TLR, NLR and other modulators as infectious disease vaccine adjuvants

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Abstract

Vaccines based on attenuated or killed viruses and bacteria are highly effective in preventing infection with a range of pathogens, but can have safety issues. Therefore, there is a move towards subunit vaccines based on recombinant proteins or naked DNA. However, protein subunit vaccines are typically poorly immunogenic when administered alone and therefore require co-administration with adjuvants to boost the immune response. For many decades there was very little progress in understanding the mechanism of action of adjuvants, but recently there have been a number of significant breakthroughs in this area. The binding of pathogen-derived molecules to different immune sensors, including Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors (NLR) and retinoic acid-inducible protein-1-like receptors (RLR), activate important innate immune pathways and provide us with not only an understanding of how current vaccines and adjuvants work, but also provide potential targets for novel adjuvant development.

Introduction

Vaccination has been an extremely powerful tool for preventing infectious diseases. Eradication of smallpox and the dramatic reduction in polio and measles throughout the world represent the most significant successes of vaccination to date. However, infectious diseases remain a leading cause of death worldwide. Traditional vaccines have mainly consisted of live attenuated pathogens, whole inactivated organisms or inactivated bacterial toxins. The development of more advanced and effective vaccines to combat infectious diseases, such as influenza, HIV, tuberculosis and malaria is a major goal of modern medical research. However, these new generation vaccines are usually based on recombinant proteins, and although safer, they are often less immunogenic than traditional attenuated or

killed vaccines. To elicit a robust immune response, protein subunit vaccines typically need a boost from an adjuvant, a substance that derives its name from the Latin '*adjuvare*' meaning 'to help'. A successful vaccine, therefore, should not only contain protective antigen(s), but also a good adjuvant that can effectively amplify the protective immune responses.

Despite the fact that alum and oil-in-water emulsions have been used for decades as human vaccine adjuvants, their mechanism of action had remained unclear. It was reported that these adjuvants acted by increasing antigen availability and uptake by immune cells, but their underlying molecular mode of action was not understood. Janeway famously referred to adjuvants as 'immunologist's dirty little secret' [1]. Janeway's hypothesis on the innate immune sensory system, and the intense research efforts invested into dissecting the molecular basis of innate immune activation, has shed much light on how adjuvants shape the quality and quantity of the immune response to foreign antigens [2].

Induction of protective immunity by vaccination

The induction of a protective immune response to a pathogen relies on a complex interplay of the cells and molecules of the innate and adaptive immune systems. Dendritic cells (DC) play a crucial role in linking the innate and adaptive immune systems. DC and other innate immune cells express pattern recognition receptors (PRRs) that have broad specificity capable of detecting common structural motifs or pathogen associated molecular patterns (PAMPs) (Figure 1). PAMPs derived from different classes of pathogens bind to diverse families of PRRs that include toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), retinoic acid-inducible gene (RIG)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These interactions decode pathogen information by triggering distinct signalling pathways to differentially activate APCs, thereby directing the adaptive effector response in a manner that is specifically tailored to the

invading microbe [2]. The recently obtained wealth of information about the innate immune system, has not only provided us with a greater appreciation of how existing adjuvants function but also provides a new set of targets for the rational development of novel adjuvants.

Vaccines based on live attenuated pathogens and inactivated whole pathogens have been extremely successful in preventing a large number of common infectious diseases. The potent immunogenicity of such vaccines can be attributed to the presence of endogenous adjuvants, namely, the high content of PAMPs, such as LPS, CpG and peptidoglycans [3,4].

Toll-like receptors

TLRs represent a family of evolutionary conserved PRRs. TLRs are type I transmembrane receptors that are characterised by leucine rich repeats (LRRs) in the extracellular portion and an intracellular Toll/IL-1 receptor (TIR) domain, which is homologous to the intracellular domain of IL-1 receptor family members [5]. Since their discovery, it has been established that TLRs play important roles in recognising specific microbial components derived from pathogens, including bacteria, fungi, protozoa and viruses. To date, ten functional TLRs have been described in humans, many of which are widely expressed by different cell types of the immune system, including DC, macrophages, NK cells, mast cells, neutrophils, B cells, T cells and non-immune cells, such as fibroblasts and epithelial cells. TLRs can be divided into subfamilies primarily recognizing similar PAMPs; TLR1, TLR2, TLR4 and TLR6 recognise lipid structures, whereas TLR3, TLR7, TLR8 and TLR9 recognise nucleic acids. Most TLRs (TLR1, 2, 4, 5, 6, and 10) are expressed on the cell surface, whereas other TLRs (TLR3, 7, 8 and 9) are present within endosomal compartments. Furthermore, TLRs are expressed as homodimers or heterodimers (TLR2 + TLR1 or TLR2 + TLR6), and each TLR recognises distinct microbial stimuli. TLRs also differ in their cellular distribution and the intracellular

signals they trigger. All signalling pathways triggered by TLRs utilise the adaptor protein, MyD88 with the exception of TLR3 which utilises TRIF. LPS binding to TLR4 activates both MyD88 and TRIF.

The TLR4 agonist, LPS was recognized as an adjuvant, capable of driving antibody responses to mixed protein antigens as early as 1955 [6], but is too toxic for use as an adjuvant for human vaccines. Monophosphoryl lipid A (MPL) is a chemically modified derivative of lipid A moiety of LPS and is considerably less toxic, but has similar immunostimulatory activity. MPL was the first TLR ligand approved for human use in the Hepatitis B vaccine, Fendrix® [7]. The formulation strategy employed appears to be critical in directing the type of immune response elicited by MPL. Although MPL aqueous formulation enhances antibody responses, MPL in oil formulation are more effective at stimulating T cell responses [8]. Many of the adjuvant systems developed by GlaxoSmithKline Biologicals (GSK) incorporate MPL. AS02 is an oil-in-water emulsion, containing MPL and QS-21 (a saponin derived immunostimulator) that induces strong antibody and Th1 responses. AS02 is being evaluated in clinical trials in vaccines against malaria, HPV, HBV, tuberculosis and HIV [9-12]. AS01 is a liposomal formulation containing MPL that induces potent humoral and cell mediated responses, including CTL responses and is being evaluated in clinical trials of vaccine against malaria.

An exciting new development in the adjuvant field was the recent announcement that the FDA has approved Cervarix, a vaccine against human papillomavirus (HPV), which causes cervical cancer. The vaccine is composed of recombinant capsid proteins from types 16 and 18 HPV as virus-like particles, with a combination of aluminium hydroxide and MPL (AS04) as the adjuvant. Cervarix had previously won EU approval in 2007, but was the first vaccine licensed by the FDA that includes a TLR agonist as an adjuvant component. A recent study has report a good safety profile for the AS04 adjuvant in over 68,000 individuals [13].

The oil-in-water emulsion adjuvant, MF59 from Novartis has been included in an EU licensed influenza vaccine (Fluad[®]) for more than 10 years, has been administered to more than 50 million people and has accumulated a significant safety database [14]. MF59 has been shown to be more potent for both antibody and T-cell responses than aluminum-based adjuvants [14]. Recently, MF59 has been shown to be an effective delivery system for a synthetic TLR4 agonist (E6020) in modulating the immune response to a subunit influenza vaccine [15]. Combining adjuvants like E6020 and MF59 allowed selective induction of the immune responses, in particular the induction of Th1 cells, which are important in protection against intracellular pathogens, such as influenza vaccines. It has been shown that MF59 activates DC, but its exact mechanism of action and possible involvement of PRR is currently unknown [16]. Mosca *et al.* demonstrated that MF59 together with CpG and alum activated innate immunity at the site of injection [17]. Using microarray analysis, the early genes induced in gluteus muscle of mice was examined by different adjuvant formulations. The cluster of genes modulated by all adjuvants, named ‘adjuvant core response genes’, were characterised by the upregulation of cytokines (IL-1 β and IL-2), chemokines (such as CCL2, CCL12 and CXCL10) and adhesion molecules, suggesting the establishment of an immunocompetent environment at the site of injection [17].

TLR3

It has been demonstrated poly I:C, a synthetic analog of double stranded RNA and an agonist for TLR3 and MDA5, has mucosal activity when co-administered intranasally with an inactivated influenza vaccine. It enhanced mucosal and systemic humoral responses, resulting in complete protection against homologous and heterologous influenza viruses, including the highly pathogenic H5N1 avian influenza virus [18]. The adjuvant effects of poly I:C require co-operative activation of TLR3 and cytoplasmic RNA helicase MDA5 pathways [19].

PolyI:polyC12U (Ampligen®), which is similar to poly I:C, has been reported to have a low incidence of clinical toxicity [20]. To date, more than 75,000 doses of Ampligen have been administered to humans and have been generally well tolerated [18].

TLR5

Flagellin, the protein component of bacterial flagella which aids bacterial mobility, is an agonist for TLR5 and is a potent adjuvant in mice, as well as cynomolgus and African green monkeys [21-23]. *Pseudomonas aeruginosa*, a Gram negative bacterium and opportunistic pathogen is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. A Phase III clinical trial of *P. aeruginosa* flagellins in CF patients demonstrated that a vaccine containing flagella subtype antigens a₀, a₁, a₂ and b was well tolerated and caused a 30% reduction in the incidence of infection [24].

TLR7/8

Single stranded RNA is a ligand for mouse TLR7 and human TLR8 [25]. Synthetic compounds that bind to TLR7/8 induce activation and maturation of DC. The small molecule nucleotide analogues imiquimod and resiquimod are ligands for TLR7 and TLR7/8 respectively [26]. Several synthetic imidazoquinolines have been shown to have potent antiviral and antitumour effects due to their ability to induce inflammatory cytokines, especially IFN- α . One of these imidazoquinoline compounds, Imiquimod, has been approved for the treatment of certain cancers and for human papillomavirus infection [27].

TLR9

Bacterial DNA, which contains unmethylated CpG dinucleotides, is a ligand for TLR9. Synthetic oligonucleotide (ODNs) containing CpG motifs are potent vaccine adjuvants and

their function is mediated through activation of TLR9 signalling pathways [28]. CpG ODNs have shown to be effective adjuvant in clinical trials in humans. However, in 2008, a clinical trial of a hepatitis B candidate vaccine, Heplisav from Dynavax, comprising recombinant hepatitis B surface antigen mixed with a synthetic oligonucleotide containing CpG as the adjuvant, was put on hold by the FDA over safety concerns. One subject in the trial developed Wegener's granulomatosis, a severe autoimmune disease, in which blood vessels become inflamed. However, a second Phase 3 trial is expected to begin in early 2010 for individuals with chronic kidney disease. A recent cancer vaccine clinical trial with CpG-ODN have also been abandoned due to unexpectedly weaker responses in humans relative to those observed in mice [29]. This was attributed to a lower frequency of TLR9 expression on human than mouse immune cells.

TLR synergy

It has become increasingly evident that molecules that activate the innate immune system through TLRs can promote adaptive immunity to vaccine antigens, but are not always effective on their own. On the other hand many TLRs have been shown to work synergistically. This principle has been applied in the development of an experimental vaccine against H1N1 and H5N1 influenza viruses. A combination of MPL and a synthetic TLR7 ligand allowed the dose of H5N1 antigen to be dramatically decreased in mice; as little as 0.1 µg of antigen when combined with the experimental adjuvant had the same effect as 10 µg antigen dose combined with alum [30].

Modulating TLR signalling to improve adjuvant activity

Although TLR ligands are capable of promoting Th1 and Th17 responses to co-administered antigens, which are required for protective immunity to many pathogens, they can also induce

Treg cells. This reflects the fact that TLR activation of DC and other innate cells results in the production of anti-inflammatory cytokines, such as IL-10, which promote Treg cells, as well as pro-inflammatory cytokines, including IL-1, IL-12 and IL-23, which promote the induction of Th1 and Th17 cells [31]. We have found that IL-10, but not IL-12, production by DC activated with CpG, LPS or poly I:C is mediated by activation of p38 MAP kinase [32]. Inhibition of p38 suppressed CpG-induced IL-10 and enhanced IL-12 production by DC. Furthermore, addition of a p38 inhibitor to an experimental acellular pertussis vaccine formulated with CpG as the adjuvant, suppressed the induction of Treg cells, while enhancing Th1 responses and protection against *Bordetella pertussis* challenge [32]

TLR independent adjuvant activity

Recent studies in mice have shown that at least for antibody responses, which are the basis of protection induced with most currently licensed vaccines, TLR signalling may not be as important as previously thought. Mice deficient in critical signalling components for TLR, such as MyD88 and TRIF were able to mount robust antibody responses to T cell-dependent antigen when administered with either alum, incomplete or complete Freund's adjuvant, and MPL [33]. In the absence of TLRs, unconventional adjuvant-containing vehicles, such as viruses and apoptotic cells could elicit efficient adaptive immune responses to a vaccine [34]. Furthermore, Sanders and co-workers demonstrated that the absence of TLR5 did not have a substantial impact upon the ability of flagellin to promote T cell dependent antibody response to itself or a bystander antigen [35]. New families of intracellular PRRs, such as RIG-like receptors (RLHs) and NOD-like receptors (NLRs) have recently been demonstrated to activate innate immune responses and the subsequent adaptive immune response and these may mediate TLR-independent adjuvant activity. Indeed the NLR, IPAF has been shown to recognise flagellin [36]. However, other studies have found that TLR activation is essential

for the adjuvant activity of certain vaccines. We have demonstrated an absolute requirement for TLR4 in protection induced with the pertussis whole cell vaccine [3]. The mechanism appears to involve TLR4-mediated activation of DC by LPS present in the vaccine, which in turn activated Th1 and Th17 cells that conferred protective immunity. It has also been demonstrated that protection with the yellow fever vaccine involves activation of plasmacytoid and other DC subtypes through multiple TLRs [4].

RIG-like helicases (RLHs)

In addition to TLRs, a number of distinct cytosolic receptors for RNAs are employed by the innate immune system to initiate antiviral responses. These include a family of RNA helicase receptors referred to as RLRs: RIG-1, melanoma differentiation factor 5 (MDA-5) and laboratory of genetics and physiology-2 (LGP2) [19]. Unlike TLRs that recognize viral nucleic acids in the endosomes, the RLRs recognise signatures of virus replication within the cytosol of infected cells. Most viruses produce dsRNA in infected cells. Initially both RIG-1 and melanoma MDA5 were identified as sensors of the synthetic analog of dsRNA, poly I:C. RIG-1 was reported to be involved in the detection of poly I:C, and the subsequent activation of transcription factors NF κ B, IFN regulatory factor (IRF) 3 and 7, leading to inflammatory cytokine and type 1 IFN production [37]. After recognition of viral RNA, RIG-1 and MDA5 bind to IPS-1 via the CARD domain. IPS-1 is localized in the mitochondria and acts as an adaptor that links RLRs to type 1 IFN production [38]. Dissecting the signalling pathways involved in type 1 IFN production has been utilized to develop a novel adjuvant. Kobiyama and co-workers generated several different IPS-1 CARD-fusion polypeptides. Administration of one such polypeptide, N'-CARD polypeptide fused to a protein transduction domain (PTD) elicited production of type I IFNs, maturation of DC and enhanced immunogenicity of the antigen by promoting Th1 responses, which conferred

protection against lethal influenza infection in mice [39]. The N'-CARD-PTD polypeptide has the ability to self-transmigrate into the nucleus and trigger activation of innate immune cells in the absence of TLRs but in the presence of NDH and TBK-1, which are ubiquitously expressed in a wide range of cell types. N'-CARD-PTD represents a novel and possible candidate adjuvant in future vaccine development. Modulation of intracellular signalling using cell permeable polypeptides is a promising technology for future clinical applications. The findings of this study also provide insights that may prove useful in the rational design of immunomodulatory agents, such as the use of constitutively active signalling of the innate immune response [39].

NOD-like Receptors (NLRs)

NLRs are a family of cytoplasmic PRRs that contain 3 distinct domains: C-terminal LRRs, mediating ligand sensing, a centrally located NACHT domain, mediating self-oligomerization and the activation of NLRs, and an N-terminal domain, mediating protein-protein interactions for initiating downstream signalling. NLRs have been grouped into several subfamilies based on the basis of their effector domains and also on the history of their NACHT domains: NODs, NALPs, IPAF and NAIPs. The NOD and IPAF families contain CARD effector domains, while the NALPs and NAIPs contain pyrin (PYD) effector domains and 3 BIR domains, respectively. While the ligands and functions of many of these PRRs are unknown, it appears their major role is to recognize cytoplasmic microbial PAMPs and/or endogenous danger signals, initiating immunological responses.

Muramyl dipeptide (MDP), the minimal unit of peptidoglycan was first recognised as the minimum effective component of complete freund's adjuvant (CFA) in 1972 [40]. Recently, the adjuvanticity of MDP was shown to be NOD2 dependent, as NOD2 deficient mice are unable to mount a normal humoral response after immunization with MDP with

antigen [41]. Interestingly, the adjuvanticity of MDP is altered depending on the formulation used; in saline solution, MDP mainly enhances humoral immunity but when used in conjunction with lipophilic carriers such as liposomes, it induces a strong cellular immune response. However, pyrogenicity problems have restricted the use of MDP for vaccination purposes in humans. In an attempt to circumvent the toxicity problems, a number of synthetic analogues of MDP have been developed, including Murabutide™ and Muramyl tripeptide dipalmitoyl phosphatidylethanolamine (MTP-PE). However, in an early clinical testing, MTP-PE proved to be reactogenic and poorly tolerated [42].

Several NLRs (NALPs and IPAF) form a caspase-1-activating multiprotein complex, termed the inflammasome, which processes pro-inflammatory cytokines, including IL-1 β . Amongst the various inflammasomes, the NALP3 inflammasome is particularly effective at sensing a plethora of diverse molecules, including bacterial and viral PAMPs, stress-associated danger signals, such as ATP or monosodium urate crystals (MSU), as well as asbestos, silica, alum and β -amyloid [43].

It has been recognised for several years that IL1 β may have an adjuvant capacity; the addition of IL-1 enhanced serum antibody production in mice immunized with protein antigens [44]. Caspase-1, also known as IL-1 β -converting enzyme, processes and activates pro-IL-1 β and pro-IL-18. Pro-IL-1 β lacks a signal peptide and so remains inside the cell. Caspase-1 cleaves pro-IL-1 β into active mature IL-1 β , which is then secreted into the extracellular space [43]. It is now generally accepted that activation and release of IL-1 β requires two distinct signals, the first signal leads to the transcriptional upregulation and synthesis of pro-IL-1 β and other components necessary for inflammasome function, such as NALP3 itself; the second signal leads to NALP3 inflammasome complex formation, caspase-1 activation and IL-1 β cleavage.

Recent reports indicate that virus infection also results in the activation of the inflammasome. Both Sendai and influenza viruses activated NALP3 inflammasome in macrophages pulsed transiently with ATP for 30 minutes *in vitro* [45]. Uric acid crystals activate NALP3 inflammasome [46] and it has recently been reported that Influenza virus can induce uric acid in bronchoalveolar lavage fluid and serum in mice [47]. Shi *et al.* demonstrated that uric acid, which is released from injured cells, stimulated DC maturation, and when co-injected with HIV-1 gp120 antigen *in vivo*, significantly enhanced CD8 T cell responses [48].

Insoluble ammonium salts, or alum is the most widely used adjuvant in human vaccines and has been included in licensed vaccines all over the world for over 50 years. Although the mechanism of action of alum is still unclear, recent reports have implicated the NALP3 inflammasome in its immunostimulatory activity [49-52]. The adjuvant action of alum was shown to require NALP3 activation in a mouse model of allergic airway disease in which alum was administered intraperitoneally with ovalbumin [50]. Mice deficient in *Nalp3*, *ASC* or *caspase-1* failed to mount a significant antibody response to an antigen administered with aluminium adjuvants, but had intact responses with CFA [50]. NALP3 has been shown to be required for the generation of antigen-specific antibody responses to antigen administered with alum as the adjuvant [51,52]. Interestingly, Li and co-workers demonstrated that the adjuvants, QuilA and chitosan also activated the NALP3 inflammasome *in vitro*, suggesting that particulate adjuvants may share a common mechanism of action [52]. Furthermore, the enhancement of IL-1 β production by DC through NALP3 appears to be a general property shared by particulate adjuvants [53]. The generation of potent antigen-specific antibody response with biodegradable poly (lactide-co-glycolide) (PLG) microparticles was not dependent on NALP3. However, NALP3-deficient

mice had defective antigen-specific cell mediated immunity when PLG was used as an adjuvant [53].

C type lectins (CLRs)

C-type lectin receptors (CLRs) encompass a large family of proteins with varied functions. They contain one or more C-type lectin domains which mediate carbohydrate binding in a Ca^{2+} dependent manner [54]. CFA, an emulsion, incorporating killed *Mycobacterium tuberculosis*, efficiently induces Th1 responses in mice, but is too toxic for use in humans. In the search for alternative adjuvants based on CFA that are both safe and effective, purified PAMPs and their synthetic analogues have been investigated. CLRs have been implicated in the recognition of certain mycobacterial cell wall components [55]. One such component, trehalose-6,6-dimycolate (TDM) has potent inflammatory activity [56] and when used alone or in combination with a TLR4 ligand has adjuvant activity in mice [57,58]. The less toxic analogue Trehalose-6,6-dibehenate (TDB) induced robust Th1 responses after immunization with the recombinant *M. tuberculosis* antigen H1, conferring protection against challenge, reducing the bacterial load as effectively as the traditional BCG vaccine [59,60]. Both the mycobacteria-derived glycolipid TDM and the synthetic adjuvant TDB selectively activate the FcR γ –Syk–Card9 pathway in APC to induce a unique innate immune activation program that directs protective Th1 and Th17 cells [55]. Interestingly, the binding of the β -glucans curdlan or zymosan to the CLR, dectin-1, activates the Syk kinase, initiating signaling via the Card9–Bcl10–Malt1 pathway, and directs Th17 cell differentiation [61]. A recent study however, ruled out dectin-1 as the receptor responsible for TDM and TDM recognition, but identified other possible CLR candidates, including dectin-2, DCAR, OSCAR and PIRA [55].

Crosstalk between PRRs

Evaluating combinations of PAMPs may be a useful strategy in future adjuvant design. Such multicomponent adjuvants could potentiate an immune response and/or skew the immune response appropriately depending on the infectious disease target. Indeed, examples of synergy and crosstalk between TLR agonists and between TLR and NOD agonists have already been reported. TLR3 and TLR4 agonists strongly synergise with agonists of TLR7, 8 and 9 leading to a Th1 polarised response [62]. TLR4 agonists have also been shown to synergise with NOD1 and NOD2 agonists to induce DC maturation [63].

Conclusion

Vaccines are crucial for the prevention of infectious diseases. A better understanding of the biological basis of existing vaccines may provide clues for optimal development strategies for future adjuvants and vaccines. It may also provide explanations for past vaccination failures. Collectively, such information should pave the way toward a new generation of highly immunogenic low risk vaccines. Although concerns have been raised about the potential safety of PRRs agonists as adjuvants in new generation human vaccines, many current vaccines are effective because they include a range of PAMPs. For example, whole bacterial vaccines such as the whole cell pertussis vaccine contain significant amounts of LPS and bacterial DNA [3]. Armed with a better understanding of the mechanisms of action of adjuvants should help in the generation of more effective and safer vaccines against infectious diseases.

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Figure 1. Mechanisms of innate immune activation induced by vaccine adjuvants.

PAMPs, which are components of many vaccines and adjuvants activate cell surface associated or intracellular TLRs or NLRs. These PRRs then interact with specific adaptor molecules culminating in NF- κ B or IRF activation. Viral derived products (dsRNA or ssRNA) can also activate endosomal TLRs, along with RLRs (such as RIG-1), which induces type I IFN production. Alum activates the cytosolic NALP3 inflammasome leading to pro-IL-1 β cleavage into bioactive IL-1 β .

Fig 1