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Recent Advances in the Discovery and Delivery of TLR7/8 Agonists as Vaccine Adjuvants

David J. Dowling

Division of Infectious Diseases, Department of Medicine, Boston Children's Hospital and Harvard Medical School, Boston, MA 02115

ABSTRACT

The need for new adjuvants is absolutely cardinal to the development of new vaccines and to further optimizing current immunization approaches. However, only a few classes of adjuvants are presently incorporated in vaccines approved for human use. Recent advances in the discovery and delivery of TLR agonists as vaccine adjuvants have begun to open up a new toolbox for vaccinologists. At the forefront of this movement is the use of synthetic small molecule TLR7/8 agonist-based adjuvants. In this review, we emphasize the importance of vaccine formulation science in driving recent developments in TLR7/8 adjuvanticity, summarize some of the most current and notable studies in this field, and discuss desirable attributes of next generation TLR7/8 adjuvants for use in enhancing vaccine responses in vulnerable populations, such as the very young. Finally, we explore advances that may further edge the development of TLR7/8 adjuvant-based vaccine formulations toward clinical human evaluation.

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INTRODUCTION

Over the past 200 years, vaccination has been the most effective medical intervention to reduce death and morbidity caused by infectious diseases, especially in childhood (1). Vaccination exploits the generation of immunological memory by the mammalian immune system following exposure to Ag (components of a pathogen), providing the immunized with the ability to respond rapidly to a subsequent encounter with the originating Ag or its related pathogenic organism without undergoing the pathological effects of natural infection (1). Vaccination therefore imprints the immune system with the experience of live pathogen exposure, resulting in the generation of strong humoral immune responses by Ag-specific memory B, which is mediated by various subsets of specialized Ag-specific CD4⁺ T cells (2). However, the efficacy of immunization varies dramatically, with the composition of

vaccine, be it live attenuated, inactivated, subunit, monovalent/multivalent, protein-, or nonprotein (e.g., polysaccharides)-based, all important factors (3).

Adjuvantation is a key tool to enhance vaccine-induced immunity (3). Adjuvants can enhance, prolong, and modulate immune responses to vaccine Ags to maximize protective immunity (3, 4) and may potentially enable more effective immunization in the very young and the elderly (5). Fittingly, the word adjuvant is derived from the Latin *adjuvare*, meaning “to help.” In the early stages of vaccinology, adjuvants were often not required, as the majority of vaccines employed live attenuated or killed organism, which themselves contained inherent adjuvant activity needed to instruct enhanced immunization (i.e., self-adjuvanted). Adjuvants currently employed in human vaccines licensed for use in Europe and/or the United States include aluminum salts (alum) (of which there are three classes), oil-in-water emulsions

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Address correspondence and reprint requests to: Dr. David J. Dowling, Division of Infectious Diseases, Enders Research Laboratories, Room 730, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail address: david.dowling@childrens.harvard.edu

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Abbreviations used in this article: alum, aluminum salt; AS, adjuvant system; BCG, Bacille Calmette–Guérin; BZN, benzonaphthyrine; DC, dendritic cell; ER, endoplasmic reticulum; GLA, glucopyranosyl lipid A; HA, hemagglutinin; HBV, hepatitis B virus; IMQ, imidazoquinoline; IRF, IFN regulatory factor; LN, lymph node; MenB, group B meningococcus; MenC, group C meningococcus; MPLA, monophosphoryl lipid A; MVA, modified vaccinia virus Ankara; NHP, nonhuman primate; PAMP, pathogen-associated molecular pattern; PCV, pneumococcal conjugate vaccine; pDC, plasmacytoid DC; PLGA, poly(D,L-lactic-co-glycolic acid); PRR, pattern recognition receptor; RSV, respiratory syncytial virus; SMIP, small molecule immune potentiator; Tfh, T follicular helper.

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(e.g., MF59 and adjuvant system [AS] 03), and TLR9 adjuvants. Until the end of the 20th century, vaccine adjuvantation was largely focused on the use of alum (6), even if alum-adjuvanted vaccines often required multiple doses for protection to be achieved (5). Similarly, several oil-in-water adjuvants have been incorporated into vaccines to increase Ab responses (3, 4). Alum- and oil-in-water-adjuvanted vaccine adjuvants have some limitations, however, often requiring multiple doses for induced protection (5), and drive Th2- over Th1-polarized immunity. However, as vaccinology moves toward the development and use of defined Ags such as inactivated, subunit, and purified recombinant proteins and peptides, which can lose much of the immunological information needed to trigger an immune response leading to enhanced adaptive immunity, the use of novel adjuvants and delivery systems to replace alum has become critical. Over the past 30 years, however, there has been a significant expansion of our understanding of how the human immune system uses pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) and thereby instruct immune responses to fight infections. PRRs are germ-line encoded receptors, and unlike T cell or BCR, they do not undergo somatic mutation or clonal distribution.

As the PRR field grows, so does the potential for the rational design of PRR-targeting adjuvants. The first described and maybe best-characterized family of PRRs are the TLRs. At the adjuvant forefront are detoxified congeners of endotoxin that stimulate TLR4 (7). A cell surface PRR, TLR4 recognizes several PAMPs, including LPSs from the outer membrane of Gram-negative bacteria, and is the target for the adjuvant monophosphoryl lipid A (MPLA). *Salmonella minnesota* MPLA is manufactured by passaging naturally derived *S. minnesota* LPS through sequential acidic and basic hydrolysis steps to generate a diphosphorylated form with significantly lower pyrogenicity/toxicity (~1000-fold lower), but which retains robust adjuvant activity (3, 4). Adjuvant combinations of MPLA with alum have been licensed and/or trialed as part of several vaccine formulations. Most prominently, AS04 includes 3-deacyl-MPLA combined with aluminum phosphate and is used in two approved vaccines: Cervarix (human papillomavirus) and Fendrix (hepatitis B virus [HBV]). Two other combination AS, AS01 and AS02, both consisting of MPLA and the purified plant bark extract/saponin QS21, are components of the Mosquirix (RTS,S) malaria vaccine (8, 9).

A growing menu of novel adjuvants targeting TLRs is now becoming available to immunologists and vaccinologists (10). Among them, TLR9 has also proven to be a successfully target via the use of adjuvant oligonucleotides (ODN) containing unmethylated CpG sequences (11). CpG-ODN adjuvants have been found to trigger B cell activation and preferentially induce Th1-like over Th2-like CD4⁺ Th immune responses but with different immune enhancement effects in different species (11). The recently licensed HEPLISAV-B HBV vaccine, containing a CpG-based TLR9 agonist as adjuvant (named 1018-ISS), preferentially induces Th1 responses and requires fewer doses in adults than HBV vaccines employing alum alone (12) with a similar safety profile (13). In addition to TLR4- and TLR9-targeting agonist adjuvants, various

other PRR agonist adjuvants have been evaluated in human clinical trials. Agonists for TLR3 (short dsRNA) and TLR5 (bacterial protein called flagellin) have also demonstrated adjuvant activity.

The addition of TLR7/8 agonists to vaccines is not a new concept historically. First-generation vaccines, including those consisting of inactivated or attenuated virus, contained inherent TLR7 and/or TLR8 adjuvant activity (7). Both the inactivated polio and Japanese Encephalitis vaccines, which were introduced in 1955 and 1968, respectively, contained ssRNA TLR7/8 agonists. Additionally, the highly effective live attenuated yellow fever vaccine 17D is reported to activate multiple dendritic cell (DC) subsets via TLR2, TLR7, TLR8, and TLR9 (14), whereas TLR8 is upregulated following phagocytosis of *Mycobacterium bovis* (Bacille Calmette-Guérin [BCG] vaccine) by THP-1 cells (15). However, none of the currently approved adjuvants uniformly or sufficiently enhance cell-mediated immune responses that are required for elimination of intracellular organisms or cancers or enhancing immune responses of at-risk populations, such as the very young. In the future, adjuvants that stimulate robust humoral and cellular immunity, including both CD4 and CD8 responses, are increasingly desired for a number of vaccines, especially those focused on the elimination of intracellular organisms. As such, vaccines containing novel TLR7/8 adjuvant formulations are increasingly reaching advanced development and licensing stages, with the potential to fill previously unmet clinical needs.

TLR7 AND TLR8

TLR7, TLR8, and TLR9 are expressed on the surface of endosomes and belong to the same subfamily of leukocyte PRRs. Human TLR7 and TLR8 are phylogenetically similar (16, 17) and were first described in 2000 (18). Together, TLR7 and TLR8 mediate recognition of purine-rich ssRNA to elicit an immune responses to pathogens that are recognized in the endosome. Naturally derived viral uridine-rich ssRNAs include those of influenza and HIV (19). TLR7 (20) and TLR8 are also implicated in the recognition of bacterial RNA (16, 21). TLR7 is primarily expressed in human plasmacytoid DCs (pDCs) and, to some extent, in T cells, B cells, eosinophils, neutrophils, and monocytes/macrophages. TLR8 is expressed in monocytes, macrophages, T cells, and most predominantly in myeloid DCs (16, 17). TLR7 and TLR8 both signal through MyD88/MAL, including IL-1R-associated kinase-4 (IRAK-4) recruitment, to mediate cytokine and IFN production through NF- κ B and IFN regulatory factor (IRF) 7, respectively (22, 23). In response to proinflammatory cytokine signaling, TLR7 and 8 transcription is induced via NF- κ B.

Upon cellular stimulation (e.g., with a small molecule) or infection, TLR7 and 8 are released from the endoplasmic reticulum (ER) to the endosome by leucine-rich repeat (LRR)-containing protein 59 (LRRC59), which promotes the ER resident membrane protein uncoordinated 93 homolog B1 (*Caenorhabditis elegans*) (UNC93B1) (24) to mediate packaging of TLR7 vesicles to exit the ER and translocate to the Golgi. Although less is known about TLR8, once TLR7 reaches the endosome, acidification of the

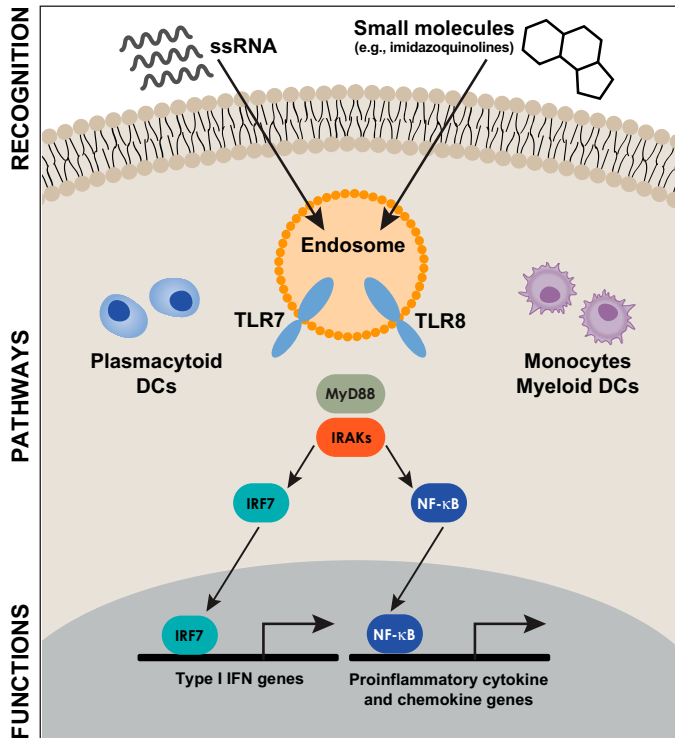


FIGURE 1. Immune responses induced by TLR7/8 ligands in primary human DC subsets.

The natural ligand of TLR7 and TLR8 is ssRNA. Small molecule IMQ compounds such as imiquimod and resiquimod (R848) also activate both receptors and are the subject of adjuvantation research. TLR7 and TLR8 agonists differ in their DC subset selectivity and IFN/cytokine/chemokine induction profile. TLR7-specific agonists activate pDCs and induce mainly IRF7-driven signaling, IFN- α , and IFN-regulated cytokines. TLR8-specific agonists activate monocytes and myeloid DCs, leading primarily to the NF- κ B activation and production of proinflammatory cytokines and chemokines, such as IL-12p70. The ability of TLR7/8 agonists to activate DCs and thus elicit Th1-like responses (IFN- γ -producing CD4 cells and IgG2-producing B cells) can be exploited to enhance the efficacy of vaccination. IRAK, IL-1R-associated kinase.

endosome leads to proteolytic cleavage of TLR7 (25). This cleavage event generates a functionally competent receptor essential for TLR7 signaling in response to ligand binding. TLR8 is documented to trigger production of proinflammatory cytokines including TNF, IL-6, IL-1 β , and IL-12p70 from human conventional DCs (Fig. 1). Importantly, IL-12p70 is a key cytokine in the enhancement of Th1-polarized immune responses and promotes cytotoxic T cell proliferation, survival, and the generation of immunological memory (26).

Although predominantly studied in APCs, the expression of endosomal TLRs by adaptive lymphocytes has also been described by several reports (27) and may be in direct opposition to the receptors' roles in innate immune cells. Specifically, TLR7- and 8-induced augmentation of lymphocyte activation and function is closely linked to TLR expression in respective T cell subsets (28).

TLR8 is exclusively expressed in human regulatory T cells, and exposure to TLR8 agonists triggers the TLR8-MyD88-IRAK4 signaling pathway, which mediates the reversal of the suppressive function of regulatory T cells (29). Correspondingly, TLR7 signaling inhibits proliferation and cytokine secretion by CD4⁺ T cells that mediates an anergic/nonresponsiveness phenotype during HIV infection (30). Although naive human B cells express low levels of TLRs, similar to pDCs, TLR9 and TLR7 are preferentially present. Activated and memory human B cells express a broader range of TLRs that, although especially prominent for TLR9, also include TLR1, TLR6, TLR10, and TLR7. Interestingly, B cell-intrinsic TLR7 signaling may play a role in optimal B cell responses during chronic infection (31), which could be leveraged to activate memory B cells and magnify humoral immune responses during immunization via coengaging of the BCR and TLR7 with hapten-protein Ag and adjuvants, respectively (32).

Small molecule agonist families have been identified which activate TLR2, TLR4, TLR7, and TLR8. In relation to TLR7 and TLR8, synthetic small molecule TLR7/8 agonist-based adjuvants include synthetic chemical agonists such as imidazoquinolines (IMQs) (33–35) and benzazepines (36). IMQs, including imiquimod and resiquimod (R848), are by far the most studied to date. IMQs are small (usually <400 Da) compounds that bear structural homology to the purine adenosine and, like naturally derived agonists, activate mammalian leukocytes via TLR7 and/or TLR8, leading to MyD88-dependent NF- κ B activation (37, 38). Whereas TLR7 agonist imiquimod activates significant antiviral and modest Th1-polarizing responses from pDCs (and monocytes), including IFN- α production, agonists with more specific TLR8 activity such as the small synthetic thiazoquinoline CL075 (also known as 3M-002) activate monocytes and myeloid DCs to induce robust cytokine production that drives adaptive immunity (39) (Fig. 1). This suggests that IMQs may hold better adjuvantation potential in humans than CpG ODN, as TLR7 and TLR8 are broadly expressed on DCs and other APCs in contrast to TLR9, which is primarily expressed on pDCs. Of note, IMQs engage the TLR7/8 pathway in a species-specific fashion. Human, pig, and nonhuman primate (NHP) TLR8 are activated by the same agonists (40), but murine TLR8 is divergent from human TLR in the expression of LRRs and therefore is not activated by agonists of human TLR8 (19). For example, humans, but not mice, detect ssRNA through TLR8 expressed on myeloid DCs (23). Such issues warrant caution in translating these findings on TLR7/8-acting molecules to potential human targeted vaccine strategies.

FORMULATION MATTERS: UNLEASHING TLR7/8 ADJUVANTICITY

Formulation science has traditionally been underappreciated in adjuvant discovery and development, often seen as secondary to traditional immunological studies. However, over the past decade, the development of more fully characterized vaccine formulations has become a major goal for many vaccinologists and pharmaceutical companies (41). The same holds true in the discovery and

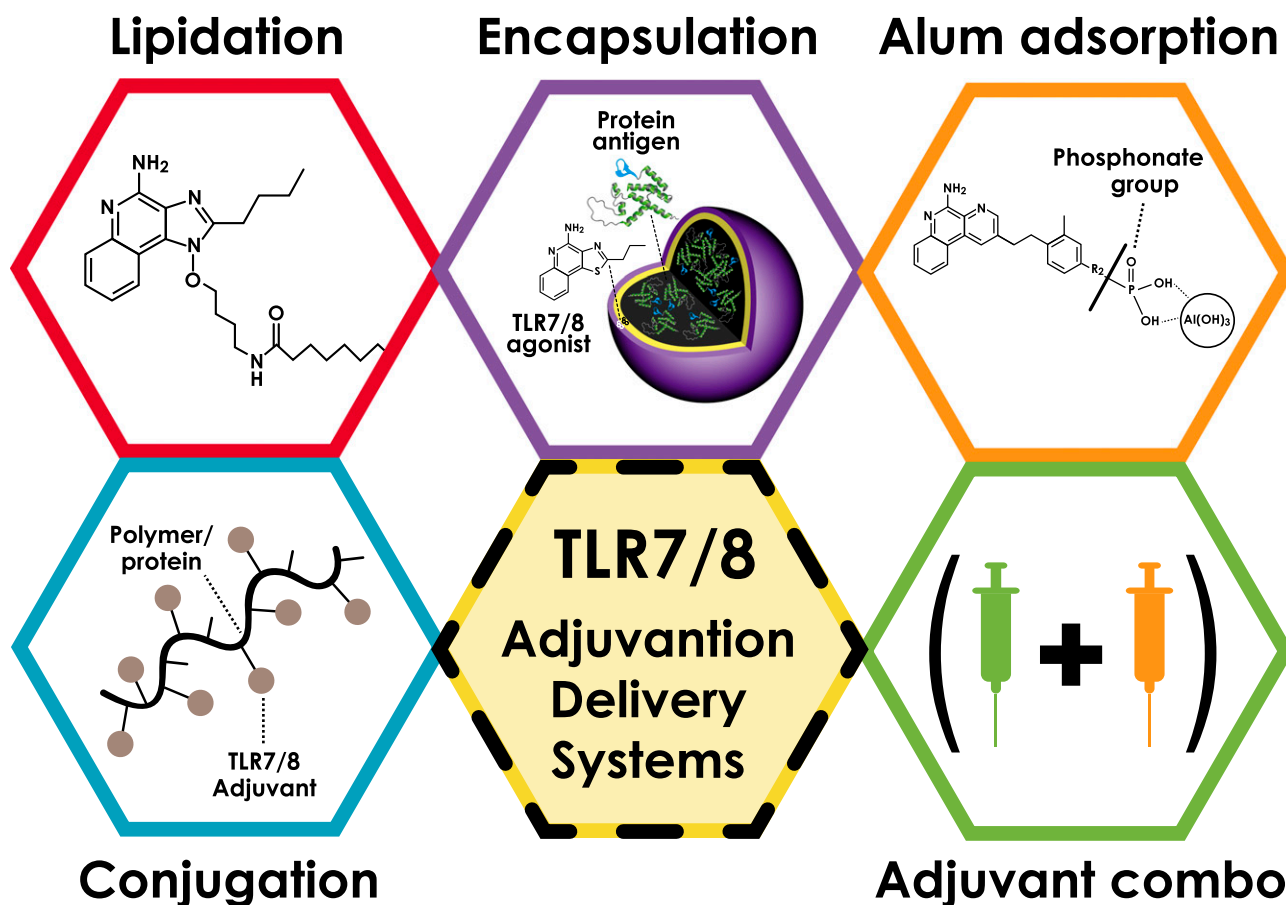


FIGURE 2. Innovations in formulation and delivery systems for TLR7/8 agonists.

Small molecule TLR 7/8 agonists have demonstrated great potential as vaccine adjuvants because they directly activate APCs and can enhance both humoral and cellular immune responses, especially Th1 responses. The most promising innovations in formulation and use of delivery systems for TLR7/8 agonists include 1) lipidation approaches, as best demonstrated by 3M-052, a TLR7/8-activating IMQ bearing a C18 lipid moiety and designed for slow dissemination from the site of injection, 2) encapsulating nanoparticles (e.g., polymersomes), 3) adsorption to alum adjuvants (TLR7 agonists are functionalized with polyethylene glycol linkers), in which terminal phosphonate groups allow for ligand exchange of hydroxyl and/or phosphate groups on the surface of aluminum hydroxide $[\text{Al}(\text{OH})_3]$ or phosphate (AlPO_4), 4) conjugation to polymers and/or protein Ags, and 5) additive/synergistic admixture combinations with other adjuvants such as TLR4 adjuvants.

delivery of TLR7/8 agonists as vaccine adjuvants. Typically, the unformulated synthetic small molecule IMQs TLR7/8 ligand adjuvants do not work well in comparison with their larger m.w. counterparts such as alum because they are prone to diffuse away from the injection site, which can also result in systemic toxicity (17). Most notably, R848 has a poor tolerability profile when tested in humans, and common systemic side effects include injection site reactogenicity and flu-like symptoms (fever, headache, and malaise) that correlate with systemic immune activation such as high concentrations of numerous cytokines in the blood (42, 43). Therefore, improving IMQ-based vaccine adjuvant properties required further formulation techniques to minimize adjuvant reactogenicity and localize the molecule in the body after administration.

The first step in this trend came indirectly through the manufacture of the synthetic small molecule imiquimod, which is

approved in a topical 5% cream formulation (under the trade name Aldara [imiquimod cream]) for treatment of human papillomavirus-mediated external genital warts, actinic keratosis, and superficial basal cell carcinoma (17). Imiquimod is a TLR7-selective analog of R848 which is 100-fold less potent. Like all IMQs, owing to imiquimod's insolubility in aqueous solutions near physiological pH, formulation success was achieved by use of fatty acids as the preferred solvent for topical imiquimod formulations (44). When applied as a topical skin adjuvant, imiquimod enhanced intradermal influenza vaccine responses (8, 45, 46). However, topical IMQs (such as imiquimod and resiquimod [R848]) have unfavorable pharmacokinetic properties that induce strong local and systemic inflammatory reactions and are poorly tolerated (42, 43); they are likely not required for their vaccine efficacy and reduce their effectiveness for inducing adaptive immunity. Secondly, the vast majority of licensed vaccines are administered

TABLE I. Studies investigating lipidated TLR7/8 agonist formulation and adjuvanticity

Adjuvant	Formulation	Model	Disease Model	Immune Response Activated	Reference
3M-052	Oil-in-water emulsion (SE), alum admixed	Neonatal NHP rhesus macaque	Pneumococcus	Overcoming hyporesponsiveness to neonatal PCV/single birth shot protection	(48)
3M-052	3M-052 adsorbed to alum via helper lipid	Mouse	Tuberculosis and HIV	Enhanced Ab and Th1-type cellular immune responses to vaccine Ags	(49)
3M-052	Liposome/SE	Mice and ferrets	Influenza	Protection from lethal H5N1 homologous virus challenge	(50)

H5N1, influenza A virus subtype H5N1; SE, stable emulsion.

admixed with Ag via a standardized i.m. route. Therefore, new formulation concepts and properties of delivery were needed to better retain the small molecule TLR7/8 adjuvants at the injection site to improve their pharmacokinetics in ways that positively influenced their activity for use in prophylactic vaccines. Encouragingly, some promising innovations in formulation and use of delivery systems for TLR7/8 agonists have recently been achieved, including 1) lipidation approaches, 2) encapsulating nanoparticles, 3) adsorption to alum adjuvants, and 4) conjugation to polymers and/or protein Ags (Fig. 2).

Lipidation

First, the lipidation approach is best demonstrated by 3M-052, a locally acting lipidated IMQ TLR7/8 agonist adjuvant bearing a fatty acid tail (C18 lipid moiety). The physical-chemical properties of 3M-052 ensure it stays at the site of vaccination, thus only inducing local adjuvant effects without inducing systemic cytokines (Table I). In contrast with R848, which is rapidly dissipated from the injection site, entering the blood within 5 min postdose and inducing significant serum TNF, pharmacokinetic and pharmacodynamic studies demonstrate that 3M-052 is not detected in serum and induces only a minimum serum cytokine signature (47, 48). 3M-052 has also been evaluated in numerous vaccine models (Table I). As compared with R848, a liposome formulations of 3M-052 admixed with hemagglutinin (HA) could enhance Th1 immunity without induction of systemic cytokines (47). Interestingly, 3M-052 could also drive robust Th1-cytokine production by human newborn leukocytes in vitro, both alone and in synergy with the alum-adjuvanted pneumococcal conjugate vaccine (PCV)13 (Prevnar 13) (48). When admixed with PCV13 and administered i.m. to infant neonatal rhesus macaques, 3M-052 dramatically enhanced generation of Th1 CRM-197-specific neonatal CD4⁺ cells and activation of newborn and infant *Streptococcus pneumoniae* polysaccharide-specific B cells as well as serotype-specific Ab titers and opsonophagocytic killing (48). The key to 3M-052 adjuvanticity lies in its ability to enhance and prolong IFN- γ production by CD4⁺ T cells to vaccine Ags (49), driving germinal center reactions and Ab subclass production associated with the development of IFN- γ -driven type 1 immunity that are endowed with higher effector functions in vivo (e.g., IgG2a/c in mice and IgG3 in human), enhancing protection to live pathogen challenge (50). Overall, 3M-052 is an excellent example of a small molecule TLR7/8 adjuvant designed for slow dissemination from the site of injection, which can be admixed

with various Ags already employed in conventional vaccine formulations while also demonstrating an improvement in dose sparing over the prototypical IMQs like R848 (47, 48).

Encapsulation nanoparticles

Advances in the field of immunoengineering, which is developing alongside vaccinology, have begun to greatly influence vaccine formulation design (51, 52). Historically, the first steps into vaccine formulation design were driven by the need to stabilize Ags (i.e., first-generation vaccines often employed Ag adsorption onto alum). Second-generation efforts employed more characterized material, such as the biodegradable synthetic polymer, poly(D,L-lactic-co-glycolic acid) (PLGA), which is a widely investigated nanoparticle adjuvant for controlled and effective delivery of vaccine Ags, including synthetic peptides. These are usually produced as solid block particles ranging from 50 to 500 nm in size, with Ags entrapped or adsorbed on the surface of the particles (53). Immunization with an R848-encapsulating PLGA nanoparticle attenuates the production of systemic inflammatory cytokines induced by free R848 while enhancing murine humoral immunogenicity to the model Ag OVA (54) (Table II). In the context of an NHP simian immunodeficiency virus model, and as compared with alum alone-adjuvanted vaccine Ags, coencapsulation of a TLR7/8 IMQ with MPLA induced a robust innate response as well as Ag-specific humoral, plasmablast, and long-lived bone marrow resident plasma cells (55). Furthermore, the combination of MPLA and the TLR7-selective IMQ R837 in PLGA nanoparticles mediated synergistic enhancement of Ab responses against influenza A virus subtype H5N1 (H5N1)-derived HA, which was mechanistically contingent on the direct activation of B cells and DCs through TLRs as well as on T cell help (56). Similarly, admixture of imiquimod and the synthetic TLR4 adjuvant glucopyranosyl lipid A (GLA) induced synergistic cytokine secretion from human whole blood in vitro, and combined liposomal encapsulating formulation enhanced innate and adaptive Th1 responses in vivo to recombinant *Plasmodium* proteins (57).

Nanoparticle vaccine delivery systems are amenable to be engineered to mimic the size, shape, and surface chemistry of pathogens (58), often referred to as pathogen-like particles. This can be even more powerful when also employing encapsulation of both the target Ag and adjuvant, enabling codelivery of both to subsets of immune cells (i.e., directly to APCs) and even specific subcellular compartments. Polymersomes, a form of nanoparticles with vesicle-like morphology, are an example of a dual adjuvant- and Ag-delivery

TABLE II. Studies investigating TLR7/8 agonist encapsulation formulation and adjuvanticity

Adjuvant	Formulation	Model	Disease Model	Immune Response Activated	Reference
R848	PLGA encapsulation	Mice	OVA	Nanoparticle encapsulation of TLR7/8 agonist R848 attenuates the production of systemic inflammatory cytokines while enhancing humoral immunogenicity	(54)
TLR7/8 IMQ	PLGA encapsulation with TLR4 agonist MPLA	NHP rhesus macaque	SIV	Induction of robust innate, Ag-specific humoral, plasmablast, long-lived bone marrow resident plasma cells compared with alum alone–adjuvanted vaccines	(55)
TLR7/8 IMQ	PLGA encapsulation with TLR4 agonist MPLA	Mice and NHP rhesus macaque	Influenza	Induction of robust innate, Ag-specific humoral responses as compared with single TLR alone–adjuvanted vaccines. Immunization protected mice against lethal avian and swine influenza virus and induced robust immunity against pandemic influenza A virus subtype H1N1 (H1N1), A/California/04/09 strain in rhesus macaques.	(56)
TLR7 IMQ (imiquimod)	Anionic liposomal (phospholipids, cholesterol) encapsulation with TLR4 agonist GLA	Mice	Malaria	Induction of robust innate, Ag-specific humoral, plasmablast, long-lived bone marrow resident plasma cells compared with alum alone–adjuvanted vaccines	(57)
IMQ CL075 (TLR8/7)	Poly(ethylene glycol)-bl-poly(propylene sulfide)–Ag85 encapsulation	Humanized TLR8 neonatal mice	Tuberculosis and HIV	Induction of adult-like innate maturation of human neonatal DCs in vitro. Single neonatal immunization induced noninferior Ag85-specific T cell responses as compared with live attenuated BCG vaccination in vivo.	(59)

system, which are also significantly more stable than liposomes (52). Polymersomes can be engineered for bioresponsive intracellular payload delivery (52), such as lysis in response to pH changes, a highly advantageous method for the specific targeting of endosomal receptors such as TLR7 and TLR8. Furthermore, such formulations can be made to mimic the immunomodulating effects of the live attenuated vaccine such as BCG. When co-loaded with the *M. tuberculosis* Ag 85B peptide 25, TLR8 agonist-containing polymersome nanoparticles are comparable to BCG in inducing Ag-specific immune responses in hTLR8-expressing humanized neonatal mice in vivo (59).

Adsorption to alum

In a third approach, benzonaphthridine (BZN) TLR7 agonists have been chemically modified with phosphonates to allow adsorption onto alum hydroxide (60, 61) (Fig. 2). The adsorption

of a TLR7/8 adjuvant to alum to create small molecule immune potentiators (SMIPs) shares some similar advantages to lipidation and encapsulation, such as 1) it exploits the inherent adjuvanticity of alum, 2) including its ability to simultaneously codeliver Ag and adjuvant, while also 3) ensuring the small molecule adjuvants stays at the site of vaccination, thereby limiting the biodistribution of the alum-adsorbed BZN TLR7 adjuvant. Often using R848 or alum as comparators, the adjuvanticity of a number of BZN-based SMIP compounds has been evaluated in vivo with promising results (Table III). SMIPs have demonstrated significantly enhanced adjuvanticity in the context of several licensed vaccine classes, including formulation with recombinant group B meningococcus (MenB) proteins alone or with the full four-component alum-adjuvanted MenB vaccine formulation (trade name: Bexsero) (60), the glycoconjugate CRM197–group C meningococcus (MenC) vaccine (trade name: Menjugate) (62), and a tetanus, diphtheria,

TABLE III. Studies investigating TLR7/8 agonist adsorption to alum

Adjuvant	Formulation	Model	Disease Model	Immune Response Activated	Reference
SMIP TLR7	BZN TLR7 agonists chemically modified with phosphonates to allow adsorption onto aluminum hydroxide	Mice	OVA, meningococcal disease, and anthrax	Significantly enhanced amount and quality of Ag-specific Ab responses, and titers and serum bactericidal activity (SBA) against MenB	(60)
SMIP TLR7	Alum-adsorbed BZN TLR7	Mice	Meningococcal disease	Improved potency of glycoconjugate. CRM197-MenC vaccine as compared with alum-adsorbed vaccine, such as increased anti-MenC Ab titers and SBA against MenC, even with a single immunization.	(62)
SMIP TLR7	Alum-adsorbed BZN TLR7	Mice	Tetanus, diphtheria, and acellular pertussis (whooping cough)	Formulation of an acellular pertussis vaccine enhanced <i>Bordetella pertussis</i> -specific Th1/Th17 responses, serum IgG2a/b, and the protective efficacy against <i>B. pertussis</i> aerosol challenge	(63)
SMIP TLR7	Alum-adsorbed BZN TLR7	Mice and rabbit	<i>Staphylococcus aureus</i>	Significant Ab titers, Th1-skewed immune response, reduction of abscess formation, and dermonecrosis	(65)
SMIP TLR7	Alum-adsorbed BZN TLR7	Adult rhesus macaques	RSV	Env-specific CD4 ⁺ T cells in the vaccine-draining LNs, which directly correlated with increased Tfh cell differentiation and germinal center formation	(66)

and acellular pertussis (whooping cough) vaccine (63). Most interestingly, SMIP TLR7 adjuvantation approaches have opened up an opportunity to shape responses to diseases not yet preventable by vaccination (64), including *Staphylococcus aureus* and HIV. In the case of the former, SMIP TLR7 adjuvantation significantly increased Ab titers, a Th1 skewed immune response, reduction of abscess formation, and dermonecrosis in an *S. aureus* rabbit model in vivo (65). Using an NHP model, the latter study demonstrates that TLR7 adjuvantation can induce a high-magnitude HIV Env-specific CD4⁺ T cell response in the vaccine-draining lymph nodes (LNs), which directly correlated with increased T follicular helper (Tfh) cell differentiation and germinal center formation (66). Tfh cells are pivotal regulators of the germinal center response and humoral immunity (2). Alum/TLR7 combinations may also, following vaccination, facilitate the expansion and sustain the generation of the memory B cell compartment within the draining LN by increasing recruitment of naive B cells (67).

Conjugation

Fourth, TLR ligands covalently coupled to vaccine Ags may have several benefits over nonconjugated Ags (68). For example, the chemical conjugation of R848 with protein Ags has shown that synchronous delivery of Ag and TLR7/8 adjuvant may be a highly efficient approach for optimizing T cell priming toward Th1 responses (40, 69) (Fig. 2). Promisingly, immunization of mice with

a SMIP TLR7 agonist conjugated to the pneumococcal RrgB Ag could extend animal survival after lethal challenge with live *S. pneumoniae*, with a 10-fold Ag dose-sparing effect (70) (Table IV). Additionally, the coupling of an Ag and an IMQ-based TLR7/8 agonist together with spontaneously nanoparticle-forming thermoresponsive polymers could also elicit vaccine-induced broad-based neutralizing Abs and CD4/8 T cell responses to immunogenic proteins, including those from HIV (71) and respiratory syncytial virus (RSV) (72). Chemical conjugation to other adjuvants is also effective, as with the IMQ-based TLR7 agonist CL307, which, when linked to a synthetic lipopeptide TLR2 agonist, enhanced maturation of human DCs in vitro and increased humoral responses against HIV-1 p24 in BALB/c mice in vivo (73). Perhaps the most interesting form of TLR7/8 adjuvant conjugation involves the use of an inactivated influenza virus (IPR8-R848) (74). Here, immunization of infant NHPs with an amine derivative of R848, conjugated to inactivated influenza virus particles, significantly increased virus-specific Ab- and cell-mediated responses and virus clearance and reduced lung pathologic condition postchallenge, as compared with the nonadjuvanted virus vaccine A/Puerto Rico/8/34 (H1N1) (74).

Adjuvant combinations

Finally, we introduce the possibility of using TLR7/8 adjuvants in combination with other non-TLR7/8 adjuvants (Fig. 2). Adjuvant combinations may have synergy/additive/inhibitory effects in

TABLE IV. Studies investigating TLR7/8 agonist conjugation formulation and adjuvanticity

Adjuvant	Formulation	Model	Disease Model	Immune Response Activated	Reference
SMIP TLR7	TLR7 agonist conjugation on RrgB pilus Ag formulated with alum	Mice	Pneumococcus	Immunization with TLR7 agonist conjugation on RrgB Ag extended animal survival after lethal challenge with <i>S. pneumoniae</i> , with 10-fold Ag dose sparing	(70)
IMQ-based TLR7/8 agonist	Particle-forming polymer-linked adjuvants	Mice	OVA and HIV	Induction of high-magnitude and persistent local innate immune activation, with associated enhanced CD8 ⁺ T cell responses and Th1-skewed Ab responses	(71)
IMQ-based TLR7/8 agonist	Coupling adjuvant, immunogenic viral protein to nanoparticle-forming thermoresponsive polymers	Mouse immunization and live viral intranasal challenge	RSV	Elicitation of neutralizing Abs to structure-dependent epitopes on RSV (and other relevant pathogenic Ags)	(72)
IMQ-based TLR7 agonist CL307	Conjugation to synthetic Pam2C lipopeptide TLR2 agonist	Mice	HIV	Enhanced maturation of human DCs in vitro increased humoral responses against HIV-1 p24 in BALB/c mice in vivo	(73)
R848	Conjugated to inactivated influenza virus (IPR8-R848)	Neonatal NHP African green monkey	Influenza	TLR7/8-adjuvanted vaccines induced significantly increased virus-specific Ab- and cell-mediated responses, virus clearance, and reduced lung pathologic condition postchallenge, as compared with the nonadjuvanted virus vaccine A/Puerto Rico/8/34(H1N1)	(74)

vitro or in vivo, which may or may not have effects on potentiating vaccine-induced responses. Therefore, the objective of optimized coadjuvant formulations may be to induce beneficial outcomes such as 1) the need for fewer vaccine doses, 2) the ability to use lower Ag doses clinically, especially on the context of multivalent vaccines, and 3) the induction of preferential immune polarization not achievable by one adjuvant alone. Perhaps the most studied combination formulation strategy involves simple coadministration TLR7/8 adjuvants with TLR4 agonists, simply admixed (75) or in liposomal and emulsion formulations (76) (Table V). The approach is not limited to TLR-based combinations. Costimulation of R848 and the macrophage-inducible CLR (Mincle) can drive synergistic maturation of human DCs with Th1 polarization abilities ex vivo (77). In the context of a therapeutic vaccination, oral administration of the TLR7 agonist GS-9620 (vesatolimod), in combination with Ad26/Modified Vaccinia virus Ankara (MVA) heterologous prime-boost vaccination, decreased levels of viral DNA in LNs and peripheral blood, improving virologic control rebound (78). This TLR7/8 adjuvantation is likely mediated in the presence (formulation mixture) of the inherent TLR2-TLR6, MDA-5, and NALP3 inflammasome adjuvant activity of MVA (79).

AGE-SPECIFIC TLR7/8 ADJUVANTICITY IN EARLY LIFE

Childhood vaccination is one of the most effective means of controlling infectious diseases (80, 81). However, adaptive immunity in newborns presents with distinct ontogeny, features, and functionality of T and B cells, making young infants highly reliant on soluble and cellular innate immune mechanisms (5). This results in decreased magnitude of immunogenicity and reduced persistence of functional Abs, both major concerns for early life immunization strategies (5). Historically, solutions to these challenges include the use of multidose pediatric immunization schedules, expanding the length of time between vaccine doses and/or administration of doses later in infancy, all of which increase immunogenicity (82). Alum-based subunit vaccines consisting of purified microbial products often lack the necessary adjuvant activity to induce and optimally shape an immune response in early life (83). Accordingly, development of rationally designed age-specific vaccine formulations, which may include adjuvants that more effectively enhance immune responses in childhood, are warranted (3, 4). In this context, TLR7/8 agonists have demonstrated unique utility. Unlike agonists of most TLRs that

TABLE V. Studies investigating TLR7/8 agonist admixture formulation/combination vaccination

Adjuvant	Formulation	Model	Disease Model	Immune Response Activated	Reference
Phospholipid-conjugated TLR7 agonist	Admixed with small molecule TLR4 adjuvant	Mice	Influenza	Combined TLR4 and TLR7 adjuvant vaccination induces Th1- and Th2-type immune responses for long-lived cellular and neutralizing humoral immunity against the viral HA	(75)
3M-052	Liposomal and emulsion formulations with GLA	Mice	Enteric protozoan pathogen	Enhancement of intestinal IgA, plasma IgG2a/IgG1, IFN- γ , and IL-17A responses, which inhibited parasites adherence to host cells	(76)
R848	Admixture and Mincle C-type lectin receptor (CLR) agonist	Human naive T cell stimulation ex vivo	N/A	Dual TLR7/8 and macrophage-inducible CLR activation of human newborn DCs synergistically drove Th1 polarization ex vivo	(77)
TLR7 agonist GS-9620 (vesatolimod)	Heterologous prime boost vaccination/immunomodulator therapy	NHP	HIV	Oral administration of TLR7 agonist in combination with Ad26/MVA prime-boost vaccination decreased levels of viral DNA in LNs and peripheral blood, improving virologic control rebound	(78)

N/A, not applicable.

elicit reduced Th1 cytokine production by newborn and infant leukocytes, IMQs induce robust Th1-polarizing responses from both neonatal and adult DCs alike (33, 36, 59). TLR7/8 stimulation also may uniquely activate B cell responses in early life (84–86).

Infant NHP models, such as the rhesus macaque (*Macaca mulata*), have proven highly useful in furthering evidence of TLR7/8 agonists as vaccine adjuvants in vivo, as TLR8 in most NHPs is highly conserved in terms of both amino acid identity (e.g., 98.6% for rhesus macaque as compared with humans) and the predicted distribution pattern of extracellular LRRs (87). Neonatal and infant NHP leukocytes have also demonstrated human-like TLR7/8 adjuvant-induced cytokine responses in vitro (33). For example, when conjugated to particulate vaccine Ags, R848 can have adjuvant activity in NHPs immunized in the first month of life (74) that may overcome waning immunity (88). Moreover, immunization of 1-d-old NHPs with the combination of TLR7/8 agonist and Prevnar 13 resulted in accelerated and enhanced anti-*S. pneumoniae* polysaccharide-specific B cells, serotype-specific Ab titers, and Ab-mediated phagocytic killing, to levels ~10–100 times greater than a single birth dose of PCV13 alone. These data suggest that appropriately formulated TLR7/8 agonist

adjuvants could enhance responses to vaccines administered even very shortly after birth (48), especially important because newborn vaccines achieve relatively high population penetration as birth is the most reliable point of health care contact worldwide (89). Maternal immunization can also negatively impact early life responses to vaccination, mainly through the transplacental transfer of vaccine Ag-specific maternal IgG (90). Furthermore, the use of TLR7/8 agonist adjuvants in neonatal immunization may have an additional beneficial effect of overcoming such maternally acquired Ab interference (90), owing to the ability to induce more robust vaccine-specific T cell responses than non or alum-adjuvanted vaccines.

TLR8: A SENSOR FOR BACTERIAL RNA

Although the relevance TLR8 in viral RNA recognition and initiation of innate immune responses is well established, its role in sensing bacterial RNA during infections has been proposed (91). Initial studies indicated that TLR8 could trigger human monocytes/macrophages responses to bacterial RNA following transfection of extracts from sonicated *Streptococcus pyogenes* (92).

Moreover, TLR8-dependent detection of bacterial RNA is crucial for triggering murine monocyte activation in response to infection with *S. pyogenes* (92). Similarly, *S. aureus* RNA is a TLR8 ligand that can induce IFN- β and IL-12 induction by human monocytes/macrophage in an IRF5-dependent fashion (93). The strongest evidence yet provided linking TLR8-driven innate immune activation by bacterial RNA and enhanced vaccine responses is in the context of live attenuated vaccines. Engagement of TLR8 on human monocytes/DCs by bacterial RNA derived from live *Escherichia coli* or the live attenuated vaccine BCG initiated Th1-polarized responses to direct Ag-specific CD4⁺ T cells in the generation of Tfh cells, which correlated with enhance Ab-secreting plasmablasts and a more robust Ab response (21). In domestic pigs, immunization with a live bacterial *Salmonella* vaccine also induced robust anti-*Salmonella* Tfh cell and Ab responses, but immunization with its heat-killed counterpart did not (21). These findings are additionally noteworthy because pigs have been identified as an appropriate in vivo model to recapitulate the response elicited in humans to TLR7/8 adjuvants (94). Overall, the identification of TLR8 as a primary human sensor of viability-associated PAMPs, known as “vita-PAMPs” (95, 96), and its role as a driver of Tfh cell differentiation, make TLR8 a promising target for Tfh cell-skewing vaccine adjuvants.

CONCLUSIONS

The persistently high global burden of infections in the very young (97) provides a compelling rationale for the continued use and development of safe and effective vaccines (89). The use of new classes of adjuvants may spur a new revolution in vaccinology (98). In this context, the recent advances in the discovery and delivery of TLR7/8 agonists as vaccine adjuvants is of particular translational promise. Small molecule TLR7/8 agonists have demonstrated great potential as vaccine adjuvants, because they directly activate APCs and can enhance both humoral and cellular immune responses, especially Th1 responses. Basic research into nucleic acid-sensing mechanisms, including those focused on TLR7/8, have revealed the central role these pathways play in mediating responses to live attenuated vaccines and how these insights could be harnessed for the design of new vaccines (99). Along with effective adjuvantation, refined vaccine delivery systems, improvements in identifying more efficacious vaccine Ag candidates, and systems vaccinology are key technological and immunological advances fueling the current transformation of vaccinology (100). Rational vaccine design approaches employing immunoengineering may allow for the controlled preparation of vaccine formulations of the desired immunostimulatory properties, particulate size, and Ag load, all of which improve safety by potentially limiting systemic toxicities by their targeted nature (64).

As always, safety considerations will be paramount. A key concern regarding adjuvanted vaccine development is reactogenicity, the propensity of a formulation to cause acute inflammatory events either locally (e.g., erythema, tenderness) or systemically as

fever. Of note, vaccine adjuvants are not licensed separately; rather, the adjuvant is a constituent of the licensed vaccine formulation. Historically, both saponin- and emulsion-based adjuvants required extensive research and engineering improvements to produce controlled preparations with less reactogenic profiles yet maintained a strong adjuvant effect (4). Similarly, detergent extraction and genetic modification are successful approaches employed to ameliorate LPS toxicity and prevent excess reactogenicity of outer membrane vesicles (101). Therefore, TLR7/8 adjuvants must be evaluated both alone and as a component of an indicated vaccine formulation. TLR7/8 adjuvant optimization may entail 1) manipulating pharmacokinetic properties that affect compound biodistribution (e.g., limit systemic exposure by covalent attachment of a hydrophobic group) and 2) facilitating interaction with formulations designed to ensure localized codelivery of the Ag and immunostimulatory compound (e.g., nanoparticle encapsulation).

Overall, there are several synthetically defined TLR7/8 adjuvanted vaccine formulations at various stages of clinical development, which may well offer significant advantages compared with current alum-based subunit vaccines. The adaptive response induced in these TLR7/8-focused adjuvant studies can be summed up as producing Th1-like response (IFN- γ -producing CD4 cells and IgG2-producing B cells) with concomitant inhibition of Th2 immunity. Continued evaluation and optimization of these TLR7/8 adjuvant approaches, including potential dose-sparing effects, improved reactogenicity profiles, long-term safety, and efficacy outcomes, are clearly merited.

DISCLOSURES

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