxic stress situations such as alterations in the intracellular redox environment or exposure to oxidants, heavy metals, alcohol, xenobiotics, or viral infection. For this reason, they are referred to as “stress proteins” and are divided into different families according to their molecular size, for example, HSP100, HSP90, HSP70, HSP60, HSP40, and HSP20. One of the most intensively studied protein families is the 70-kDa HSP70 [85] (Fig. 12.1). As major stress-inducible proteins, the HSP70 family consists of ubiquitous HSP73 and of HSP72, which is inducible by heat shock, oxidative stress, and infection.

12.2.3.2 Passive Release of Heat Shock Proteins

Initial studies from Gallucci et al. [86] demonstrated that DCs are stimulated by endogenous signals received from stressed, virally infected, or necrosis-induced cells but not by healthy cells or cells undergoing apoptosis. The Srivastava group subsequently demonstrated that necrotic but not apoptotic cell death leads to the release of HSPs including gp96, HSP90, and the inducible HSP70 family member, HSP72 [87]. These investigators showed that exposure of DCs to necrotic but not apoptotic cells resulted in the nuclear translocation of NF- κ B and subsequent maturation of DCs. In more recent times, these early studies were confirmed by several

groups. For example, in *in vitro* experiments on cell lysates, HSP70 released from necrotic monocytes/macrophages was shown to function as an endogenous danger signal to augment the pro-inflammatory responses in monocytes/macrophage [88]. In other lines of studies, HSP70 was found to be passively released from necrotic human prostate carcinoma cells lines treated with hyperthermia [89]. Together, there is sufficient evidence documenting passive release of HSPs from necrotic cells.

12.2.3.3 The Binding Step: Recognition of Heat Shock Proteins Through Pattern Recognition Molecules

Heat shock proteins such as HSP70 are known to bind to both classical and non-classical PRMs expressed on innate immune cells [90]. For example, as reviewed [5, 91], HSP72 has been shown to bind selectively and with high specificity and affinity to DCs, macrophages, ILCs, and peripheral blood monocytes. Of utmost importance in regard to the initiation of TLR-triggered signalling pathways was the early discovery that HSP72 is recognized by TLR2 and TLR4, together with their cofactor CD14 [92–94].

To date, the list of putative HSP receptors has grown and now includes the scavenger receptor CD36 [95–97], the costimulatory molecule CD40 [98], the LDL-receptor-related protein CD91 [99, 100], and LOX-1 [95, 101], as well as SRA and the class F scavenger receptor expressed by ECs-1 (SREC-1), that is, other members of the scavenger superfamily [96, 102, 103] (for these receptors, also compare Part II, Sects. 5.2.7.3 and 5.3.3) (for scavenger receptors, see Part II, Sect. 5.3.3).

12.2.3.4 Function of Heat Shock Proteins

That its structure has been widely conserved throughout evolution suggests an essential role in the survival of the organism. In mammalian cells, some HSP family members are present constitutively (“pre-packaged”), act as chaperone molecules, and function as key mediators of proteostasis by controlling protein synthesis, folding, assembly, trafficking, and degradation (“proteostasis” = protein homeostasis). In addition, they contribute to the activity cycle of hormone receptors, including steroid hormones. Through all these functions, they control the quality of newly synthesized proteins and participate in cell homeostasis (for further reading, see Refs. [81, 104–107]).

The presence and expression of HSPs were originally interpreted as a danger signal for cell stress. Today, we can conclude that the extracellular presence of HSPs in their function as DAMPs, but not their intracellular expression, signals dangerous tissue damage. Indeed, it is now recognized that when present in the cell, they are protective molecules utilized in cellular repair in response to different types of injury, to prevent damage resulting from the accumulation and aggregation of non-native proteins such as oxidized proteins.

Once passively released or actively secreted in the extracellular space, HSPs operate as constitutive DAMPs (for secreted inducible HSPs, see below Sect. 14.2.2.2). Basically, extracellular HSPs appear to function in influencing the inflammatory and immunological balance in tissues. Thus, on the one hand, HSPs, in particular, HSP70

through interaction with TLR4, were demonstrated to promote pro-inflammatory responses [108]; on the other side, they were shown to possess anti-inflammatory properties, thereby qualifying them as so-called DAMPERS or SAMPs [109].

The role of HSPs in the promotion of adaptive immune responses has also two sides. On the one hand, HSPs—when associating with antigenic peptides—reportedly contribute to mounting an adaptive immune response, for example, an anti-tumor immune response by promoting antigen cross-presentation by DCs (reviewed in [90]) (for cross-presentation, see Part VIII, Sect. 31.3.5). On the other hand, HSPs were found to be critical in the induction, proliferation, suppressive function, and cytokine production of Tregs, which maintain peripheral tolerance [110] (for Tregs, see Part VIII, Sect. 33.4.3).

Regarding these dual functions of extracellular HSPs, Calderwood et al. [90] conclude: *“Their effects on the immune system are, thus, bivalent. In the presence of PAMPs or tissues undergoing necrosis, Hsp70, in particular, becomes a strong inflammatory agent. The precise nature of the responses elicited by extracellular HSPs may, therefore, depend upon the particular tissue milieu within which they are released and the identities of the receptors on the surfaces of immune cells that encounter them.”*

12.2.3.5 Concluding Remarks

Strikingly, HSPs operating as DAMPs in the extracellular space are characterized by their bivalent function. In this regard, the conclusion by Calderwood et al. [90] nicely matches with our conclusion made in the discussion of a role of HSP70 in innate alloimmunity [6]: *“Altogether, the context-dependent, even contradistinctive activities of HSP70 reflect the biological phenomenon that, throughout evolution, mammals have developed an elaborate network of positive and negative regulatory mechanisms, which provide balance between defensive measures against dangerous bacterial and viral pathogens and protective measures against unwarranted destruction of the host by the activated immune system. Fine-tuning of TLR signaling in amplitude, space, time, and character is a key aspect of inflammatory reactions in health, homeostasis, and pathology. What is becoming more and more apparent is that positive and negative regulators within immune responses do not work as a single entity, but rather, similar to an orchestral score, each component is reliant on its other tools such as HSP70 to produce a harmonious melody instead of a crashing cacophony.”*

12.2.4 Other DAMPs Released from Necrotic Cells (Subclass IA-1 DAMPs)

12.2.4.1 General Remark

Besides HMGB1 and HSPs, other molecules have been described to be passively released from necrotic cells and to function as extracellular DAMPs. As passively released molecules with chemically different structures, they play expectably various functions. Without mentioned all those contemplable molecules, a few of them are briefly touched in the following.

12.2.4.2 Passive Release of S100 Proteins

The family of S100 proteins, or calgranulins, is composed of 25 members, and they are named according to their solubility in 100% saturated solution of ammonium sulfate at neutral pH [111] (Fig. 12.1). Of note, several S100 family members have been shown to be released from necrotic cells to acting as critical DAMPs in the promotion of inflammatory responses. The most prominent proteins include S100A8 (also known as calgranulin A or MRP-8), S100A9 (calgranulin B or MRP-14, which can form a dimer with S100A8 extracellularly), S100A12 (calgranulin C or EN-RAGE), and S100B protein (reviewed in [9, 112–116]). The proteins S100A8 and S100A9 are expressed in cells of myeloid origin, including neutrophils and monocytes as well as keratinocytes and epithelial cells under inflammatory conditions. Both proteins can complex, and as a heterocomplex of S100A8/A9, they are often called calprotectin. This complex is highly abundant in human neutrophils and constitutes a large part of the total protein content of these cells. The protein S100A12 binds both zinc and copper and is highly abundant in innate immune cells such as macrophages and neutrophils [117]. The protein S100B is expressed in astrocytes, certain neuronal populations, Schwann cells, melanocytes, chondrocytes, adipocytes, skeletal myofibers and associated satellite cells and DCs and lymphocyte populations, and a few other cell types [118].

The Binding Step to TLR4 and RAGE Receptor

After release, extracellular S100A8 and S100A9 were found to interact both with TLR4 and RAGE to exhibit their pro-inflammatory effects, thereby promoting cell migration, proliferation, and differentiation as well as adaptive immune responses [9, 119, 120]. Notably, recent studies were published indicating that CD14 is a co-receptor of TLR4 in the S100A9-induced cytokine response [121]. On the other hand, S100A12 and S100B are reportedly recognized by RAGE, whereby S100A12 has also recently been found to bind to TLR4 to induce monocyte activation [122]. Following recognition, both interactions are known to result in NF- κ B-mediated production of pro-inflammatory cytokines [123, 124]. Interestingly, a model was recently proposed, whereby conformational flexibility in the RAGE receptor allows the adoption of a binding conformation for interaction with the stable hydrophobic groove on the surface of S100B [124]. Besides TLR4 and RAGE, other receptors have been identified to sense S100 proteins including GPCRs, scavenger receptors, or heparan sulfate (HS) PGs and N-glycans (reviewed in [125]) (for GPCRs and scavenger receptors, see Part II, Sects. 5.3.3 and 5.3.5).

Intracellular Function of S100 Proteins

Intracellularly, S100 proteins are involved in aspects of regulation of proliferation, differentiation, apoptosis, Ca²⁺ homeostasis, energy metabolism, inflammation, and migration/invasion through interactions with a variety of target proteins including enzymes, cytoskeletal subunits, receptors, transcription factors, and NAs [125, 126]. For example, S100A8 and S100A9 were demonstrated to play a role in Ca²⁺-dependent interactions between the cytoskeleton and the plasma membrane. In addition, a functional correlation was discovered between the S100A8/A9

heterotetramer and microtubules, promoting polymerization of microtubules in resting phagocytes, which is reversed by the phosphorylated form of S100A8/A9 [9].

Concluding Remarks

Together, as concluded elsewhere [125], “*S100 proteins are only expressed in vertebrates, and their expression and/or activities appear to be mechanistically linked to the refinement or fine tuning of cell-specific gene expression and responses to external stimuli. Intracellular and extracellular functions of S100 proteins are beginning to be described in detail, making these proteins less enigmatic than in the past. Cardiac function, tissue repair/regeneration, inflammation (including neuroinflammation), infection and cell growth and differentiation, are processes in which certain S100 proteins are active players.*”

In fact, their identification as vital DAMPs to promote pro-inflammatory responses is even more critical as they have been shown to be actively secreted by stressed innate immune cells (for details see Sect. 14.2.2.4).

12.2.4.3 Nucleic Acids

General Remarks

Recognition of viral NAs by PRMs is known to be a vital part of our immune defense response to viral infections. Such a response to our own endogenous self NAs is kept in check by several mechanisms under homeostatic conditions because uncontrolled induction of a vigorous innate inflammatory and/or autoimmune response to host-derived NAs might end up with a collateral “suicidal” catastrophe. On the other hand, modern immunological research has revealed that endogenous NAs can nevertheless act as DAMPs in certain circumstances to induce and amplify sterile inflammatory innate immune responses which may transition into autoimmune diseases. Such conditions that reflect a disturbed homeostasis include an oversupply or defects in the NA-degradation machinery (see below) or a misplacement/mislocation of NAs.

Passive Release of Nucleic Acids

An oversupply of NAs occurs when, like other cellular components, they are passively released in large amounts from numerous destroyed necrotic cells, which may have undergone ACD or RCD. These NAs include nuclear DNA (nDNA), mitochondrial DNA (mtDNA) containing CpG-DNA repeats (for CpG, see below), and nuclear and cytosolic RNA. For example, the release of endogenous DNA has been observed in the context of NETosis, a subroutine of RCD in which neutrophils actively release their DNA as NETs [127] (for NETs and NETosis, see Part III, Sect. 8.2.3, and Part V, Sect. 19.3.6).

Once released into the extracellular space in these situations, NAs get usually already degraded and—as shown for mtDNA—are oxidatively modified [128, 129]. Following, they are engulfed as cell-extrinsic NAs by neighbored phagocytosing cells such as macrophages, leukocytes, and DCs and subsequently delivered into the endolysosomal system or may even be directly delivered into the cytoplasm. There,

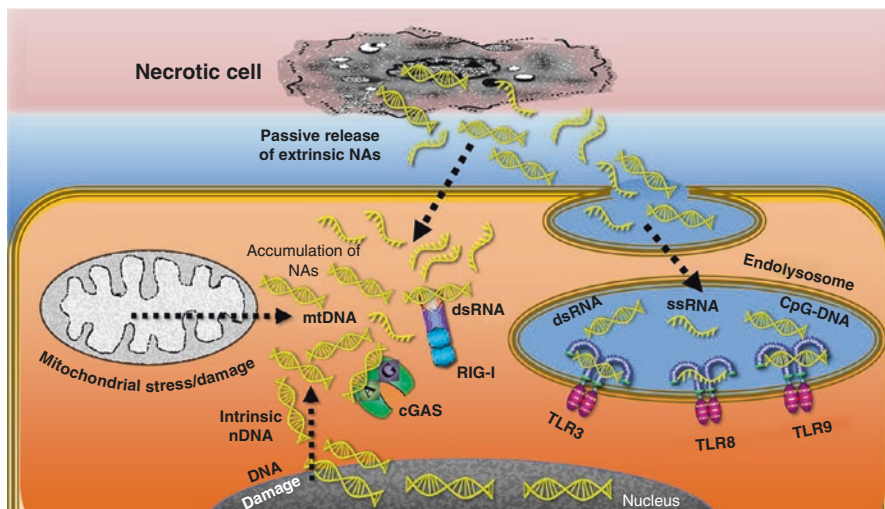


Fig. 12.3 Scenario model of cell-extrinsic and cell-intrinsic emission of nucleic acids. Cell-extrinsic emission of nucleic acids occurs as passive release from necrotic cells. Degraded nucleic acids enter phagocytic cells directly or via endocytosis associated with their accumulation in the endolysosome (where they are recognized by TLR3, TLR8, and TLR9) or in the cytosol (where they are recognized by RIG-I or cGAS). Dyshomeostatic cell-intrinsic emission/accumulation of (degraded) nucleic acids in the cytosol can occur in case of DNA damage or mitochondrial stress/damage. *cGas* cyclic GMP-AMP synthase, *CpG-DNA* cytosine phospho-bound guanine DNA, *dsRNA* double-stranded RNA, *mtDNA* mitochondrial DNA, *NAs* nucleic acids, *nDNA* nuclear DNA, *TLR* toll-like receptor, *RIG-I* retinoic acid-inducible gene (protein) I, *ssRNA* single-stranded RNA. Sources: Refs. [128–133]

they are recognized by and bind to a heterogeneous group of PRMs such as TLRs (TLR3, TLR7 → TLR9) and specific DNA and RNA receptors (Fig. 12.3). In the following a few more (oversimplified) details of these sensing procedures are added, guided by recent comprehensive reviews published by Ablasser et al. [131], Roers et al. [130], Miyake et al. [132], and Hartmann [133], where the reader will find much more detailed information.

The Binding Step of Engulfed Nucleic Acids

Once arrived after engulfment as cell-extrinsic-emitted DAMPs in the endolysosomal system, NAs are recognized by and bind to lysosomal membrane-bound TLRs, whose NA-binding domains face the lumen of the endolysosomal compartment (also compare Part II, Sect. 5.2.2.4). Thus, DNA is sensed by TLR9, whereby this receptor recognizes a specific sequence pattern, that is, unmethylated CpG dinucleotides (CpG-DNA) called the “CpG motif,” a hexamer, containing a cytosine triphosphate deoxynucleotide (“C”) followed by a guanine triphosphate deoxynucleotide (“G”). The “p” refers to the phosphodiester link between consecutive nucleotides. Long dsRNA is sensed by TLR3, a receptor that was the first *bona fide* PRR identified as a sensor for dsRNA. TLR3 is not present in the cytosol of healthy cells and thus is

regarded as receptor sensing dsRNA coming from outside the cell. Further, TLR7 senses even short RNA, preferentially double-stranded and containing guanine and uridine. The TLR8 functions as receptors for uridine-rich RNA molecules which are likely to be degraded from ssRNA fragments. Notably, TLR7 is expressed in B cells, pDCs, and macrophages, whereas TLR8 is expressed in macrophages and DCs.

Of note, recent attention has been paid to an emerging role of the processing of NAs and TLRs as a mandatory prerequisite to allowing initiation of innate immune responses to NAs (reviewed in [132]). In fact, NA degradation by DNases and RNases has been recognized as a key role in preventing hazardous activation of NA sensors that may become deleterious for the host. For example, DNA degradation by lysosomal (and cytoplasmic) DNases is now known to prevent deleterious homeostatic activation of cytosolic DNA-sensing pathways. As a matter of fact, crude NAs are not effective for stimulating NA receptors. Intriguingly, lysosomal TLRs are also dependent on NA processing in lysosomes. Thus, digestion of dsDNA by DNase II was shown to be required for TLR9 response to ssDNA. Furthermore, TLR7 and TLR8 respond to ribonucleosides and ODNs, instead of ssRNA itself, indicating a requirement for RNA processing. Nucleic acid-sensing TLRs themselves need to be processed by lysosomal proteases. Without processing, TLR8 and TLR9 were found to fail to form dimers. Together, as stressed [132], “*the processing of NAs and TLRs has the key role in keeping NA sensors responsive to microbial NAs without inducing hazardous responses to endogenous NAs.*”

In case cell-extrinsic NAs are directly delivered into the cytoplasm, they behave as cell-intrinsic NAs and bind to specific cytosolic NA receptors. This scenario is described below in Sects. 13.4.3 and 13.4.4).

Extracellular Function of Nucleic Acids

Nucleic acids possess immunostimulatory capacities and are regarded as the keys to defend against viral infections [134]! Hard to believe but already more than 100 years ago, the Polish-German surgeon von Mikulicz-Radecki treated patients with severe surgical peritoneal infections with NAs and other similar substances. The aim was to induce massive hyperleukocytosis, which would increase natural resistance to the bacteria involved. Von Mikulicz-Radecki reported his clinical trial findings at the 33rd Congress of the German Society of Surgeons in 1904 (cited in [135]). Many years later, one of the first reports was published that historically documented the immunostimulatory potential of DNA, in fact, depending on a TLR-independent phenomenon [136]. Still, the mechanism of action of NAs was not understood until a couple of years ago. Today it is known that upon recognition of NAs, the endolysosomal TLRs and cytosolic sensors activate signalling cascades that culminate in the production of type I IFNs, which primarily include numerous subtypes of IFN- α and a single IFN- β , as well as pro-inflammatory cytokines such as TNF and IL-1 β . Type I IFNs then—now acting as DAMPs—induce a vast plethora of antiviral genes through the activation of the *Janus kinase (JAK)-signal transducer and activator of transcription (STAT)* pathway. Moreover, NAs have been shown to be involved in promoting sterile inflammation upon non-pathogen-mediated traumatic tissue injury [137]. The scenarios are described in more detail in Part VI, Sects. 22.3.3, 22.3.6, and 22.3.7.

12.2.4.4 Extracellular Histones and Nucleosomes

General Remarks

Histones and nucleosomes are critical nuclear proteins that contribute to the structural organization and stability of chromatin. When released from dying cells (succumbing to ACD or RCD such as NETosis) into the extracellular space, histones, that is, free histones and DNA-bound histones (nucleosomes), have been recently recognized as candidates of the DAMP family (for further reading, see Refs. [57, 138–144]). As known, in eukaryotic cells, DNA is packaged into chromatin, the basic repeating unit of which is a nucleosome. A nucleosome consists of 147 bp nucleotides wrapped around a histone octamer, which is composed of two copies each of histone H2A, H2B, H3, and H4. Nucleosomes are packaged into progressively higher-order structures to form chromosomes ultimately. Notably, unstructured NH₂-terminal histone tails that protrude from the nucleosome are subject to covalent chemical modifications, which impact chromatin organization and function (for more details, see Part VI, Sect. 24.2.2, and Fig. 24.1).

Binding Step of Histones and Nucleosomes

In *in vitro* and *in vivo* studies on TLR2- and TLR4 knockout (KO) mice, extracellular histones were found to induce immunostimulatory signalling in cells such as DCs, ECs, and renal tubular cells as indicated by secretion of TNF, IL-6, and IL-10 [140, 145]. In addition to histone-induced pro-inflammatory innate immune responses via TLR2 and TLR4, subsequent experiments on an *in vivo* model of hepatic IRI demonstrated that TLR9 KO mice were protected from histone-mediated IRI; these studies encouraged the authors to propose that exogenous histones may have acted as a cofactor that amplified the TLR9-mediated signalling brought about by endogenous circulating DNA released from dying cells [139]. However, as discussed elsewhere [142, 144], these unexpected results might have been influenced by remnant DNA bound to histones, thereby reflecting a DNA → TLR9-dependent DAMP effect.

Notably, as recently discussed by Marsman et al. [142], DNA and histones are organized in nucleosomes in the nucleus, and evidence suggests that nucleosomes are released as such from necrotic cells to exert TLR9-independent immunostimulatory capacities as well. Indeed, nucleosomes have been demonstrated to activate human and murine DCs [146]. Given that nucleosomes were repeatedly found to bind to the plasma membrane, the existence of a nucleosome-specific receptor has been proposed, but this receptor has thus far not been identified. Therefore, further experiments are needed to clarify this unusual phenomenon.

Extracellular Function of Histones and Nucleosomes

Besides their immunostimulatory capacities in inducing innate inflammatory responses, extracellular histones were shown to cause direct TLR-independent cytotoxicity to epithelial and endothelial tissue. The exact mechanism of this unique cytotoxic effect is not quite clear. However, current theories suggest that extracellular histone binds to phospholipid–phosphodiester bonds, similar to their

DNA-binding sites, an effect that alters membrane permeability and instigates calcium ion influx (reviewed in [144]). As such, extracellular histones have a central role in necroinflammation [147].

Due to their cytotoxic and pro-inflammatory effects, extracellular histones have been shown to contribute to excessive and overwhelming cell damage and death, thus contributing to the pathogenesis of both sepsis and ARDS which may be associated with MOF [148]. More recent findings from experiments in mice reportedly indicate that extracellular histones induce multiple organ injuries in two progressive stages, direct injury to ECs followed by subsequent release of other DAMPs [149]. Besides this acute life-threatening pulmonary event, extracellular histone/nucleosome DAMPs have been shown to be involved in the pathogenesis of various other acute or chronic diseases including cerebral vascular events, myocardial infarction, drug-induced hepatic sterile inflammation, acute kidney injury, and Alzheimer's disease (reviewed in [144]).

Concluding Remarks

In fact, there is an emerging role of histones in their function as DAMPs to mount necroinflammation—as concluded by Silk et al. [144]: *“It is clear that both free and DNA-complexed histone have important roles in mediating pro-inflammatory signalling in sterile acute organ injury. Released during periods of cell death and immune activation, histone, nucleosomes and NETs induce cytotoxicity by altering cell membrane permeability to calcium ions, activating TLRs on innate immune cells, stimulating NLRP3 inflammasome and complement systems, resulting in a sterile pro-inflammatory environment. There are three distinct pharmacodynamic approaches to target histone-mediated inflammation, by reducing the release, neutralising or blocking histone signal transduction. Although these approaches have proven to provide significant protection from mortality in animal models of acute organ injury, further research is necessary to warrant their safe application in a clinical setting.”*

12.2.4.5 Nuclear Pro-Forms of Interleukin-1 Family Members

Interleukin-33

The DAMP IL-33 is constitutively expressed as a nuclear protein in multiple cell types, in particular, ECs, epithelial cells, and fibroblasts, though its precise function within the nucleus is not fully understood. Nevertheless, IL-33 was found to be able to associate with chromatin by tethering to histones H2A/H2B, via a short chromatin-binding motif, located in its N-terminal nuclear domain [150]. Biologically active full-length IL-33 can be immediately released in the extracellular space after traumatic cell death (necrotic cell death or mechanical injury) enabling instant alarm or danger signalling without the need for further processing or modification (see Refs. [151–154]). Once released, the cytokine has been reported to operating as a canonical DAMP [155]; regarding this function, other authors denoted IL-33 as an “alarmin” [156] or “dual function-alarmin” [157]). Following emission, IL-33 elicits

extracellular effects by binding to the *suppressor of tumorigenicity 2* (ST2) receptor (see Part VI, Sect. 22.5.6.4). Although full-length IL-33 is biologically active, cleavage generates mature forms of IL-33 with up to tenfold more biologically active compared to the full-length protein [158].

Of note, IL-33 has been shown to be actively secreted by dying cells or by severely stressed cells, qualifying it as an inducible DAMP (for details, see below, Sect. 14.2.4.2).

Interleukin-1alpha Precursor (Pro-Form)

By contrast to the pro-form of IL-1 β , the pro-form of IL-1 α (= IL-1 α precursor/pro-IL-1 α) is bioactive and thus operates as a DAMP [159]. The protein is constitutively expressed as an IL-1 α precursor in the nuclei of a range of resting non-hematopoietic cells such as EpCs and ECs and in a range of tissues including the lung, liver, and kidney [160] (for more details, see Part VI, Sect. 22.5.6.2). Like IL-33, pro-IL-1 α can be immediately released into the extracellular space after necrotic cell death enabling prompt initiation of sterile inflammatory responses without the need for further processing or modification [154, 159]. For example, studies on necrotic cell lysates in mice revealed that IL-1 α is a vital DAMP released from necrotic cells to trigger recruitment of neutrophils on neighboring mesothelial cells [161]. These earlier studies were confirmed by studies on necrotic retinal pigment epithelial cells demonstrating IL-1 α to trigger pro-inflammatory cytokine and chemokine secretion [162], as well as by more recent experiments showing IL-1 α to be released from necrotic corneal epithelial cells, capable of triggering inflammatory responses at the ocular surface [163].

Once extracellular, pro-IL-1 α reportedly binds to the IL-1R1 to initiate downstream pro-inflammatory signalling, thereby propagating and extending an inflammatory milieu [164] (see also Part VI, Sect. 22.5.6.2). Of note, in stimulated cells, pro-IL-1 α is processed by the membrane-bound protease calpain, before active release as an inducible DAMP into the extracellular space through an unconventional vesicular pathway (for more details, see below, Sect. 14.3.2.2).

12.2.4.6 Mitochondrial N-Formylated Peptides and Cytochrome C

General Remarks

Mitochondria are evolutionary endosymbionts derived from bacteria. Thus, it is not unexpected that they bear molecules as mtDNA and FPs, which were found to be encoded only by bacterial or mitochondrial genes due to initiation of protein synthesis with N-formyl methionine. They are generally cleavage products of bacterial and mitochondrial proteins. Although these peptides play a crucial role in the protein synthesis of bacteria and mitochondria [165], they are not used in cytosolic protein synthesis of eukaryotes. Regardless of their different origin, FPs are known to play a role in the initiation of inflammatory responses by activating the FPRs [166, 167]. In this regard, one may discuss that FPs, like LPS, can operate as both endogenous and exogenous DAMPs (see above, Sect. 11.2.6) (for further reading, see Refs. [168–172]).

The Binding Step and Function of N-Formylated Peptides

Formyl peptides are known to bind to FPRs that have been identified as a subfamily of GPCRs (see also Part II, Sect. 5.3.5.3). In human, members include FPR1, FPR2, and FPR3. Through binding with FPRs, FPs serve as potent chemoattractants, which also include activated complements and chemokines, in recruiting and guiding leukocytes to the site of infective and sterile tissue damage. In addition, after binding to FPs, these receptors promote pro-inflammatory responses including cell adhesion, directed migration, granule release, and superoxide production. In recent years, the cellular distribution and biological functions of FPRs have expanded to include additional roles in homeostasis of organ functions and modulation of inflammation (reviewed in [173]).

Earlier studies have already shown that FPs are involved in the pathogenesis of multiple inflammatory diseases such as ulcerative colitis and Crohn's disease (further reviewed [173]). Recent clinical findings are in support of these early observations showing that the plasma concentration of FPs is increased in trauma patients with SIRS and/or sepsis when compared to control trauma group [11, 167]. In *in vitro* and *in vivo* experiments with rats, the investigators observed FPs-induced concentration-dependent contraction in the trachea, bronchi, and bronchioles [172]. Giving these data, the authors concluded that their findings provide a new and different way of considering the role of FPs in acute lung injury and airway contraction following trauma.

Role of Cytochrome C as a DAMP

Interestingly, mitochondria-derived cytochrome C has recently been proposed to act as a putative DAMP [171]. In fact, from experimental and clinical studies, cytochrome C is known to be released from dying cells. Evidence in support for a role of cytochrome C as a DAMP was deduced from observations in patients showing that serum cytochrome C levels increase during systemic inflammatory conditions. However, the most convincing proof for this proposal derived from a targeted experimental study showing that *in vitro* stimulation of murine spleen cells with exogenous cytochrome C results in activation of NF- κ B associated with increased production of neutrophil- and monocyte-triggered pro-inflammatory cytokines and chemokines into the culture medium [174]. More recently reported supportive studies on microglia-like cells suggest that the inflammatory effect of cytochrome C is at least partially mediated by TLR4-triggered signalling [175]. Additional studies are required to define cytochrome C as a *bona fide* DAMP as well as to identify its sensing PRMs precisely.

Concluding Remarks

Interestingly, new studies provided evidence proposing the possibility that many so-called cryptides, that is, fragmented peptides derived from various mitochondrial proteins and encoded by mtDNA, may also act as mitochondrial DAMPs to promote sterile inflammation via activation of neutrophils [169]. These observations demonstrate that the story of mitochondrial DAMPs involved in traumatic diseases and sepsis has just begun. For example, the cooperation of mitochondrial FPs with

bacterial FPs in case of bacterial sepsis following trauma will become an attractive subject of future clinical SIRS research. In the course of such studies, further mitochondria-derived DAMPs may be discovered and could be considered as putative targets for the treatment of respiratory failure and sterile inflammation.

12.2.4.7 F-Actin

Another molecule that has recently raised remarkable attention to operating as a DAMP refers to F-actin that was found to trigger sterile inflammation and, in vertebrates, adaptive immunity (comprehensively reviewed by Reis e Sousa's group in Refs. [176–178]). F-actin is an evolutionarily conserved, highly abundant and ubiquitous cytoskeletal protein that usually provides structural support to the cell. In mammals, F-actin is exposed on and released by necrotic cells that have lost plasma membrane integrity to bind to the PRM DNGR-1, a receptor that is expressed selectively by DCs [179]. Like Dectin-1, DNGR-1 is a transmembrane CLR that samples the extracellular and endosomal space and signals via Src and Syk (see Part II, Sect. 5.2.7.3). Following phosphorylation by Src-family kinases, DNGR-1 recruits the tyrosine kinase Syk to promote DC cross-presentation of dead cell-associated antigens.

12.2.4.8 Heme

Another player in the field of passively released DAMPs refers to red cell-derived heme (for reviews, see [180, 181]). Indeed, a potential role of heme as a DAMP potentially operating in the pathogenesis of transfusion-related acute lung injury (TRALI) was already suggested by Land [182].

The hydrophobic heme is an ancient and ubiquitous molecule present in organisms of all kingdoms, composed of an atom of iron linked to four ligand groups of porphyrin. In situations of hemolysis, large quantities of heme (like other DAMPs such as eATP) are released into the circulation. A high amount of free heme was shown to operate as a potential inducer and amplifier of the inflammatory response. For example, the molecule elicits multiple inflammatory responses, activating leukocytes and their migration, up-regulating adhesion molecule and cytokine expression, and augmenting oxidant production and lipid peroxidation [183–185]. Of note, heme, but not porphyrins without iron, was demonstrated to operate as a DAMP promoting the formation of the NLRP3 inflammasome in LPS-primed macrophages [186] (see Part VI, Sect. 22.4.2). Interestingly, in these experiments, the activation of NLRP3 by heme was observed to require spleen tyrosine kinase, NADPH oxidase-2, mitochondrial ROS, and K⁺ efflux but was independent of heme internalization, lysosomal damage, or ATP release.

In addition, in other lines of studies, heme released in murine sickle cell disease was found to trigger the TLR4-mediated pathway resulting in endothelial cell activation associated with WPB degranulation and vaso-occlusion in murine sickle cell disease [180, 187] (for WPB, see Sect. 14.2.5.3).

In this context, it should not be forgotten that heme acts as an activator of the C3 convertase and thus has been shown to activate complement [188], a topic that will be resumed in Part VI, Sect. 23.2.3.3.

12.2.4.9 Concluding Remarks

This brief overview is just a snapshot of the world of passively released DAMPs. Some of those molecules have not mentioned here; some others wait for future discovery. It should be noted that the passive release of large amounts of DAMPs is often the consequence of regulated necroptotic cell death that in its own is induced by DAMPs earlier emitted in the course of the injury. The scenario resembles the phenomenon of deadly avalanches when initial local emission of moderate amounts of DAMPs results finally in the generation of large quantities of DAMPs released into the systemic circulation (for more aspects of this phenomenon, compare Part V, Chap. 20).

12.2.5 DAMPs Indirectly Activating the NLRP3 Inflammasome (Subclass IA-2 DAMPs)

12.2.5.1 General Remarks

A class of stress-/injury-induced molecules that provide the second signal for activation of the canonical NLRP3 inflammasome is denoted here and throughout both volumes of the book as Class IA-2 DAMPs. As will be outlined in detail in Part VI, Sect. 22.4, inflammasomes are macromolecular protein complexes that are composed of inflammasome-initiating receptors/sensors and inflammatory caspases, in the presence or absence of the inflammasome adapter *protein* ASC. A typical feature of members of this class of DAMPs is the fact that they do not directly activate inflammasomes via physical binding to NLRP3 but act through the promotion of molecular homeostatic perturbations in an innate immune cell which are then sensed by NLRP3 (compare Part II, Sect. 5.2.3.3). Such DAMPs include eATP at millimolar concentrations, K⁺ ionophores [189] and crystalline/particulate substances (e.g., MSU, cholesterol crystals, silica, alum), or other factors that cause lysosomal destabilization [190, 191]. As said, these activators are indirectly sensed without direct binding to NLRP3 to trigger inflammasome assembly, IL-1 β /IL-18 release, and pyroptosis [192–195] (for pyroptosis, see Part V, Sect. 19.3.4).

Here, this class of DAMPs is briefly addressed, whereas the activation mechanism of the NLRP3 inflammasome is outlined in more detail in Part VI, Sect. 22.4.

12.2.5.2 Adenosine-5'-Triphosphate

General Remarks

Adenosine-5'-triphosphate can be regarded as the prototype of DAMPs indirectly activating the NLRP3 inflammasome. Like other nucleotides, ATP has both intra- and extracellular functions [196, 197]. Intracellularly, these nucleotides are well known for their function as a universal energy source which drives the biological reactions that allow cells to function and life to flourish. Here, this topic is not further discussed.

Passive Release of ATP and the Binding Step

In events of necrosis, ATP is passively released in large quantities. For example, in studies on pressure-disrupted or freeze-thaw-treated necrotic B16 cells, mitochondria-derived ATP was shown to be capable of activating the NLRP3 inflammasome [198]. Once released into the extracellular space, ATP like ADP is rapidly metabolized to adenosine monophosphate (AMP), which in turn is metabolized to adenosine. This nucleotide phosphohydrolysis involves a two-step enzymatic process regulated by ectoenzymes. In the first step, ATP and ADP are both converted to AMP through the ectonucleoside triphosphate diphosphohydrolase 1 (CD39) (reviewed in [199]). To function as a DAMP, eATP initially triggers signalling through the activation of purinergic P2 receptors [200]. As outlined in Part II, Sect. 5.3.4.3, these receptors have a widespread expression throughout different tissues and are involved in innate and adaptive immune responses. As also mentioned in Sect. 5.3.4.3, P2 purinoceptors can be further subdivided into metabotropic P2YRs, which are G-protein-coupled, and ionotropic P2XRs, which are non-selective nucleotide-gated ion channels. Of note, it is the P2X7 receptor through which eATP leads indirectly to canonical NLRP3 activation under involvement of hemichannels of pannexin-1 [192, 194, 201, 202].

Extracellular ATP Activates the NLRP3 Inflammasome Indirectly and Functions as a “Find-Me” Signal

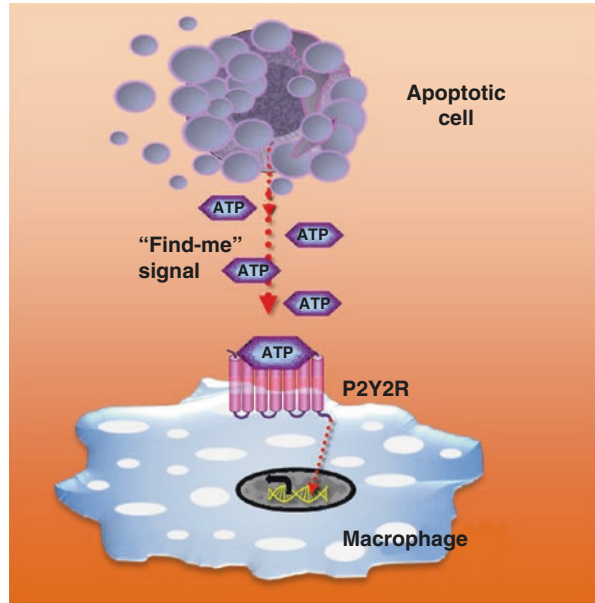
In fact, activation of the canonical NLRP3 inflammasome qualifies eATP as a Class IA-2 DAMP. The exact mechanism of this phenomenon is still not fully understood. However, current notions hold that eATP may initially engage P2X7 to change cellular ion composition, in particular, K⁺ efflux and Ca²⁺ influx (detailed in Part VI, Sect. 22.4.2.2).

Besides its role in activating the inflammasome indirectly through P2X7 signalling, eATP-triggered P2Y2R signalling has been identified as a “find-me” signal for leukocytes, promoting phagocytic clearance of apoptotic cells or bacteria by macrophages and neutrophils, thereby contributing to the resolution of inflammation (Fig. 12.4). Other lines of studies have indicated that P2Y2R signalling contributes to fundamental leukocyte functions such as migration and mediator production by neutrophils, eosinophils, DCs, and macrophages (for reviews, see [200, 203]).

Concluding Remarks

Together, the dual role of eATP-triggered signalling in both indirect activation of the NLRP3 inflammasome and promotion—as a “find-me” signal—of phagocytic processes confers eATP a unique feature among all members of the DAMP family that may qualify this molecule as a “hybrid DAMP.” Of note, both functions are not executed by eATP after its passive release from necrotic cells alone but also via active secretion by stressed or dying cells; a scenario that will be alluded to below in Sect. 14.2.2.3.

Fig. 12.4 Schematic diagram: the “find-me” signal. Extracellular ATP secreted by apoptotic cells triggers P2Y2R signalling, thereby providing a “find-me” signal for phagocytes to promote phagocytic clearance of apoptotic cells. *ATP* adenosine-5'-triphosphate, *P2Y2R* P2Y purinoceptor 2. Sources: Refs. [192, 200, 203]



12.2.5.3 Monosodium Urate Crystals

General Remarks

Like ATP, uric acid has been shown to activate the NLRP3 inflammasome indirectly. This molecule is produced when purines, for example, from DNA or RNA, are oxidized by xanthine oxidase, an enzyme found in peroxisomes of most cells. As a regular constituent of healthy cells, where it is believed to possess antioxidant properties, uric acid is released into biological fluids such as blood. In patients suffering from hyperuricaemia as a consequence of too much intake of purines and/or genetic predisposition, uric acid saturates body fluids and may be converted to MSU crystal when deposited in joints and other tissues, thereby causing gout and other inflammatory diseases (reviewed by Rock et al. [204]).

Function of Monosodium Urate Crystal as a DAMP

Of note, the biologically active form of uric acid is thought to be crystallized MSU that forms when intracellular stores of uric acid are released into the extracellular environment. In 2003, uric acid was identified by the Rock group as a major DAMP and later on shown in mice to promote an acute inflammatory response when released as crystals from dying cells [205, 206]. Once released into the extracellular space and engulfed by phagocytosing cells such as macrophages, the MSU crystals/particles are sensed by NLRP3 resulting in activation and assembly of the NLRP3 inflammasome. It is suggested that the MSU particles cause the rupture or leakage of lysosomes or phagosomes associated with release of cathepsin B into the cytosol, thereby leading to molecular perturbations of the cell sensed as dyshomeostatic

DAMPs by NLRP3 [190, 207, 208]. This process has been known as “frustrated phagocytosis” wherein the phagocytosed crystals cannot be engulfed entirely or digested, thereby leading to changes in the structure of the Golgi complexes like fragmentation and reorganization of Golgi membranes [209]. These perturbations reflect *bona fide* dyshomeostatic DAMPs which are genuinely sensed by NLRP3. Yet, other mechanisms are also discussed to contribute to NLRP3 activation, such as MSU crystal-promoted K⁺ efflux reflecting also molecular perturbations that are, as mentioned above, thought to be involved indirectly in ATP sensing (for dyshomeostatic DAMPs, see below, Sect. 13.4.5; for mechanisms of NLRP3 activation, including particulate-mediated activation, see Part VI, Sect. 22.4.2.2).

Concluding Remarks

Clearly, via indirect activation of the NLRP3 inflammasome, uric acid represents a potent DAMP capable of inducing inflammatory responses. This implies that in terms of uncontrolled inflammatory pathways, this effect can lead to both acute and chronic inflammatory diseases. As mentioned above, gout disease can be regarded as a classical disorder which can be associated with clinical manifestation of a number of co-morbid conditions including renal disease, hypertension, diabetes mellitus, metabolic syndrome, cardiovascular disease, lipid disorders, and respiratory symptoms (reviewed in [204]). Interestingly, it has been speculated that these comorbidities may pathogenetically be elicited by uric acid in its role as a DAMP—as other DAMPs of this class like cholesterol do, which were shown to contribute to other sterile inflammatory disorders such as atherosclerosis and metabolic syndrome. Future clinical trials will probably give an answer to this burning issue.

Together, uric acid metamorphoses into a potent immunostimulatory DAMP when it undergoes a phase change by nucleating into crystals of MSU that obviously—when ingested by phagocytes as a particle—causes intracellular perturbations to indirectly lead to NLRP3 inflammasome activation. Pathogenetically, the gout disease is regarded as a typical disorder induced via this sketched pathway. Whether or not gout-associated comorbidities are induced by the same path is an interesting speculation but remains to be proven in future clinical trials.

12.2.5.4 Cholesterol Crystals

General Remarks

The phenomenon that irritant crystals/particles can be highly pro-inflammatory is not limited to MSU crystals; indeed, many other crystals and particles operate as “NLRP3-activating DAMPs” to stimulate inflammation and cause disease, such as cholesterol and beta-amyloid (see below), as well as calcium phosphate crystals, alum, crystalline silica, and asbestos [191, 210, 211]. These DAMPs are not released from necrotic cells; instead they use various mechanisms to reach the extracellular space as endogenous DAMPs or as exogenous DAMPs when used as vaccines or inhaled as airborne pollutants.

Here, we focus on cholesterol crystals. Of note, under homeostatic condition, cholesterol biosynthesis, transport, metabolism, and secretion are tightly controlled

[212, 213]. Inborn (familial hypercholesterolemia) or fatty diet-mediated defects in these pathways can result in pathological accumulation of free, unesterified cholesterol in the circulation, which can lead to the formation of toxic cholesterol crystals when nucleated in phagocytic cells or arterial wall [214, 215]. For this scenario, cholesterol is well known to play a critical role in the progression of atherosclerosis that is now regarded as a chronic innate immune inflammatory disease induced by chronic-repetitive infective or sterile injury of the vessel wall. The disorder is further characterized by lipid deposition, leukocyte and macrophage infiltration, and proliferation of VSMCs [216–218] (for VSMCs, see Part III, Sect. 9.4.3). The mechanistic role that cholesterol plays in this scenario was not clarified for a long period of time. However, studies during recent years have revealed that its major action can be seen in the activation of the NLRP3 inflammasome.

Function of Cholesterol as a DAMP

The first report showing a critical role of the NLRP3 inflammasome in the progression of atherosclerosis was published by Duewell et al. referring to studies on murine macrophages [219]. The authors demonstrated that crystalline cholesterol acts as an endogenous DAMP and its deposition in arteries or elsewhere is an early cause rather than a late consequence of inflammation. Interestingly, these studies also revealed that OxLDL contributes to cholesterol crystallization concomitant with NLRP3 inflammasome priming and activation suggesting that OxLDL may operate as the priming step (signal 1) in NLRP3 assembly (for priming step in NLRP3 activation, see Part VI, Sect. 22.4.2.2). Remarkably, these data were confirmed by other studies on human macrophages demonstrating that cholesterol crystals are able to induce NLRP3 inflammasome activation via lysosomal destabilization [220].

The precise mechanism of how cholesterol crystals are sensed by NLRP3 is not entirely clear. One may again suggest as proposed for MSU and shown by data from human macrophages [220] that these particles after phagocytosis by macrophages induce phagolysosomal destabilization and rupture. As already mentioned above for MSU crystals, the process is known as “frustrated phagocytosis” leading to changes in the structure of the Golgi complexes in terms of like fragmentation and reorganization of Golgi membranes [209]. As intracellular molecular perturbations, these changes reflect *bona fide* dyshomeostatic DAMPs through which cholesterol is indirectly sensed by NLRP3.

Concluding Remarks

At the time being, an increasing number of publications on the role of inflammasomes in atherogenesis can be noticed. Plausibly, cholesterol as an old well-known contributor to this systemic vascular disease has taken center stage. This the more as it has recently become evident that not only innate immune but also adaptive auto-immune processes are involved in the progression of atherosclerosis [221, 222]. This is reason enough to dedicate an own chapter to this topic in Volume 2 of the book.

12.2.5.5 Lipotoxic Species (Ceramide, Palmitate)

General Remarks

The metabolic syndrome (MetS) is a growing public health and clinical challenge worldwide due to escalating urbanization, surplus energy intake, increasing obesity, and sedentary life habits. The syndrome is associated with a fivefold increase in the risk of T2D and twofold the risk of developing cardiovascular disease (CVD) over the forthcoming years [223, 224]. Notably, recent investigations have uncovered a role of the DAMP-activated NLRP3 inflammasome in the pathogenesis of the MetS.

Function of Lipotoxic Species

First evidence for a role of DAMPs in obesity-induced inflammation and insulin resistance were reported from studies in mice showing that obesity activates the NLRP3 inflammasome by sensing the lipid molecule ceramide, which is composed from sphingosine and fatty acid [225, 226]. Other lines of studies in rodents confirmed these observations by uncovering further metabolic stress-induced lipid species acting as NLRP3-activating DAMPs. Thus, in a working model in which free fatty acids activate inflammasome-dependent IL-1 β secretion from myeloid cells, the investigators found that that elevated fatty acid caused by a high-fat diet was capable of activating the NLRP3 inflammasome in macrophages via a ROS-dependent signalling pathway. Remarkable from these experiments was the observation that IL-1 β induced by fatty acid prevents normal insulin signalling in multiple insulin target tissues, ultimately resulting in insulin resistance [227]. Doubtlessly, for clinicians, this finding explicitly explains the development of insulin resistance in obese patients, at least partially. Also, in experiments on murine LPS-treated macrophages, it could be demonstrated that ingested palmitate, a saturated fatty acid ester, activates the NLRP3 inflammasome resulting in a lysosome-dependent release of IL-1 β [228]. In their conclusion, the authors also discuss a potential mechanistic role of lysosome destabilization and cathepsin B.

Of note, recent evidence from studies on circulating immune cells of patients with T2D suggests that ceramide- or palmitate-induced ER stress in macrophages may promote upregulation of the ROS \rightarrow TXNIP axis that is known to contribute to the activation of the NLRP3 inflammasome [229] (for ER stress, see Part V, Sect. 18.5; for ROS \rightarrow thioredoxin-interacting protein (TXNIP) axis, compare Sect. 13.4.6.3, but also Part VI, Sect. 22.4.2.2).

Concluding Remarks

Together, increasing evidence indicates the importance of DAMP-induced activation of the NLRP3 inflammasome in the pathophysiology of MetS. Future intervention studies are expected to reveal whether or not inhibition of NLRP3 inflammasome activation can efficiently prevent the deleterious effects of this metabolic disease.

12.2.5.6 Beta-Amyloid

Notably, the DAMP A β is increasingly discussed to contribute to the pathogenesis of Alzheimer's and Parkinson's disease and T2D. Amyloids are proteins with cross- β -sheet structure produced by both bacteria and humans that contribute to pathology and inflammation in complex human diseases, including Alzheimer's disease, Parkinson's disease, T2D, and secondary amyloidosis. Pathologically, for example, via Alzheimer's disease-induced mutations, the membrane-bound amyloid precursor protein (APP) is sequentially cleaved abnormally into a small peptide fragment, A β , and secreted into extracellular fluid where they may be highly self-aggregating [230]. Once extracellularly released, the fragments/aggregates operate as an endogenous DAMP.

Today, the pro-inflammatory effects of these proteins are thought to be predominantly mediated by their property to activate the NLRP3 inflammasome. This property was described in an early report by Halle et al. [231], who provided first evidence for a role of A β in the pathogenesis of Alzheimer's disease. Remarkably, already in this early article, the authors suggested that "frustrated phagocytosis" of engulfed A β aggregates may contribute to the loss of lysosomal integrity under involvement of the enzyme cathepsin B. Again, today, one may discuss again that, mechanistically, the molecular changes associated with frustrated degradation reflect *bona fide* dyshomeostatic DAMPs through which A β is indirectly sensed by NLRP3.

The increasing relevance of A β for the pathogenesis of Alzheimer's disease is mirrored by a growing list of publications in the international literature (for three examples, see Refs. [230, 232, 233]).

A similar sequela of events is currently discussed for the pathogenesis of Parkinson's disease. Thus, recent studies showed that amyloid deposits of *α -synuclein*—known to be the main pathological feature of this disease [234]—induced inflammation through activation of TLR2 and NLRP3 inflammasome only when folded as amyloid fibrils [235].

Also, aggregation of islet A β deposits primarily comprised of islet amyloid polypeptide (IAPP) has been found to contribute to beta-cell dysfunction in T2D via upregulation of the NLRP3 inflammasome activation [236–239]. Interestingly, detailed mechanistic investigations on bone marrow-derived macrophages revealed that IAPP species generated during the early stages of aggregation act as stimuli for TLR2-dependent pro-IL-1 β expression, whereas species produced later during aggregation (amyloid fibrils) serve as stimuli for NLRP3-dependent IL-1 β secretion reflecting final activation of the inflammasome [238].

Concluding Remarks

Intriguingly, it has become apparent that one single DAMP, here A β , is involved in several human diseases which at first glance—though all representing chronic inflammatory disorders—have no common pathogenesis and, at least partially, are manifested in different organs. In Volume 2 of the book, the reader will encounter this interesting innate immune phenomenon several times.

12.2.5.7 Concluding Remarks

It is evident that the subclass of DAMPs described in this section consists of molecules with different structures and functions. The reason for this particularity is reflected by the heading of this section: their property to act as signal 2 necessary to fully activate the canonical NLRP3 inflammasome indirectly. Due to this trait of perturbing the intracellular homeostasis, they are believed to provoke the generation of dyshomeostatic DAMPs which are ultimately sensed by NLRP3 (see below, Sect. 13.4.5). In other words, it is this “double DAMPs axis” that activates the NLRP3 inflammasome to play a leading role in mounting both infective and sterile inflammatory milieus.

12.2.6 Résumé

The phenomenon of passive release from necrotic cells of a plethora of diverse DAMPs with various pro-inflammatory functions has led to the creation of the new term “necroinflammation.” The remarkable “clou” of this event is that bacterial/viral infection-caused necrosis of cells—via emission of large amounts of endogenous DAMPs—promotes a robust host defense against the virus or the bacterium concerned and not the pathogen per se (for more information about DAMPs as origin and consequences of necroinflammation, see Ref. [50]).

12.3 DAMPs Exposed at the Cell Surface (Class IB DAMPs)

12.3.1 Introductory Remarks

The assignment of exposed DAMPs to a separate class is owed to their particularity that they are not passively released from necrotic cells but function as critical DAMPs when exposed at the cell surface, predominantly on stressed or dying cells. In this position, they may carry out entirely different functions. Thus, members of this class of endogenous DAMPs can facilitate phagocytic processes by interacting with scavenger receptors, can activate ILCs and unconventional T cells as ligands for the NKG2D receptor, and can promote cell adhesions in their role as adhesion molecules. Here, in this subchapter, some relevant examples are briefly discussed.

12.3.2 Phagocytosis-Facilitating Molecules (“Chaperones”) (Subclass IB-1 DAMPs)

12.3.2.1 General Remarks

Phagocytosis-facilitating molecules in terms of chaperones can operate as DAMPs exposed at the plasma membrane via promotion of engulfment of antigenic material by phagocytes such as DCs. In fact, such DAMPs can be considered to be capable of “scanning” the antigenic pattern of stressed or dying cells such as cancer cells on

encountering processed antigenic peptides. These DAMPs subsequently meet professional phagocytes such as DCs and macrophages, where they are recognized as “eat-me” signals by scavenger receptors, for example, by the scavenger receptor CD91 (also called the LDL-receptor-related protein 1 or LRP1) that has recently gained special attention as an important receptor on DCs to facilitate engulfment of antigens (compare Part II, Sect. 5.3.3; and for reviews, see Refs. [240–243]). Of note, another “eat-me” signal is lysophosphatidylserine that acts as an inducible inflammation-resolving DAMP and will be described below in Sect. 14.4.5.

12.3.2.2 Calreticulin

General Remarks

A crucial Class IB-1 DAMP recognized by CD91 is CALR, an ER-based chaperone that when outside of the ER as “ecto-CALR” has emerged to exert an explosion of crucial functions from the cell surface and extracellular environment [244, 245].

Calreticulin—as an “eat-me” signal for phagocytes—is a member of immunogenic HSPs family. The protein is a highly conserved, ER-resided, Ca^{2+} -binding chaperone protein that plays a key role in the activity and regulation of Ca^{2+} homeostasis/signaling, and through the interaction with the isomerase ERp57 (= a protein disulfide isomerase that catalyzes disulfide bonds formation of glycoproteins as part of the calnexin and calreticulin cycle), CALR facilitates proper folding of ER-chaperoned proteins (reviewed in [246]). The molecule also contributes to the correct assembly of MHC-I molecules and insures efficient loading of antigens [247].

Of note, when stressing insults provoke ER stress, the serine/threonine kinase PERK becomes activated and phosphorylates eIF2 α to elicit a UPR that—when unsuccessful—results in apoptotic cell death (for details of UPR, see Part V, Sect. 18.5). In the course of the UPR, before completion of apoptosis, the complex CALR/ERp57 is translocated to the plasma membrane to get exposed (Fig. 12.5). Importantly, both the action of PERK and the phosphorylation of eIF2 α are required for this translocation. In addition, CALR/ERp57 appears on the surface of stressed, mostly apoptotically dying cells as a result of exocytosis, following a classical pathway in which Golgi apparatus-derived vesicles fuse with the plasma membrane in a SNARE-dependent manner (for details, see Ref. [248]).

Extracellular Functions of Calreticulin and Its Cognate Binding Receptors

Calreticulin when outside of the ER and translocated to the plasma membrane (now called ecto-CALR) exerts multiple functions and plays a critical role in the phagocytic removal of apoptotic cells. With respect to this function, surface-exposed CALR acts as a DAMP on apoptotic cells, thereby generating an engulfment signal that stimulates the uptake of apoptotic corpses and the presentation/cross-presentation of the corresponding antigens by DCs (reviewed in Refs. [249–251]) (for antigen presentation and cross-presentation by DCs, see Part VIII, Sects. 31.3.4 and 31.3.5). This scenario plays an eminent role in engulfment of tumor-associated antigens (TAAs) that—via the process of ICD—contributes to a vigorous anti-tumor immune response [252].

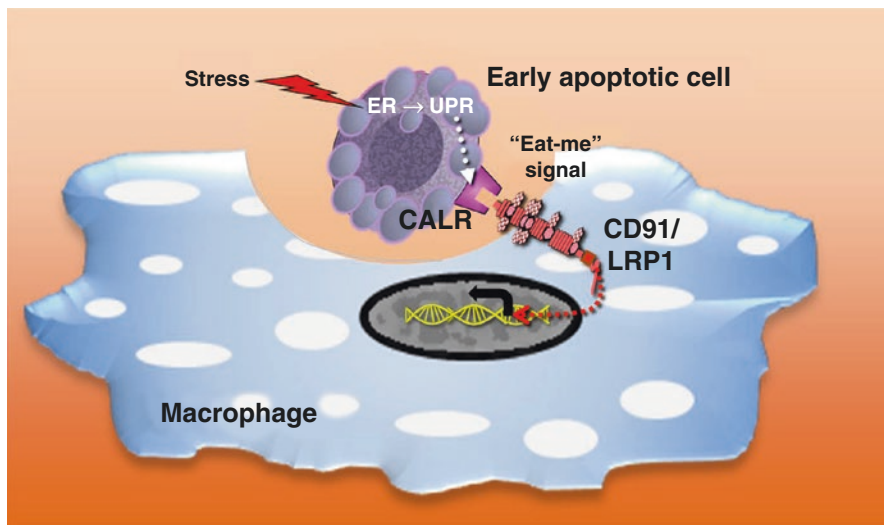


Fig. 12.5 Schematic diagram: the “eat-me” signal. Calreticulin exposed on the surface of early apoptotic cells triggers CD91/LRP1 signalling to provide an “eat-me” signal for phagocytes to promote engulfment of these dying cells. *CALR* calreticulin, *ER* endoplasmic reticulum, *LRP1* low-density lipoprotein receptor-related protein 1, *UPR* unfolded protein response. Sources: Refs. [240–243, 246]

The phagocytosis-facilitating function is mainly executed through binding and activation of the CD91 receptor on the phagocytes [249] (compare Part II, Sect. 5.3.3.3). Importantly, it is this receptor on DCs which allows for cross-priming/cross-presentation of the chaperoned peptide antigen [253]. This action is of immense relevance for cross-presentation of TAAs.

Notably, the CD91 receptor is a transmembrane protein, acting as a co-receptor for the bridging molecule opsonin C1q, a complement component. Hence, CALR together with CD91 is critically essential for C1q-mediated phagocytosis of opsonized pathogens and apoptotic bodies. Moreover, besides CD91 and C1q, some other CALR’s partners are critical in modulating the whole phagocytosing process including bridging or/and signalling molecules phosphatidylserine (PS; “eat-me” signal) (Fig. 12.5), CD47 (“don’t eat-me” signal), and the scavenger receptor SRF-I (also called SREC-I or SCARF1) (reviewed in [254]) (for SRF-1, also see Part II, Sect. 5.3.3.2; for C1q see Part VI, Sect. 23.2.2.2).

It is worth adding here that CALR was found to be actively secreted by cancer cells to act as a soluble DAMP. Thus, clinical studies in patients suffering from RA, SLE, and lung cancer showed that CALR in its soluble form circulates in the periphery, in lung cancer patients before and—in much higher concentration—after chemotherapy. As such, the DAMP was proposed to use clinically as a potential diagnostic biomarker in lung cancer patients [255]. Subsequent studies of the same group provided evidence suggesting the scavenger receptor SRA to sense the soluble form of CALR, thereby triggering macrophage activation [256].

Concluding Remarks

There is emerging evidence suggesting a critical role of CALR in inducing increased immunogenicity in cancer cells. As mentioned above, the DAMP serves as a phagocytic signal on cancer cells following induction of ICD, thereby contributing to the maturation of immunogenic DCs, which elicit—via efficient cross-presentation of TAAs—a potent anti-tumor CD8⁺ T cell response. This scenario makes tumors susceptible to immunotherapy-based anticancer strategies. For this reason, CALR is now regarded as one of the most potent target DAMPs for developing new anticancer therapeutics (more details of ICD will be presented in Volume 2).

12.3.2.3 Heat Shock Proteins as DAMPs Exposed at the Cell Surface

Heat shock proteins operate as DAMPs when passively released or actively secreted into the extracellular space (see above, Sect. 12.2.3, and below Sect. 14.2.2). Like CALR, HSPs can also be exposed at the cell membrane. At this position, they are known to support engulfment of antigenic peptides by phagocytosing cells, whereby CD91 also serves as the key endocytic receptor. For example, HSP70 is considered to “sample” the antigenic milieu of cancer cells on encountering processed peptides *in vivo* and can be used to carry this sample into the APC during immunization [90]. Similarly, HSP70 and HSP60 exposed on stressed apoptotic cells were found to increase their immunogenicity, thereby generating a T cell-mediated specific anti-tumor response [257]. In other lines of studies, severe heat shock-treated cancer cells were shown to expose, besides CALR, HSP70 and HSP90, thereby contributing to immunogenicity of a tumor [258]. Again, like the interaction of CALR with CD91, the HSP70 → CD91 axis was shown to be required for cross-presentation of HSP-chaperoned peptides to elicit a potent anti-tumor immune response (reviewed in [253]).

Concluding Remarks

Together, these findings show that HSPs in their role as chaperones for antigenic peptides can function as strong DAMPs to facilitate engulfment of antigenic peptides, for example, derived from TAAs. It is for this reason that tumor-derived HSPs have been proposed to use for anticancer immunotherapy [259].

12.3.2.4 Concluding Remarks

Molecules such as CALR and HSPs, for example, expressed on tumor cells, can function as endocytosis-promoting DAMPs by facilitating engulfment of antigenic peptides recognized by cognate PRMs such as CD91 and TLRs. As already mentioned, this phenomenon, that is, HSPs complexed with antigenic peptides, is currently being used to prepare effective vaccines in virology and oncology to elicit strong antiviral and anti-tumor immunity [260, 261]. Calreticulin is of special importance because it has been demonstrated to act as a mandatory DAMP to increase immunogenicity of cancer cells via the phenomenon of ICD. In Volume 2, the important function of the ER-associated chaperones CALR and HSPs will be resumed in their role as DAMPs to fight against cancer growth.

12.3.3 Major Histocompatibility Class I Chain-Related Molecules (Subclass IB-2 DAMPs)

12.3.3.1 General Remarks

Another class of DAMPs which exert their function as danger signals when exposed on the cell plasma membrane refers to molecules here sorted into IB-2 DAMPs. Though these DAMPs are characterized by numerous and highly variable genetic, structural, and biochemical features, they all belong to the major histocompatibility complex Class I gene superfamily (MHC-I-related proteins) and bind to a single, invariant, receptor, the NKG2D receptor (compare Part II, Sect. 5.3.7.4). This activating receptor is expressed on the surface of NK cells, iNKT, $\gamma\delta$ T cells, CD8⁺ MAIT, CD8⁺ T cells, and subsets of CD4⁺ T cells (compare Part VII, Sects. 27.2.2, 28.2.2, and 28.4.2). The receptor NKG2D serves as a major recognition receptor for this class of DAMPs to detect and eliminate cells, which are stressed as transformed or infectively and sterily damaged cells. On healthy cells, these endogenous IB-2 DAMPs (also called NKG2D ligands) are expressed at a low level only, but their expression is markedly induced on such stressed cells, whereby they translocate (probably from the stressed ER [262]) to the plasma membrane to get anchored and exposed.

Two families of IB-2 DAMPs, mainly expressed on epithelial cells and ECs, have been identified in humans: (1) the stress-inducible molecules non-conventional MICA and MICB, which could be considered prototypical members of this class, and (2) the stress-inducible UL16 binding proteins 1, 2, and 3 (ULBPs 1, 2, 3), together with two novel members of this family, the *ULBP/retinoic acid early inducible protein 1* (RAET1) gene cluster, ULBP4/RAET1E and ULBP4/RAET1G (for reviews, see Refs. [263–269]).

Of note, among these danger signals, MICA and MICB are the most polymorphic. To date, 100 alleles are known for MICA and 40 for MICB. This corresponds to 79 and 26 unique protein sequences, respectively. Although these numbers are far smaller than those for classical HLA Class I molecules (close to 10,000 alleles and 7000 proteins), one has to bear in mind that our current knowledge of HLA diversity spans from genotyping of over 20 million individuals (current estimate of the size of international bone marrow registries) (competently reviewed by Carapito and Bahram [263]). The proteins ULBP/RAET1 seem to be less polymorphic than MICs. This may be due, however, to sampling, as fewer than 300 individuals have been sequenced. Among the six *ULBP/RAET1* genes, four have been found to be polymorphic in exon 2 and exon 3 coding for the $\alpha 1$ and $\alpha 2$ domains, respectively [263].

12.3.3.2 Regulation of MHC-I Chain-Related Molecules

Mechanisms of regulation of members of this subclass of DAMPs have been comprehensively reviewed by Raulet et al. [270]. Cell stress stimuli induce generation of these DAMPs, which are regulated at several levels of biogenesis by various types of such signals that are worked out in distressed, infected, or transformed cells. The regulatory processes have been found to occur discerned at the transcriptional,

post-transcriptional, translational, and post-translational levels. The signals that regulate the various steps of DAMP biogenesis are induced in the course of stress responses such as the oxidative stress response, the heat shock response (HSR), the DDR, and the ER stress-initiated UPR (for these stress responses, see Part V, Sects. 18.3–18.6). Thus, in a way and broader sense, they can be also regarded as DAMPs. For example, in the case of cancer, numerous independent pathways such as the DDR and the UPR (under involvement of transcription factor E2F) that are activated in proliferating cancer cells are likely to collaborate in the induction of DAMPs (for E2F, see Box 12.1). In case of infective cell injury, the HSR pathway, at least in part, has been shown to play a role in the induction of MICA and MICB at the transcriptional level. Moreover, there is some evidence suggesting that (DAMP?) → TLR-triggered activation of the transcription factors TBK1 and IRF3 is implicated in the production of these DAMPs. Sterile cell injury, such as oxidative stress, can also induce the generation of IB-2 DAMPs, although little is known concerning the underlying mechanisms. Nevertheless, there is some evidence suggesting that the DAMP OxLDL (see below, Sect. 13.3.2) sensed by TLR4 is involved in generation of members of this class of DAMPs at the transcriptional level (mostly reviewed in [270]).

Box 12.1 What About E2F factors?

E2F is a group of genes that codifies a family of transcription factors (TF) in higher eukaryotes. Three of them are activators: E2F1, E2F2, and E2F3a. Six others act as suppressors: E2F3b, E2F4–8. All of them are involved in cell-cycle regulation and synthesis of DNA in mammalian cells. E2Fs as TFs bind to the TTTTCGCGC consensus binding site in the target promoter sequence of genes.

Further reading: Dimova DK, Dyson NJ. The E2F transcriptional network: old acquaintances with new faces. *Oncogene* 2005;24:2810–26.

Finally, the newly, in the course of stress responses, generated molecules translocate through the cytosol via the ER and the Golgi apparatus to become anchored on the plasma membrane. In addition to exposure, the DAMPs can also be secreted, or excreted in vesicles such as exosomes, or shed via proteolytic cleavage. In this case, the molecules may interact with NKG2D on the surface of NK cells and unconventional T cells, thereby blocking the receptors on these cells in a manner that inhibits their interactions with target cells resulting in reduced cytotoxicity (reviewed also in [270]).

12.3.3.3 Function of MHC-I Chain-Related Molecules

This subclass of DAMPs is critical for the activation of NKG2D-expressing cells that execute killing activities; they include ILCs such as NK cells and unconventional T cells such as iNKT, CD8⁺MAIT, and $\gamma\delta$ T cells (see Part III, Sects. 8.4.3.3 and 8.5.2–8.5.4; as well as Part VII, Sects. 27.2.2, 28.2.2, 28.3.2, and 28.4.2). For further details of the function of these cells in viral infections and tumor cell surveillance, the reader is referred to these sections.

12.3.3.4 Concluding Remarks

The subclass of DAMPs briefly addressed in this section play an increasing role in viral infections and tumor growth. Regarding the many possibilities of which viruses and tumors have devised mechanisms to evade detection and elimination by NKG2D-bearing killing cells, therapeutic strategies are considered to restore or enhance NKG2D-dependent activation of these cells, in particular, NK cells. On the other hand—as splendidly outlined by Lanier [264]—where DAMP-activated NKG2D-bearing cells contribute to aggravate autoimmune responses, suppression of expression of these DAMPs in inflammation provides an attractive therapeutic target. As concluded by Lanier [264], “*A better understanding of the cell-intrinsic and -extrinsic mechanisms that regulate the expression of the NKG2D ligands and the intracellular signals controlling NKG2D-induced responses in T cells and NK cells is needed to take full advantage of this potent immune pathway.*”

12.3.4 Résumé

It becomes apparent to the reader that the molecules sorted in this subchapter possess different structures, different locations, and various functions. Indeed, one could classify them in their role as DAMPs choosing other ways. The common feature here chosen is their exposure on the cell surface, though some of them have been shown to be secreted as well. The critical role of Subclass IB-1 DAMPs is their function in facilitating antigen uptake. For example, CALR, as mentioned, has a vital impact on engulfment of TAAs to increase the immunogenicity of cancer cells via the phenomenon of ICD. On the other hand, MICS, here MICA, have gained center stage by documenting that it has the requisite attributes of a *bona fide* transplantation antigen. Thus, recent studies by Carapito et al. showed that matching for the non-conventional MHC-I MICA gene significantly reduces the incidence of acute and chronic graft-versus-host disease (GVHD) [271]. The authors concluded from their findings—besides others—that “*The tight linkage disequilibrium between MICA and HLA-B renders identifying a MICA-matched donor readily feasible in clinical practice.*”

12.4 Outlook

Certainly, the list of endogenous constitutively expressed native DAMPs as selected and presented in this chapter refers to the more prominent DAMPs and thus is not complete. Of particular importance are the Class IA DAMPs because, as native molecules passively released from necrotic cells, they are now known to promote necro-inflammatory responses (reviewed by Sarhan et al. in Ref. [50]). In fact, the phenomenon of necroinflammation as a common consequence of necrosis, in particular, RN such as necroptosis and ferroptosis, gains increasing attention, and growing evidence suggests a significant *in vivo* impact on human diseases [272, 273]. The authors conclude “*that targeting regulated necrosis in vivo represents a novel concept that requires the establishment of first-in-class compounds for clinical use.*”

Compared to Class IA DAMPs, Class IB DAMPs have been neglected for a while. On the other hand, as mentioned, they are now recognized as DAMPs, which are emitted by stressed but not necessarily dying cells. As such, these DAMPs may take over an important innate immune defending function together with damage-modified molecules (Cat. II DAMPs) in moderately stressful situations, in which no necrotic cell death occurs.

Together, endogenous constitutively expressed native molecules represent an impressive category of DAMPs with extraordinary properties. In Volume 2, their negative, detrimental role in the pathogenesis of many human diseases will be thoroughly outlined.

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