Danger signals: SOS to the immune system
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The activation of dendritic cells, necessary for the initiation of primary and secondary immune responses, can be induced by endogenous danger signals – released by tissues undergoing stress, damage or abnormal death – and also by exogenous danger signals elaborated by pathogens. Some endogenous danger signals that recently have been discovered are heat-shock proteins, nucleotides, reactive oxygen intermediates, extracellular-matrix breakdown products, neuromediators and cytokines like the IFNs. We propose that allergy may be initiated by the direct damage of dendritic or other cells by toxic chemicals and allergenic proteases, and suggest that the triggering of danger signal receptors by exogenous pathogen-derived molecules may be more to the advantage of the pathogen than to the host.

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Abbreviations

APC	antigen-presenting cell
CD40-L	CD40 ligand
DC	dendritic cell
Hsp	heat-shock protein
LPS	lipopolysaccharide
TLR	Toll-like receptor

Introduction

Danger signals were first postulated in 1994 as part of a model of immunity that suggests that the immune system responds to substances that cause damage, rather than to those that are simply foreign [1]. They consist of molecules or molecular structures, released or produced by cells undergoing stress or abnormal cell death, that are perceived by resting antigen-presenting cells (APCs) and that induce the APCs to become activated, to offer co-stimulatory signals, and, thus, to initiate immune responses. In this review we will discuss recent evidence regarding endogenous danger signals and some of the pathogen-derived signals that might mimic them (for some nice reviews on exogenous danger signals, see [2••,3•]). We will concentrate on studies using dendritic cells (DCs) as APCs [4,5••] because these are the true 'professionals' [6], able to stimulate naïve T cells and thus initiate primary immune responses; however, many of the findings also apply to B cells and macrophages, which can re-stimulate memory T cells and thus initiate secondary responses.

We can divide danger signals into two large subclasses, according to their source: endogenous signals are produced by the organism that generated the susceptible DCs; exogenous signals are produced by different organisms. Among the endogenous signals, some are truly primal initiators and others give positive-feedback signals to enhance or modify an ongoing response. Some danger signals are intracellular whereas others are extracellular or secreted products. Some are constitutive whereas others are inducible and require neosynthesis or modifications before they can function to activate DCs. There are several that bridge these distinctions.

Primal danger signals versus feedback molecules

Primal danger signals are mediators, made available during tissue damage, whose elaboration does not require the previous activation of APCs. Feedback signals are those made by the activated DCs themselves or by other cells that have been stimulated by activated APCs. Although several known danger signals fit into both categories, and others are purely primal, we know of no substance yet that is purely a feedback mediator.

The first endogenous danger signals to be characterized — CD40 ligand (CD40-L) [7], TNF-α and IL-1β [8] — are good examples of dual-purpose signals. All three are both primal and feedback molecules in that they are expressed not only by non-immune cells undergoing one form of stress or another but also by activated cells of the immune system. CD40-L, for example, is upregulated on activated T cells and is best known for its role in T cell communication with B cells and DCs. In these roles, CD40-L is a positively enhancing immunostimulant rather than a primal alarm signal because its expression on T cells requires previous activation of DCs. However, CD40-L is also expressed by activated platelets [9]. Under normal conditions, platelets and tissue-resident DCs do not come in contact with each other. But in injured and bleeding tissue the platelet-expressed CD40-L could serve as an early source of DC stimulation to quickly initiate immune responses to any noxious agents found there.

TNF-α and IL-1β made by activated T cells or activated DCs are positive-feedback signals. However, both are also primal danger signals. For example, IL-1β is induced in keratinocytes by inflammatory stimuli [10], and TNF-α is made by injured myocardium [11]. Such injury-related elaboration of a DC activator may explain why auto-antibody is often seen in patients after acute myocardial infarctions [12]. It is difficult to induce B cell tolerance to intracellular components, and T cell tolerance to peripheral antigens is an ever-present but ever-incomplete process. Thus alarm signals expressed by injured tissues can lead to transient production of tissue-specific autoimmune responses. If the injury were chronic, such autoimmune responses could contribute to long-lasting disease. It

would perhaps be interesting to scan the target tissues in tissue-specific autoimmune diseases (e.g. using microarrays for stress-related mRNAs) to determine whether the immune response might be due to a non-immune injury.

Constitutive danger signals

For some time after their discovery, DCs were thought to be constitutively active APCs. Indeed Janeway invoked this feature to suggest that, unlike macrophages, DCs did not need to be stimulated by conserved, evolutionarily distant, bacterial products and thus might be the APCs involved in responses to viruses (which are not so evolutionarily distant) or allergens (which are not 'pathogenic bacteria') [13]. In the past two years, however, it has become clear, from analyses of DCs studied immediately *ex vivo* [14] or generated *in vitro* [15], that they generally exist in a resting state from which they can be roused by many different stimuli.

One of the reasons DCs were thought to be constitutively active is that the procedures used to isolate them from tissues [16], or to enrich them *in vitro* [17], cause enough damage to unleash both constitutive and inducible danger signals. Although there was some speculation that injury might induce DC activation [14], the idea did not gain general acceptance until the publication of two studies, one in mouse [18•] and one in human [19•], showing that cells killed by acute necrotic death can activate resting DCs whereas cells dying by physiological apoptotic death do not. In this way only pathological cell death stimulates the immune system; physiological apoptotic death, which happens continuously in the body, is neutral and may sometimes be anti-inflammatory [20].

Although any substance that is normally found only inside cells, and that can be released upon damage to the plasma membrane, could in principle act as a danger signal, only a few candidates have yet been identified. Intracellular nucleotides like ATP and UTP, which function in energy metabolism and which are normally stored in the cytosol, are released from a variety of cells under conditions of hypoxia, ischemia, inflammation or even mechanical stress [21] and can activate DCs by themselves or in conjunction with TNF-α [22,23]. The effect of these nucleotides is mediated by triggering of the purinergic receptor, P2Z/P2X7 [24], shown to be present on resting DCs [25].

For some time it has been known that unmethylated CpG sequences in microbial DNA are exogenous danger signals that activate APCs (reviewed in [26••]). Though mammalian DNA also has CpG islands, these were thought to be inactive because they were methylated and had the 'wrong' sequences. There is now a hint, however, that unmethylated CpG sequences in mammalian DNA can indeed serve to activate DCs (K Ishii, D Klineman, personal communication), as long as the DNA is double-stranded and not cut into short pieces (the latter might be expected in apoptotic cells).

Heat-shock proteins: bridging the categories of constitutive and inducible danger signals

Heat-shock proteins (Hsps) are evolutionarily ancient and highly conserved families of proteins, found in all prokaryotes and eukaryotes, that are involved in protein folding, protection and transport. Each Hsp is localized in a restricted compartment: Hsp28, Hsp70 and Hsp90 in the cytosol and nucleus; Hsp60 and a subset of Hsp70 in mitochondria; grp78 and gp96 in the endoplasmic reticulum. Considered to be intracellular soluble proteins because they have no transmembrane domains, they can be translocated to the plasma membrane upon cellular stress [27].

Recently Basu *et al*. [28••] have shown that necrotic cells, but not apoptotic cells, can release Hsps, and several groups have shown that different Hsps can activate DCs [28••,29••,30] and macrophages [31,32], making the Hsps the first large family of endogenous danger signals. Because some Hsps are constitutively present whereas others are upregulated by various types of stress (including the temperature shock that gave them their name), they comprise both constitutive and inducible signals. It is not yet known if the pre-packaged Hsps have different effects on DCs from those that are neo-synthesized upon cellular stress.

Studies on the receptors mediating Hsp stimulation of the APCs indicate that Hsps may bind different families of receptors, with the same functional outcome — APC activation. gp96 binds CD91, a protein related to the low-density lipoprotein receptors [33] and it may also bind the scavenger receptor, CD36 [29••]. The receptors for Hsp60 and Hsp70 have instead been linked to the Toll-like receptor (TLR) family [3•]. The TLR co-receptor CD14 has been shown to be necessary for human monocyte activation by Hsp70 [30] and for human macrophage activation by Hsp60 [32]. Among mice, the C3H/HeJ strain, which is deficient for tlr-4 and is consequently resistant to lipopolysaccharide (LPS)-induced B cell activation, is also resistant to Hsp60-induced macrophage activation [34••].

Inducible danger signals
Newly synthesized molecules

Inducible danger signals come in at least two categories. Some are newly synthesized upon cellular stress or damage and some pre-exist but are modified when damage occurs (see below). In the first category we can place the type-I IFNs which, in addition to their antiviral effects, are also known to have immunomodulating functions (reviewed in [35•]). There is both direct and indirect evidence that IFNs, or other virally induced signals, can act as danger signals. For example, infection with influenza virus or treatment with Poly I:C, a synthetic mimic of double-stranded RNA that can induce production of type I IFNs, can activate resting DCs [36]. Influenza infection, through an as-yet unknown mechanism, can push the DCs even further, into a superactivated state that bypasses the requirement for T cell help in the stimulation of cytotoxic T lymphocytes [37]. IFN-α itself, like TNF-α, can act as

both a primal and a positive feedback signal. As a primal activator, we showed that IFN-α activates resting mouse DCs [18•] and others showed that it activates human monocytes [38,39]. As a feedback immunostimulant, it has been shown that a small subset of activated DCs produces large quantities of IFN-α [40,41] and that IFN-α can synergise with TNF-α to support DC activation [42]. There is also evidence that a subset of DCs derived from CD14+ precursors are not sensitive to type I IFNs [43], suggesting specificity in IFN-induced stimulation.

Other inducible danger signals released by metabolically stressed cells are reactive oxygen intermediates [44], which are known in flies and mammalian cells to activate NF-κB. There is some evidence, in fact, that reducing the level of free radicals can lower the incidence of transplant rejection [45].

Both constitutively present and newly synthesized danger signals have the possibility of fulfilling more than one role. IFN-α, for example, signals DC activation, as well as the synthesis of antiviral transcription inhibitors in neighboring tissue cells. Vasoactive intestinal peptide (VIP) — a neuromediator that is found in the gut and in the anterior chamber of the eye and that can also be released by nerve endings in case of tissue damage — not only induces DC activation (whereas other neuropeptides such as Somatostatin and substance P do not [46]; S Gallucci *et al.*, unpublished data) but also is involved in the control of IgA class-switching in B cells [47]. Tissues may thus be able to use different danger signals to inform the immune system of the type of pathogen present, and/or the type of immunity that is best in that location.

Constitutive molecules that are modified by stress or damage

In injured or inflamed tissues, the activation of extracellular proteases or the release of intracellular proteases can lead to the cleavage of components of the extracellular-matrix into small fragments, and some of these have been reported to activate DCs or macrophages. For example, intradermal injection of mice with the matrix-proteolytic enzyme metalloproteinase-9 induces phenotypic changes in skin Langerhans cells that are characteristic of the activation/maturation process [48]; similarly, the degradation products of heparan sulfate — a linear copolymer component of proteoglycans that is ubiquitously distributed on cell surfaces and extracellular matrices — have been shown to activate DCs *in vitro* [49,50]. Because heparan sulfate has chemical analogy to peptidoglycans, and because bacterial peptidoglycans bind to the Toll receptor, it has been suggested that heparan-induced DC activation may be another of the endogenous danger signals that act through the TLR family [49].

Some cellular adhesion molecules on DCs might serve dual functions: to localize DCs in the normal structure of a tissue by binding their 'regular ligands' and as activation receptors when they bind the degradation products of those ligands. For example, cross-linking of CD43 [51] or CD44 [52] induces DC activation and cytokine production. CD44 binds to hyaluronan, and small breakdown products of hyaluronan have also been shown to induce DCs to become activated and produce pro-inflammatory cytokines, especially TNF-α [53]. Thus the simple view would be that CD44 is an activation receptor for fragments of hyaluronan. However, the picture is not yet entirely clear because this activation, which is blocked by anti-TNF-α antibodies, is intact in CD44 knockout mice.

Direct damage to DCs as a danger signal?

We can hypothesize that DCs might have a default mechanism that induces them to activate whenever an insult hits them. Such a mechanism might explain the ability of simple compounds like $NiCl_2$, $MnCl_2$, $CoCl_2$ or $SnCl_2$ to induce DC maturation [54•]. These inorganic substances have been shown in other systems to block cell membrane ionic channels and interfere with the cell's energy metabolism, inducing 'cell suffering'.

Another category of DC activators that might induce direct DC damage — as well as release of danger signals by other types of cell — are compounds like TNCB and DNCB [54•], which are well-known experimental allergens. Although natural allergens are often thought to be innocuous substances, to which the immune system responds inappropriately, new evidence suggests that at least some of them are not innocuous at all. Der p1, the main allergen from the house dust mite *Dermatophagoides*, is a cysteine proteinase that is able to cleave surface receptors like CD25 and the IgE receptor, CD23 [55], and to disrupt the architecture of lung epithelia by cleaving tight-junction adhesion proteins [56]. We agree with McFadden and Basketter [57] that the damage done by the irritants helps the development of an allergy. Whether the allergens damage DCs themselves or the surrounding tissues, we predict that each major allergen may either itself do damage or it will be packaged (for example in bee venom) with other substances that do.

Conclusions and anomalies

The wealth of recently accumulated evidence gives an emerging picture of a rich and complex set of communication lines between cells of the body and cells of the innate and adaptive immune systems. The alarm signals sent by stressed, damaged or parasitized cells (some of which have non-immunological function) are a diverse panoply of constitutive and inducible molecules that can initiate different kinds of immunity in different tissues and to different pathogens.

There are two anomalies that are worth discussing further: the response of mice to human IgG; and the binding of microbial molecules to the receptors for the endogenous danger signals (REDS).

Immunity without danger

Mice respond to aggregated human IgG (but not to the unaggregated form) in the absence of any apparent danger signals. The recent finding that Fc receptor aggregation stimulates DCs [58••] offers an explanation. We would like to suggest that Fc receptors on DCs might act as a memory devices for past dangers. After a primary response has cleared a pathogen, antibodies continue to persist for some time. Should the pathogen return, and be bound by residual antibody, the antigen–antibody complexes could serve to stimulate a rapid secondary response, before the pathogen has time to generate substantial damage. Thus, if human IgG aggregates resemble mouse IgG–antigen complexes, they might initiate immune responses by triggering Fc receptors on DCs.

Are danger signal receptors exploited by pathogens?

Members of the TLR family have been shown in flies and in mammals to respond to both endogenous and exogenous stimuli. As mentioned above, tlr-4 combined with CD14 has been shown to mediate the effect of LPS, the most well known exogenous danger signal; similarly, tlr-4 also responds to endogenous Hsp60. Because the studies showing the activating effect of Hsps include rather convincing controls showing that the results are not due to LPS contamination of the Hsp preparations, we are led to conclude that the same receptors can recognize both endogenous and exogenous danger signals. One conclusion from these results could be that the immune system has been able to maximize its danger-recognition devices such that a small number of receptors can respond to a wide variety of endogenous and exogenous danger signals. Another view might be that microbes have found an evolutionary advantage in having molecules that mimic the endogenous activators. This would not be the first time that pathogens have found a way to exploit the immune system and it makes intuitive sense that a pathogen might want to suppress immunity. *Plasmodium malariae* has recently been shown to prevent the activation of DCs [59] and certain strains of non-pathogenic salmonella can prevent the translocation of NF-κB and dampen inflammation [60].

But what could be the advantage for parasites in *activating* the immune system by interacting with REDS? One advantage could come from indiscriminate and widespread activation that prevents the outgrowth of lymphocytes specific for the pathogen; this was recently suggested as the primary outcome of DC activation that is mediated by bacterial CpG [61]. Another is that the activation of APCs is quickly followed by death. Bacterial lipoproteins have been shown to induce apoptosis of macrophages *in vitro* [62], and LPS kills DCs *in vivo* [63] and *in vitro* (S Gallucci, L Graham, R Caricchio, P Matzinger, unpublished data), even at very low doses. Although it is beyond the scope of this review to cover the strategies employed by parasites to evade or control the immune response, it is sufficient to say that the more

we understand endogenous danger signals the more pathogen strategies we may uncover.

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