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Natural endogenous adjuvants

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Abstract It has long been known that immunization with a protein by itself is often not sufficient to stimulate immunity, and may instead induce tolerance. To elicit productive immune responses exogenous adjuvants need to be co-injected with an antigen. One important class of adjuvants are the unique (non-mammalian) components of microbes. It is now believed that an adjuvant is required for immunity because the immune system evolved to respond to dangerous situations such as infections, and the presence of an adjuvant is the mechanism used to identify these situations. However, there are some circumstances where immune responses are generated in the apparent absence of any microbial or other exogenous adjuvant. Such situations include immune responses to transplants, tumors, autoimmunity and possibly certain viral infections. It has been postulated that in these situations the danger signals come from endogenous adjuvants that are released from dying cells. There is abundant evidence that dead cells are immunogenic, and recently it has been shown that cells contain endogenous adjuvant activities that are released after death. Some actual and putative endogenous adjuvants, such as monosodium urate and heat shock proteins, have been identified and there are others whose identities are not yet known. The potential biological roles of this class of adjuvants are discussed.

Keywords Adjuvants · Immunity · Danger signals · Heat shock proteins · Uric acid

History and introduction

In 1925, Ramon [83] discovered that anti-toxin responses were markedly enhanced when an animal was immunized with the toxin admixed with other substances. Interestingly, these first immune-enhancing compounds included bread crumbs and tapioca (which probably weren't sterile) as well as other agents such as saponin and starch oil. Thus was born the concept of adjuvants (from the latin word "adjuvare", to help), molecules that have the ability to augment immune responses to an antigen. One year after Ramon's dis-

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covery, Glenny et al. [37] reported that aluminum salts had adjuvant activity, and, remarkably, alum (aluminum hydroxide) was the only FDA-approved adjuvant for use in man throughout the 20th century. Subsequent work identified many other agents with adjuvant activity, including detergents, oils and a number of microbial products such as killed *M. tuberculosis*, bacilli Calmette-Guerin, *Corynebacterium parvum*, Pertussis toxin, and lipopolysaccharide (LPS).

The discovery of adjuvants arose from empirical observations and the underlying mechanisms of how they worked were unknown. It was postulated that adjuvants helped to make a long-lived depot of antigen that could continually stimulate responses [36], and might promote migration of leukocytes to the site of immunization [30]. These mechanisms may indeed be some of the ways in which certain adjuvants, such as oil emulsions, help to promote immune responses. However, Dresser [23] noted that the adjuvant effect of *Bordetella pertussis* organisms lasted for only a few days, while these bacteria stimulated phagocytosis for weeks. Such data suggested that adjuvants might also enhance immune responses in ways other than simply making a depot of “appetizing” antigen.

In many of the early experiments, the inclusion of adjuvants in an immunization enhanced the magnitude of immune responses, but responses were still obtained in the absence of adjuvants. However, Dresser [21, 22] reported that this was not the case with purified soluble protein immunogens. In this situation, injection of the soluble protein antigen by itself not only failed to stimulate an antibody response, but actually tolerized animals [22]. However, if a bacterial adjuvant was admixed with the protein immunogen, then immune responses were generated [19, 21]. This led to an early concept that two signals were needed to stimulate immunity. Dresser thus described that “adjuvanticity can be looked on as a property of a substance which can act as a physiological switch, directing at least some immunologically competent cells to respond by making antibody rather than by becoming immunologically paralyzed by the antigen” [23]. He envisioned that the adjuvant acted directly on the B cell itself. Of course, the possibility that adjuvants might act on other cells was not considered at the time because many of the other key players involved in generating immune responses to proteins, such as antigen-presenting cells (APCs), T helper cells and costimulation, were not yet elucidated or well understood.

The concept that a second signal was needed to generate immune responses to antigen was subsequently proposed in other contexts. In considering what might limit the activation of autoreactive B cells, Brescher and Cohen [14] hypothesized that a second signal was needed to help activate B cells. In separate considerations of why xenogeneic cells were weaker stimulators than allogeneic ones, Lafferty and Cunningham [64] postulated that T cell activation required two signals from an APC, the second, antigen-independent signal being species restricted. Experimental evidence that the T cell required two signals to be activated came from a number of studies, including ones analyzing the requirements for thymocytes to be activated by mitogens [34, 67], and for T cell clones to respond to mitogen or TCR stimulation [35, 54, 85]. As in the experiments of Dresser for antibody responses [22, 23], and as predicted by Lafferty for T cells, Jenkins and Schwartz [50] and Quill and Schwartz [80] found that a T cell clone was tolerized when it received only antigen stimulation without a second signal. Subsequent studies ultimately identified costimulatory receptors on T cells (e.g., CD28) and their ligands on APCs (e.g., CD80 and CD86) that provided the second signal for T cell activation [52, 94].

Strangers and danger

Despite considerable progress in the field of immunology, adjuvants remained poorly understood and were not really incorporated into the paradigms that attempted to explain immune responses; they remained the Immunologist's "dirty little secret" [48]. The finding that proteins were often not immunogenic without adjuvants, and that T cells required two signals to be activated led Janeway to propose that: "the immune system has evolved specifically to recognize and respond to infectious microorganisms, and that this involves recognition not only of specific antigenic determinants, but also of certain characteristics or patterns common on infectious agents but absent from the host" [48]. He proposed that there were non-clonally distributed receptors on APCs for non-mammalian microbial molecules, which, when triggered, induce the expression of costimulatory molecules. Consequently, infectious agents, but not self proteins, would generate the second signal needed for T cell activation. This model has been nicknamed by some as the "stranger hypothesis" because its central tenant is that the immune system looks for and only responds to strangers (microbes) (Fig. 1).

Fig. 1 Exogenous adjuvants and the immune response to infectious non-self. The "stranger" model proposes that the immune system has evolved to detect and mount responses to infectious agents. The host initially detects infections through receptors, such as the TLRs, on APCs that bind non-mammalian microbial molecules, such as LPS and microbial cell wall components. As a consequence of this interaction, the APC matures, expresses costimulatory molecules and can stimulate T cell responses. It is believed that this is how exogenous adjuvants function. In the absence of an adjuvant, the APCs fail to stimulate immunity and may instead induce tolerance. For simplicity, only three of the many TLR are shown (TLR Toll-like receptor, APC antigen-presenting cell, LPS lipopolysaccharide)

Fig. 2 Cell death and endogenous adjuvant. It has been postulated, and emerging data supports the concept, that cells contain endogenous adjuvant molecules, such as HSP and uric acid, that are released upon cell death. These adjuvants, like exogenous ones, stimulate APCs to mature and become immunostimulatory. Since many pathological situations cause cells to die, this process alerts the immune system to potential danger and helps to stimulate a protective immune response (*HSP* heat shock protein)

In this stranger model, adjuvants are the non-mammalian microbial molecules that function by activating APCs to become fully stimulatory. Receptors for conserved microbial molecules were first discovered in drosophila [10] and their mammalian homologues, the toll-like receptors (TLRs), were subsequently identified in mammals [70]. Various TLRs recognize a variety of conserved non-mammalian microbial molecules [110]. These receptors are stimulated by known adjuvant molecules, such as LPS, and, when triggered, can activate APCs (Fig. 1).

Janeway's hypothesis that "the immune system evolved to discriminate infectious non-self from noninfectious self" [49] doesn't explain how immune responses are generated to tumors, transplants, autologous tissues (in autoimmunity) and potentially to some viruses (which are synthesized by the host and may lack unique molecular patterns). In considering these issues, Matzinger [68] proposed an alternative hypothesis that the immune system evolved to discriminate dangerous antigens from non-dangerous ones. In this theory, the danger signal arises from injured cells of the host. This signal then activates APCs to become effective stimulators of T cells (Fig. 2). In other words injured cells produce an adjuvant activity that is endogenous and distinct from the exogenous adjuvants of microbes. This Danger hypothesis also postulated that endogenous danger signals are the only factors that control immune responses, and that the immune system does not otherwise distinguish between foreign and self molecules [68]. Infections would stimulate immune responses by causing the release of endogenous adjuvants (interestingly, the notion that some adjuvants might work by damaging cells was suggested in the early 1960s [21]). However, an alternative view is that both models are operative with endogenous and exogenous adjuvants contributing to the generation of immune responses.

Injured cells stimulate immune responses

It had been known for years that animals generate immune responses when immunized with cells bearing a foreign antigen. For example, tumor immunity could be elicited by in-

jecting tumor cells [29, 79]. From such studies it was apparent that immune responses can be generated against many cell-associated antigens including intracellular ones. The cells used for these immunizations were typically injured (e.g., by irradiation) and even if they were not intentionally injured, they may have been damaged during experimental manipulations and/or died after injection into an ectopic site. Moreover, experimentally inducing death of some cancer cells *in situ* promotes the generation of tumor immunity [6, 16, 20, 74, 75]. In fact, in many cases injury and death of such antigen-bearing cells is probably essential for these immune responses to develop. This is because we now know that immunity only develops after intracellular antigens are released and cross-presented by professional APCs [12, 40, 43, 98]. Many studies have shown that dendritic cells (DCs) can acquire and present antigens from dying cells [1, 41, 45, 88].

When cells were used as immunogens, an adjuvant was often not necessary to elicit immunity. However, for many years it was not really considered why these preparations were immunogenic. Were the cells simply a source of antigen, was the antigen in a physical form (e.g., cell debris) that was more readily acquired by APCs, were preparations contaminated with a microbial adjuvant such as LPS, or was something else promoting responses such as an endogenous adjuvant?

Clear evidence that cells do indeed contain endogenous adjuvant activities came from immunizations in which cells were admixed with antigen and used as a source of adjuvant rather than as the source of antigen. Using this approach Galluci et al. [31] first demonstrated that a delayed-type hypersensitivity response (an immune response of CD4 T cells) was primed in mice immunized with soluble ovalbumin mixed with killed syngeneic cells, but not in ones injected with antigen alone. It was possible in these experiments that the added cells were simply adsorbing the soluble antigen and converting it to particulate form, which is known to be more immunogenic [59]. However, Shi and colleagues [96, 97] similarly found that both CD4 and CD8 T cell responses were markedly increased when mice were immunized with particulate antigen admixed with syngeneic cells, and that the injected cells augmented responses even when they were physically separated from the antigen *in vivo* [97]. Moreover, the T cell responses were antigen specific and not primed in animals injected with cells without antigen [97]. Therefore, the co-injected cells were providing a classical adjuvant effect.

Both cultured and primary cells of several different histotypes were tested and all contained endogenous adjuvant activity [31, 97]. Therefore, this endogenous adjuvant activity appeared to be an inherent property of cells generally. Cells that were killed instantly by freeze-thawing had adjuvant activity when injected *in vivo* [31, 97]. This suggested that the adjuvant activity in cells is constitutively present. In addition, the amount of adjuvant increased by about 10-fold when cells were stressed or injured for several hours before dying [97]. Therefore, the level of endogenous adjuvant in cells is regulated and can increase in response to injury.

Effect of dying cells on DCs

The danger hypothesis proposed that dying cells would stimulate APCs to become stimulatory and, as discussed above, adjuvants are thought to work at least in part by this mechanism. Do dying cells stimulate APCs? This question was first investigated by Galluchi et al. [31], who found that lysates of syngeneic cells stimulated cultured immature DCs to

mature and express costimulatory molecules. These APCs, but not ones that were untreated, could present antigen and stimulate a T cell clone [31]. Therefore, dying cells can stimulate DCs, at least in vitro.

Are APCs similarly affected by dying cells in vivo? This is an important question because mouse DCs are highly prone to mature in culture, even spontaneously, so that their behavior in vitro may not be identical to that in vivo. Shi and Rock [96] investigated the effect of dying cells on APCs that were acquiring antigen in vivo and migrating to draining lymph nodes. To identify these APCs, mice were injected subcutaneously with fluorescent microspheres. The APCs that ingested these particles and then migrated from the skin to the draining node could then be visualized and quantified by flow cytometry. Co-injection of dying cells increased the numbers of “antigen-bearing” APCs that had migrated to the node. Interestingly, about 50% of these APCs were DCs and the other 50% were probably macrophages. The expression of costimulatory molecules on the DCs was as high, but not higher, than on mature migrating cells from control mice. These results indicate that dying cells in vivo can stimulate APCs to acquire antigen and/or mature and migrate to lymph nodes.

A surprising finding was that dying cells increased the migration of APCs containing injected particulate but not soluble labeled antigen [96]. In this kind of experiment, it is possible that the small number of migrating APCs labeled with fluorescent soluble antigen could be obscured by larger numbers of resident APCs, if the latter acquired the soluble antigen from draining lymph fluid. Interestingly, however, Shi also found in vivo that dying cells functioned as an adjuvant for T cell responses to particulate antigen, but not soluble ones. Therefore, it is possible that dying cells selectively promote the activity of APCs that are acquiring particulate antigen, which is precisely the kind of antigen that they would encounter from dying cells. In other words, dying cells could be sending a signal for the immune system to “look” closely at the antigens released from dying cells. However, under some circumstances endogenous adjuvants also promote responses to soluble antigens. Galluchi et al. [31] found that dying cells functioned as an adjuvant for T cell responses to a soluble protein.

It is apparent from these studies that dying cells can stimulate APCs both in vitro and in vivo. This stimulation can cause the APCs to mature, up-regulate costimulatory molecules and migrate into secondary lymphoid organs. These effects are likely to underlie, at least in part, the ability of dying cells to augment immune responses (Fig. 2). Whether they influence immune responses through other mechanisms remains to be determined.

Signals from necrotic versus apoptotic cells

In the danger hypothesis [68], Matzinger theorized that because necrosis was pathological, and therefore potentially dangerous, it should transmit a danger signal to the immune system. In contrast, it was hypothesized that apoptosis should not produce a danger alarm because many cells die by apoptosis in non-pathological situations. This difference between necrosis and apoptosis was important because this model additionally proposed that the immune system did not distinguish self from foreign antigen but rather dangerous from innocuous ones. Consequently, it was envisioned that if danger signals were released from normal (non-pathologic) dying cells, then autoimmunity might be provoked. It also made sense that apoptotic cells might not release a danger signal because they retain membrane

integrity (as least initially), can be rapidly cleared by phagocytes, and often do not stimulate inflammation (which can be a sign that innate immunity has been stimulated). On the other hand, there are many pathological situations in which cells die by apoptosis, e.g., in many infections and tumors, and teleologically it would seem useful for the immune system to be alerted to such dangerous situations. Moreover, if the immune system is in fact tolerant to self antigens in normal cells, then there would be no risk of autoimmunity when the immune system is called to investigate “false alarms” (cells dying normally by apoptosis) unless perhaps the individual had inherited autoimmune susceptibility genes [82]. Finally, apoptotic cells often undergo secondary necrosis and release their intracellular contents [47, 66]. This process clearly occurs *in vivo* because there are many situations where apoptotic cells release measurable amounts of cytosolic enzymes into the serum. There are also many situations where cells dying by apoptosis stimulate inflammation *in vivo* [47]. These arguments as to whether or not apoptotic cells should be immunostimulatory are theoretical. Experimentally, there is now evidence both for and against a role of apoptotic cells in signaling danger.

There is limited evidence that apoptotic cells are not immunostimulatory, at least under certain conditions. Some studies have reported that apoptotic cells failed to stimulate DCs to mature *in vitro* [31, 51, 89, 102]. It has also been suggested that apoptotic cells stimulate DCs to produce anti-inflammatory rather than proinflammatory cytokines [73, 90, 107]. In a few studies, injection of apoptotic spleen cells or DCs pulsed with apoptotic cells has been reported to induce tolerance instead of immunity [26, 105]. The lack of immunogenicity in these various studies raised the possibility that apoptotic cells lack endogenous adjuvant activity. Galluchi et al. [31] investigated this issue, and reported that when co-injected with antigen, apoptotic cells lacked adjuvant activity to help the priming of T cell responses *in vivo*.

On the other hand there are more extensive data indicating that in many circumstances apoptotic cells are immunostimulatory. In a large number of studies, DCs exposed to apoptotic cells *in vitro* are stimulated to mature [15, 24, 38, 44, 46, 78, 81, 86, 87]. Moreover, in most studies dendritic cells pulsed with apoptotic cells can stimulate T cell responses *in vitro* [17, 27, 41, 58, 88, 92, 93, 103, 108]. Similarly, immunization with apoptotic cells themselves [18, 24, 55, 58, 75, 84, 91, 106] or injection of an agent that induces apoptosis of a tumor *in situ* [6, 16, 20, 74, 75] stimulated T cell responses *in vivo*. The ability of apoptotic cells to stimulate both DCs and T cell immunity suggests that they may contain endogenous adjuvants. Indeed, Shi et al. [97] found that apoptotic cells contained potent adjuvant activities that supported the priming of T cell responses to particulate antigens *in vivo*. Interestingly, cells that were induced to die by apoptosis were actually about tenfold more potent as an adjuvant compared to necrotic cells. This may correlate with the findings in several studies that apoptotic cells are actually more immunogenic than necrotic ones [41, 91, 92, 93, 106].

It is not clear why some studies found apoptotic cells to be non-immunostimulatory, while others found them to be immunogenic and producing endogenous adjuvant activity. It is formally possible that the immunostimulatory activity in some apoptotic cell populations was actually due to contaminating necrotic cells. However, if only a subpopulation of cells were responsible for the adjuvant activity then the apoptotic cell populations should be substantially less potent than necrotic cells—yet precisely the opposite was observed in several studies [41, 91, 92, 93, 106]. It is also possible that the differences could be due to whether the apoptotic cells underwent secondary necrosis and released their endogenous adjuvants. Consistent with this notion, it has been reported that late stage apop-

otic cells (which will be undergoing secondary necrosis), but not ones in the early stages of apoptosis, stimulate DCs [15, 78]. Yet other possibilities for the discrepant results in the literature are that the particular type of cell, its physiological state and/or the specific mechanism leading to apoptosis (e.g., transcription-dependent versus transcription-independent), determines whether endogenous adjuvant activity is generated and released. For example, it has been reported that cells that are stressed before undergoing apoptosis become more immunogenic [24, 25], and that certain triggers of apoptosis, such as chemotherapeutic agents, increase immunogenicity of cells more than others [46, 81]. Despite these uncertainties the preponderance of the data currently indicates that in many situations apoptotic and necrotic cells are immunostimulatory and both contain endogenous adjuvant activity.

Molecular basis for endogenous adjuvant activity?

While it is clear that dying cells release molecules that influence the immune system, the molecular identity of these factors is incompletely understood. Recently, some endogenous molecules with adjuvant activity have been identified and there are probably others yet to be discovered. The present discussion focuses exclusively on endogenous adjuvants released from injured and dying cells, and does not consider cytokines, some of which have adjuvant activity (e.g., IL-12), but are generated by living cells.

One potential set of endogenous adjuvant molecules are heat shock proteins (HSPs). The immunogenic potential of cellular HSPs was first discovered in studies attempting to identify tumor antigens. A tumor antigen isolated from a murine sarcoma was identified as an HSP and, surprisingly, found to be of normal (unmutated) sequence [104]. Nevertheless, injection of the tumor-derived HSP into mice could induce tumor-specific immunity. Interestingly, many different HSPs, including HSP70, grp96 and HSP90 were found to have immunogenic potential [104]. Subsequent studies found that the HSPs could prime tumor-specific immunity because they had bound peptides derived from tumor antigens, and could target these peptides into APCs [104, 113]. In these studies, the HSPs were immunogenic even without adjuvants. Several groups have reported that HSPs can stimulate DCs to mature *in vitro* [8, 11, 28, 60, 72, 99, 100, 117], and mutant HSPs that lack peptide binding still have immune-stimulating potential *in vivo* [5]. Moreover, injection of HSPs *in vivo* have been described to result in a massive increase in DCs in the draining lymph node [13]. The immunogenicity of cells in some studies has been correlated with their content of HSPs [24, 25, 71]. Together, these data suggested that HSPs have adjuvant activity.

To prove that a purified or recombinant endogenous molecule has adjuvant activity, it is necessary among other things to exclude the possibility that the preparation is contaminated with an exogenous microbial adjuvant. Molecules such as LPS are very common contaminants of many reagents, e.g., endotoxin is present in many sources of distilled water and washed glass ware. The possibility that microbial contaminants are responsible for adjuvant activity is particularly a concern for HSPs because these molecules are now known to signal through the LPS receptor (TLR4/MD2/CD14) [3, 4, 56, 57, 76, 114, 116] and its associated signaling pathway [114, 115] (see below). Two observations initially argued against LPS being responsible for the immunostimulatory activity of HSPs. First, this activity was heat sensitive (while LPS is relatively heat-resistant) and, second, it was not eliminated by treatment with polymyxin B, an LPS-binding molecule [3, 56, 115,

116]. However, it was recently reported that low concentrations of LPS can be inactivated by heating and that LPS in HSP preparations is incompletely removed by polymyxin B [9, 32, 33]. Furthermore, it was found that LPS-free HSP60 and HSP70 were inactive and their activity could be restored by the addition of very small amounts of LPS [9, 32, 33]. Therefore, at the present time it is not clear whether HSPs truly contain endogenous adjuvant activity. Moreover, to prove that HSPs are important endogenous adjuvants, it will be necessary to show that their elimination or neutralization blocks the adjuvant activity of cells *in vivo*; this has not yet been tested.

A number of other endogenous molecules, including fragments of fibronectin [77], fibrinogen [101], hyaluronic acid [111] and lung surfactant protein [39], have been reported to stimulate macrophages or DCs through TLR4. Since some exogenous adjuvants, such as monophosphoryl lipid A, stimulate through TLR4, it is possible that these endogenous molecules will have adjuvant activity *in vivo*, although this possibility has not been formally tested. However, as discussed for the HSPs, molecules that are found to stimulate through TLR4 need to be rigorously evaluated for the possibility that microbial contaminants are responsible for their biological activity.

Other endogenous adjuvants have been detected and purified from cells. Shi et al. [97] found that cytosol of cells contained adjuvant activities that augmented the priming *in vivo* of T cell responses to particulate antigen. These cytosolic adjuvant activities could be separated by size exclusion chromatography into two distinct fractions, one of high and the other of low molecular weight [95]. The molecule(s) responsible for activity in the high molecular weight fractions has not yet been identified. However, the low molecular weight fraction was purified to homogeneity and identified as uric acid [95]. The adjuvant activity of uric acid was eliminated by treatment with uricase (a highly specific uric acid oxidase), indicating that the active principal was uric acid and not a microbial contaminant [95]. Consistent with this observation, uric acid was fully active in LPS-unresponsive (TLR4^{-/-}) mice [95]. Eliminating uric acid from injured cells reduces their adjuvant activity by about tenfold [95]. This indicates that uric acid is a major endogenous adjuvant in cells.

As with other adjuvants, uric acid may work, at least in part, by stimulating DCs to mature. *In vitro*, uric acid stimulated immature DCs to express costimulatory molecules [95]. Moreover, *in vivo* it augments response to immunizations with particulate antigen, but not with peptide-pulsed activated DCs; this later result suggests that it may be working by stimulating immature DCs (as it is not needed once DCs are mature). However, further studies are necessary to resolve its mechanism of action.

Uric acid is generated when cells degrade their purines and in humans is the end product of this process. Since cells continually turn over their nucleotides, uric acid is a normal constituent of the cytosol of cells. Therefore, when any cell dies and loses membrane integrity, uric acid will be released to potentially alert the immune system. Moreover, when cells are injured they often degrade their RNA and DNA, producing increased amounts of uric acid. The elevated levels of uric acid correlate with the observation that stressed cells have higher amounts of adjuvant activity [97], and provides a mechanism for the immune system to better identify stressed cells.

Since uric acid is continually produced, it is a normal constituent of serum and eventually excreted by the kidney. It was therefore surprising that such a ubiquitous molecule could function as an endogenous adjuvant. However, it was noted that *in vitro*, uric acid stimulated DCs to mature only at concentrations where crystals of monosodium urate

(MSU) began to nucleate [95]. Moreover, the concentrations of uric acid needed to provide adjuvant activity *in vivo* were supersaturating for biological fluids. Similarly, the concentration of uric acid in cytosol is extremely high, and when released will create locally a highly supersaturated condition, which in the presence of extracellular sodium should favor crystallization. It was subsequently found that preformed crystals of MSU were active *in vitro* and also as an adjuvant when injected *in vivo* [95]. These observations led to the hypothesis that a danger signal is generated when this normal “self” molecule undergoes a chemical phase transition into a crystal. Consistent with this hypothesis, high concentrations of uric acid are asymptomatic unless they nucleate into crystals, creating the inflammatory disease of gout.

It is important to point out that it is likely that there are several distinct endogenous adjuvants. This possibility is supported by the biochemical separation of more than one activity in cells (see above). These factors may be functionally redundant but it is also possible that different adjuvants will have different biological effects. If the latter is true, then different assays may preferentially detect different adjuvants.

Receptors for endogenous adjuvants

It is presently believed that many of the known exogenous adjuvants work by stimulating TLRs [53]. In mice, there are 11 TLR genes that encode transmembrane proteins which recognize features of highly conserved microbial products that are distinct from mammalian molecules. When the TLRs are stimulated, they signal through a series of intracellular adaptor molecules and kinases to activate the transcription factor nuclear factor-kappa B and in some cases interferon regulated factors. These transcription factors stimulate the expression of many key molecules that control immune responses, including costimulatory molecules, proinflammatory cytokines, anti-microbial molecules, and vascular adhesion molecules. For in depth discussion of these receptors and their signaling pathways readers are referred to recent reviews [7, 110].

An issue of fundamental importance is whether endogenous and exogenous adjuvants work via same receptors and pathways. This question is important because it has implications for whether endogenous adjuvants function equivalently to exogenous ones, or whether they exert distinct biological effects. As discussed above, several endogenous molecules like HSPs are reported to mediate their effects through TLR4, although there is controversy as to whether this is actually due to microbial contaminants. In contrast, MSU stimulates responses in TLR4^{-/-} cells and mice, and, therefore, does not stimulate through TLR4 [95]. Since TLR7, 8 and 9 are stimulated by nucleotide or nucleotide-like structures, such as RNA and imiquimod, it is possible that these TLRs might recognize MSU; however, preliminary experiments argue against this possibility (Shi et al., unpublished data). Whether MSU stimulates other TLRs is not presently known.

Biological roles for endogenous adjuvants

Endogenous adjuvants have been postulated to play a central role in alerting the immune system to potential danger. Their release from injured cells has been hypothesized to play an important role in generating immunity to pathogens generally, even ones that contain

exogenous adjuvants [68, 69]. Moreover, it has been proposed that endogenous adjuvants may be essential in situations where immune responses are generated to cellular antigens in the absence of microbial or exogenous adjuvants [68, 69]. One of these situations is transplantation. Transplantation of tissues between individuals of the same species can elicit a robust immune response to polymorphic gene products, yet the transplanted tissues do not contain pathogen-associated molecular patterns (PAMPs). Other similar situations occur in autoimmunity and tumor immunity. In autoimmune diseases and in some cancers, immunity develops to autologous tissues that obviously lack exogenous adjuvants. Yet another such situation may be infections with certain viruses. Viral gene products are all synthesized by the infected organism's own cells and may lack unique, non-mammalian structures, although in at least some cases viral components, including proteins and RNA, may be able to stimulate TLRs [2, 61, 62, 65, 109]. For all of these responses the release of endogenous adjuvants from injured tissues could provide the necessary stimulus to promote the initiation of immune responses.

Central to these concepts is the notion that in these various situations cells would be injured and release adjuvants, which would then help stimulate immunity. Does such cell death actually occur in these settings? Clearly, viruses can be cytopathic and kill cells. Similarly, apoptosis or necrosis is often observed in tumors. In transplantation, tissues are often injured during the isolation and transplant procedure or may die when introduced into ectopic locations. For example, it is estimated that only about 50% of islet cells survive when injected into the portal vein of recipients. Whether in fact cell death plays a role in promoting immune responses to viral infections and transplantation is unknown. However, there is some evidence that dying cancer cells can help to trigger tumor immunity (see above). It may seem less obvious that cell death would precede the onset of autoimmune diseases. However, there is now good evidence that the death of some islets of Langerhans triggers the development of autoimmune diabetes in NOD mice [112]. Similarly, in transgenic mice expressing a foreign antigen in their pancreatic islets cells, injury of the islet cells can lead to the stimulation of autoreactive T cells [42, 63]. Therefore, it is possible that cell death and the attendant release of endogenous adjuvants is important to the initiation of many of these immune responses.

However, it is important to emphasize that at the present time, the actual role of endogenous adjuvants *in vivo* and their contribution to the generation of various immune responses is largely unknown. It is clear that endogenous adjuvants can promote the generation of T cell responses when injected together with antigens [31, 97]. Moreover, injecting dead cells that have been depleted of MSU reduces the immune response to the antigens within the dead cells (Shi, unpublished data). These findings strongly suggest that endogenous adjuvants will similarly help the immune system respond to antigens released by dying cells *in situ*. Nevertheless, the contribution of endogenous adjuvants, if any, to autoimmunity or immunity to transplants, tumors or viral infections remains to be determined.

Potential uses of endogenous adjuvants

Regardless of the physiological role of endogenous danger signals, these agents might be useful as adjuvants for vaccines. Vaccines attempt to safely elicit immunity to pathogens that is ideally robust, protective and long lived. However, current formulations of many subunit vaccines give weaker and shorter-lived immunity than natural infection. While it

is clear that adjuvants can be used to boost immunity, alum (aluminum hydroxide) is the only adjuvant presently available for clinical use in the USA. Alum is a weak adjuvant and one that may bias responses to effector responses (Th2) that are not protective against many pathogens.

Endogenous adjuvants need to be evaluated for their potential use in vaccines. In theory, they may allow vaccinations to safely mimic the pathway that naturally triggers immunity to many pathogens. These agents also promote CD8 T cell immune responses, which are important in immunity to many pathogens, such as viruses and tumors, but not elicited by most subunit vaccines. If the endogenous adjuvants work differently than exogenous ones, and there is limited data that this may be the case [96], then they may be unique therapeutics to manipulate immune responses. Moreover, they would then have the potential to be combined with microbial adjuvants to further boost immune responses or to change the quality of the response in unique ways. However, there are presently no data that addresses these possibilities. As endogenous adjuvants become better defined, this will clearly be an important area of investigation.

Conclusions

It is now clear that most, if not all, cells contain endogenous adjuvant molecules that can promote immune responses. These factors appear to work at least in part by stimulating DCs to mature and become immunostimulatory. These molecules have been proposed to play key roles in communicating a danger signal to the immune system and promoting responses in transplants, tumors, infections and autoimmunity. However, this is a relatively new and poorly understood field. To better understand these adjuvants and their biological roles, they need to be identified molecularly and there has been initial progress in this area. Endogenous adjuvants are also of considerable interest as novel adjuvants for use in vaccines.

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