Membrane repair and immunological danger

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Antigens are able to elicit productive immune responses only when second signals are provided by adjuvant molecules. It is well established that exogenously acquired, pathogen-associated molecular patterns fulfil this adjuvant role when recognized by specific receptors on antigen-presenting cells. Recent evidence points to the existence of another class of adjuvant, which is apparently released from injured cells. Such endogenous adjuvants, referred to as 'danger' signals, could alert the immune system to situations that cause cell damage, but not necessarily those that involve infections. Endogenous adjuvants provide a good explanation for immune responses generated against tumours and autologous tissues, but it has been difficult to explain how a constant activation of the immune system is avoided, considering the frequency at which cells are injured *in vivo*. Here, we suggest that the efficiency with which cells reseal wounds in their plasma membrane might be an important factor in the balance between tolerance and autoimmunity. Recent observations in synaptotagmin-VII-deficient mice suggest that defective membrane repair could lead to autoimmunity in tissues that are more susceptible to mechanical injury. Keywords: adjuvant; autoimmunity; immunity; membrane repair; synaptotagmin VII

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Introduction

The generation of effective immune responses against soluble proteins requires the co-injection of molecules with adjuvant activity (Dresser, 1961, 1968). In the absence of adjuvants, injection of purified proteins can induce tolerance (Dresser, 1962). Although it has been postulated that some adjuvants enhance immune responses by retaining the antigen in a long-lived form *in vivo*, their main mechanism of action is thought to be the induction of co-stimulatory signals that can be recognized by T cells. This concept is well established in the case of adjuvants of microbial origin, such as lipopolysaccharide (LPS), due to an explosion of information in recent years. Receptors for conserved molecules from infectious organisms have been identified, and the signalling pathways by which they induce antigen-presenting cells (APCs) to express co-stimulatory molecules have been characterized (Beutler, 2004;

Takeda *et al*, 2003). These findings have, in large part, confirmed predictions that the immune system evolved to recognize and respond to patterns that are common on infectious agents, but absent from the host (Janeway, 1989). Because these are exogenously acquired molecules, this concept has been referred to as the 'stranger' hypothesis (Rock *et al*, 2005).

However, there is plenty of evidence that the immune system is also able to mount effective responses in the absence of 'stranger' molecules. Examples of such situations include immunity to transplants and to tumours and autoimmunity. To explain how these responses occur, it was hypothesized that the immune system could discriminate 'danger' signals from injured host cells (Matzinger, 1994; Rock *et al*, 2005). This scenario is consistent with the fact that many adjuvants are tissue irritants (Dresser, 1961). This review focuses on recent studies that are beginning to provide evidence for the existence of endogenous adjuvants. Given that these molecules are released from injured cells, the regulation of their effect by plasma-membrane repair on immune responses is also discussed.

The 'danger' hypothesis

In 1994, Polly Matzinger proposed an alternative to the widely established concept that the immune system primarily discriminates self from non-self. This alternative hypothesis suggested that the immune system is mainly adapted to recognize and respond to signals generated in a 'dangerous' situation that involves cell damage (Matzinger, 1994). This model, which became known as the 'danger' hypothesis, is based on the idea that the crucial signals for the initiation of immune responses are endogenous, not exogenous (Matzinger, 2001). According to this hypothesis, the power lies with the tissues: the activation state of APCs would depend on the health of cells in their neighbourhood.

What is the evidence in support of this idea? It has been extensively demonstrated that recognition of microbial molecular patterns by Toll-like receptors (TLRs) initiates immune responses that are essential for the control of infections (Kopp & Medzhitov, 2003). However, there is also evidence that TLR-mediated microbial recognition can lead to very different outcomes, depending on whether the stimulus originates from pathogenic or non-pathogenic organisms. Non-pathogenic commensal bacteria induce TLR-mediated signalling in the gut (Rakoff-Nahoum *et al*, 2004), but under normal conditions this does not trigger an inflammatory response comparable with that caused by pathogenic bacteria (Didierlaurent *et al*, 2002). The exacerbated response to pathogens might be explained, at least in part, by the ability of these

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organisms to penetrate into deeper tissue layers and produce effectors that directly stimulate inflammation (Galan, 2001). However, it has also been suggested that the absence of injury and/or the anti-inflammatory environment of the gut induces tolerance, because antigen presentation by dendritic cells occurs in the absence of co-stimulation (Sierro *et al*, 2001). Thus, in many instances the initiation of immune responses seems to require tissue damage, which is a speciality of pathogenic microorganisms. In addition to having mechanisms for invading and crossing cell barriers, pathogenic microbes produce a large number of membrane-damaging molecules, such as pore-forming toxins and specialized secretion systems, that puncture the membranes of eukaryotic cells. In addition to facilitating the contact of pathogens with deeper layers of mucosal surfaces, these mechanisms might promote the release of endogenous adjuvants from host cells.

There are several examples of situations in which injured cells stimulate immune responses in the absence of microorganisms. Immunity against tumours can be generated by experimentally inducing the death of cancer cells *in situ* (Barba *et al*, 1994; Caruso *et al*, 1993). CD4 T-cell responses are induced when mice are immunized with ovalbumin mixed with syngeneic cells that have been killed, but not when ovalbumin is injected alone (Gallucci *et al*, 1999). Although the exact mechanism by which injured cells influence immune responses is still not clear, they were shown to induce dendritic-cell maturation (Gallucci *et al*, 1999) and migration to draining lymph nodes *in vivo* (Shi & Rock, 2002). Thus, accumulating evidence suggests that both exogenous and endogenous adjuvants contribute to the initiation of immune responses by activating APCs (Rock *et al*, 2005).

Endogenous adjuvants

Although evidence indicates that injured cells release molecules with the capacity to modulate immune responses, little is known about the identity of these molecules. Heat-shock proteins (HSPs) were proposed to be endogenous adjuvant molecules due to their capacity to prime antigen-specific immunity in the absence of other adjuvants (Srivastava & Maki, 1991). However, subsequent studies raised the possibility that the observed immunostimulatory activity was a consequence of LPS contamination. LPS-free HSP60 and HSP70 were inactive, and very small amounts of LPS were able to restore adjuvant activity (Bausinger *et al*, 2002; Gao & Tsan, 2003). Additional studies in which HSPs are specifically inactivated or eliminated are required to clarify this issue. Such evidence has become available recently for the high mobility group box 1 (HMGB1) protein, which is both a nuclear factor and a secreted cytokine mediating responses to infection, injury and inflammation (Lotze & Tracey, 2005). Necrotic cells from *HMGB1*^{-/-} mice were reported to have a reduced ability to promote inflammation, suggesting that release of this protein on cell death is a signal to alert the immune system (Scaffidi *et al*, 2002). Recently, two fractions with adjuvant activity were identified in the cytosol of ultraviolet-irradiated 3T3 mouse fibroblasts (Shi *et al*, 2003). Although the composition of the high molecular weight fraction is still unknown, the low molecular weight fraction was purified to homogeneity and identified as uric acid. The adjuvant activity of the purified fraction was abolished by treatment with the highly specific uric acid oxidase uricase, which eliminates the possibility of microbial contamination (Shi *et al*, 2003; for an excellent review, see Rock *et al*, 2005).

Maintenance of membrane integrity

The existence of endogenous adjuvants raises a puzzling question: what prevents the immune system from remaining in a state of constant activation, considering that cells are constantly dying and being replaced *in vivo*? One explanation is that pathological and non-pathological forms of cell death differ fundamentally in their capacity to trigger the release of endogenous adjuvants (Matzinger, 2001). In non-pathological situations cells die by apoptosis, a programmed death pathway in which cells, at least initially, maintain their membrane integrity. The large number of apoptotic cells that are generated *in vivo* are cleared rapidly by phagocytes in a non-inflammatory process (Henson *et al*, 2001). By contrast, necrotic death is characterized by plasma-membrane disruption and the release of intracellular contents, including the molecules that are responsible for sending 'danger' signals to the immune system. It is therefore not surprising that necrosis is usually associated with pathology and can lead to autoimmunity (Gallucci *et al*, 1999).

Both necrotic and apoptotic cells can be immunostimulatory (Rock *et al*, 2005). However, these observations are not inconsistent with the view that plasma-membrane disruption is required for the release of endogenous adjuvants. In most experimental settings, injury during cell manipulation cannot be ruled out, which possibly explains why apoptotic cell uptake by dendritic cells induces T-cell responses in some studies (Hoffmann *et al*, 2000), whereas in other studies only necrotic cells show immunostimulatory activity (Sauter *et al*, 2000). Apoptotic cells can also undergo secondary necrosis, with a concomitant release of their intracellular contents (Majno & Joris, 1995). This finding is consistent with the view that a delay in the clearance of apoptotic cells *in vivo* can lead to autoimmunity (Pisetsky, 2004). Apoptotic cells contain potent adjuvant activity (Shi *et al*, 2000), and so the maintenance of plasma-membrane integrity seems to be crucial for the prevention of undesired immune responses.

Plasma-membrane injury *in vivo*

Necrotic death is not the only mechanism by which endogenous adjuvants can be released *in vivo*. There is extensive evidence that the plasma membrane of cells in many tissues is injured and subsequently repaired without leading to cell death. In most of these studies, plasma-membrane wounding was monitored through the detection of membrane-impermeable molecules that entered the cytoplasm of intact cells. After imposition of a mechanical force, serum albumin, propidium iodide, lucifer yellow or fluorescent dextran were trapped inside viable cells from the skin (McNeil & Ito, 1990), lung (Doerr *et al*, 2005), skeletal muscle (McNeil & Khakee, 1992) and cardiac muscle (Clarke *et al*, 1995). Similar *in vivo* plasma-membrane wounds were detected in cells from experimentally undisturbed gastrointestinal tract (McNeil & Ito, 1989), endothelia (Yu & McNeil, 1992) and hair cells of the auditory papilla (Mulroy *et al*, 1998). The most pronounced detection of *in vivo* wounding was in the triceps muscle of mice subjected to downhill running, which reinforces the view that mechanical force is the main agent responsible for producing membrane disruptions *in vivo* (McNeil & Khakee, 1992). These studies show that injury to the plasma membrane of cells is physiologically a frequent event *in vivo* and they highlight the need for mechanisms to prevent endogenous adjuvant release and the development of autoimmunity.

Fig 1 | Resealing efficiency and release of endogenous adjuvants. Recent evidence suggests that the cytosol of mammalian cells contains molecules with adjuvant activity that are able to stimulate maturation of antigen-presenting cells (APCs) and activate naive T cells. Plasma-membrane repair might have a role in preventing immunostimulation, by limiting the release of cytosol from accidentally injured cells. (Modified from Rock *et al*, 2005).

Exocytosis-mediated plasma-membrane repair

Given the frequency by which cells are injured *in vivo*, it could be argued that this poses a problem for the containment of cytosolic molecules with strong immunostimulatory activity. However, it is important to realize that animal cells have a very efficient Ca^{2+} -dependent mechanism for the rapid repair of wounds in their plasma membrane (Heilbrunn, 1956). In fibroblasts injured by a micro-needle puncture, Ca^{2+} influx triggers plasma membrane resealing within 10–30 s (Steinhardt *et al*, 1994). Release of the cytosolic enzyme lactate dehydrogenase (LDH) is observed immediately after contraction wounding of fibroblasts, but further cytosol loss is rapidly stopped by membrane resealing (Chakrabarti *et al*, 2003; Reddy *et al*, 2001). Thus, the rapid repair of lesions in the plasma membrane could be a key mechanism for preventing the activation of immune responses under steady-state conditions (Fig 1).

It has long been known that the influx of Ca^{2+} occurring during injury to the plasma membrane triggers a resealing process that is mediated by exocytosis (McNeil & Steinhardt, 1997). This observation initially came as a surprise, as Ca^{2+} -regulated exocytosis was largely regarded as a specialization of certain cell types (Stinchcombe & Griffiths, 1999). However, it soon became apparent that non-specialized cells also contained Ca^{2+} -regulated exocytic vesicles (Coorsen *et al*, 1996; Ninomiya *et al*, 1996). Subsequent studies identified lysosomes as the major vesicular population that fuses with the plasma membrane in response to Ca^{2+} influx (Jaiswal *et al*, 2002; Rodriguez *et al*, 1997).

A search for potential Ca^{2+} sensor molecules that regulate lysosomal exocytosis led to the detection of synaptotagmin VII (Syt VII) on lysosomes (Martinez *et al*, 2000). Dominant-negative inhibition or gene ablation showed that blocking Syt VII function inhibits both

lysosomal exocytosis and membrane resealing (Chakrabarti *et al*, 2003; Martinez *et al*, 2000; Reddy *et al*, 2001). A recent report of the effects of the small molecule vacuolin 1 suggested that plasma-membrane repair could still occur in the absence of lysosomal exocytosis (Cerny *et al*, 2004). However, subsequent work showed that vacuolin 1, despite altering the morphology of lysosomes, does not inhibit the Ca^{2+} -dependent fusion of lysosomes with the plasma membrane (Huynh & Andrews, 2005). Lysosomes therefore remain the most important candidates for the exocytic vesicular population that mediates membrane repair. Nevertheless, one aspect was apparently difficult to reconcile with such a role. Imaging experiments detected lysosomal exocytosis 30–150 s after stimulation with a Ca^{2+} ionophore (Jaiswal *et al*, 2002, 2004), which is slower than the kinetics of membrane resealing (Steinhardt *et al*, 1994). It is important to note, however, that ionophores cause a sharp decline in the ATP content of cells as a result of increased plasmalemmal Ca^{2+} ATPase activity and the dissipation of mitochondrial proton gradients (Gmitter *et al*, 1996). ATP depletion inhibits Ca^{2+} -triggered lysosomal exocytosis (Rodriguez *et al*, 1997), which is expected given the requirement for the hexameric ATPase *N*-ethylmaleimide-sensitive factor (NSF) in all intracellular membrane-fusion events (Littleton *et al*, 2001). Thus, the kinetics and properties of Ca^{2+} ionophore-triggered exocytosis should not be considered representative of what occurs under physiological conditions. This conclusion is reinforced by the fact that lysosomal exocytosis is reduced in *Syt VII*^{-/-} cells stimulated by wounding (Chakrabarti *et al*, 2003) or by the receptor-mediated agonist bombesin (Jaiswal *et al*, 2004), but not after exposure to a Ca^{2+} ionophore (Jaiswal *et al*, 2004). A direct determination of the kinetics of lysosomal exocytosis triggered by membrane wounding should be possible as soon as the current technical obstacles are overcome.

Membrane repair: a defence against autoimmunity?

If plasma-membrane repair is important for limiting the release of endogenous adjuvants from cells, one prediction is that resealing defects might cause unregulated immunostimulation. There are two mouse models in which deletion of a single gene results in defective membrane repair: *Syt VII*^{-/-} (Chakrabarti *et al*, 2003) and *dysferlin*^{-/-} (Bansal *et al*, 2003) mice. Remarkably, *Syt VII*^{-/-} mice develop an inflammatory process in the skin and skeletal muscle, with many similarities to the human autoimmune diseases polymyositis/dermatomyositis. Infiltration of inflammatory cells and foci of muscle-fibre destruction are observed in association with elevated serum creatine kinase activity, loss of muscle strength and the production of anti-nuclear antibodies—all of which are diagnostic features of autoimmune inflammatory myopathy. Interestingly, these animals also have a marked increase in skin collagen fibre content, consistent with what is observed in a number of human autoimmune syndromes (Chakrabarti *et al*, 2003). Other tissues show no detectable alterations, which suggests that the inflammatory process is restricted to tissues under mechanical stress. It is tempting to speculate that impaired plasma-membrane resealing in *Syt VII*^{-/-} mice enhances the release of endogenous adjuvants in tissues in which injury is more frequent and that this results in local activation of the immune system. Testing this hypothesis requires the development of tools to inactivate endogenous adjuvants *in vivo*. There is already good evidence that dendritic cells activated by injured pancreatic- β cells can prime self-reactive T cells (Turley *et al*, 2003).

Defective membrane repair has also been shown in myofibres from *dysferlin*^{-/-} mice (Bansal *et al*, 2003). Although present in other cell types, dysferlin is most abundantly expressed in skeletal and cardiac muscle. Interestingly, the cytosolic domain of dysferlin contains six C₂ domains, some of which have predicted Ca²⁺ binding properties similar to the C₂A and C₂B domains of synaptotagmins (Bansal & Campbell, 2004; Davis *et al*, 2002). Dysferlin cycles between the sarcolemma and a population of intracellular vesicles the nature of which is still poorly understood. When muscle fibres are injured, Ca²⁺ binding to dysferlin C₂ domains is thought to trigger the exocytosis of these vesicles, promoting membrane resealing (Bansal & Campbell, 2004; Bansal *et al*, 2003). Mutations in dysferlin are linked to two clinically distinct muscle-wasting diseases: limb-girdle muscular dystrophy type 2B and Miyoshi myopathy. Although autoimmunity is rare in human patients with these and other forms of muscular dystrophy (Confalonieri *et al*, 2003; Funauchi *et al*, 2002), *SLI/J* mice that also carry mutations in dysferlin are considered a model for experimental autoimmune myopathy (Hart *et al*, 1987; Vafiadaki *et al*, 2001). It will be interesting to investigate how muscle fibres compare with other cell types, such as fibroblasts, regarding the amount of immunostimulatory molecules they contain and the rate by which these adjuvants are released on cell injury. Further studies using *Syt VII*^{-/-} and *dysferlin*-deficient mice should provide important additional insights into the intriguing relationship between plasma membrane injury and repair, and how the immune system decides when to 'leap into action'.

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