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Correlates of GLA family adjuvants' activities.

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Introduction

Although adjuvants have been the subject of intense research and development for the past three decades, the unmet need fulfilled by adjuvants for ushering in a new generation of vaccines has only been demonstrated relatively recently. This brief overview will focus on TLR4 ligands, which have the longest history of clinical development among the TLRs or PAMPs.

Edgar Ribi, during his long career at the Rocky Mountain Laboratories of National Institutes of Health's National Institute of Allergy and Infectious Diseases in Hamilton, Montana and later at the company he founded, Ribi Immunochem, performed the early analysis of bacterial lipopolysaccharides, focusing on separating the adjuvant activity from the toxic effects, an effort that led to the development of monophosphoryl lipid A, or MPL [1–3]. Ribi Immunochem developed a variety of MPL-based adjuvants and the research community validated several of these in a variety of animal models and in veterinary applications. The company was eventually sold to Corixa Corp., which was later sold to GSK which continues to produce MPL in the Hamilton site today. Although safe and effective, the product potential of MPL was not fully realized until GSK, over the period of several years, invested in formulation technology.

The importance of formulation in maximizing efficacy while minimizing toxicity will be addressed in the next chapter. MPL-based adjuvants developed by GSK for commercial products include MPL-alum (AS04, found in the products Fendrix® and Cervarix®) and liposomal QS21-MPL (AS01 series found in the malaria vaccine RTS,S and the shingles vaccine Shingrix®). Each of these formulations has distinct adjuvant properties. IDRI has developed several formulations of the synthetic TLR4 agonists GLA (Phad) and SLA. These formulations include the agonists with alum, oil/water emulsion (SE), liposomal QS21, and, more recently, nano-alum. QS-21 is purified from saponin, derived from the bark of *Quillaja saponaria*, and serves as a potent adjuvant through the promotion of CD8 T-cell responses and the generation of IgG1 and IgG3 antibodies. Formulations are chosen based on the type

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of immune response desired, age of recipient, route of administration, and nature of the antigen. In the following sections, we will discuss what we have learned in the past two decades in both animal models and in clinical studies about how TLR4 agonists can be used to safely generate effective immune responses.

1. From Molecule to Adjuvant: Product-Enabling Formulations of TLR4 Agonists

While several decades of effort resulted in increased understanding of the structure-function relationship of TLR4 agonists as well as novel candidate molecules, it was not until later that the effects of formulation of the TLR4 ligands were fully appreciated. Indeed - given the highly insoluble nature of almost all TLR4 ligands discovered to date - some type of formulation is absolutely necessary to achieve a pharmaceutically acceptable preparation. The situation is complicated by the fact that many formulation platforms (e.g. squalene emulsions, aluminum salts) act as adjuvants even in the absence of TLR4 ligands due possibly to their particulate nature, the molecules contained within that are inflammasome activators on their own, and other structural properties. Thus, when considering formulation effects on TLR4 ligand adjuvant activity, one must also take into account the added adjuvant activity that may be inherent in the formulation vehicle itself.

A classic illustration of these principles is the early clinical development of GSK's RTS,S malaria vaccine where MPL® was formulated in multiple adjuvant formation platforms, including AS04 (MPL® adsorbed to aluminum oxyhydroxide), AS02 (MPL® and the saponin QS-21 in oil-in-water emulsion containing squalene and α-tocopherol), and AS01 (MPL® and QS-21 in phosphatidylcholine liposomes). In the initial Phase 1 comparison involving a sporozoite challenge, the vaccine containing AS02 protected 6 of 7 subjects from sporozoite challenge compared to 2 of 7 and 1 of 8 recipients who received the vaccine containing AS03 (emulsion without MPL and QS-21) or AS04, respectively [4]. Subsequent trials focused on comparing AS02 to AS01, and demonstrated that AS01 consistently provided greater protective efficacy and antigen-specific antibody titers [5,6]. Moreover, when combined with a recombinant TB antigen, AS01 generated significantly higher Th1-type CD4+ T cell responses compared to the same antigen administered with AS02 [7].

More recent reports have compared the AS series of adjuvants in the context of HBV and HPV antigens. In a Phase II evaluation, Burny *et al.* ranked AS01>AS03>AS04>Alum in terms of ability to enhance adaptive and innate response (as well as reactogenicity prevalence) when administered to human subjects with recombinant HBV surface antigen [8]. Furthermore, subjects receiving a tetravalent HPV vaccine with AS01 generally showed the highest antigen-specific antibody, memory B-cell, and CD4+ T cell responses compared to the same antigen with AS04 or AS02 [9]. It appears clear from the above mentioned clinical studies that AS01 is a more effective formulation for delivering MPL and QS-21 compared to an oil-in-water emulsion. Preclinical studies came to the same conclusion [10]. Nevertheless, each vaccine is a unique case, and many factors may influence which adjuvant formulation is most suitable, including route of delivery, desired immune response, target population, dose, stability, etc.

Formulation effects are clearly evident even using simple *in vitro,* cell-based experiments, although the ranking may be quite different from *in vivo* models. Thus, when the synthetic TLR4 ligand GLA is formulated as an aqueous nanosuspension (GLA-AF), oil-in-water emulsion (GLA-SE), liposome (GLA-LS), or adsorbed to aluminum oxyhydroxide (GLA-Alum), there are dramatic differences in the magnitude of cytokine production from stimulated human cell lines or whole blood, with GLA-AF generally generating the highest levels [11]. In contrast, GLA-AF is typically the weakest of the formulations in terms of *in vivo* adaptive Th1 responses in preclinical models [11]. Results from Phase 1 clinical comparisons of different formulations of GLA (oil-in-water emulsion vs. QS-21-containing liposomes) with recombinant TB and malaria antigens will be available in the near future (NCT02647489, NCT02508376).

Yet another factor to consider in appropriate adjuvant formulation approaches is the interaction of the adjuvant formulation with the vaccine antigen. In the case of AS01 or AS04, it appears clear that co-localization of the antigen with the adjuvant, within certain temporal and spatial constraints, is necessary to achieve optimal responses [12,13]. However, these studies did not address whether direct association of the antigen (e.g. surface conjugation, electrostatic association, etc.) with the adjuvant formulation platform affects the immune response. In the case of aluminum salts, it is clear that adsorption of the antigen (or lack thereof) and the strength of binding influence the resulting immune responses [14]. Likewise, associating antigen to liposomes through surface conjugation or encapsulation is reported to shape immune responses [15]. Whether the added benefit, if any, of direct antigen-adjuvant formulation association justifies the requisite manufacturing and stability challenges of such compositions warrants further discussion.

Regardless of the adjuvant formulation platform, it is difficult to overstate the importance of thorough manufacturing quality control and physicochemical characterization approaches. Ensuring consistent physicochemical properties such as particle size and TLR4 ligand concentration/conformation is essential for reproducible biological activity. In this regard, Haensler *et al.* showed that different manufacturing methods used to incorporate the synthetic TLR4 ligand E6020 into a squalene oil-in-water emulsion significantly altered the safety (pyrogenicity) of the formulation, attributable to the different levels of partitioning of the molecule between the oil and aqueous phases depending on the method of manufacture [16]. Another example concerns the preparation of aqueous nanosuspensions of TLR4 ligands, including the concentration ratio of TLR ligand/excipient and the method of mixing [17,18]. Changes in these parameters clearly affected *in vitro* cytokine stimulation activity.

In the development of pharmaceutically acceptable formulations of TLR ligands, it is perhaps advisable to focus on established platforms [19]. Thus, oil-in-water emulsions, liposomes, and aluminum salts, which are already widely employed in approved drug or vaccine products, have demonstrated the most success in terms of advancement of TLR4 agonists through the clinical and product development pathway (e.g. AS01, AS04, GLA-SE). Less established platforms such as polymeric particles have demonstrated promise in preclinical models but can be hampered by manufacturing challenges, including raw material sourcing, terminal filterability, scalability, and/or stability. Nevertheless, established platforms may benefit from optimization approaches made feasible by modern

manufacturing and excipient tools. For example, traditional aluminum salts can be altered to achieve tunable nanoparticle sizes, potentially enhancing adjuvant properties [20,21]. Other efforts have achieved the thermostability of established formulation platforms through judicious design of excipient composition combined with drying technologies. Thus, lyophilized formulations of GLA (with aluminum oxyhydroxide or squalene-in-water emulsion) maintained adjuvant properties even after exposure to several months of elevated temperatures [22–24]. Such progress should help facilitate distribution and use of vaccines containing advanced adjuvant formulation technology in resource-poor areas. In the next section we will discuss specific applications of formulated TLR4 ligands.

2. TLR4 Agonists Enable a New Generation of Vaccines For Global Health

2.1 Influenza

Influenza viruses have been a burden on society in the last centuries causing annual morbidity and mortality. Sporadic pandemic outbreaks can be devastating with the outbreak in 1918 causing millions of deaths and dropping the average life expectancy in the US by about 10 years [25]. They are particularly difficult to combat with a single antigen formulation since the constant emergence of drifted and shifted viruses allows escape from established immunity necessitating re-formulation of the vaccine each year [26].

Inclusion of TLR4 agonists into vaccine formulations enables the activation of AID causing accelerated hypermutation and broadening of the immune response [27]. Properly formulated TLR4 agonists will trigger caspase-dependent release of IL18 which synergizes with the type 1 interferons induced by the TLR agonist and leads to powerful stimulation of immune effector cells [28]. This leads to more families of antibodies being generated in the immunized individuals allowing better recognition of pathogen serovars and neutralization of strains not found in the vaccine formulation [29].

For these reasons, formulated TLR4 agonists are promising candidates for inclusion into flu vaccine formulations – especially those indented to induce “universal” flu responses that either target specific neutralizing regions of the influenza hemagglutinin, or relay on broad immune responses that cross react with proteins from different viruses [30]. Additionally, as discussed below, the inclusion of TLR4 agonists can lead to the induction of cellular responses to other targets like the more conserved M or NS proteins of the virus and thereby initiate protective cellular immunity. Human studies combining TLR4 agonists with unadjuvanted influenza vaccines have found strong boosting of immune responses and demonstrated that antigen dosages could be spared by over 40-fold [31]; this could help with stretching stockpiled vaccine supplies and allow protection of immune impaired sub populations like the elderly [32]. A recent study showed that a TLR4-based adjuvant, GLA-AF, developed for intradermal application was effective in enhancing antibody production in a clinical study of healthy volunteers.

2.2 Tuberculosis

Demonstration of protective efficacy of recombinant in TLR-4 based adjuvants has been most extensively demonstrated with the tuberculosis (TB) vaccine candidate 72f (later M72).

Both AS01 and AS02, MPL-containing adjuvant formulations, protected against disease progression in pre-clinical models. AS01 was chosen for extensive clinical development, and results of these trials are expected soon [33–35].

Another candidate has been developed using GLA-SE adjuvant, a vaccine candidate developed for the prevention and treatment of TB, “ID93 + GLA-SE”. ID93 is a fusion of four *Mycobacterium tuberculosis* antigens with diverse roles that are recognized by T-cells isolated from TB-exposed individuals and lack human sequence homology. ID93 + GLA-SE is being developed for two indications: as an immunotherapeutic agent to improve the outcome of drug treatment for active TB, and as a prophylactic vaccine to prevent infection with TB. Two phase 1, and one phase 2a clinical trial of ID93/GLA-SE in healthy adults in the United States and South Africa have been completed. Results from these studies demonstrated the induction of a broad, polyfunctional T-cell response, an increase in multi-functional antibody, and robust CD4+ T-cell responses in IGRA+ adults, suggesting that the vaccine boosts the immune response to natural infection [36]. The phase 2a trial, in which vaccination occurred at the end of TB treatment, demonstrated encouraging CD4+ T-cell and antibody responses to vaccination. These studies also demonstrated an acceptable safety profile for ID93/GLA-SE in more than 200 research participants.

2.3 Malaria

Malaria is caused by the *Plasmodium* family of protozoan parasites that have a complex life cycle with a mosquito vector. Vaccine approaches to these parasites vary in the desired immune response to be generated. Current approaches include: (1) Transmission blocking vaccines (“TBVs”): these vaccines target the stage of the parasites’ life cycle that occurs in the mosquito. The idea is to inhibit the parasite inside the insect vector thereby preventing spread of the disease [37]. (2) Blood-stage antigen directed approaches include proteins like apical membrane antigen 1 (AMA1) and the merosorite surface proteins (MSPs). Here the idea is to prevent the parasite from invading host cells by having antibodies circulate that block functional receptors [38]. (3) Liver-stage antigens are more targeted towards eliciting cellular responses that would kill liver stage parasites. The most advanced malaria vaccine candidate, Mosquirix®, falls into this category. In this vaccine the antigen contains portions of the circumsporozoite protein (CSP) [39].

Recombinant protein-based antigens for the first two approaches benefit from the inclusion of a TLR4 agonist for the same reason the influenza vaccine candidates do: enhanced affinity and broadness of recognition allows targeted neutralization of critical sites on the parasite as well as cross-reactivity to strains of the parasite not included in the vaccine formulation. For TBV approaches, the durability of the response and weak immunogenicity of some constructs are additional factors where inclusion of a TLR4 directed adjuvant can enhance the durability of the response, providing the high titers of antibody required for transmission blocking activity over a longer period of time as shown in non-human primates [40]. Other approaches like certain live attenuated parasites or viral vectors may not benefit from an adjuvant at the time of injection since this may neutralize the delivering vector. However, in prime/boost scenarios the addition of an adjuvant to the purified antigen prime or boost can greatly expand T cell help providing a more powerful regimen [41]. For this

reason, a number of groups building on the potential success of Mosquirix® and are using TLR4 agonists in their adjuvants when targeting malaria [42–46].

2.4 HIV

There has been excitement since the Rv144 “Thai” trial of an HIV vaccine around the possibility that an effective HIV vaccine may be possible. In this prime/boost trial where canarypox-based viral vector primed subjects were boosted with recombinant gp120 proteins, for the first time, efficacy was demonstrated with an early separation between vaccinated and placebo groups [47,48]. The short-lived duration of protection invited speculation that durability of a potent response may have been the reason why the vaccine did not demonstrate better efficacy and if inclusion of more powerful adjuvants could address the durability problem. As vaccines for another viral pathogen, the problems facing an HIV vaccine are similar to those for influenza vaccines. However, since the target of this virus is CD4 which is present on T cells, HIV vaccines are unique in the challenges they present: it has been argued that expanding helper T cells using TLR4 based adjuvants could expand the target population for the virus. While the overall number of CD4 positive cells may not change following in response to adjuvant administration, it could be argued that T cell activation could enhance viral infection of these cells. For these reasons, many researchers still consider using TLR agonists in the formulation but argue that the formulation needs to balance the quantity of CD4 positive cells with the enhanced quality of the immune response [49]. Nonetheless, promising data in the presence of TLR4 based adjuvants are being generated and future trials will likely demonstrate that enhanced neutralizing antibody titers will outweigh any risk of more CD4 positive cells arriving at the site of infection [50].

2.5 Schistosomiasis

Schistosoma species of worms cause debilitating diseases throughout the world. Of these, the intestinal species *S. japonicum* and *S. mansoni* cause a majority of the morbidity burden [51]. While there no approved vaccine, recent promising approaches include TLR4-based adjuvants in their late-stage preclinical and early clinical work [52]. There are numerous reasons to include a powerful TLR4 based adjuvant in approaches to these worms. For one, the parasite appears to evade the immune system by evading the Th2 response initiated by the host. Additionally, IgE responses can be primed in individuals in endemic countries [53] and could potentially be boosted by the vaccine potentially causing anaphylactic reactions in immunized individuals. Since TLR agonists - when included in a vaccine - can both skew the immune response to a more Th1 profile, unmasking the parasite and alter an IgE response away from the allergic profile [54], inclusion of these can be critical to successful schistosome vaccination.

A recombinant protein vaccine against a *Schistosoma mansoni* antigen (“Sm14”), when adjuvanted with GLA-SE, induced durable antibody responses and an increase in cytokine-producing CD4 T-cells [52]. A Phase II trial in Senegalese children and adolescents will begin in late 2018. Another very promising schistosomiasis vaccine candidate is Sm-p80, for which the inclusion of a TLR-based adjuvant, GLA-SE, was critical to vaccine efficacy in pre-clinical models, including non-human primates. The properties of the adjuvanted

vaccine provided candidate cross-species protection, durable responses, and therapeutic responses in infected animals [55–57].

2.6 Leishmaniasis

The leishmaniases are a group of vector-borne diseases transmitted by phlebotomine sand flies that regurgitate then inoculate *Leishmania* parasites during blood meals. Estimates are that up to 350 million people are at risk of infection and subsequent development of leishmaniasis in one form or another [58,59]. Visceral leishmaniasis (VL) results from infection with either *L. infantum* or *L. donovani* and can be fatal in a substantial subset of patients if left untreated. Up to 90% of *L. donovani* infections in humans remain sub-clinical, however, and do not cause symptoms [60]. Asymptomatic *Leishmania* infections that resolve without manifesting VL are thought to be controlled when an effective antigen-specific T cell response producing IFNγ is generated [61–63]. In contrast, active VL disease is associated with a depressed response and infected macrophages are rendered unresponsive to activating cytokines by the presence of IL-10 [61,64–68]. These data indicate the importance of a potent Th1 response, rather than a Th2 or suppressive microenvironment, to protection [69].

Experimental *Leishmania* infection of mice has both enhanced our understanding of helper T cells during an infection and allowed dissection of the Th1/Th2 paradigm [70–72]. As such, they also provide systems with which to evaluate immune biasing by adjuvants and multiparameter flow cytometry analyses of the immune responses after immunization have indicated that the degree of protection against *Leishmania* infection in mice largely corresponds with the frequency of CD4+ T cells simultaneously producing IFNγ, IL-2 and TNF [73]. These effector cells are also unique in their capacity to produce high amounts of IFNγ. *Leishmania* infection models have accordingly been utilized to demonstrate that various TLR4L preferentially support the development of high quality antigen-specific Th1 responses.

When we used either GLA-SE or SLA-SE in conjunction with the *Leishmania* vaccine antigen we observed that - when they were incubated with antigen - spleen cells from immunized mice secreted large quantities of IFNγ and TNF, but not IL-5. Furthermore, approximately three quarters of the antigen-specific CD4 T cells in mice immunized with LEISH-F3 / GLA-SE or LEISH-F3 / SLA-SE had a polyfunctional Th1 phenotype, producing double and triple combinations of IFNγ, IL-2 and TNF [74]. In agreement with the increase in these cells that correlate with protection - relative to unimmunized mice - animals immunized with the LEISH-F3 antigen formulated in GLA-SE or SLA-SE had reduced *L. donovani* burdens following infectious challenge. In a subsequent phase I clinical trial, antigen-specific immune responses of volunteers who received three study injections of LEISH-F3 with either GLA-SE or SLA-SE were evaluated. Injection of antigen alone did not generate responses, indicating the importance of adjuvant in promoting antigen-specific CD4 T cell responses. At day 35 (i.e., 7 days following the second immunization), the percentage of CD4 positive T cells positive for IFNγ, TNF and IL-2 in response to LEISH-F3 antigen stimulation was higher in subjects immunized in the context of 10 μg of SLA-SE than 10 μg GLA-SE, and this significant difference was retained at day 84. T cell responses

were still detected at day 168. Together, these data indicate that both LEISH-F3 + GLA-SE and LEISH-F3 + SLA-SE are safe in humans and induce antigen-specific cellular responses that could protect against *Leishmania* infection.

2.7 Leprosy

Leprosy (Hansen's disease), caused by *Mycobacterium leprae* infection, is principally a dermatological and peripheral neurological disorder. Manifestation varies dramatically across a wide array of symptoms and various forms that correlate with the quality of the immune response. Similar to the outcome of *Leishmania* infections in mice, the Th1/2 paradigm dictates the presentation of leprosy in patients. Multibacillary (MB) leprosy patients classically present with strong Th2 supported humoral responses that do not limit *M. leprae* replication and disseminated skin lesions and large bacterial burdens ensue. In contrast, anti-*M. leprae* responses in paucibacillary (PB) leprosy patients are skewed toward Th1 cells and replication and dissemination of *M. leprae* is limited. Many contacts of leprosy patients also exhibit *M. leprae* antigen-specific inflammatory T cell responses and the majority do not develop disease. Thus, the ideal vaccine against leprosy would induce strong, long-lasting T cell responses directed against *M. leprae* antigens that would limit infection and prevent disseminated disease.

Several killed or attenuated vaccines have been evaluated for their protective efficacy against *M. leprae* but, besides the continued use of BCG, which confers only partial protection [75–77], a vaccine is not currently available. In evaluations of crude *M. leprae* preparations as vaccines to limit bacterial replication, TLR4L, but not agonists of TLR7 or TLR9, permitted ~100-fold reducing quantity of heat killed *M. leprae* required to significantly reduce bacterial burdens in mice (unpublished data). The potential of a TLR4L to adjuvant a defined subunit vaccine was recently demonstrated in the therapeutic administration to *M. leprae*-infected armadillos of LEPF1, a chimeric fusion protein, with GLA-SE. As shown in the next section, the preclinical studies relating to TLR4 ligands have led to the development of licensed products in human vaccines.

3. Current Successful Vaccines Enabled by TLR4 Adjuvant Formulations

Modern adjuvant formulations have enabled a number of successful commercially-licensed vaccines over the last two decades. The first vaccines licensed with new adjuvant formulations were approved with a relatively narrow indication, and were intended for use in populations for whom traditional vaccine adjuvants provided incomplete protection. However, as the number of individuals receiving novel adjuvants has increased and a track record of safety has been established, the use of approved vaccines with modern adjuvants has extended to healthy adults and children around the world. Experience with each of these recently-licensed vaccine products is informative and summarized below.

3.1 Fendrix

In 2004, regulatory officials licensed the first vaccine containing the TLR4-agonist adjuvant, MPL. Fendrix™, from GlaxoSmithKline (GSK), contains a recombinant Hepatitis B Virus (HBV) surface antigen (sAg) combined with the adjuvant system "AS04" (MPL and

aluminum hydroxide). Worldwide, more than 300 million persons are infected with HBV, and public health authorities now recommend universal HBV vaccination at birth and among individuals at high risk of HBV infection such as HIV-infected persons and patients undergoing hemodialysis. The implementation of universal HBV vaccination has already reduced some of the long-term complications of HBV infection [78]. Importantly, however, as many as one in 10 healthy individuals immunized with the traditional HBV vaccine containing an aluminum hydroxide adjuvant will not mount a protective immunologic response [79]. The proportion of people failing to respond to the aluminum-adjuvanted HBV vaccine is even higher among the elderly (40%) [80] and patients with end-stage renal disease (ESRD). This latter group has a high risk of HBV infection, with up to 60% of ESRD patients being carriers of HBV prior to widespread vaccination, with few options to assure protection. Prior to the licensure of Fendrix™, the most common option for achieving protective antibody titers after vaccination was to double the dose of HBV sAg and to supplement the 3 shot vaccination series with an additional fourth injection at 2 months after the last injection.

Initial studies comparing Fendrix™ to the aluminum-adjuvanted HBV vaccine in healthy participants demonstrated that a higher proportion of individuals vaccinated with Fendrix™ achieved protective antibody titers, showed antibody titers to be significantly higher in the Fendrix™ group, and exhibited dose-sparing, with a vaccination schedule at 0- and 6-months to be equivalent to the aluminum-adjuvanted group receiving injections at 0-, 1- and 6-months [81]. A greater proportion of individuals receiving Fendrix™ reported pain, swelling and redness at the injection site, though the proportion reporting these to be “serious” was less than 5% and did not differ significantly between vaccination groups.

These studies were followed by comparisons between Fendrix™ (two-injection vaccination schedule) and high-dose aluminum-adjuvanted HBV vaccine (four-injection vaccination schedule) in hemodialysis patients. In these trials, a non-significant trend was observed where a greater proportion of Fendrix™ recipients achieved protective antibody titers (91%) compared with the high-dose standard vaccine (84%). More notable was the fact that 80% of Fendrix™ recipients maintained a protective antibody titer 3 years post-vaccination, while only 51% of patients in the high-dose standard vaccine arm retained protective titers [82,83]. Research is ongoing to see if a Fendrix™ can provide protection to other groups at high risk of both HBV infection and incomplete response to standard vaccine formulations, such as HIV-infected individuals [84].

3.2 Cervarix

Five years after the approval of Fendrix™, GSK received approval from the United States Food and Drug Administration (FDA) for the use of a modern adjuvant vaccine against human papillomavirus (HPV). Cervarix™ is comprised of virus like particles (VLP) of the HPV L1 epitope from high risk HPV strains 16 and 18, combined with the same adjuvant system in Fendrix™ (ASO4). Initial studies compared Cervarix™ to Gardasil™, a similar vaccine from Merck that contains HPV VLPs from strains 16 and 18 as well as the non-oncogenic strains 6 and 11, together with aluminum hydroxyphosphate adjuvant.

In healthy women and adolescent girls, there were notable differences between both vaccine formulations. Both vaccines induced high-geometric titer antibody responses to HPV 16, which were found to be durable. Antibody levels remained elevated for more than 8 years in Cervarix™ recipients, but declined to levels induced by natural infection as early as 3 years in those receiving Gardasil™ [85,86]. Antibody levels are not a surrogate for protection, as women with antibody levels below limit of detection remaining protected against acquisition. Cervarix™ vaccination also resulted in significantly higher pseudovirion-based neutralization assay (PBNA) titers, a higher retention of antibodies to HPV 18, stronger serum binding antibody responses, and superior cellular immune responses (proportion of CD4 T-cell responders to HPV 16 / 18 and memory B cell responses to HPV 18) [87].

HPV vaccines were initially evaluated for both immunogenicity and efficacy against one or more of a number of endpoints, including prevention of HPV DNA persistence, progression to anogenital dysplasia, or the development of cancer. Gardasil™ and Cervarix™ both provided >90% protection from persistence of the HPV strains included in each vaccine [88,89]. Notably, both vaccines induced varying degrees of protection from persistence of strains not included in the vaccine. More than 60% of Cervarix™ recipients were protected from persistence of other high-risk HPV strains, including 31, 45 (clade A9, along with HPV 16), and a minority of women were protected from persistence of HPV 33, 35 and 52 (also clade A9). Less than half of women who received Gardasil™ were protected from non-vaccine strains, including nearly 50% protection from HPV 31, and modest protection against HPV 33, 35, 52 and 59 [90,91]. The mechanisms by which these two vaccines protect against HPV strains outside of the vaccine formulation remain unclear, but it has been suggested that the adjuvant formulation plays a strong role [90]. Evidence to support this concept may come from studies with influenza, showing that adjuvant formulations extended protection beyond vaccine strains [30,92]. Among women who were HPV-negative as baseline, both Cervarix™ and Gardasil™ provided more than 90% protection against cervical dysplasia [93,94]. Consistent with studies of the ASO4-adjuvanted HBV vaccine, Cervarix™ administration was frequently associated pain, redness and swelling at the injection site, with 16% of participants having the pain characterized as serious. Frequencies of injection site reactions were similar with Gardasil™, though less than 1% of participants were characterized as having a serious event [95].

In 2014, the FDA licensed a new version of Gardasil™ that included nine high-risk HPV strains, and by 2016 GSK stopped supplying Cervarix™ to US markets due to very low demand for the bivalent vaccine formulation.

3.3 Shingrix

Varicella zoster virus (VZV) is the cause of varicella (also known as chickenpox) following primary infection and herpes zoster (HZ, also known as shingles) following reactivation of viral disease after latency. Prior to widespread vaccination against varicella, nearly 90% of the North American population acquired VZV, and approximately 20% of adults would develop HZ over the course of their lifetimes. With the introduction routine use of live-attenuated varicella vaccine (LAVV) in childhood, complications from varicella have

dropped significantly, though notably the incidence of HZ has increased markedly. It is estimated that in the US alone there are more than one million annual cases of HZ.

Two vaccines have been licensed for the prevention of HZ. First, a higher dose of LAVV was developed for the prevention of HZ and associated post-herpetic neuralgia (Zostavax™). Then in 2018, a new VZV glycoprotein E (gE) adjuvanted subunit vaccine was approved for the prevention of HZ in immunocompetent adults (Shingrix™). The adjuvant system used in Shingrix™ is known as “ASO1B”. It contains 50 micrograms of MPL with a liposomal QS-21. The comparison between Zostavax™ and Shingrix™ is not as similar as the previous comparisons, as the two differ in not just adjuvants but also antigen presentation. However, it is illustrative to examine the experience with both vaccine constructs.

Shingrix™ was similarly approved for the prevention of HZ in adults ≥50. The data on immunology, efficacy and safety of Shingrix™ differed substantially from Zostavax™. Among adults 50 and over, overall vaccine efficacy against HZ was 97.2% and notably did not differ by age decile [96]. Both CMI and antibody titers to gE increased exponentially after vaccination, in both people >60 and >70 years of age. Peak gE-specific T-cell responses were similar in both age groups [97]. Both CMI and antibody levels were durable for up to 9 years post vaccination. While the data on efficacy, immunogenicity and durability are quite impressive, experts noted the somewhat high frequency of adverse events in vaccine recipients [98]. Nearly 80% of participants reported pain in the vaccine arm (compared with 10% in the placebo group), and approximately 7% found the symptoms from the vaccine to be serious.

Within the first half-year after the introduction of Shingrix™, the vaccine had captured nearly 90% of the US market [99], showing the commercial success of adjuvanted protein subunit vaccines.

3.4 Mosquirix

Fendrix™, Cervarix™, and Shingrix™ all highlight the promise of novel adjuvants enabling new or more effective vaccines for adults and adolescents. However, in part because of the extreme caution with which new vaccine formulations are introduced into infants and young children, there had not been any vaccines adjuvanted with TLR4 agonists approved for use in this population. A promising place to start exploring the use of novel adjuvants in pediatrics emerged as vaccine developers showed that new adjuvants could enable successful vaccines against infections that had previously proved elusive to effective vaccines but were enormous global health problems. One such example came in the form of malaria. Nearly half a million people die from malaria each year, including a disproportionate number of children. Many years of research suggested that antibodies to the *Plasmodium falciparum* CS protein may be protective against malaria infection, and preclinical and early-phase clinical trials reported suboptimal immunologic responses to vaccine constructs containing the CS protein produced by recombinant yeast on a HBV sAg particle (RTS,S) adjuvanted with alum [100]. The addition of MPL boosted antibody responses to this vaccine formulation and in healthy adults was not associated with any safety concerns. After carefully deescalating the age at which the vaccine was provided to children in malaria-endemic areas and showing safety down to 1 year of age, a dose was selected and in a Phase

IIb study, RTS,S with MPL and QS-21 (AS01) reduced the prevalence of malaria by 37% and provided protection against severe malaria in 58% of children immunized [101]. More adverse events were noted in the control group than recipients of the adjuvanted RTS,S vaccine. In the definitive Phase III study in children (5–17 months) and young infants (6–12 weeks), RTS,S + AS01 reduced clinical malaria cases by 26% when given as a 3-dose primary series and 39% when a fourth booster dose was provided [102]. A small number of children (<0.3%) who received the adjuvanted vaccine reported central nervous system adverse events, primary fever, meningitis or seizures; this was attributed to chance by European Regulatory Authorities, who also required follow-up in Phase IV studies, but approved the vaccine for use in malaria-endemic regions in 2015.

Conclusions

In the nearly 15 years since the first TLR4 adjuvant was approved for clinical use, millions of adults and children around the world have safely received vaccines enabled by adjuvant formulations of these TLR4 ligands. A robust clinical pipeline and an increasing array of options for adjuvant formulations provides optimism that solutions to global infectious diseases that have been here-to-fore elusive will come within reach in the near future.

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