

# Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox

Anna Rubartelli<sup>1</sup> and Michael T. Lotze<sup>2</sup>

<sup>1</sup> Cell Biology Unit, National Cancer Research Institute, Largo Rosanna Benzi 10, 16132 Genova, Italy

<sup>2</sup> Departments of Surgery and Bioengineering, G.27A Hillman Cancer Center, University of Pittsburgh Cancer Institute, 5117 Centre Ave, Pittsburgh, PA 15213, USA

**Immune responses are initiated and perpetuated by molecules derived from microorganisms pathogen-associated molecular-pattern molecules or from the damage or death of host cells [damage-associated molecular-pattern (DAMP) molecules]. Many DAMPs are nuclear or cytosolic proteins with defined intracellular function that, when released outside the cell following tissue injury, move from a reducing to an oxidizing milieu resulting in their functional denaturation. Here, we discuss the consequences of DAMP oxidation on the outcome of acute inflammation. We also suggest that, outside the cell, DAMPs might adopt novel conformations or alter the redox of the extracellular environment to more closely mimic the internal one, thereby avoiding oxidation-mediated inactivation and promoting pathology. We propose that chronic inflammation associated with autoimmunity, chronic viral infection and cancer is probably mediated by persistent release and function of DAMPs, promoting and promoted by a disordered redox environment.**

## Introduction

In the setting of infection, microorganisms initiate a series of host events promoted by their derived products, so-called pathogen-associated molecular-pattern (PAMPs) molecules. PAMPs are recognized by membrane-bound or vesicular (endosomal) pattern-recognition receptors (PRRs), including the Toll-like receptors (TLRs), the NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). PRRs 'sense' bacterial products and activate intracellular cascades that lead to an inflammatory response [1,2]. Bacterial infections associated with tissue injury release host intracellular components. Some of the factors released by damaged or dying cells have been termed damage-associated molecular-pattern (DAMP) molecules because they contribute to the induction of inflammation (Figure 1) and interact with PRRs. Even in the absence of pathogens, disrupted or injured cells recruit innate inflammatory cells by releasing DAMPs. Interestingly, many of the receptors for DAMPs and PAMPs are shared, either membrane bound (e.g. TLR4) or intracellular (e.g. TLR3, TLR7–9, all NLRs and RLRs), indicating that similarities

exist between pathogen-induced and noninfectious inflammatory responses [3].

The extracellular delivery of DAMPs depends on two characteristics: their production by injured cells and the extent of tissue injury. In addition to intracellular factors, DAMPs include hyaluronan and heparan sulfate present in the extracellular matrix, as well as degraded matrix components [4]. Therefore, the actual repertoire of DAMPs in damaged tissues can vary greatly depending on the type of cell (epithelial or mesenchymal) and injured tissue. Many intracellular proteins secreted actively through non-classical pathways and endowed with inflammatory activity [so-called leaderless secretory proteins (LSPs)] [5,6] can be released by dying cells and are thus DAMPs. The chromatin-associated protein high-mobility group box 1 (HMGB1) is a prototypical LSP, which can promote inflammation as a DAMP [7]. Interestingly, some LSPs, such as thioredoxin and macrophage-inhibitory factor (MIF), are oxide reductases over-expressed in several pathological states, including autoimmunity and cancer [6]. Their extracellular function as inflammation mediators depends at least in part on their enzymatic activity. Notably, in healthy tissues, intracellular redox is highly reducing but the normal extracellular environment is substantially oxidizing. Following injury, however, oxidoreductases and nonprotein thiols, usually confined within cells, can be released, thereby modifying the extracellular redox state. Here, we consider that, in addition to the type and amount of DAMPs, the redox state of the injured tissues might influence the outcome not only of an innate but also of an adaptive immune response. Recruited cells, including macrophages, myofibroblasts, mast cells and eosinophils, promote healing depending on the site and nature of the insult. It is likely that the polarized patterns of subsequent adaptive immune responses are modulated by such environmental cues, including the presence of DAMPs and PAMPs as well as the redox state of the injured tissue.

## Thiol redox state: inside and outside the cell

The cytosol and the extracellular milieu are highly diverse environments. An important difference between them is the thiol redox status. The cytosol is highly reducing thanks to a group of thiol-regulating enzymatic systems,

Corresponding author: Rubartelli, A. ([anna.rubartelli@istge.it](mailto:anna.rubartelli@istge.it)).

Available online 12 September 2007.

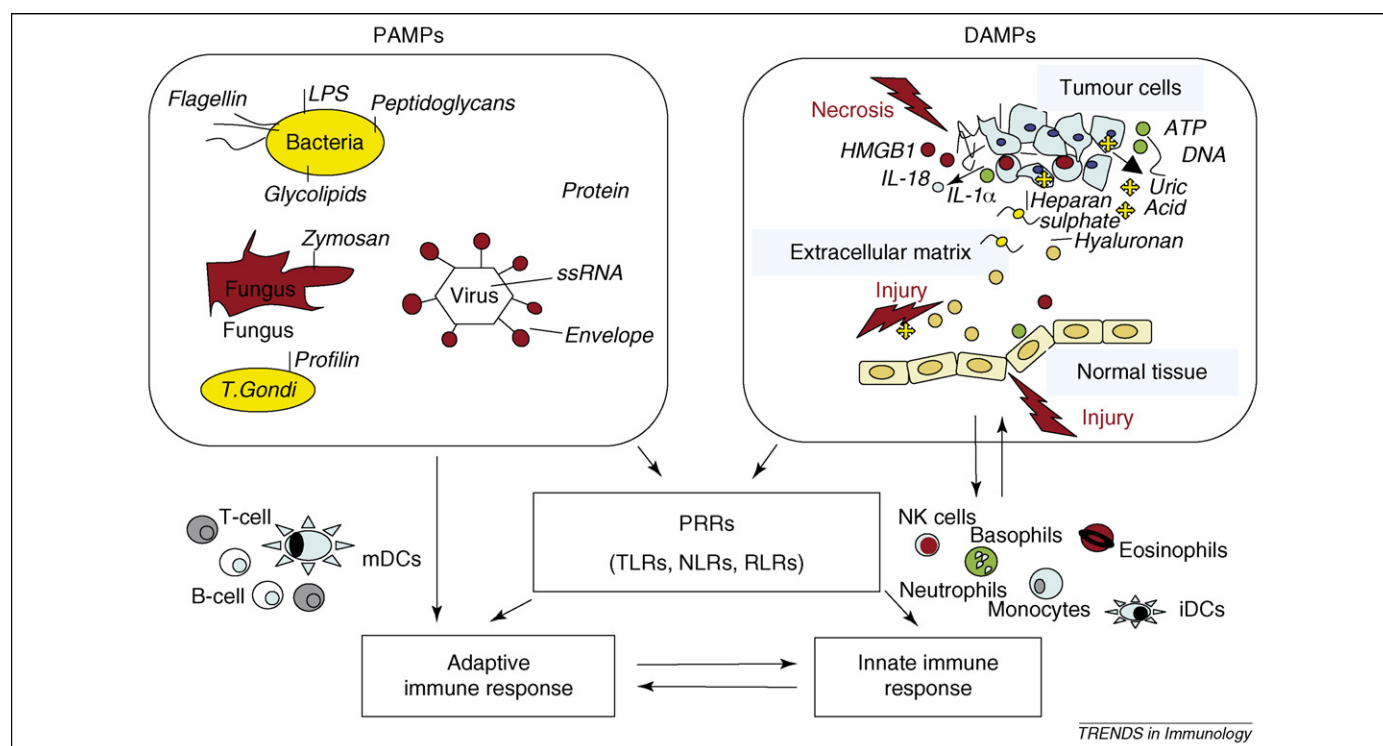


**Figure 1.** Classes of molecules that initiate innate and adaptive immune responses. The initiating events in inflammation and the resultant adaptive immune response include both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These are protein (e.g. HMGB1) or non protein (e.g. ATP, uric acid) molecules which in healthy conditions are confined within cells but are delivered outside upon cell damage, such as necrosis, and in some instances secreted by cells. The levels of released DAMPs depend on the abundance of intracellular DAMPs and are often higher and enduring in neoplastic tissues than in normal tissues. This leads to persistent inflammation. Other sources of DAMPs include degraded extracellular matrix, such as heparan sulphate and hyaluronan. Many of the receptors so far identified for DAMPs and PAMPs are shared, and belong to the family of Pattern Recognition Receptors, PRRs. Therefore, both sterile and non sterile inflammation signals converge on a common pathway. Matzinger has suggested a unique integrating hypothesis that all molecules signaling danger or damage are DAMPs, with hydrophobic portions of molecules (HYPOs) derived either from dead and dying host cells or pathogens. Abbreviations: LPS, Lipopolysaccharide; ssRNA, single strand RNA; *T. gondii*, *Trypanosoma gondii* (protozoan parasite); HMGB1, high mobility group box 1 protein; PRRs: pattern recognition receptors; TLRs, Toll-like Receptors; NLRs, NOD1-like Receptors; RLRs, RIG-I-like receptors.

including the thioredoxin-thioredoxin reductase [8] and glutaredoxin-glutathione systems [9]. Conversely, the extracellular space is strongly oxidizing owing to the paucity of reducing enzymes and the abundance of oxidizing agents [i.e. oxygen as well as other transiently produced gaseous mediators, including nitric oxide (NO)] (Figure 2). Nonprotein thiols, such as the small cysteine-containing tripeptide glutathione and the amino acid cysteine, which assist thiol-regulating enzymes, are mostly reduced intracellularly and predominate outside of cells in the disulfide form [10–12]. However, not all the intracellular compartments are reducing. Indeed, the lumen of all the secretory pathway components, including the ER, is highly oxidizing. A complex system of thiol-disulphide oxidoreductases is present in the ER and promotes disulfide-bond formation [13]. Unlike the cytosol, in which the reduced and oxidized glutathione molecules are maintained in a ratio of approximately 100:1 (highly reducing), the corresponding ratio within the ER lumen is approximately 3:1 (more oxidizing) [14].

The reduced or oxidized form of proteins, for these reasons, depends on the compartments where their biosynthesis occurs (Figure 2). Cytoplasmic proteins are synthesized within the cytosol and, therefore, their cysteine residues have typically free sulphhydryl groups. These free cysteines are usually located in binding pockets of substrates, coenzymes or metal cofactors, thereby

taking part in catalytic reactions directly. They are highly susceptible to inactivation by oxidation [15] and are maintained in a reduced state by the cytoplasmic thiol-regulating enzymatic systems. Proteins that must be delivered outside the cell are endowed with an N-terminal signal sequence (the leader sequence) that directs their targeting to the endoplasmic reticulum (ER), within which their biosynthesis is completed. Owing to the oxidizing conditions of the ER lumen, most cysteine residues of secretory and membrane proteins are linked by disulphide bonds that ensure stability in the extracellular milieu [13,14].

### LSPs: inside and outside

In the early 1990s, it became evident that some proteins that are active biologically outside of the cell are synthesized within the reducing environment of the cytosol, avoiding passage into the ER lumen, and are released through nonclassical pathways [16]. Lacking the secretory leader peptide, these proteins have been termed LSPs. The first LSP identified and studied was interleukin (IL)-1 $\beta$  [17]. Since then, an increasing number of LSPs have been identified [6] (Table 1). Some of them, such as IL-1 $\beta$ , IL-16 and IL-18, function primarily outside of the cell. Conversely, others have apparent functions both inside and outside of the cell. These include cytosolic enzymes (such as thioredoxin), calcium-binding factors (proteins of

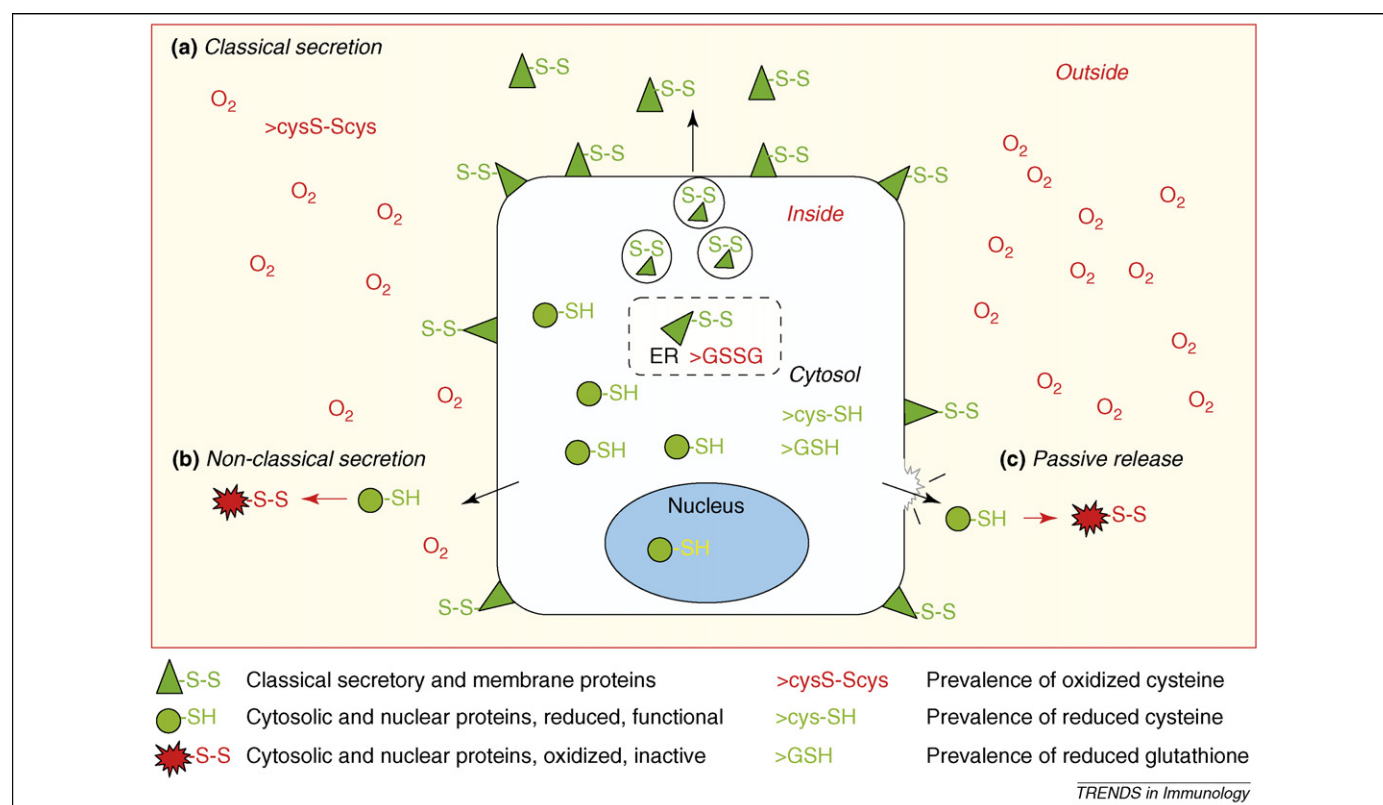


**Figure 2.** Protein folding is influenced by the redox state. Intracellular proteins can leave the cells in three different ways. **(a)** Classical secretion, which occurs along the exocytotic route and is restricted to proteins destined to reach the cell surface or the extracellular space. They are targeted to the lumen of the endoplasmic reticulum (ER) by the secretory signal peptide and complete their synthesis within the ER. Most of the cysteine residues of membrane and secretory proteins synthesized there are captured in disulfide bonds (S-S), which are essential for the stability of these proteins in the oxidizing extracellular milieu. **(b)** Nonclassical secretion, which is restricted to leaderless secretory proteins (LSPs), a group of proteins that, in spite of their predominantly extracellular function, lack secretory signal peptides and are synthesized within the cytosol. For these proteins, active and regulated secretion by specialized cells through nonclassical mechanisms has been demonstrated. **(c)** Passive release as a result of plasma membrane injury, which enables exteriorization of soluble cytosolic and nuclear proteins. The cysteine residues of these proteins, like that of all cytosolic and nuclear proteins, have typically free sulphhydryl groups (SH). When cytosolic or nuclear proteins are externalized, either through active secretion or passive release, they undergo oxidation that, in most cases, drives inactivation.

the S100 family) and nuclear proteins (HMGB1 and engrailed-2). Not all cells are able to secrete LSPs. For instance, HMGB1 and thioredoxin are produced abundantly by most cells but are secreted only by certain cell types and at given maturation states [18–21]. Most LSPs are mediators of inflammation and/or immune responses [6]. This is true not only for what are considered conventional cytokines [IL-1, IL-18, fibroblast growth factor (FGF) and MIF] but also for proteins, such as HMGB1 or thioredoxin, with a primary intracellular function. HMGB1, secreted by hemopoietic cells through a lysosome-mediated pathway [19], is a major mediator of endotoxic shock [18]. It acts on several immune cells to trigger inflammatory responses [7]. Extracellular thioredoxin also mediates several cytokine- and chemokine-like functions [22] and contributes to T-cell activation [23]. Interestingly, thioredoxin, as well as other LSPs, including MIF and its analogue cysteinylated glycosylation inhibitory factor (GIF) [6], are endowed with the active enzymatic redox site C–X–X–C (where X denotes any amino acid), which is required for at least some of their cytokine-like functions [8,24].

In keeping with their primary cytoplasmic or nuclear localization, LSPs in principle require reducing conditions to maintain their correct protein folding and undergo inactivation in the oxidizing extracellular milieu [15]

(Figure 2). Some LSPs can avoid oxidation or, alternatively, still function in an oxidizing milieu. For instance, IL-1 $\beta$  and IL-18 are synthesized as precursor proteins that display free cysteines, which are prone to oxidation [25,26]. In the mature secreted form, following caspase-1 processing, the cysteines, although not disulfide-linked, are buried within the hydrophobic interior and are thus less accessible to oxidation, enabling the cytokines to function both locally and systemically. The extracellular half life of IL-18 is short, however, secondary to oxidation-mediated inactivation. It is prolonged if the cysteine residues are replaced by serine [26]. A different mechanism is exploited by the lectin galectin-1 that delays the oxidation of its six cysteine-reduced residues by binding sugar moieties on the cell surface or extracellular matrix immediately following secretion [27]. Conversely, galectin-1 is an example of an LSP in which oxidation does not inactivate but rather modifies its function. Reduced galectin-1 functions as a regulator of both innate and adaptive immune responses. When oxidized, it serves as an essential factor for peripheral-nerve regeneration [28]. Similarly, the extracellular activities of the astrocytic protein S100B, as a factor promoting neurite outgrowth and neuronal survival, relies on the oxidation of its cysteine residues. When oxidation is prevented, S100 induces proinflammatory responses by both astrocytes and microglia [29]. Evolution has thus

**Table 1. Most leaderless secretory proteins (LSPs) act as cytokines**

| Protein <sup>a</sup>                            | Extracellular function                                                                       | Intracellular function                          |
|-------------------------------------------------|----------------------------------------------------------------------------------------------|-------------------------------------------------|
| <b>Cytokine</b>                                 |                                                                                              |                                                 |
| IL-1 $\alpha$                                   | Proinflammatory immune mediator                                                              | Activator of transcription                      |
| IL-1 $\beta$                                    | Proinflammatory immune mediator                                                              | No known function                               |
| IL-16                                           | Proinflammatory immune mediator; T-lymphocyte chemoattractant                                | No known function                               |
| IL-18                                           | Proinflammatory immune mediator                                                              | No known function                               |
| FGF-1                                           | Growth, angiogenic and motility factor                                                       | Growth regulator (nuclear)?                     |
| FGF-2                                           | Growth, angiogenic and motility factor                                                       | Growth regulator (nuclear)?                     |
| FGF-9                                           | Neurotrophin, growth survival factor                                                         | No known function                               |
| Annexin I                                       | Anti-inflammatory immune mediator                                                            | Vesicular traffic                               |
| Galectins                                       | Pro/anti-inflammatory factors                                                                | Phagocytosis                                    |
| CNTF                                            | Neurotrophin, growth survival factor                                                         | No known function                               |
| S100                                            | Neurotrophin, inflammation mediator                                                          | Ca <sup>2+</sup> -binding, regulatory functions |
| <b>Enzyme or cytokine</b>                       |                                                                                              |                                                 |
| MIF                                             | Proinflammatory immune mediator, shock factor                                                | Transcription-factor modulator                  |
| EMAP-II                                         | Proinflammatory factor                                                                       | Multisynthetase complex protein                 |
| PGI/AMF                                         | Growth motility factor                                                                       | Glycolytic enzyme                               |
| TRX/ADF                                         | Immune mediator, chemotactic factor; involved in implantation and establishment of pregnancy | Major cellular disulfide reductase              |
| <b>Enzyme</b>                                   |                                                                                              |                                                 |
| TRX reductase                                   | Enzyme reducing oxidized TRX?                                                                | Enzyme reducing oxidized TRX                    |
| Caspase I (ICE)                                 | No known function                                                                            | IL-1/IL-18-converting enzyme                    |
| Transglutaminase                                | Protein cross-linking                                                                        | Apoptosis protein cross-linking                 |
| Coagulation factor XIII                         | Coagulation                                                                                  | No known function                               |
| <b>Nuclear factor or differentiating factor</b> |                                                                                              |                                                 |
| Engrailed-2                                     | Transcription factor by intercellular transfer?                                              | Homeodomain transcription factor                |
| <b>Nuclear factor or cytokine</b>               |                                                                                              |                                                 |
| HMGB1                                           | Proinflammatory shock factor                                                                 | Chromatin component                             |

Abbreviations: CNTF, ciliary neurotrophic factor; EMAP-II, endothelial monocyte-activating polypeptide-II; FGF, fibroblast growth factor; ICE, interleukin-1 $\beta$ -converting enzyme; PGI/AMF, phosphoglucose isomerase/autocrine motility factor; TRX/ADF, Thioredoxin/adult T-cell leukemia-derived factor.

<sup>a</sup>See [5,6] for comprehensive reviews of leaderless secretory proteins.

economized, providing additional redox-dependent functions to a single protein.

In addition to their rapid oxidation and inactivation within the oxidizing extracellular milieu, there are other features that limit the activity of LSPs both temporally and spatially. First of all, secretion of leaderless proteins is usually slow and limiting, with a positive inside:outside concentration ratio. Moreover, many LSPs have 'sticky' domains, such as basic or lectin domains, which mediate their binding to several ubiquitous binding partners or sites [6]. These include heparan sulphates and sugars moieties, which are distributed abundantly on the cell surface or in the extracellular matrix, thereby preventing the diffusion of secreted molecules. In some settings, such as the cross-talk between immune cells, nonpolarized cells secrete LSPs in a polarized fashion. DCs, interacting with T lymphocytes or NK cells, enable clustering of IL-1 $\beta$  or IL-18 at the immunological synapse and promote polarized secretion within the cleft. This limits cross-talk outside of the synapse, enabling immune-cell activation but preventing the spread of inflammation [20,30]. The net effect is that short-range activities of LSPs predominate over the systemic effects (Figure 3a).

Most LSPs are powerful mediators that stimulate immunity coordinately with other cytokines, regulating the onset, progression and termination of inflammation. Indeed, dysregulation of cytokine networks leads to chronic inflammation and emergence of autoimmune disorders. On this basis, the apparent constraints of LSPs might actually be advantageous biologically. The slow, inefficient, short-range secretion and the rapid extracellular oxidation and inactivation in the course of acute inflammation might favor self-limiting activity, thereby enabling

control of otherwise potentially noxious effects. LSPs can function over a short range, until oxidized. This restricts the site of action temporally and spatially, thereby limiting prolonged stimulation and controlling inflammation and associated tissue dysfunction.

### LSPs act as DAMPs

The danger hypothesis was first articulated fully by Polly Matzinger [31]. She suggested that the immune response is initiated when innate immune cells recognize signs of tissue distress, typically when molecules that are only found inside cells normally are released into the extracellular space following damage. Such endogenous DAMPs could alert the immune system to cell damage even in the absence of infection, thereby providing a ready means to recruit inflammatory cells and elicit an inflammatory response to damaged autologous tissues. Damage arises in the setting of cellular nutrient, oxidative, or genomic stress, resulting in tissue dysfunction and, when prolonged, in tumor formation. The tissues thus drive not only the innate response but also probably mediate the earliest events in the transition to the adaptive immune response with polarization of T helper cells into Th1, Th2 and Th17 phenotypes.

Several DAMPs that can activate the immune system have been identified. They include intracellular proteins, such as heat-shock proteins [32] or HMGB1 [7], proteins derived from the extracellular matrix that are generated following tissue injury, such as hyaluronan fragments [4], and nonprotein purinergic molecules, such as ATP [33,34] and uric acid [35]. DAMPs activate cells of innate immunity by triggering TLRs or other alarm-signal receptors [2], therefore inducing an inflammatory response. If

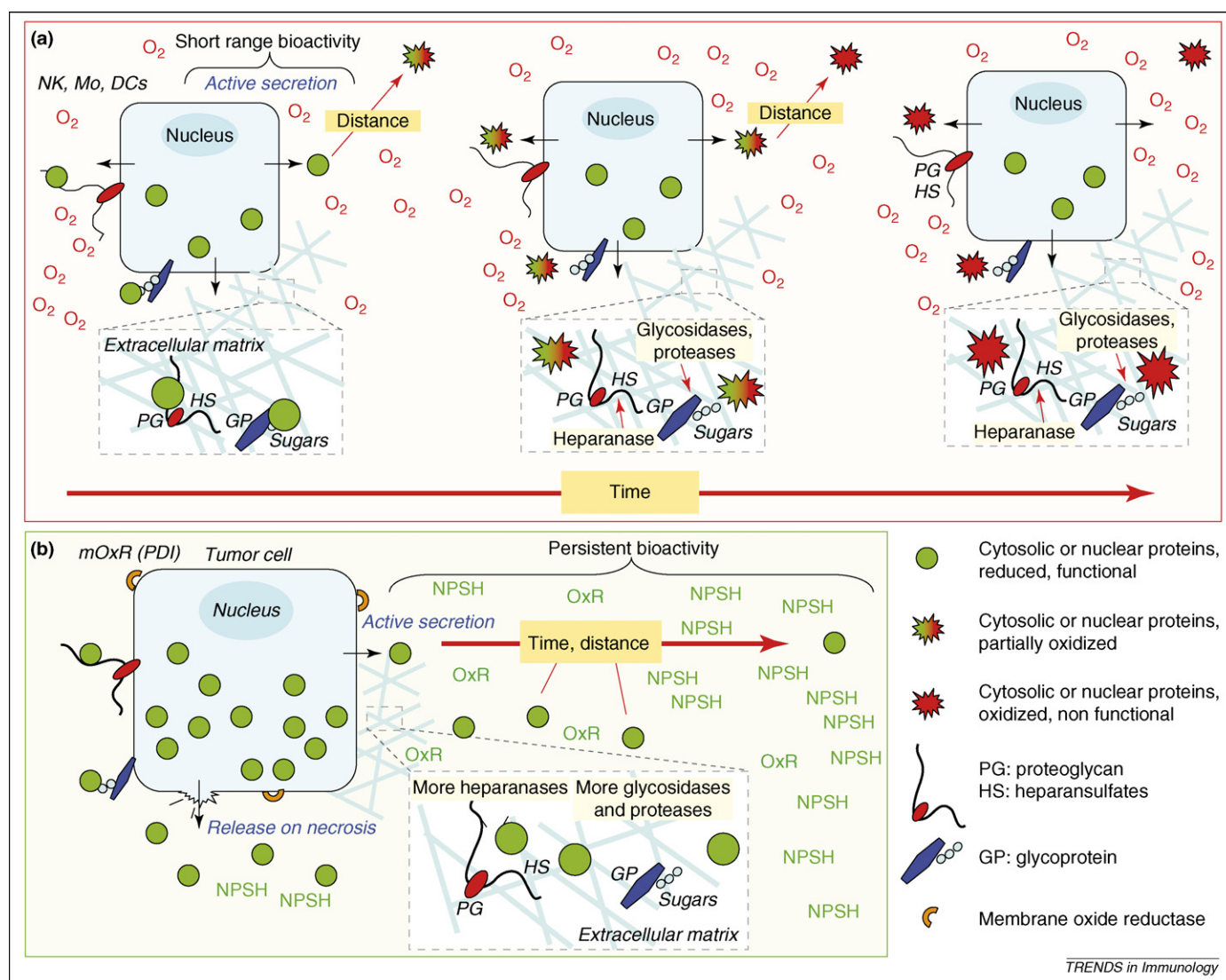


**Figure 3.** Oxidation-dependent inactivation of LSPs and DAMPs can be prevented by a pathological extracellular microenvironment. Many LSPs (e.g. FGF, HMGB1, engrailed-2) possess basic amino acids that mediate their binding to heparan sulphates. Others are lectins (e.g. galectins) that bind galactoside residues. These features account for the short-range secretion of many leaderless proteins, which adhere to their binding sites at or near the producing cell. Most LSPs require free thiols for their activity, either because they bear the active enzymatic redox site C-X-X-C (such as thioredoxin and MIF) or because intra- or inter-chain disulphide bond formation causes conformational changes that are detrimental for their function (FGF1, galectins and HMGB1). Together, these structural features may provide a means to regulate and limit the extracellular activity of LSPs. Under physiological conditions, such as acute inflammation, LSPs are secreted but can function only briefly and locally before they become inactivated by the oxidizing extracellular milieu. This restricts their range of action both temporally and spatially. This control might be lost in pathological conditions, such as in the neoplastic microenvironment. **(a)** LSPs are secreted actively by inflammatory cells (such as NK cells and monocytes). Once externalized, they bind specialized receptors or dissipate. The extracellular milieu would cause their oxidation and hence their inactivation as a function of time. Oxidation might also occur as a function of distance because cells can express on their membrane or release small quantities of oxidoreductases and nonprotein thiols [24,63] that might create a gradient of reduction, maintaining proteins in a reduced state and hence active in the proximity of cells. **(b)** In pathological states, such as cancer, autoimmunity and chronic viral infections, this control could be lost, owing to conversion of the extracellular milieu into a reducing environment. This might be caused by the presence outside cells of functional oxidoreductases and increased levels of free thiols [60,61], thereby supporting prolonged activity of the LSPs. Other tumor-related changes, such as overexpression of LSPs by tumor cells and the presence within the local microenvironment of high levels of enzymes that can detach LSPs from the extracellular matrix, might confer ready diffusion of these proteins, thereby determining their long-range bioactivity. Abbreviations: GP, glycoproteins; HS, heparansulphates; Mo, monocyte; mOx-R, membrane oxidoreductase; NPSH, nonprotein thiols; PDI, protein disulfide isomerase; PG, proteoglycans.

inflammatory responses expand beyond the local site, it causes the loss of tissue function and an exuberant systemic inflammatory response. To counteract excessive collateral tissue damage, the injured tissue might indeed induce the release of other DAMPs that provoke anti-inflammatory responses. Thus, the outcome will be determined by a balance between proinflammatory DAMPs (e.g. HMGB1) [7] and anti-inflammatory DAMPs (e.g. adenosine) [34].

Interestingly, some of the DAMPs identified recently are LSPs. HMGB1 has been characterized as a DAMP [36]

because it is released by necrotic but not by apoptotic cells and necrotic cells from *Hmgbl1*<sup>-/-</sup> mice have a reduced ability to promote inflammation when compared with wild-type mice [7]. Also, galectin-3, a leaderless protein secreted by cells through the nonclassical pathway and able to exert proinflammatory functions, has been revisited recently in light of the emergent understanding of DAMPs and has been proposed to act as a danger signal [37]. The difference between LSPs and DAMPs relies on the means by which they exit the cell. When released passively by dying cells expressing the protein, they are considered DAMPs. When

secreted actively and, in some cases, limited to specialized cells, they are considered LSPs. From a functional point of view, the highly regulated secretion of LSPs enables a tighter control of inflammation than the release of DAMPs. If we extend these observations, we can, in principle, postulate that almost all LSPs could function as DAMPs, with few exceptions. Among these, IL-1 $\beta$  and IL-18 are synthesized as inactive precursors and are secreted rapidly following processing by caspase-1, the mature forms being virtually absent from the cell. Therefore, injured cells can only release the biologically inactive form of the cytokine [17]. IL-1 $\alpha$ , conversely, also belongs to the family of DAMPs as it is released by dying cells and is active with or without processing [38]. DAMPs are externalized following damage to the plasma membrane. They are released by cells dying by necrosis, which are characterized by cellular disruption, but not by apoptotic or autophagic cells, which, at least initially, maintain their membrane integrity. Thus, molecules harbored within the soluble cytosol and similarly soluble and mobile nuclear proteins, such as HMGB1, have a greater likelihood of emerging than molecules that are confined within intracellular organelles. These are protected by an additional membrane and require greater injury to be delivered outside the cell. Obviously, the relative amounts of the released molecules also depend on their cytosolic concentrations within the injured cell. Classical secretory proteins are confined to the exocytotic route, without ready access to the cytosol, and are secreted promptly. Their intracellular content is comparatively low in contrast to their extracellular delivery. Conversely, LSPs accumulate within the cytosol and are generally long-lived. For these reasons, although cytokines secreted by the classical ER–Golgi pathway [IL-2, IL-12, interferon (IFN)- $\gamma$  and many other immune mediators] are unlikely to function as endogenous DAMPs, LSPs with cytokine-like functions can be considered to act as such (Figures 2 and 3).

As discussed above, the rapid extracellular inactivation of an oxidation-sensitive proinflammatory leaderless protein can be beneficial during an inflammatory response. The protein, secreted in low amounts and in a regulated fashion by inflammatory cells alerts the innate immune system and its biological function is then abrogated by oxidation, thereby restricting the inflammatory response locally (Figure 3a). Conversely, enhanced release of the protein on cell necrosis and/or modification in the redox state outside of the cell can prolong the extracellular half-life of the protein, with potentially pathological consequences (Figure 3b).

### DAMPs and redox in chronic inflammation

During chronic inflammation (or stress), cells that limit and repair the local tissue damage are recruited to the site of inflammation or infection. Cells, such as regulatory macrophages, mast cells, eosinophils, plasmacytoid DCs and myofibroblasts, are involved in coating, ingesting and eliminating damaged molecules and cells, thereby supporting tissue regeneration and wound healing. When DAMPs are released persistently, thus signaling ongoing cellular stress, a carefully choreographed integration of innate and adaptive inflammatory cells with stromal and epithelial components promotes containment and fibrosis. During

continued DAMP release, eosinophils promote the removal of debris and damaged cells [39], whereas mast cells induce local-tissue edema and vasodilatation, inhibit T and NK effectors and favour Th2 responses [40,41]. This happens in many chronic inflammatory conditions, including autoimmunity, atherosclerosis, obesity, graft-versus-host disease and chronic viral and bacterial infections, and during the frequent end-stage of chronic inflammation in adults, cancer [42].

Not only does chronic inflammation increase the risk of cancer but, also, when cancer develops, inflammation persists within the tumor microenvironment through unscheduled nonapoptotic and nonautophagic death. This has been explored and well defined in the studies of Karin [43–45] and White [46–48], and is mediated in part through NF- $\kappa$ B signaling pathways. Indeed, cancer cells are often defective in the processes of apoptosis and autophagy, instead undergoing necrotic death frequently. The emergent notion of the role of nonapoptotic death driving persistent inflammation during carcinogenesis [49] points to necrosis, release of DAMPs and the locally reduced environment promoted by a loss of intracellular contents into the external milieu as major factors in sustaining the inflammatory process. Furthermore, many tumors over-express LSPs, including HMGB1 [50], FGF [51], S100 [52,53], thioredoxin-thioredoxin reductase [30,54] and MIF [55]. Therefore, necrosis of tumor cells results in the copious release of LSPs, in high abundance owing to cellular ‘piecemeal’ necrosis within the tissues, rather than the ordered engulfment of apoptotic or autophagic corpses. These released DAMPs are responsible for recruiting proinflammatory cells and in turn induce inflammation, secretion of cytokines and chemokines, activation of metalloproteases and production of angiogenic factors. This setting further promotes tumorigenesis and tumor progression. Given that thioredoxin and MIF are oxidoreductases, it is tempting to speculate that their release within the tumor microenvironment at high concentrations, in concert with oxidation-sensitive LSPs, might prolong the activity of these proteins and tumorigenic inflammation.

Interestingly, redox modifications in the tumor microenvironment correlate with hypoxia. This is a common finding in advanced solid human tumors that are associated with poor prognosis and resistance to radiation and pharmacological therapy [56]. Low oxygen tension induces several responses at the level of the tissues, mediated in large part by hypoxia-inducible factor 1 (HIF-1), which modifies energy metabolism, pH, survival, vascularization and cell migration [56]. Hypoxic areas within tumors also over-express thioredoxin [57], which in turn increases HIF-1 activity [58]. Thioredoxin is not the only oxidoreductase that is over-expressed in tumors. MIF is also increased in several tumor types [55] and is up-regulated in glial tumor cells during hypoxic stress [59]. Disulfide-isomerase expression and externalization is related to the invasive properties of malignant glioma [60]. In addition, tumor tissues contain more nonprotein thiols than nonmalignant tissue [61], demonstrating a consistent relationship between tissue hypoxia and nonprotein thiol levels [62].

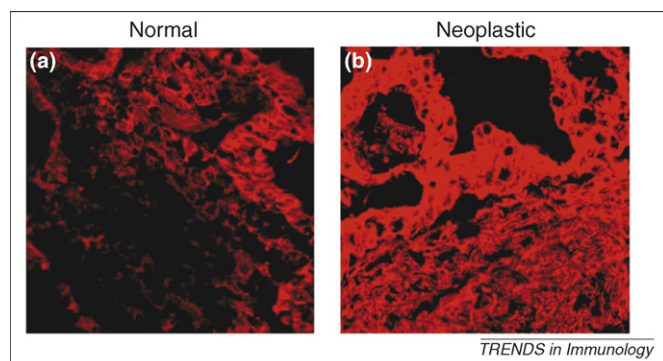


**Figure 4.** Neoplastic tissues contain abundant free thiols. Illustrative of some of the principles considered here is the finding that tumors, which are sites of chronic inflammation, contain more nonprotein thiols than non-neoplastic tissues. This could provide a mechanism by which DAMPs produced within the tumor microenvironment could be maintained in a nonoxidized (active) state and hence engender chronic inflammation. Mercury orange staining [60,61] of specimens from (a) normal and (b) neoplastic tissue from the same patient who had undergone surgical resection of a colon carcinoma. The staining shows that higher levels of nonprotein thiols (red) are detected in the neoplastic tissues than in its normal counterpart. A.R., unpublished.

Neoplastic cells in hypoxic areas eventually undergo necrosis. Because intracellular oxide reductases are up-regulated in hypoxic areas, this process leads to the release of large quantities of these enzymes that could prolong the activity of the oxidation-sensitive DAMPs present in the microenvironment. Interestingly, we have observed that viable tumor cells that over-express thioredoxin and MIF actively release micromolar amounts of nonprotein thiols (i.e. cysteine or glutathione) (A.R., unpublished). Furthermore, staining of neoplastic tissues with a reagent specific for nonprotein thiols is apparently not restricted to the inside of cells but, rather, is also present within the intercellular space (Figure 4). The presence of oxidoreductases and nonprotein thiols usually confined within cells turns the physiological extracellular milieu 'upside-down' and switches the microenvironment from an oxidizing to a reducing state. A nonphysiological extracellular reducing milieu might promote the generation of chronic inflammation by prolonging the extracellular function of the redox-sensitive DAMPs and proinflammatory LSPs secreted by recruited inflammatory cells, which are otherwise short-lived outside of cells. One can speculate that, in the setting of other types of necrosis (for example, those involving ischemia or reperfusion and infarction) low molar quantities of extracellular thiols and oxidoreductases might account for the short-range activity of DAMPs released by dying cells, resulting in attenuated inflammation and tissue damage.

## Conclusions

To initiate and maintain an inflammatory response, suitable integrated signals must be received and inflammatory cells must be recruited and activated. Whether a chronic local adaptive immune response or a wound-healing response ensues is mainly dictated by the presence and the type of PAMPs, the type and number of stressed cells and the intracellular DAMPs that are released but also by other elements present within the extracellular milieu. These include the presence of hypoxia, high or low pH, redox, gaseous mediators, such as NO and carbon

monoxide, and degraded matrix components. The inflammatory microenvironment usually promotes resolution and control of a pathogen or injurious agent rapidly. Futile cycles of cell death in tumor cells, autoimmune targets or virally infected cells with blocks in apoptotic or autophagic death might lead to chronic inflammation, associated with persistent release of DAMPs and redox alterations. The exaggerated release of DAMPs, possibly over-expressed by dying cells, as well as a permissive (reducing) redox environment, might sustain and prolong inflammation. Identifying the crucial targets in disorders associated with DAMP release and persistence might enable the development of novel pharmacological interventions in chronic inflammatory states.

## Acknowledgements

We thank all the members of our laboratories for helpful discussion. This project was funded, in part, by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Italian Health Ministry and Istituto Superiore Sanità (to A.R.), the Pennsylvania Department of Health and 1 PO1 CA 101944-01A2 "Integrating NK and DC into Cancer Therapy" (to M.T.L.).

## References

- Martinon, F. and Tschopp, J. (2005) NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* 26, 447–454
- Bianchi, M.E. (2007) PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* 81, 1–5
- Adib-Conquy, M. and Cavaillon, J.M. (2007) Stress molecules in sepsis and systemic inflammatory response syndrome. *FEBS Lett.* 581, 3723–3733
- Scheibner, K.A. et al. (2006) Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J. Immunol.* 177, 1272–1281
- Nickel, W. (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur. J. Biochem.* 270, 2109–2119
- Chimini, G. and Rubartelli, A. (2005) Novel pathways of protein secretion. In *Molecular Chaperones and Cell Signalling* (Henderson, B., ed.), pp. 45–60, Cambridge, Cambridge University Press
- Scaffidi, P. et al. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418, 191–195
- Arner, E.S. and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* 267, 6102–6109
- Fernandes, A.P. and Holmgren, A. (2004) Glutaredoxins: glutathione dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid. Redox Signal.* 6, 63–74
- Pastore, A. et al. (2003) Analysis of glutathione: implication in redox and detoxification. *Clin. Chim. Acta* 1, 19–39
- Mansoor, M.A. et al. (1992) Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal. Biochem.* 200, 218–229
- Ashfaq, S. et al. (2006) The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J. Am. Coll. Cardiol.* 47, 1005–1011
- Ellgaard, L. (2004) Catalysis of disulphide bond formation in the endoplasmic reticulum. *Biochem. Soc. Trans.* 32, 663–667
- Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* 4, 181–191
- Leichert, L.I. and Jakob, U. (2006) Global methods to monitor the thiol-disulfide state of proteins *in vivo*. *Antioxid. Redox Signal.* 8, 763–772
- Muesch, A. et al. (1990) A novel pathway for secretory proteins? *Trends Biochem. Sci.* 15, 86–88
- Rubartelli, A. et al. (1990) A novel secretory pathway for interleukin-1 $\beta$ , a protein lacking a signal sequence. *EMBO J.* 9, 1503–1510
- Wang, H. et al. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285, 248–251
- Gardella, S. et al. (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep.* 3, 995–1001

- 20 Semino, C. *et al.* (2005) NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood* 106, 609–616
- 21 Rubartelli, A. *et al.* (1992) Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* 267, 24161–24164
- 22 Wakasugi, N. *et al.* (1990) Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc. Natl. Acad. Sci. U. S. A.* 87, 8282–8286
- 23 Angelini, G. *et al.* (2002) Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1491–1496
- 24 Nguyen, M.T. *et al.* (2003) A 16-residue peptide fragment of macrophage migration inhibitory factor, MIF-(50-65), exhibits redox activity and has MIF-like biological functions. *J. Biol. Chem.* 278, 33654–33671
- 25 Kamogashira, T. (1988) Site-specific mutagenesis of the human interleukin-1  $\beta$  gene: structure-function analysis of the cysteine residues. *Biochem. Biophys. Res. Commun.* 150, 1106–1114
- 26 Yamamoto, Y. *et al.* (2004) Generation of highly stable IL-18 based on a ligand–receptor complex structure. *Biochem. Biophys. Res. Commun.* 317, 181–186
- 27 Camby, I. *et al.* (2006) Galectin-1: a small protein with major functions. *Glycobiology* 16, 137R–157R
- 28 Inagaki, Y. *et al.* (2000) Oxidized galectin-1 promotes axonal regeneration in peripheral nerves but does not possess lectin properties. *Eur. J. Biochem.* 267, 2955–2964
- 29 Koppal, T. *et al.* (2001) S100B proteins that lack one or both cysteine residues can induce inflammatory responses in astrocytes and microglia. *Neurochem. Int.* 39, 401–407
- 30 Gardella, S. *et al.* (2001) CD8(+) T lymphocytes induce polarized exocytosis of secretory lysosomes by dendritic cells with release of interleukin-1 $\beta$  and cathepsin D. *Blood* 98, 2152–2159
- 31 Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12, 991–1045
- 32 Panayi, G.S. *et al.* (2004) Stress cytokines: pivotal proteins in immune regulatory networks. *Curr. Opin. Immunol.* 16, 531–534
- 33 Boeynaems, J.M. and Communi, D. (2006) Modulation of inflammation by extracellular nucleotides. *J. Invest. Dermatol.* 126, 943–944
- 34 Bours, M.J. *et al.* (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol. Ther.* 112, 358–404
- 35 Shi, Y. *et al.* (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 425, 516–521
- 36 Lotze, M.T. and Tracey, K.J. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* 5, 331–342
- 37 Sato, S. and Nieminen, J. (2004) Seeing strangers of announcing “danger”: galectin-3 in two models of innate immunity. *Glycoconj. J.* 19, 583–591
- 38 Mosley, B. *et al.* (1987) The interleukin-1 receptor binds the human interleukin-1 $\alpha$  precursor but not the interleukin-1 $\beta$ . *J. Biol. Chem.* 262, 2941–2944
- 39 Lotfi, R. *et al.* (2007) Eosinophilic granulocytes and damage associated molecular pattern molecules (DAMPs). *J. Immunother.* 30, 16–28
- 40 Lu, L.F. *et al.* (2006) Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 442, 997–1002
- 41 Kitawaki, T. *et al.* (2006) IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. *Int. Immunol.* 18, 1789–1799
- 42 de Visser, K.E. *et al.* (2006) Paradoxical roles of the immune system during cancer development. *Nat. Rev. Cancer* 6, 24–37
- 43 Sakurai, T. *et al.* (2006) Loss of hepatic NF- $\kappa$ B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10544–10551
- 44 Karin, M. *et al.* (2006) Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 124, 823–835
- 45 Karin, M. (2006) Nuclear factor- $\kappa$ B in cancer development and progression. *Nature* 441, 431–436
- 46 Degenhardt, K. and White, E. (2006) A mouse model system to genetically dissect the molecular mechanisms regulating tumorigenesis. *Clin. Cancer Res.* 12, 5298–5304
- 47 Jin, S. and White, E. (2007) Role of autophagy in cancer: management of metabolic stress. *Autophagy* 3, 93–95
- 48 Degenhardt, K. *et al.* (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 10, 51–64
- 49 Vakkila, J. and Lotze, M.T. (2004) Inflammation and necrosis promote tumour growth. *Nat. Rev. Immunol.* 4, 641–648
- 50 Lotze, M.T. and DeMarco, R.A. (2003) Dealing with death: HMGB1 as a novel target for cancer therapy. *Curr. Opin. Investig. Drugs* 4, 1405–1409
- 51 Pardo, O.E. *et al.* (2006) FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKC $\epsilon$ . *EMBO J.* 25, 3078–3088
- 52 Diederichs, S. *et al.* (2004) S100 family members and trypsinogens are predictors of distant metastasis and survival in early-stage non-small cell lung cancer. *Cancer Res.* 64, 5564–5569
- 53 Zou, M. *et al.* (2005) S100A4 (Mts1) gene overexpression is associated with invasion and metastasis of papillary thyroid carcinoma. *Br. J. Cancer* 93, 1277–1284
- 54 Powis, G. *et al.* (2000) The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic. Biol. Med.* 29, 312–322
- 55 Mitchell, R.A. (2004) Mechanisms and effectors of MIF-dependent promotion of tumorigenesis. *Cell. Signal.* 16, 13–19
- 56 Pouyssegur, J. *et al.* (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441, 437–443
- 57 Hedley, D. *et al.* (2004) Up-regulation of the redox mediators thioredoxin and apurinic/apyrimidinic excision (APE)/Ref-1 in hypoxic microregions of invasive cervical carcinomas, mapped using multispectral, wide-field fluorescence image analysis. *Am. J. Pathol.* 164, 557–565
- 58 Csiki, I. *et al.* (2006) Thioredoxin-1 modulates transcription of cyclooxygenase-2 via hypoxia-inducible factor-1 in non-small cell lung cancer. *Cancer Res.* 66, 143–150
- 59 Bacher, M. *et al.* (2003) Up-regulation of macrophage migration inhibitory factor gene and protein expression in glial tumor cells during hypoxic and hypoglycemic stress indicates a critical role for angiogenesis in glioblastoma multiforme. *Am. J. Pathol.* 162, 11–17
- 60 Goplen, D. *et al.* (2006) Protein disulfide isomerase expression is related to the invasive properties of malignant glioma. *Cancer Res.* 66, 9895–9902
- 61 Vukovic, V. *et al.* (2000) Microregional heterogeneity of non-protein thiols in cervical carcinomas assessed by combined use of HPLC and fluorescence image analysis. *Clin. Cancer Res.* 6, 1826–1832
- 62 Moreno-Merlo, F. *et al.* (1999) Association between tissue hypoxia and elevated non-protein sulphhydryl concentrations in human cervical carcinoma xenografts. *Br. J. Cancer* 81, 989–993
- 63 Jordan, P.A. and Gibbins, J.M. (2006) Extracellular disulfide exchange and the regulation of cellular function. *Antioxid. Redox Signal.* 8, 312–324