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Rationally-designed vaccine adjuvants: separating efficacy from toxicity

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TABLE OF CONTENTS

1. Abstract
2. Introduction: Need for effective, safe vaccine adjuvants
3. Immunological basis of adjuvant efficacy and adjuvant toxicity
- *3.1. Basic principles*
- *3.2. Dendritic cells, innate immune system activation, and successful vaccination*
- *3.3. Regulation of the transition from innate to adaptive immunity*
- *3.4. Engagement of signaling pathways for inflammation*
- *3.5. Off-target effects*
- *3.6. Innate-immune tolerance and memory in adjuvant responses*

4. Theoretical and practical approaches to minimize adjuvant toxicity: Forks in the TLR signaling pathways and potential to favor costimulation
5. Conclusion
6. Acknowledgements
7. References

1. ABSTRACT

Adjuvants, substances included in many vaccines in order to improve immune responses, are challenging to develop and license because adjuvant compounds that stimulate strong protective immunity also frequently induce significant toxicity. Adjuvant design and development has until recently been largely empirical; but with the current knowledge that most adjuvants act via receptors of the innate immune system, molecular-based approaches are rapidly advancing the field. Data support the concept that proinflammatory pathways induced by innate immune receptor triggering underlie many of the observed toxic effects. Importantly, the cellular signaling pathways that lead to inflammation are known, for a number of innate immune receptors, to be distinct from those that are involved in the costimulation of protective adaptive immune responses, leading to approaches for attenuating inflammatory signaling that should lead to safer and more effective vaccine adjuvants. This article addresses whether there is a clear rationale for the separation of toxicity from efficacy in the function of adjuvants based upon innate immune receptor ligands.

2. INTRODUCTION: NEED FOR EFFECTIVE, SAFE VACCINE ADJUVANTS

Vaccine research and development appropriately focuses upon defining and perfecting immunogens, the pathogen-derived protein or polysaccharide targets of T and B cells, but generally has paid less attention to another critical aspect of immunization, the activation of the innate immune system; however, that focus is changing. Primary antibody and cellular responses are weak or nonexistent unless immunization also effectively triggers the innate immune system, a vaccine property known as its "adjuvant activity." Undoubtedly, the vast majority of effective vaccines depend on adjuvant activity, whether intrinsic, as with complex vaccines that contain molecules of microbial origin; or added, such as the aluminum hydroxide or aluminum phosphate (alum) included in purified component vaccines. The increased use of added adjuvants appears to be inevitable for a number of reasons. For instance, the immunogen-sparing capacity afforded by adjuvants may be necessary to sufficiently extend vaccine supplies for a global response to an influenza pandemic. Additionally, adjuvants can reduce the number of booster

injections required to achieve protection, as well as result in a higher proportion of recipients achieving seroconversion. Beyond such quantitative improvements, adjuvants also offer the prospects for qualitative control as well—selectively skewing adaptive immunity toward Th1, Th2, or cytotoxic T cell responses, allowing effective immunization by distinct routes such as via the skin or mucosa, and potentially, eliciting optimal responses in the very young and very old, populations in whom most contemporary vaccination strategies are not optimally effective. Adjuvants currently in use may not provide the efficacy and safety needed for the aforementioned vaccination requirements that involve different delivery routes and elicitation of distinct types of immunity, as well as targeting different populations. The alum adjuvants in general vaccine use in the US stimulate antibody responses and have little toxicity as used, but are limited in terms of the antigens for which they provide adjuvanticity and the types of immune responses they elicit (*1*), (2). Developing adjuvants to meet these broader needs is a major goal involving immunology and vaccinology researchers today.

What are the safety and efficacy standards required of vaccine adjuvants? According to the US Food and Drug Administration (FDA) guidelines, proposed adjuvants must meet generally accepted standards of purity and quality, and all constituent materials must be included in a certificate of analysis in order to obtain Investigational New Drug (IND) approval. Pre-IND guidance information is available at: http://www.fda.gov/cber/gdlns/combvacc.pdf. The IND submission should address such issues as:

- The identity and purification process of the raw materials as well as the stability of the final adjuvant configuration;
- Information on biological activity with regards to choosing a particular adjuvant, ratio of adjuvant to antigen, and immunogenicity studies with and without adjuvant;
- Toxicology results on adjuvant and antigen/adjuvant formulations intended for clinical application.

As is stated in FDA regulation 21 CFR 610.15, "An adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not affect adversely the safety or potency of the product." These early stages of development must demonstrate the added value of the adjuvant in the adjuvant/vaccine complex and include comparison of adjuvanted vaccine to placebo.

Thorough nonclinical safety assessments of adjuvants need to be conducted in order to identify potential local or systemic reactions (*3*). To provide guidance in making these assessments, the World Health Organization has developed a reference for nonclinical evaluation of vaccines: www.who.int/biologicals/publications/nonclinical_evaluation_vaccines_nov_2003.pdf. Outlined in these materials are global recommendations and international regulatory expectations for various phases of vaccine product development with the overarching goal of maximizing the benefit-to-risk ratio.

3. IMMUNOLOGICAL BASIS OF ADJUVANT EFFICACY AND ADJUVANT TOXICITY

3.1. Basic principles

Historically, adjuvants have almost always been associated with toxic reactions, and devising approaches to reduce toxicity while achieving desired efficacy is a central goal in adjuvant research. Most early adjuvants were irritants that provoked considerable local inflammation— a well known but poorly understood fact, termed by the late Charles A. Janeway, Jr., as the "immunologists' dirty little secret" (*4*). The unanswered question is whether toxicity is an inevitable consequence of adjuvant activity. In some cases, structural modifications of an adjuvant molecule that decrease toxicity may also lead to a concomitant partial or full loss of adjuvant activity (*5*). In contrast, other studies have demonstrated that the moieties responsible for toxicity could be inactivated, leaving much of the adjuvant activity intact (*6*). However, with most adjuvants the source of toxicity and the potential role of toxic effects in its essential function have not yet been resolved. Numerous studies have associated the adjuvant activity of various substances of microbial origin with their ability to trigger receptors of the innate immune system; chief among them are the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) receptors (*7*) (*8*). It should be noted, however, that activation via TLR pathways is not essential for the function of every adjuvant. (*9*). A recent study showed that alum adjuvant, as well as complete and incomplete Freund's adjuvants, and a mixture of monophosphoryl lipid A plus trehalose dicorynomycolate enhanced antibody responses in mice genetically-engineered to be unable to signal through the major TLR adapters MyD88 and TRIF. The signaling pathways utilized by these non-TLR-based adjuvants are not known and it remains unclear whether there are specialized receptors for these adjuvants (*9*). Regardless of whether TLR-dependent or independent pathways are involved, several biological processes clearly contribute to adjuvant activity (Table 1): 1) upregulation of antigen processing and presentation and increased expression of Major Histocompatibility Complex (MHC) class I and class II molecules; 2) enhanced expression of costimulatory molecules, in particular CD80 and CD86; and 3) expression of cytokines such as interleukin (IL)-12 and IL-18 that promote adaptive immunity. Where does toxicity enter in? Studies of TLR ligand recognition and responses shed considerable light on this question. In addition to triggering costimulatory molecule expression and other responses required for adaptive immunity, TLRs also activate defensive measures against microbial invaders, including release of inflammatory cytokines such as IL-1, IL-6, and TNF-alpha, secretion of antimicrobial peptides, and release of neutrophil chemoattractants. Because of this parallel induction of inflammation and costimulation, there is a close association of toxicity with TLR-based adjuvant efficacy, an association that presents an apparent roadblock to improved adjuvant development. As will be discussed below, strategies that use adjuvants to stimulate innate immunity also rely upon natural processes to down-modulate innate immune responses as adaptive immune responses progress. Additionally, localized or targeted

Table 1. Adjuvant mechanisms in primary immune responses

Immune Mechanism	Comment	Refs.
Costimulatory molecules	CD80, CD86, CD40, CD70 upregulated on APC	55
Antigen processing and presentation	Upregulated MHC and processing mechanisms in APC	13, 56
Cytokines	Work in concert with costimulatory molecules, e.g. IL-12, IL-18	57
Apoptosis	Caspase upregulated; role in indirect antigen presentation	58, 59
Downregulation of Treg, inhibitory cells	Potential requirement for adjuvant function; possible novel strategy	42, 60
Antigen depot effect?	Hypothetical mechanism but controversial; may be tolerogenic	61,62

Table 2. Potential sources of toxicity in adjuvants that act via TLR

Toxic activity	Molecular Basis
Inflammatory cytokine release	NF-KappaB activation by MyD88 pathway
Off-target effects	Expression of relevant TLR on tissues not involved in immune responses
Poor transition from innate to adaptive immunity	Suboptimal downregulation of innate immune system activation

delivery of the adjuvant/immunogen is an important strategy to ensure that TLR-expressing bystander cells and tissues do not become involved (Table 2). Because alum and the adjuvants tested by Gavin *et al.* (*9*) appear not to activate TLR pathways that could lead to substantial toxicity, understanding how they work may provide insights into alternative pathways that may avoid toxicity altogether, enabling activation of "pure" costimulatory/antigen presentation pathways. However, at present the cellular receptors and pathways utilized by these non-TLR-dependent adjuvants are not well defined. Therefore, TLR, NOD, and other innate immune receptor-associated ligands are currently the best prospects for rational adjuvant design. Indeed, a number of TLR ligands are currently in clinical studies [reviewed in (10) (11)].

3.2. Dendritic cells, innate immune system activation, and successful vaccination

Many studies have shown that the most efficient route to a successful immune response is via antigen uptake and presentation by activated dendritic cells (DC) (12). Triggering of DC by microbial ligands or adjuvants profoundly alters the status of DC with respect to antigen processing and presentation. Tissue-resident immature DC express low levels of costimulatory molecules and MHC class II, but actively sample their local environment, internalizing external antigens, but not efficiently processing and presenting them. Upon triggering, DC lose their ability to efficiently acquire external antigens, but shift into the mode of processing and presenting material already taken into the cells. The activated DC then migrate to draining lymph nodes and gain the ability to activate naïve T cells by expression of high levels of costimulatory molecules such as CD40, CD80, CD86, and CD70 (13) (14). The capability to fine-tune the immune response is additionally furthered by the heterogeneity of responding DC types and the effect of signaling by distinct ligands (15). Taken together, these insights suggest that DC are the principal targets for improved vaccine adjuvants.

3.3. Regulation of the transition from innate to adaptive immunity

Unlike invertebrates that rely solely upon innate immunity, vertebrates must activate adaptive immune responses in order to clear most infections. Current data indicate that the initial innate immune response must be limited and shifted away from an inflammatory cascade for the development of effective adaptive immunity (16). Recently, Kelvin and collaborators reported that SARS patients with poor clinical outcomes exhibited a persistently high level of innate immune activation without transition to adaptive immune responses. The converse was true for those patients who resolved SARS infection, in which innate immune responses typified by interferon (IFN) alpha declined as antibodies to the SARS coronavirus spike protein developed (17). Emerging information suggests that such a change in defensive focus from innate to adaptive immunity involves multiple pathways with the potential to inhibit innate immunity, but currently how these pathways are activated and work in concert with adaptive immune responses is not well understood. For example, the cytokine IL-21, produced by activated T cells, diminishes NK cell responses while stimulating adaptive immune responses (18), and may initiate immunosuppressive activity in DC (19). Indeed, there are multiple mechanisms that prevent unbridled activation of the innate immune system (20). These include negative signaling pathways tied to TLR triggering, involving the signaling inhibitors IRAK-M and tollip, a transcriptionally-controlled negative regulator comprising a shortened form of the TLR adaptor MyD88, and the molecule termed suppressor of cytokine signaling-1, which is induced by LPS and CpG treatment. Whether all of these mechanisms function in the orderly transition from innate to adaptive immunity is not fully understood. It seems likely that adjuvant toxicity may result, in part, from the triggering of early inflammatory effects that may potentially be subject to down-modulation. If the transition to adaptive immunity can be facilitated by rational adjuvant design, the resulting vaccine adjuvants may possess greatly improved adjuvanticity/toxicity profiles.

3.4. Engagement of signaling pathways for inflammation

Because the innate immune system responds immediately to infection, TLR triggering activates multiple signaling pathways that involve innate-immune defensive measures as well as the activation of adaptive immunity. For example, TLR signaling via the adapter molecules MyD88 and TRIF can, at least in certain cell types, lead ultimately to NF-kappaB activation and synthesis of inflammatory cytokines such as TNFalpha, IL-1beta, IL-6, and various chemokines (21, 22). These molecules are critical in defense against many pathogens, calling into play neutrophils and other inflammatory cells to limit infection. However, when elicited by a vaccine with no potential for infectivity, manifestations of these responses may result in unintended tissue damage.

3.5. "Off-target" effects

While DC serve as the principal antigen presenting cells of a primary vaccine response, largely by being triggered through their TLR and other innate immune receptors, many other cell types not involved in promoting adaptive immunity also express innate immune receptors. For example, Yang *et al*. (23) showed that a functional form of TLR4 is expressed on human vascular smooth muscle cells, presenting the possibility that TLR4 agonists might induce undesirable inflammation in sensitive tissues. Indeed, tissues that are especially sensitive to inflammation may protect themselves by omitting the expression of specific TLR molecules or their co-receptors (24) (25). Some adjuvants may signal through DC, but not through other APC, minimizing off-target effects. For example, TLR9, the receptor for CpG motif-containing oligodeoxynucleotides, is expressed in humans mainly on plasmacytoid DC and B cells (26). Other approaches that may prove fruitful include targeting adjuvants to B cells (27) and DC via the cell receptor DC Sign (28) or DEC205 (29). Such approaches have the potential to lower the amount of antigen required, and to decrease off-target toxic effects. Because of the potency of the inflammatory mediators induced by TLR activation and the widespread presence of TLR in many tissues, the safe use of adjuvants will require approaches to reduce off-target effects. Note that the distribution of TLR and other innate immune receptors can differ significantly between mice and humans; therefore it is important to address species differences in expression as regards targeting effects.

3.6. Innate-immune "tolerance" and "memory" in adjuvant responses

A long-known but poorly-understood aspect of innate immune responses is the development of a state of tolerance to repeated stimulation, best characterized in the setting of endotoxin exposure, including experimental administration of endotoxin under controlled conditions in humans (30) (31). The potential for similar physiological control mechanisms (depending on dosing and route of administration) to influence adjuvant activity is largely unknown (32). In this regard, the intravenous injection of CpG into mice was recently demonstrated to suppress adaptive immune responses by induction of indoleamine oxidase (IDO), the enzyme mediator of suppression by tryptophan catabolism (33). A previous study of intravenous CpG injection had shown induction of IDO in the lung, small intestine, and colon, which may have mediated inhibition of experimental asthma in a murine model (34). Other experimental evidence indicates that prior exposure to a ligand for the same or a different TLR can lead to a transient state of unresponsiveness to a given TLR ligand (32). These effects appear to result from a wide range of yet-to-be characterized changes in the tolerized cells that may include downregulation of TLR expression and significant alterations in the signaling pathways (20). Additionally, a recent report showed that TLR stimulation induced chromatin modifications that transiently silenced genes related to inflammation while simultaneously priming genes involved in antimicrobial responses (35). The potential to temporarily dampen inflammation via effectors at the level of chromatin would, if manipulatable, offer a powerful means to separate toxicity from the desirable effects of adjuvants.

4. THEORETICAL AND PRACTICAL APPROACHES TO MINIMIZE ADJUVANT TOXICITY: FORKS IN THE TLR SIGNALING PATHWAYS AND POTENTIAL TO FAVOR COSTIMULATION

Although the signaling pathways activated following TLR activation lead to both inflammatory cytokine production and the expression of costimulatory molecules, recent reports show that it is possible to design adjuvants that favor adaptive immunity over inflammation.

LPS activation of TLR4 engages at least two distinct pathways: the MyD88-dependent pathway, which uses the adaptor proteins MyD88/MAL; and the MyD88-independent pathway that uses the TRIF/TRAM protein complex. The principal inflammatory cytokine-inducing pathway is largely, albeit not entirely, associated with MyD88 engagement, which elicits the majority of NFkB activation and thus TNF (21, 22). Conversely, in macrophages, LPS signaling through the MyD88-independent (i.e. TRIF/TRAM) pathway activates the phosphorylation and dimerization of the transcription factor IRF-3, thereby inducing expression of the gene encoding IFN-beta and initiating downstream chemotactic T cell effects and costimulatory molecule upregulation (36, 37). With respect to adjuvant development, TLR activation should therefore be skewed away from the MyD88-dependent avenue and towards the MyD88-independent, TRIF-dependent pathways (21). It is worth noting that as many as 70% of the macrophage genes induced in response to LPS or *E. coli* bacteria reflect the engagement of the TRIF/TRAM-dependent pathway (38). Similarly, TLR4 triggering of murine DC revealed that approximately 50% of responsive genes were dependent upon TRIF, underscoring the significant role of that pathway in TLR4 responses (39).

Importantly, a TLR4-targeting vaccine adjuvant candidate monophosphosphoryl lipid A (MPLA), which is well-advanced in product development, including testing in human vaccine trials (40), was recently shown to stimulate adaptive immunity in the absence of significant inflammation (41). This property was based upon the ability of MPLA to engage the TRIF-dependent pathway following TLR4 ligation rather than the MyD88 pathway. MPLA induced only low levels of inflammatory cytokines such as IL-6, while at the same time inducing strong T cell activation with secretion of the TRIF-dependent chemokines RANTES, IP-10 and MCP-1, along with IFN-beta. A low level of proinflammatory cytokine IL-6, may contribute to the adjuvanticity of MPLA by temporarily inhibiting regulatory T cells (42).

Dissection of NF-kappaB responses has further illuminated the potential to eliminate pro-inflammatory responses to adjuvants. Beg and coworkers (43) found that DC utilize distinct NF-kappaB components in inflammatory versus T cell stimulatory responses to TLR

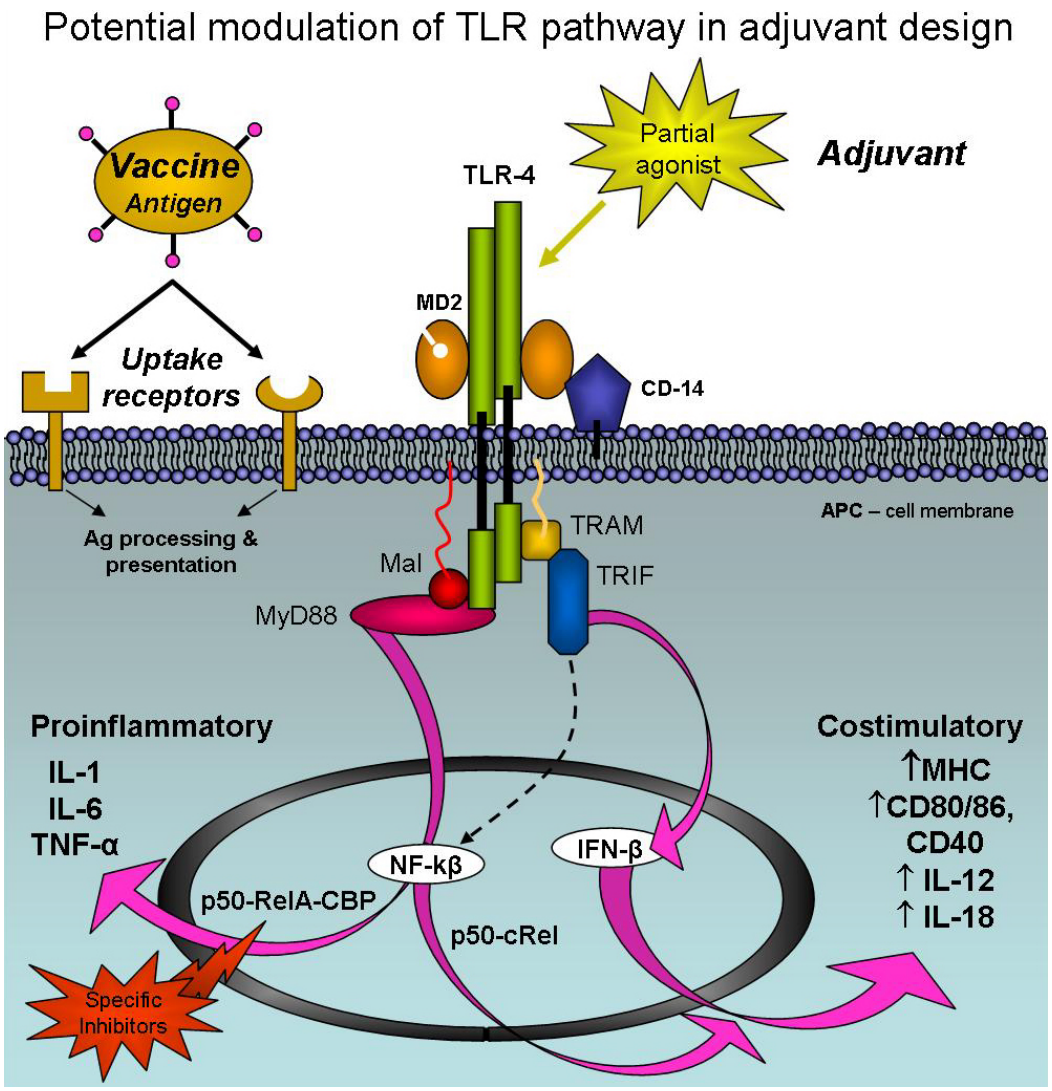

Figure 1. Potential modulation of TLR pathways in adjuvant design. Recent evidence indicates that proinflammatory responses to TLR signaling result from a separation of the NF-kappaB pathways from those which lead to costimulatory responses. This suggests that adjuvant strategies can be designed to modulate pathway usage. For example, use of partial agonists which favor the TRIF pathway or including specific inhibitors of the NF-kappaB-RelA pathway. Monophosphoryl Lipid A adjuvant appears to be a partial agonist of TLR4 (41).

triggering. NF-kappaB p50 and c-Rel were shown to function in CD40, IL-12, and IL-18 expression related to T cell costimulation, while RelA, in particular by association with the transcriptional coactivator CREB-binding protein, activated inflammatory cytokines. Another potential target for blocking TLR-mediated NF-kappaB activation is the molecule B cell leukemia-3 (Bcl-3) which interacts with homodimers of p50 and p52. Bcl-3 inhibits cytokine gene transcription by stabilizing the p50 complex. This molecule may provide a further target for limiting inflammation in the TLR signaling pathway (44). This distribution of function between different NF-kappaB subunits provides theoretical underpinnings for practical methods to engage the costimulatory pathways while leaving the inflammatory genes silent (Figure 1).

It may also be possible for an adjuvant to engage TLR4 without activation of MyD88 and only a partial dependence upon TRIF, at least in certain cell types, highlighting the potential of yet another approach to reduce toxicity while eliciting immune activation. For example, Georgel *et al.* (45) recently showed that bone-marrow derived macrophages responded to the glycoprotein G of vesicular stomatitis virus (VSV) via TLR4, leading to upregulation of IFN-beta expression via IRF-7 by utilizing the adapter TRAM. Whether this pathway can be exploited by designing adjuvants that mimic structural motifs of the VSV glycoprotein requires additional research.

Knowledge of the three-dimensional interactions of TLR-ligands and their receptors is providing important new insights into adjuvant design (46, 47). Recently, the

TLR4 co-receptor MD-2 was co-crystallized with the LPS antagonist for human TLR4, tetra-acetyl lipid A (48). The structure revealed that MD-2 plays a major role in positioning LPS-related compounds for interaction with TLR4, and may enable the rational development of lipid A analogues that, like MPLA, are partial agonists of TLR4 but that lack significant pro-inflammatory properties. Other observations may provide additional insights that could lead to novel adjuvant approaches. For example, the dose of LPS affects the quality of immune responses when administered intranasally. Whereas low levels of inhaled LPS plus antigen signaling through TLR 4 induce Th2 responses, high levels of LPS lead to antigen-specific Th1 responses (49). This raises the possibility that TLR-ligand interactions are governed by affinity thresholds that channel responses through distinct signaling pathways. These affinity thresholds may be dependent upon TLR co-receptors such as CD14 and MD-2. Such effects may eventually be quantified and mapped according to particular signaling pathways and manipulated in new adjuvant molecule or delivery systems. A recent report showed that TLR9 can signal allosterically depending on the specific ligand (50), further underscoring the emerging concept that different ligands can interact in distinct ways with TLRs, and opening the door to the rational design of adjuvants with distinct activity/toxicity profiles.

As an alternative to using an altered ligand as an adjuvant, it may be possible to co-deliver adjuvants with compounds that inhibit inflammatory segments of the NF-kappaB or other pathways while leaving the activation of costimulatory avenues intact (Figure 1). Several approaches to such modulation of NF-kappaB signaling are under investigation, including the use of small molecules that specifically target components of the NF-KappaB pathway within cells (51, 52).

5. CONCLUSION

Emerging concepts of innate immune signaling are providing both theoretical and practical directions for the rational design of vaccine adjuvants. Approaches include the design of adjuvants that are partial TLR agonists and methodology to block internal pathways with small molecule inhibitors in order to modulate adjuvant responses. New knowledge about the complexity of intracellular signaling initiated at the TLR, and emerging concepts regarding the role of extracellular co-receptors in TLR activation all support the eventual feasibility of strategies to discriminate therapeutically between the protective and inflammatory activities of vaccine adjuvants.

Ultimately, adjuvants that intrinsically lack much or all of their ability to induce toxic responses should readily meet the FDA requirements for clinical testing and approval. In order to be economically feasible, such adjuvants must also be practical to produce, stable, and applicable to multiple vaccine needs. An emerging consideration for adjuvants that target a single TLR is the possibility that polymorphisms of TLR genes in the human population could affect responses on an individual basis. This may require a genetic screening component in clinical trials and attention to the recipients' genotypes in vaccination (53). Adjuvants that activate adaptive immunity without causing problematic inflammation should also make vaccination more efficient, potentially requiring less antigen per dose, fewer booster immunizations, and achieving higher and more durable levels of immunity. It should be noted that along with development of innate immune receptor-based adjuvants is the use of co-delivered or vector-expressed cytokines that represent another important strategy to eliminate toxicity while enhancing vaccination responses (54). Such properties may be of particular importance in certain populations (e.g., young children, the elderly, and the immunocompromised) in whom current vaccine responses are less than optimal.

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