Immunity
Review

Vaccine Adjuvants: Putting Innate Immunity to Work

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Adjuvants enhance immunity to vaccines and experimental antigens by a variety of mechanisms. In the past decade, many receptors and signaling pathways in the innate immune system have been defined and these innate responses strongly influence the adaptive immune response. The focus of this review is to delineate the innate mechanisms by which adjuvants mediate their effects. We highlight how adjuvants can be used to influence the magnitude and alter the quality of the adaptive response in order to provide maximum protection against specific pathogens. Despite the impressive success of currently approved adjuvants for generating immunity to viral and bacterial infections, there remains a need for improved adjuvants that enhance protective antibody responses, especially in populations that respond poorly to current vaccines. However, the larger challenge is to develop vaccines that generate strong T cell immunity with purified or recombinant vaccine antigens.

Introduction

Many of the most effective and safe vaccines are live, attenuated variants of the targeted pathogen. Their administration results in mild, usually asymptomatic, infection, but generates long-lived immunity similar to that observed in individuals who recover from natural infection. For many pathogens, however, attenuated vaccines have not been successfully developed. For others, such as influenza, they are impractical, because natural infection itself does not confer adequate immunity. To vaccinate against such organisms, nonliving antigens are used, ranging from whole, inactivated viruses and microorganisms to single recombinant antigens.

Nonliving vaccine antigens, especially purified or recombinant subunit vaccines, are often poorly immunogenic and require additional components to help stimulate protective immunity based on antibodies and effector T cell functions. These additional components, termed adjuvants, provide the ''help'' (from *adjuvare,* to help) needed to enhance the immunogenicity of vaccine antigens. Adjuvants currently in widespread use, either in man or in animals, have for the most part been developed empirically, without a clear understanding of their cellular and molecular mechanisms of action. However, recent data suggest that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system, rather than by direct effects on the lymphocytes themselves (McCartney et al., 2009; McKee et al., 2007, 2010; O'Hagan and De Gregorio, 2009).

Why Use an Adjuvant?

Adjuvants have been traditionally used to increase the magnitude of an adaptive response to a vaccine, based on antibody titer or ability to prevent infection, but a second role for adjuvants has become increasingly important: guiding the type of adaptive response to produce the most effective forms of immunity for each specific pathogen (Kenney and Cross, 2010; Pulendran et al., 2010). Thus, there are two distinct reasons to incorporate an adjuvant into a vaccine. Adjuvants are currently used clinically to: (1) increase the response to a vaccine in the general population, increasing mean antibody titers and/or the fraction of subjects that become protectively immunized; (2) increase seroconversion rates in populations with reduced responsiveness because of age (both infants and the elderly), disease, or therapeutic interventions, as in the use on the MF59 adjuvant to enhance the response of older subjects to influenza vaccine (Beran, 2008; Podda, 2001); (3) facilitate the use of smaller doses of antigen (Banzhoff et al., 2009; Boyle et al., 2007; Schwarz et al., 2009), because the ability of an adjuvant to permit comparable responses with substantially lower amounts of antigen could be important in circumstances in which large-scale vaccination is urgent and production facilities limiting, as in the emergence of a pandemic influenza strain; and (4) permit immunization with fewer doses of vaccine. The requirement of many vaccines for multiple injections presents compliance issues and, in much of the world, significant logistic challenges. Adjuvants can reduce the number of doses required to achieve protection (Banzhoff et al., 2009; Halperin et al., 2006; Schwarz et al., 2009).

The second reason for incorporating an adjuvant into a vaccine is to achieve qualitative alteration of the immune response. For vaccines currently under development, adjuvants are increasingly used to promote types of immunity not effectively generated by the nonadjuvanted antigens. For example, adjuvants have been used in preclinical and clinical studies to: (1) provide functionally appropriate types of immune response (e.g., T helper 1 [Th1] cell versus Th2 cell, $CD8^+$ versus $CD4^+$ T cells, specific antibody isotypes) (Table 1); (2) increase the generation of memory—especially T cell memory (Galli et al., 2009b; Leroux-Roels et al., 2010; Vandepapeliere et al., 2008); (3) increase speed of initial response, which may be critical in a pandemic outbreak of infection (Galli et al., 2009a; Huleatt et al., 2007; Khurana et al., 2010); and (4) alter the breadth, specificity, or affinity of the response (Khurana et al., 2010; Malherbe et al., 2008).

Table 1. Triggering of the Innate and Adaptive Components of the Immune System by Major Adjuvants

Adjuvant	Major Immunostimulatory Component(s)	Innate Receptors or Pathway Activated	Principal Immune Responses Stimulated
Licensed Adjuvants			
Alum	aluminum salts	NLRP3 inflammasome (?)	Ab, Th2 (+ Th1 in humans)
MF59 and AS03	squalene-in-water emulsions	tissue inflammation (no receptors defined)	Ab, Th1 + Th2
AS04	MPL plus alum	TLR4 and inflammasome (?)	Ab, Th1
Adjuvants in Widespread Experimental Use or in Late Stage Clinical Development			
Poly-IC (also Poly-ICLC)	synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, $CD8^+$ T cells
MPL and formulations (AS01, AS02)	MPL and QS-21	TLR4 (MPL), ? (QS21)	Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Flagellin from *S. typhimurium*	TLR5	Ab, Th1 + Th2
Imiquimods	imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, $CD8^+$ T cells (when conjugated)
CpG oligodeoxynuceotides and formulations (IC31, QB10)	synthetic phophorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, $CD8^+$ T cells (when conjugated)
CAF01	trehalose dimycolate (cord factor)	Mincle	Ab, Th1, Th17
ISCOMS and ISCOMATRIX	saponins	mechanism undefined	Ab, Th1+ Th2, $CD8^+$ T cells
IFA (and Montanide formulations)	mineral or paraffin oil + surfactant	mechanism undefined	Ab, Th1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17

The principal immune response stimulated is based on results from human and mouse studies, although it may be limited to one species in some cases. Where indicated, conjugation of TLR ligand to antigen is necessary to obtain significant $CD8^+$ T cell responses.

Challenges for Vaccine Adjuvant Development: Humoral Versus Cellular Immunity

Most current vaccines confer protection primarily through humoral immunity (Plotkin, 2010). Responses are elicited by a variety of vaccine platforms that include live attenuated, recombinant protein, toxoids, or polysaccharide-protein conjugates. Antibody responses to many current vaccines are long-lived and require infrequent or no additional boosting to sustain protection (Amanna et al., 2007). Despite the impressive success of such vaccines, there are substantial groups of people for which current vaccines, even those using alum adjuvant, do not achieve adequate seroconversion rates or protective antibody titers. Moreover, responses to vaccines begin to decline in healthy adults after 40–50 years of age (Chen et al., 2009) and as a result of health conditions such as chronic kidney disease (Beran, 2008). The addition of an adjuvant to an existing vaccine, as has been done for influenza (Podda, 2001), or a switch from alum to a more effective adjuvant, as for hepatitis B virus (HBV) (Beran, 2008; Halperin et al., 2006), represents a substantial benefit for these groups.

For polarization of helper T cell, there are striking differences in the type of response preferentially stimulated by different adjuvants. Adjuvants such as MF59 and ISCOMs (Table 1), as well as Toll-like receptor 2 (TLR2) and TLR5 ligands, enhance T cell and antibody responses without altering their Th1/Th2 cell balance of the specific antigens. In contrast, more polarized Th1 cell responses are elicited by adjuvants that incorporate agonists of TLR3, TLR4, TLR7-TLR8, and TLR9. Complete Freund's adjuvant (CFA) and CAF01 induce mixed Th1 and Th17 cell responses. Thus, selection of an appropriate adjuvant is influenced by the type of $CD4^+$ T cell response required for protection.

A more daunting challenge is developing adjuvants that will generate protective $CD8^+$ T cell responses to soluble proteins. Here, the type of vaccine is dictated by the particular processing pathway of MHC class I presentation. Vaccines that lead to direct infection of cells, such as viral vectors or DNA, induce $CD8^+$ T cell immunity through the endogenous class I presentation pathway; however, exogenous protein vaccines require cross-presentation. To promote differentiation of functional $CD8^+$ T cells, a successful adjuvant must be given with a protein formulated in a manner that facilitates entry into the MHC class I processing pathway, trigger dendritic cell (DC) activation, and induce type-I interferon (IFN) production.

The difficulty in generating potent and durable T cell immunity with current vaccines and adjuvants has profound clinical implications for a variety of diseases. There are still no fully effective vaccines against many widespread infectious diseases, including HIV-AIDS, malaria, and tuberculosis. Although humoral immunity has a clear role in preventing infection by HIV (Mascola et al., 2000) and can influence certain stages of malaria infection (Moorthy and Ballou, 2009), there is compelling evidence that Th1 cells, $CD8^+$ T cells, or both also have a critical role in preventing or controlling these infections. More challenging still is the task of developing adjuvants for therapeutic treatment of cancers and chronic viral infections, where it will be necessary to generate potent and perhaps multifunctional T cell responses in patients who respond poorly to the relevant tumor or viral

antigens as a result of multiple layers of immune regulation (Gale and Foy, 2005; Rosenberg et al., 2004). For such vaccines, the major hurdles for an adjuvant will be to stimulate $CD8^+$ T cells and to circumvent the regulatory mechanisms that limit the host response to the tumor or pathogen. Together, these examples underscore the critical need to develop vaccines capable of inducing potent and durable T cell immunity in man.

Innate Immunity

Microbial detection by the innate system relies heavily on pattern recognition receptors (PRRs), which recognize molecular structures common to large groups of microbes (Beutler, 2009; Takeuchi and Akira, 2010). PRRs are encoded in the germline and not subject to somatic variation, unlike the antigen receptors of T and B lymphocytes, which are somatically generated and clonally distributed. PRRs do not have the exquisite specificity of the T and B cell antigen receptors of adaptive immunity, but permit detection of a wide range of potential pathogens by a small number of receptor genes undergoing constant evolutionary selection. However, discussions of the innate and adaptive immune systems often present them as two coexisting systems, whereas, in reality, the sophisticated recognition systems of adaptive immunity are evolutionarily superimposed upon the innate immune system and are fully integrated with it. Thus, both T and B cells express multiple innate recognition receptors and many of the ultimate effector functions of adaptive responses utilize cells and molecules of the innate system.

Innate responses are typically more rapid than adaptive responses, with the generation of substantial host defense occurring within minutes to hours of infection, rather than days to weeks as is typical of adaptive responses. However, innate responses wane rapidly, the result of multiple mechanisms of feedback regulation, in order to limit the tissue damage that can result from these potent, relatively nonspecific effector mechanisms. Unlike T and B cell responses, innate responses typically do not lead to memory, meaning that a subsequent encounter with a microbial pattern does not produce a faster or more intense response than the initial one.

The past 20 years have seen a revolution in our understanding of the cells, receptors, and molecules that contribute to innate immunity and in the ways that the innate response directs the subsequent adaptive immune response (Fearon and Locksley, 1996; Iwasaki and Medzhitov, 2010). The list of agents that have been used to enhance the immunogenicity of vaccines and experimental antigens is extensive (Kenney and Cross, 2010; Pulendran et al., 2010). In this review, we will focus on two categories of adjuvants: established ones that are widely used clinically or experimentally and newer adjuvant candidates for which substantial mechanistic data are available. As additional innate pathways of immunity are discovered, novel adjuvants need be developed with a clear appreciation of the cellular and molecular events responsible for their activity and a reasonable hypothesis for the type of responses needed to confer protective immunity to the target pathogen. It is no longer sufficient to develop an adjuvant solely on empirical studies, forgoing a basic understanding of its mechanism of action until long after it has been approved for human use.

How Do Adjuvants Engage the Innate Immune System?

Adjuvants in widespread clinical or experimental use have long been regarded as either immunostimulatory agents or as passive depots or vehicles. Most immunostimulatory adjuvants are ligands for PRR, although some (not discussed in this review) act by providing a key component of the innate response (cytokines) or by stimulating an activation pathway directly, bypassing the innate receptor (toxins). However, it is now becoming clear that adjuvants once thought to act primarily as depots or formulations, such as alum and emulsions, trigger innate responses and these responses are central to their adjuvant activity (Maraskovsky et al., 2009; Marrack et al., 2009; McKee et al., 2010; Mosca et al., 2008). For these widely used adjuvants there are extensive efficacy data, and substantial human safety databases for vaccines with alum, MF59, AS03, and AS04. For this reason, it is important to define the innate receptors and pathways utilized by these existing, empirically derived adjuvants and to try to establish correlations between observed safety and efficacy and mechanisms of action.

Members of nearly all of the PRR families are potential targets for adjuvants. These include TLRs recognizing lipids, lipoproteins, nucleic acids, and proteins; NOD-like receptors (NLR, also defined as "nucleotide-binding domain and leucine-rich repeat containing" receptors) responding to multiple ligands such as peptidoglycan species, flagellin, toxins, and ATP; helicases (RIG-I-like receptors, RLR) triggered by cytoplasmic RNA; and C-type lectin receptors (CLRs) recognizing carbohydrates and lipids (Beutler, 2009; Iwasaki and Medzhitov, 2010; Kawai and Akira, 2010; Takeuchi and Akira, 2010). They signal through pathways involving distinct adaptor molecules and intermediates such as MyD88, TRIF, RIP2, CARD9, and IPS-1 that partially dictate the outcome of receptor-ligand interaction. Two key transcriptional programs involving the transcription factors NF-κB, IRF-3, and IRF-7 are activated by these signaling circuits, resulting in the induction of genes encoding cytokines, chemokines, and costimulatory molecules that play a key role in priming, expansion, and polarization of immune responses (O'Neill and Bowie, 2010).

Signaling pathways triggered by constituents of damaged or dying host cells can also contribute to the function of adjuvants. This process occurs in part through the inflammasome, a molecular complex that activates caspase 1, which in turn cleaves pro-interleukin-1 β (IL-1β) and pro-IL-18 into their bioactive forms (Martinon et al., 2009). The inflammasome complex is formed upon triggering of NLR such as NLRP3 and NLRC4. This can occur through recognition of microbial ligands such as flagellin or through indirect mechanisms such as host lysosomal damage resulting from the phagocytosis of crystalline particles (e.g., alum and uric acid). Necrotic cells release ATP and uric acid, which activate the NLRP3 inflammasome, thereby linking cellular damage to an inflammatory response (Hornung et al., 2008; Iyer et al., 2009).

Mechanism of Action of Empirically Derived Adjuvants

Freund's Adjuvant

Complete Freund's adjuvant (CFA) is a mixture of paraffin oil and surfactant with heat-killed *Mycobacterium tuberculosis* or *M. butryicum* in which aqueous antigen solutions are emulsified. Although CFA is unacceptable for human use, studies on the

mode of action of this potent adjuvant can provide useful lessons for vaccine design. Immunization with protein antigens in CFA results in strong Th1 and Th17 cell responses that are dependent on the mycobacterial component and require host MyD88 signaling (Shenderov et al., 2010; Su et al., 2005). Although mycobacteria contain potent TLR2, 4, and 9 ligands, signaling through IL-1R rather than TLR largely explains the requirement for MyD88 in the enhancement of T cell responses. As might be predicted, the inflammasome is also necessary for processing of the IL-1 that triggers this IL-1R-mediated pathway (Shenderov et al., 2010). In contrast, the effects of CFA on the humoral response are inflammasome independent and the requirement for MyD88- or TRIF-dependent signaling pathways varies in different experimental models (Eisenbarth et al., 2008; Gavin et al., 2006). At present, the non-TLR PRR and ligands involved in the induction of pro-IL-1β and the inflammasome activity required for its processing are not clearly defined. One mycobacterial component with potent adjuvant activity is trehalose dimycolate (cord factor) recognized by Mincle, a CLR that signals through the Syk kinase-CARD9 pathway (Ishikawa et al., 2009; Schoenen et al., 2010). A synthetic ligand for Mincle formulated in liposomes (CAF01) shows promise as an adjuvant for tuberculosis vaccines (Gram et al., 2009). Mycobacterial peptidoglycan components previously shown to stimulate NOD receptors are also important candidates (Fritz et al., 2007). In addition, IL-12 p40 induction is also required for the Th1 cell polarizing effects of CFA, and this activity probably depends on a series of redundant signals delivered by TLR, NLR, and CLR.

Though the mycobacterial component of CFA plays a major role in the stimulation of cell-mediated immunity, emulsification of antigens in paraffin oil or surfactant alone (i.e., incomplete Freund's adjuvant; Montanide) can sustantially boost antibody responses. The mechanism of action of oil emulsion adjuvants is poorly understood, although one study suggested a partial requirement for NOD2 (Moreira et al., 2008). Nevertheless, because these emulsions are likely to cause cellular damage upon injection, it is tempting to speculate that endogenous signals released during necrotic cell death may also contribute to their adjuvant activity.

Aluminum Salts

The clinically approved alum adjuvants consist of precipitates of aluminum phosphate and aluminum hydroxide to which antigens are adsorbed. Although traditionally thought to function primarily by forming a long-lasting depot for antigen and by promoting their uptake by antigen-presenting cells (APCs), it is now clear that innate immune stimulation plays a primary role in the adjuvant activity of alum (Lambrecht et al., 2009; Marrack et al., 2009). Alum is used primarily to enhance antibody production and does not utilize TLR for its function in vivo (Gavin et al., 2006). In humans, responses to proteins with alum tend to be a mix of Th2 and Th1 cells (Didierlaurent et al., 2009); however, in mice alum induces a profoundly polarized Th2 cell response, with Th2 cell-dependent antibody isotypes, to nearly all protein antigens. Studies in vitro employing macrophages and DCs have demonstrated that, after lipopolysaccharide (LPS) priming, alum can activate the NLRP3 inflammasome to produce mature IL-1β (Li et al., 2007). This process appears to involve phagocytosis of alum crystals and lysosomal release of cathepsin B into the cytoplasm, where the enzyme localizes at the site of caspase-1-associated inflammasome activity (Hornung et al., 2008). Although the data supporting NLRP3 inflammasome triggering by alum in vitro are compelling, there is considerable controversy surrounding the role of this pathway in the adjuvant activity of alum in vivo (Lambrecht et al., 2009; Marrack et al., 2009). These conflicting findings may relate to the different alum-antigen formulations and/or immunization protocols used by the different laboratories involved. Whether the inflammasome-caspase-1-dependent processing of IL-1 and IL-18 plays a role in the strong Th2 cell polarization triggered by alum in mice is also not clear. Stimulation of the Th2 cell-promoting cytokines IL-4, IL-6, and IL-25 from innate cells by alum has been proposed as an alternative explanation for the strong Th2 cell polarization observed in mice (Lambrecht et al., 2009; Marrack et al., 2009; Serre et al., 2008).

In addition to inflammasome activation by alum itself, the adjuvant can also trigger necrotic cell death and the release of the endogenous danger signal uric acid. Indeed, injection of uricase has been shown to block the immunopotentiating effect of alum administered by the intraperitoneal route (Kool et al., 2008; Lambrecht et al., 2009). The current controversies concerning the mechanism of action of alum adjuvants underscore the need to determine which subset of the innate responses provoked by an adjuvant are specifically required for enhanced antibody or T cell responses.

Oil-in-Water Emulsions: MF59 and AS03

MF59 (Novartis) and AS03 (GlaxoSmithKline) are both oil-in-water emulsions based on squalene, an oil that is more readily metabolized than the paraffin oil used in Freund's adjuvants. MF59 is licensed in most of Europe for use with seasonal flu vaccines in the elderly, and both are used in approved pandemic flu vaccines. As a result, there are considerable human data comparing flu vaccination with these adjuvants to the same vaccine without adjuvant or with alum (Mbow et al., 2010). These emulsions stimulate stronger antibody responses, permit fewer doses and antigen dose sparing, and generate marked memory responses, with a mixed Th1-Th2 cell phenotype (Ott et al., 1995). MF59 induces substantial local stimulation, recruitment of DCs, granulocytes, and differentiation of monocytes into DCs (Seubert et al., 2008), as well as increased uptake of antigen by DCs (Dupuis et al., 1998). Intramuscular injection of MF59 leads to a pattern of induced genes that is both larger and distinct from that induced by either alum or a TLR9 agonist (Mosca et al., 2008).

Saponin-Based Adjuvants, ISCOMs

Immunostimulatory complexes (ISCOMs) are cagelike nanoparticles composed of saponins purified from the bark of a South American tree, *Quillaja saponaria*, formulated with cholesterol, phospholipid, and antigen. Vaccine antigens need not be incorporated into the particles, and most current applications use a mixture of soluble antigens and the antigen-free particle, such as ISCOMATRIX. ISCOMs do not act through any identified PRR; however, they enhance antigen uptake and prolong retention by DCs in draining lymph nodes, induce activation of DCs, and lead to strong antibody and T cell responses (Maraskovsky et al., 2009). Although ISCOMs are potent enhancers of Th cells, they do not impose a bias to either a Th1 or Th2 cell response. Unlike most other adjuvants, ISCOMs enable substantial MHC

class I presentation and induce both $CD8^+$ and $CD4^+$ T cell responses to a variety of soluble protein antigens in man (Davis et al., 2004) and experimental animals. ISCOMs appear to destabilize the endosomal membrane, allowing greater cytoplasmic access for codelivered antigens compared to other forms of antigen delivery (Schnurr et al., 2009). A heterogenous fraction of saponins, Quil A, is widely used for veterinary vaccines, and a highly purified species, QS-21, is currently being tested in human studies with several vaccine candidates.

Adjuvants Targeting Pattern Recognition Receptors

In contrast to the complex and still incompletely understood adjuvants described above, an increasing focus has been to use natural ligands or synthetic agonists for well-defined PRRs as adjuvants, either alone or with various formulations. A number of these are now in clinical or late preclinical stages of development for multiple applications and have been the subject of research to clarify the basis of their adjuvant activity.

TLR3 and RLR Ligands

The discovery that double-stranded viral RNA (dsRNA) is a potent activator of innate immunity was a seminal finding for understanding host immunity against viral infection (Alexopoulou et al., 2001). Synthetic analogs of dsRNA (i.e., Poly IC) have been used as adjuvants (Longhi et al., 2009; Stahl-Hennig et al., 2009; Trumpfheller et al., 2008) and can act through two distinct types of PRRs. Viral or synthetic dsRNA activates TLR3 in endosomes (Alexopoulou et al., 2001) or through cytosolic ribonucleic acid (RNA) helicases (RLR), such as retinoic acid-inducible gene-1 (RIG-I) and melanoma differentiation associated gene 5 (MDA5) (Kato et al., 2006). TLR3 mediates its effects through the adaptor TRIF (Alexopoulou et al., 2001), whereas RLR signal through the adaptor IFN-B promoter stimulatory-1 (Kato et al., 2006).

TLR3 activation in DCs induces IL-12 and type I IFN and improves MHC class II expression and cross-presentation (Davey et al., 2010; Jongbloed et al., 2010; Kadowaki et al., 2001; Lore et al., 2003; Poulin et al., 2010; Schulz et al., 2005; Wang et al., 2010). Stimulation of MDA-5, most notably from non-hematopoietic cells (Longhi et al., 2009; Wang et al., 2010), strongly enhances production of type I IFNs. Type I IFNs play a critical role in enhancing T and B cell immunity with dsRNA through a variety of mechanisms that include activation of DCs, NK cells, and direct effects on T cells (Blanco et al., 2001; Le Bon et al., 2006; Longhi et al., 2009). Several synthetic analogs of dsRNA (Poly IC, Poly ICLC, and Poly $IC_{12}U$) have been used as adjuvants with soluble proteins, DC targeting constructs, or inactivated viral vaccines (Gowen et al., 2007; Stahl-Hennig et al., 2009; Trumpfheller et al., 2008). Poly IC activates both TLR3 and MDA, whereas Poly IU signals through TLR3 only. Activation of both TLR3 and MDA5 optimizes the magnitude and durability of Th1 cell immunity and $CD8^+$ T cell immunity compared to either pathway alone. This highlights a central feature of the potency of Poly IC by inducing TLR3 activation of DCs directly and type I IFNs through MDA-5 (Longhi et al., 2009).

The formulation of Poly IC has a critical influence on its potency. Thus, long dsRNA is required to activate MDA-5 (Kato et al., 2008). Furthermore, complexing Poly IC with poly-L-lysine and carboxymethylcellose (poly ICLC) prolongs the adjuvant effect in vivo (Levy et al., 1975; Stahl-Hennig et al., 2009). Collectively, an optimally formulated Poly IC is an effective adjuvant for inducing broad-based adaptive immunity through both TLR and RLR signaling pathways.

TLR4 Ligands

Bacterial lipopolysaccharides have long been recognized as potent adjuvants, but their pyrogenic activity has precluded use as an adjuvant in man. Pioneering work from Ribi (Qureshi et al., 1982) led to the development of less toxic preparations of LPS, and ultimately to the substantially detoxified derivative monophosphoryl lipid A (MPL). MPL, principally formulated with antigens and alum, is now a component of licensed vaccines for HBV and papilloma and has proven to be both safe and effective (Casella and Mitchell, 2008). Both LPS and MPL are recognized specifically by TLR4, but MPL leads to signaling only through the TRIF adaptor, whereas LPS leads to TLR4 activation through both the TRIF and MyD88 pathways (Mata-Haro et al., 2007), the latter pathway resulting in high levels of many inflammatory cytokines, prominently TNF-α. MPL formulated on alum (AS04) stimulates a polarized Th1 cell response in contrast to the mixed Th1-Th2 cell response of alum alone (Casella and Mitchell, 2008; Didierlaurent et al., 2009). Much of the adjuvant activity of this mixture can be attributed to the MPL component, although alum helps prolong stimulation by MPL (Didierlaurent et al., 2009).

TLR5 Ligands

Bacterial flagellin has long been known to be a potent T cell-independent antigen, but the finding that flagellin from many species was a ligand for TLR5 suggested its potential as an adjuvant. Although flagellin itself can be an adjuvant when mixed with antigens, current application is primarily by generation of fusion proteins of recombinant vaccine antigens and flagellin (Huleatt et al., 2007). Unlike many other TLR agonists, flagellin tends to produce mixed Th1 and Th2 cell responses rather than strongly polarized Th1 cell patterns (Huleatt et al., 2007). Antibody production to fusion proteins requires TLR5 expression (McDonald et al., 2007), but optimum adjuvant effect in mice requires expression of the TLR signaling adaptor MyD88 in both hematopoietic and nonhematopoeitic (radioresistant) cell types (Sanders et al., 2008). Bacterial flagellins can also signal through inflammasomes that contain Nlrc4 (also known as IPAF) (Miao and Warren, 2010), although it is not known whether this pathway contributes to the adjuvant activity of flagellin.

TLR7 and TLR8 Ligands

Guanosine- and uridine-rich ssRNA were first identified as natural agonists for TLR7 and 8 (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). Because ssRNA is rapidly degraded by extracellular RNases, using it as an adjuvant without substantial modification or formulation is unpromising. However, a number of small synthetic compounds originally developed as type I IFN inducers, including imidazoquinolines (Imiquimod, TLR7 and Resiquimod, TLR7-TLR8) and guanosine and adenosine analogs, have been shown to activate TLR7, TLR8, or both (Gorden et al., 2005; Heil et al., 2003; Hemmi et al., 2002). TLR7 and TLR8 are expressed in endosomes, but not on the cell surface, and both mediate their effects through MyD88-dependent signaling (Hemmi et al., 2002).

Important differences exist between mice and humans with regard to tissue expression and function of TLR7 and TLR8. In both species, TLR7 is expressed in B cells, neutrophils, and plasmacytoid DCs (pDCs); however, in mice TLR7 is expressed by

macrophages and $CD8^-$, but not $CD8^+$, DC subsets (Iwasaki and Medzhitov, 2004). TLR8, in contrast, is expressed by monocyte lineage cells and myeloid DCs in man, whereas it may not be a functional receptor in mice (Jurk et al., 2002). Activation of TLR7 and TLR8 in human pDCs and mDCs, respectively, increases the expression of costimulatory molecules and production of type I IFN and IL-12 (Jarrossay et al., 2001; Kadowaki et al., 2001; Lore et al., 2003). A bispecific TLR7-TLR8 agonist may be more effective than a monospecific agonist by activating multiple DC subsets and B cells to induce cytokines optimal for Th1 cell immunity, cross-presentation, and antibody production. Small TLR7 or 8 agonists are not very effective as adjuvants when simply mixed with antigens, but can be substantially improved by formulation with or conjugation to the antigen (Wille-Reece et al., 2005, 2006; Wu et al., 2007).

TLR9—CpG-ODN and Formulated DNA

TLR9 is the only endosomal PRR specific for DNA and mediates a potent innate response to bacterial and viral DNA (Blasius and Beutler, 2010). Sequence motifs containing the CpG dinucleotide are preferentially recognized; however, specific base sequences only partly account for TLR9 binding. The sugar-phosphate backbone is also integral to recognition by TLR9 (Haas et al., 2008). Synthetic 18–25 base oligodeoxynucleotides (ODN) with optimized CpG motifs (CpG-ODN) have been studied extensively as adjuvants, either soluble or formulated in nanoparticles (Marshall et al., 2004) or virus-like particles (Jennings and Bachmann, 2009). CpG-ODN enhance antibody responses and strongly polarize Th cell responses to Th1 and away from Th2 cell responses (Kobayashi et al., 1999; Tighe et al., 2000). TLR9 has a relatively restricted cellular distribution, especially in man, with the two major APC types being B cells and pDCs (Campbell et al., 2009). Studies with a DC-specific deletion of TLR signaling in mice indicate that DC recognition is much more important for the antibody-enhancing activity of CpG-ODN than B cell expression (Hou et al., 2008). However, in primates, myeloid DCs, thought to be the principal antigen-presenting DCs, are TLR9 negative, suggesting either that activated PDC are sufficient for the adjuvant effect of CpG-ODN or that myeloid DCs become activated in the lymph node by indirect means (Teleshova et al., 2006).

What Have We Learned from Studies of Vaccines and Adjuvants?

Codelivery of Antigens and PRR Ligands Enhances Effectiveness

The immune system is optimized to generate adaptive responses to microbial antigens delivered to APCs in intimate association with PRR ligands, as would be the case for viral and microbial infections and live attenuated vaccines. For subunit vaccine candidates, codelivery has been accomplished by covalent coupling of TLR7-TLR8 (Wille-Reece et al., 2005; Wu et al., 2007) and TLR9 (Tighe et al., 2000) to purified proteins or by constructing recombinant fusion proteins consisting of antigen and the TLR5 ligand flagellin (Huleatt et al., 2007). In these examples, the potency of the linked vaccine is 10–100 times that of a comparable mix of separate components. In the case of CpG-ODN conjugates, coupling of an ODN enhances antigen uptake and cross-presentation in DCs, although the enhanced uptake is not TLR9 dependent (Heit et al., 2003). Co-delivery of antigens and PRR ligands can also be accomplished by association—covalent or noncovalent—of both within a larger particulate structure. Examples include virus-like particles (Jennings and Bachmann, 2009) and synthetic nano- and microparticles (O'Hagan and De Gregorio, 2009).

The enhanced efficiency of this codelivery may be simply quantitative—uptake of enough linked antigen for effective presentation will inherently provide a stimulatory amount of the linked PRR ligand, and enhanced uptake would lead to preferential presentation of the linked antigen. However, codelivery may also lead to preferential handling of antigens associated with PRR ligands, by facilitating antigen presentation at the level of individual lysosomes (Iwasaki and Medzhitov, 2010). A number of vaccine candidates with this strategy have reached early stage clinical studies, and this represents one of the most promising new directions in vaccine development.

Adjuvants Can Work through Both Direct and Indirect Actions on APCs

A critical scientific and practical aspect of rational vaccine design is whether DCs presenting antigen need to be synchronously activated for optimal antigen presentation and effective Th1 and $CD8^+$ T cell priming. Several key studies (Blander and Medzhitov, 2006; Joffre et al., 2009; Sporri and Reis e Sousa, 2005) have shown that TLR activation in the same DC presenting the antigen is critical for $CD4^+$ T cell activation and Th1 cell differentiation. Moreover, mouse (Bedoui et al., 2009; Schulz et al., 2005) or human (Jongbloed et al., 2010; Poulin et al., 2010) DC subsets specialized for cross-presentation show increased $CD8^+$ T cell immunity when Poly IC is used to activate cells through TLR3. However, more recent evidence shows production of type I IFNs from DCs or nonhematopoietic stromal cells not presenting the antigen can profoundly influence Th1 (Hou et al., 2008) and $CD8^+$ T cell immunity (Longhi et al., 2009; Wang et al., 2010). Induction of these type I IFNs can result from TLR-dependent activation of pDCs or MyD88-independent production from non-DCs (Hou et al., 2008) or through MDA-5 from nonhematopoietic stromal cells (Longhi et al., 2009; Trumpfheller et al., 2008; Wang et al., 2010). Overall, codelivery of antigen and adjuvant to the same DC and a bystander production of type I IFN may be required for optimizing T cell immunity. Thus, Poly IC and TLR7-TLR8, which induce an optimal cytokine milieu (IL-12 and type I IFN-α) and are able to directly activate DC subsets specialized for CD4 activation and cross-presentation, offer great promise as adjuvants (Figure 1).

Multiple Innate Stimuli Can Be Better than One

One important lesson from studies of live attenuated vaccines is that activation of multiple innate receptors may be more effective than activation of a single pathway (Querec et al., 2006). This is logical, because redundant pathways of innate responsiveness would increase the likelihood of dealing successfully with an infection via a limited number of PRRs. Studies in vitro with defined combinations of TLR ligands support this idea (Trinchieri and Sher, 2007) and suggest combinations that may be especially useful for adjuvants. The very effective adjuvant systems developed by GlaxoSmithKline take this approach, combining MPL and alum (AS04) or MPL, QS-21, and either oil-in-water emulsion (AS02) or liposomes (AS01), and many more combinations are in late preclinical or early clinical stages of development.

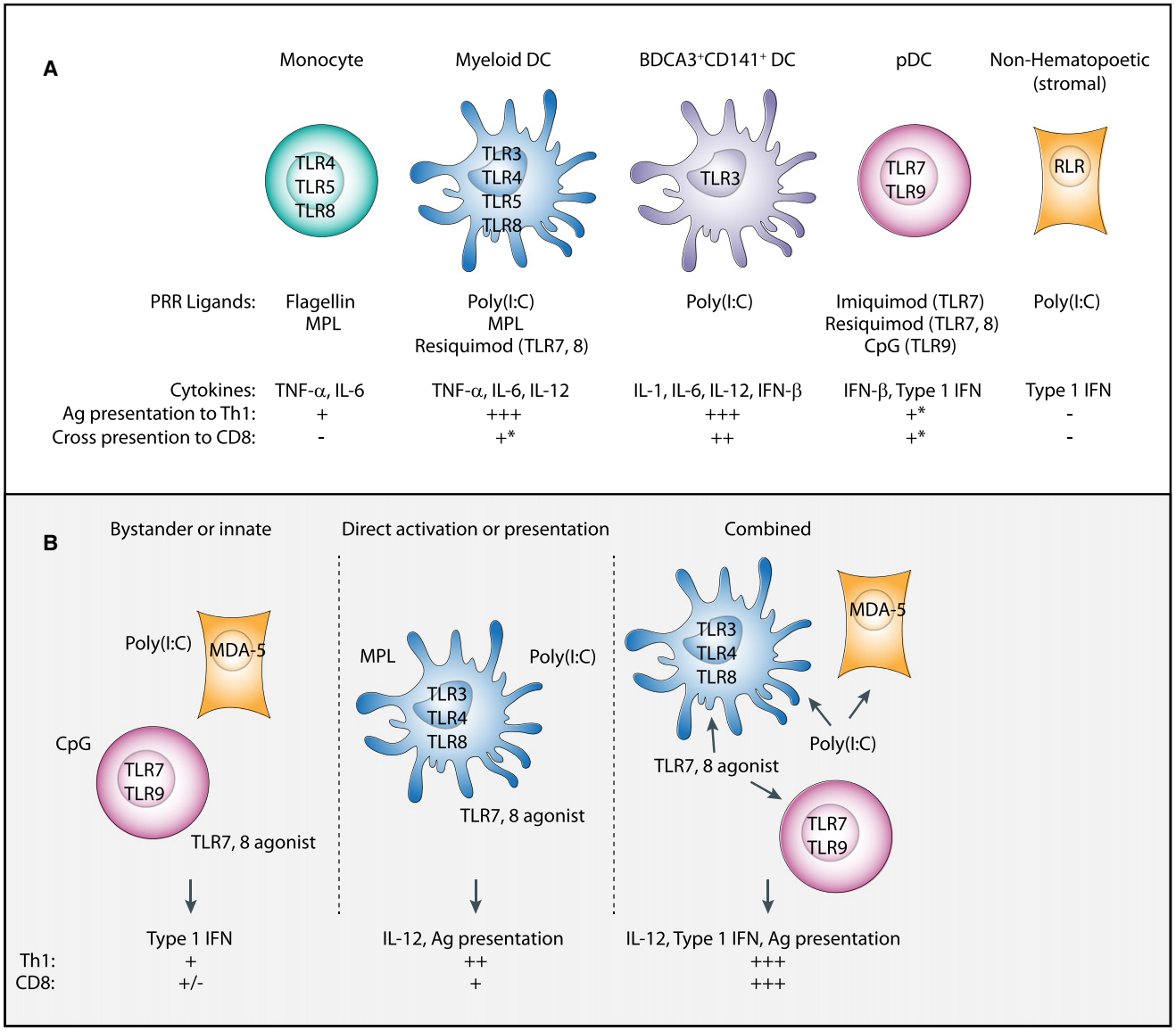

Figure 1. PRR Ligands Shape Adaptive T Cell Immunity through Direct and Indirect Effects on Dendritic Cells

(A) Expression of PRRs in human antigen-presenting and nonhematopoietic cells. The ability of the corresponding PRR ligands to produce cytokines and induce Th1 cell and cross-presentation from such cells is depicted below. MPL-based adjuvants (ASO1, ASO2, ASO4) activate monocyte and myeloid DCs, whereas TLR7 (Imiquimod) and TLR9 (CpG) ligands activate pDCs. Such adjuvants induce Th1 cell and low-level $CD8^+$ T cell responses. In humans, it is not clear whether these cells contribute to cross-priming in vivo.

(B) The influence of direct innate activation and antigen presentation, bystander innate immunity on antigen presentation, or both together are shown. The relative potency of Th1 cell and $CD8^+$ T cell immunity from these respective pathways are derived from in vivo mouse studies. Optimal Th1 cell and $CD8^+$ T cell immunity is elicited by direct activation of antigen-presenting cells and bystander production of type I IFN. This can be achieved by using Poly IC and TLR7-TLR8 ligands as adjuvants, with the source of type I IFN derived from nonhematopoetic and pDCs, respectively.

Formulations and PRR Ligands Can Be Combined to Develop the Most Appropriate Response

Rational vaccine design is guided by understanding the immune correlates of protection and then selecting vaccines or adjuvants to elicit such responses. In Table 1, the type of adaptive immune responses induced in mice and/or humans with various adjuvants and formulations are shown. The clearest example in man of how alterations in vaccine formulation improve immunogenicity and protection is the RTS,S vaccine. RTS,S is a complex formulation comprised of a circumsporozoite protein and hepatitis B surface antigen fusion protein. Initial studies showed that RTS,S administered with alum elicited circumsporozoite-specific antibody, but did not confer protection (Stoute et al., 1997). However, when RTS,S is mixed with the TLR4 ligand, MPL, QS-21, and oil-in-water (AS02A) or liposome (AS01B) formulation, there is increased antibody production and induction of Th1 cell immunity with ~30%–50% protection (Kester et al., 2009). Moreover, both Th1 cell

and humoral immunity are increased with AS01B compared to AS02A.

Animal Models and In Vitro Systems Have Important Limitations

The most widely used tools for both preclinical evaluation and mechanistic studies of adjuvants are activation of antigen-presenting cells in vitro and immunity in experimental rodent models. As noted in previous sections, there can be substantial discordance in the responses of rodents and humans to complex adjuvants or defined PRR ligands. Differences in the cellular expression patterns of PRR can often account for this, as illustrated by the functional consequences of distinct rodent and human patterns of TLR9 expression (Campbell et al., 2009). Many studies defining receptors and pathways of adjuvant activity employ genetically modified mice; however, the experimental conditions used often do not reflect the practice of human vaccination. In particular, many such mouse studies use intraperitoneal or intravenous injection rather than the subcutaneous or intramuscular routes used in clinical application. Different routes can lead to differential antigen presentation by specific DC subsets.

For example, dermal DCs and Langerhans cells play distinctive roles in adaptive immunity (Klechevsky et al., 2008). In addition, antigen doses used in mice are frequently higher that would be used for a typical recombinant vaccine in humans, and variables such timing and experimental readout can differ between mouse and human studies. The contribution of these variables to the interpretation of mechanistic experiments in mice has not been carefully examined. However, the choice of the experimental antigen itself can clearly affect the outcome of such studies. The dependence of several adjuvants on MyD88- and TRIF-linked signaling pathways can be quite different between studies with haptenated versus those with unmodified protein antigens (Palm and Medzhitov, 2009). Ovalbumin, an antigen widely used in mouse studies, tends to be cross-presented more readily than most other proteins. Hence the impressive cross-priming of $CD8^+$ T cells with ovalbumin may not necessarily represent results obtainable with more typical vaccine antigens.

Similarly, studies with blood cells in vitro have substantial limitations in evaluating adjuvants. Cell cultures are not useful for studying formulations that rely on inflammatory cytokine responses from noncirculating tissue cells. Adjuvants that act in part by altering the anatomical distribution of antigen or its persistence at the injection site may likewise not be easily studied in cell cultures.

An important consideration in selecting adjuvants for clinical use is to establish a model that will be predictive for responses in humans. Although humanized mice can be used to assess the effectiveness of adjuvants on innate immunity, such mice may be limited at present to assess adaptive responses over a prolonged period of time. A second tool is to develop mice that have similar cell-specific expression of innate signaling pathways as humans. The remaining alternative is to use non-human primates (NHP). Because NHP are more similar to humans than are mice with regard to DC subsets and PRR expression, they may offer a useful alternative for evaluating both potency and mechanism of novel adjuvants. The ability to do more invasive sampling and to evaluate adjuvants that have not yet met the regulatory requirements for testing in humans makes mechanistic studies in non-human primates an excellent bridge between rodents and humans. The major issue for using NHP is the availability of animals and the high cost of maintaining them, which often limits the size of animal groups.

Innate Immunity and Adjuvant Safety

The adoption of new adjuvants into licensed vaccines has been slowed by a variety of hypothetical safety concerns, especially the possibility of an increased risk of autoimmune disease. These concerns are based in part on two sets of observations. Infections can trigger or exacerbate some autoimmune diseases, and this can often be tied to elements of the innate response. For example, type 1 IFNs are important in the pathogenesis of lupus, and disease flares are often triggered by viral infections (Zandman-Goddard and Shoenfeld, 2005). Second, PRR ligands are capable of breaking tolerance in animal models, for example, by overcoming inhibition by regulatory T cells (Pasare and Medzhitov, 2003). Repeated injection with IFN-inducing PRR ligands can also enhance the growth and pathogenicity of *M. tuberculosis* in mouse models (Antonelli et al., 2010).

Consideration of several important differences between live infections and adjuvanted subunit vaccines can put these theoretical concerns in perspective. Innate immune stimulation with nonliving vaccines is short-lived and focused on a local injection site and its draining lymphatic. Second, adjuvants are engineered to enhance the response to immunogenic non-self antigens and few, if any, provide all of the activities needed to render a self-antigen sufficiently immunogenic to trigger autoimmunity, even if autoreactive T cells are present. Perhaps the most compelling argument is the fact that many of the most widely used and safest vaccines—the live, attenuated viral and bacterial vaccines—rely on activation through multiple PRR, yet have not been linked to an increased risk of any autoimmune disease. Similarly, the large human safety databases obtained with vaccines using either MF59 (Pellegrini et al., 2009) or AS04 (Verstraeten et al., 2008), both approved for human use in multiple countries, as well as more limited experience with several advanced experimental vaccines, have failed to support an increase in autoimmune or infectious diseases with these newer adjuvants.

Conclusion

Adjuvants have long been of great interest to vaccine developers but considered a necessary, if uninteresting, convenience to basic immunologists. Advances of the past decade in understanding innate immunity have brought a wider interest in understanding how existing adjuvants work and how they might be improved. All adjuvants appear to stimulate components of the innate immune system, but the diversity of mechanisms used by even a short list of well-studied adjuvants is impressive. Adjuvants currently used in humans enhance humoral immunity, but many new adjuvants in clinical or preclinical development are focused on enhancing specific types of T cell responses and generating the multifaceted immune responses that may be needed for challenging diseases such as malaria and HIV-AIDS. Although well-defined ligands for PRR have attracted most of the attention, it is clear that strategies for formulation and delivery of subunit vaccines can profoundly influence T cell immunity, most

notably by facilitating cross-presention of antigen by DCs. Along the path of development of new vaccines and adjuvants lies an unparalleled opportunity to study the immune responses of large populations of basically healthy humans. No other form of defined ''experimental'' challenge can be as easily and ethically given to humans, and mechanistic studies incorporated as part of the clinical development of new adjuvants can teach us a great deal about the human immune system.

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