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New adjuvants for human vaccines

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Despite their obvious benefits, decades of research and hundreds of pre-clinical candidates, only a handful of adjuvants are approved for prophylactic vaccination of humans. The slow pace of development is due to a number of knowledge gaps, the most important of which is the complexity involved in designing adjuvants that are both potent and well tolerated. Recent advances in our understanding of innate immunity have led to the identification of immune pathways and adjuvant formulations more suitable for clinical advancement. One area of particular interest is the discovery of agonists that target the toll-like receptors. This review highlights recent progress of clinically approved vaccine adjuvants and identifies potential novel adjuvants that can broaden the development of new vaccines against infectious diseases.

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Introduction

Prophylactic vaccines play an instrumental role in preventing a variety of infectious diseases. For example diseases such as polio are near eradication owing to the availability of effective vaccines to prevent infection [1]. While there are many success stories in prophylactic vaccine development, significant challenges still remain in terms of improving the effectiveness of prophylactic vaccines and developing therapeutic vaccines against important infectious diseases, cancer and Alzheimer's disease. With recent advances in genomics and proteomics several candidate subunit vaccines are under investigation for clinical applications [2]. Subunit vaccine antigens are usually better tolerated compared to inactivated or live-attenuated pathogens, however, they are in general less immunogenic and often require the addition of an adjuvant to achieve protective immune responses. Identification and development of new adjuvants is necessary because the small number of currently

approved vaccine adjuvants do not always elicit the desired protective and sustained immune response against different target pathogens. For example, there is an acute need to develop effective and safe vaccines against important infectious diseases including HIV, malaria and tuberculosis. Thus the identification of vaccine adjuvants that can help to elicit a broad and sustained immune response in immunologically hyporesponsive populations such as the elderly, immunocompromised and the pediatric populations is acutely needed. Additional benefits in identifying effective and safe adjuvants encompass antigen dose sparing and a means to shift the immune response toward a specific T cell phenotype (Th1, Th2 or Th17) to mount the appropriate response against defined pathogens. Despite the need of new adjuvants for subunit vaccines very few compounds that have been selected in pre-clinical vaccine studies have been licensed for human use (Table 1). Failures of adjuvants during the development phase are related to the manufacturing process (lack of a reproducible formulation, negative impact on antigen stability) or to local or systemic adverse events [3]. It must be highlighted that the acceptable safety profile is different in the case of therapeutic or prophylactic vaccines. In most cases preventive vaccines are given to a large number of healthy children in the first years of age, therefore they have to be extremely safe and well tolerated.

Currently licensed vaccine adjuvants

Very few vaccine adjuvants have been licensed for prophylaxis in human. Among them alum (aluminum salts) has been widely used for more than 70 years and until recently represented the only adjuvant approved in the United States. Oil in water emulsions (MF59 and AS03) are licensed for adjuvanted influenza vaccines in Europe. AS04, a combination adjuvant composed of monophosphoryl lipid A (MPL) adsorbed to alum is approved for HBV and HPV vaccines in Europe and has been recently licensed in the USA. Tables 1 and 2 adapted from O'Hagan *et al.* [3] describe licensed adjuvants and adjuvants that have been evaluated in the clinic. This section will focus on the mechanisms of action of human licensed adjuvants (Figure 1) and their impact on vaccination.

Alum

Many vaccine formulations contain aluminum salts which increase stability and immunogenicity of antigens following adsorption onto the alum particulates. Alum is the most widely used adjuvant and is found in numerous vaccines in use today, including HAV, HBV, HPV, Diphtheria and Tetanus (DT), Haemophilus influenza

2 Vaccines

Table 1

Licensed adjuvants

	Company	Class	Indications
Alum	Various	Mineral salts	Various
MF59	Novartis	O/W emulsion	Influenza (Fluad)/pandemic flu
AS03	GSK	O/W emulsion + a tocopherol	Pandemic Flu (Pandemrix)
AS04	GSK	MPL + alum	HBV (Fendrix), HPV (Cervarix)
Liposomes	Crucell	O/w emulsion	HAV, Flu (EU)

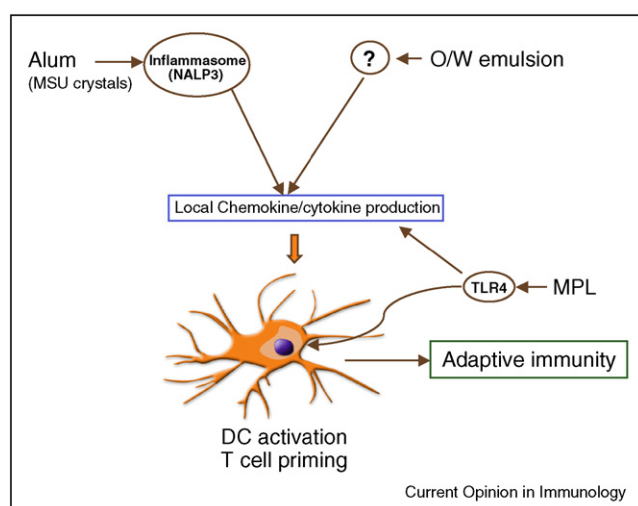
Table 2

Adjuvants in clinical development

	Company	Class	Indications	Stage
Montanide	Various	O/W emulsion	Malaria, cancer	Phase III
PLG	Novartis	Polymeric microparticles	DNA vaccine (HIV)	Phase I
Flagellin	VaxInnate	Flagellin linked to antigen	Flu	Phase I
QS21	Antigenics	Saponin	Various	Phase I
AS01	GSK	MPL + liposome + QS21	Malaria, TB	Phase II
AS02	GSK	MPL + W/O emulsion + QS21	Malaria	Phase II
RC529	Dynavax	Synthetic MPL + alum	HBV	Phase II
Iscom	CSL, Isconova	Saponins + cholesterol + phospholipids	Various	Phase I
IC31	Intercell	Peptide + oligonucleotide	TB	Phase I
CpG	Coley/Pfizer, Novartis, Idera	Oligonucleotide + alum, oligonucleotide + MF59, oligonucleotide	HBV, malaria, HVC, cancer	
MF59 + MTP-PE	Chiron/Novartis	Lipidated MDP + O/W emulsion	HIV, Flu	Phase I
ISS	Dynavax	Oligonucleotide alum	HBV	Phase II

type B (HIB) and pneumococcal conjugate vaccines. The mechanism of action of alum has only been under investigation recently [4–6]. In general, alum absorption increases antigen uptake and stability at the site of injection [7]. In addition, alum induces a local pro-inflammatory reaction that can increase immunogenicity [8,9].

Figure 1



Overview of the immune pathways involved in the adjuvant effect of key licensed adjuvants.

Several independent studies have shown that alum activates NALP3, a component of the inflammasome complex, implicated in the induction of several pro-inflammatory cytokines including IL1 β . *In vivo* studies conducted in NLRP3 deficient mice have shown that direct activation of NLRP3 contributes to alum adjuvanticity [10–12]. However, the view that the inflammasome/NALP3 pathway is required for the adjuvant activity of alum has been challenged by results demonstrating that the NALP3 pathway is dispensable for alum-mediated adjuvant activity *in vivo* [13,14]. The exact explanation of this discrepancy is unclear and could rely on the different model systems used and the possibility that redundant pathways can modulate the activity of alum in the host. Thus, pinning down precisely all of the immunological and molecular pathways involved in the adjuvant activity of alum has proved difficult. The key advantage of alum is that it has been used for over 70 years and has a proven safety record. However, pre-clinical and clinical studies have shown that alum is often less potent than other adjuvants, like oil in water emulsions. In addition, alum is a poor inducer of protective Th-1 associated immune responses, which are crucial for the development of vaccines against intracellular pathogens.

Oil in water emulsions

The squalene-based oil-water emulsion MF59 has been licensed in Europe for adjuvanted Flu vaccines since

1997 for its capacity to increase flu immunogenicity in the elderly [15]. More recent data have shown that MF59 enhances hemagglutination inhibition (HI) titers and cross-protection also in young children [16]. MF59 and a similar squalene-based oil in water emulsion called AS03 have been licensed in Europe for pandemic flu vaccines and have been widely used for the 2009 H1N1 pandemic flu campaign [17]. Before the H1N1 pandemic flu outbreak, clinical trials demonstrated that avian H5 pandemic flu vaccines containing oil in water emulsions are superior to alum-adjuvanted or non-adjuvanted H5 vaccines in the induction of protective antibody titers [18]. In particular, AS03 and MF59 allow for antigen dose sparing and increased seroconversion and cross-protection after only two doses [19,20]. A recent study has demonstrated that MF59 promotes an early CD4⁺ T cell response that predicts long term persistency of protective antibodies against the H5N1 influenza strain [21]. It has also been shown that priming with avian H5 vaccines containing MF59 or AS03 allows for better recall responses in individuals boosted many years after their primary vaccination [22,23]. Priming with adjuvanted vaccines was effective even when diverse clades of H5 were used for priming and boosting and led to a rapid rise in cross-neutralizing antibody titers and memory B cells [22]. Many reports have addressed the mechanism of action of MF59 recently. The emerging picture is that MF59 acts by inducing a local immunostimulatory environment at the injection site characterized by the up-regulation of cytokines, chemokines and other innate immunity genes, which promote the recruitment of CD11b⁺ and MHCII⁺ cells in the muscle [24]. An overlapping subset of chemokines is induced by MF59 *in vitro* in human granulocytes, macrophages and monocytes [25]. In addition to its immunostimulating activity MF59 is also known to enhance antigen uptake by dendritic cells [26].

MPL

A new class of vaccine adjuvant based on targeting the TLR pathways is emerging as an important element in the design of effective and safe vaccine adjuvants. The discovery of TLRs as key pathogen sensors that modulate the host innate and adaptive immune systems has triggered interest in exploiting this pathway to identify potential vaccine adjuvants and therapeutics agents. The concept behind TLR-based adjuvants stems from the fact that many successful vaccines demonstrate 'built-in adjuvanticity' from motifs that are now known to stimulate TLR and other innate pathways. For example, pathogen products from live-attenuated or heat killed vaccines can stimulate TLR and non-TLR pathways, thereby providing a key signal to boost antigen presenting cells and T and B cell responses directly and indirectly [27,28]. One of the most effective vaccines, the yellow fever vaccine, activates multiple subsets of DCs by signaling through TLR2, 7, 8 and 9 [29]. Therefore, it is

reasonable to hypothesize that defined TLR agonists or combinations thereof could be beneficial not only in improving existing vaccines but in developing novel vaccine formulations for prophylactic and even therapeutic interventions. In fact, a TLR4 agonist (3-O-desacyl-4'-monophosphoryl lipid A/MPL) adsorbed to alum known as AS04 is currently approved for use against human papilloma virus [30] and hepatitis B [31]. Recent studies to elucidate the mechanism of action of AS04 revealed a transient and local activation of NF- κ B activity and cytokine production, thus providing an innate immune signal for optimal activation of APC [32]. No synergistic effect between MPL and alum was noted, rather results from Didierlaurent *et al.* suggest that alum prolongs the cytokine response of AS04 at the injection site.

The contribution of MPL in human vaccines is further elucidated based on results from the head-to-head trial of the alum-MPL adjuvanted Cervarix vaccine versus the alum-adjuvanted Gardasil vaccine [30,33]. Both vaccines exploit virus-like particle technologies for the production of antigens and are highly efficacious in preventing HPV-16/18 associated cervical intraepithelial neoplasia in women from 15/16 to 25/26 years of age that are seronegative for the relevant vaccine type before vaccination [30]. However, Cervarix produced significantly higher neutralizing antibody titers for HPV-16 compared to Gardasil (3.7-fold higher) and previous reports have uncovered an increase in memory B cells at 1 month after the 3 doses of cervarix in HPV-16/18 seronegative women 18–30 years of age compared to Gardasil [34].

New TLR agonists in pre-clinical or clinical trials

TLR9 agonists represent perhaps the most advanced candidates in terms of potential as vaccine adjuvants [35]. Immunostimulatory sequences (ISS) containing repeating sequences of cytosine phosphoguanosine (CpG) dinucleotides targeting TLR9 are being evaluated as vaccine adjuvants. A synthetic oligodeoxynucleotide (ODN) containing these motifs, resulting in an unmethylated cytosine and phosphoguanosine ODN called 1018 ISS is being evaluated as an adjuvant to the recombinant hepatitis B surface antigen vaccine [36]. Clinical trials, to date, have shown that Heplisav produces rapid, high titer, sustained seroprotection in healthy adults and vaccine hyporesponsive populations [36].

Another class of TLR9 agonist based adjuvant called IC31 consists of a cationic peptide KLKL(5)KLK vehicle and the immunostimulatory oligodeoxynucleotide ODN1a sequence [37]. IC31 was found to promote highly efficient Th1 responses through a MyD88, TLR9-dependent manner [38]. The complex between the anti-bacterial peptide KLK and the immunostimulatory ODN results in a depot effect at the injections sites, thus contributing to prolonged retention of the antigen-adjuvant

4 Vaccines

formulation and subsequent amplification of an immune response. Furthermore, the presence of the antimicrobial peptide reduces the amount of ODN needed to elicit an immune response. IC31 is currently undergoing human clinical trials in a vaccine against tuberculosis (TB) on the basis of pre-clinical data showing that the combination of the TB antigens Ag85B-ESAT-6 and IC31 exhibited significant levels of protection in the mouse aerosol challenge model of tuberculosis [39,40]. Furthermore, the combination of Ag85B-ESAT-6/IC31 was found to confer efficient protection in the guinea pig aerosol model of tuberculosis infection [40].

The TLR7/8 pathway, specialized in recognition of single stranded viral RNA pathways, has demonstrated promising pre-clinical results as a target for potential vaccine adjuvant. Small molecules agonist of TLR7 (e.g. imiquimod) or TLR7/8 (resiquimod) were shown to improve the immunogenicity of a variety of vaccine adjuvants if adequately formulated or directly conjugated to protein antigens [41,42]. Importantly, it has recently been discovered that the TLR8 pathway might be an attractive pathway to design vaccine adjuvants in the neonatal population [43]. This contention stemmed from findings that TLR8 agonists, unlike TLR7 agonists, induced a robust production of Th1-associated cytokines, TNF- α and IL-12, from neonatal APC, lending support to the hypothesis that TLR8 agonist based adjuvants could prove beneficial as vaccine adjuvants in human newborns. Moreover, combined TLR3/8 or TLR4/8 agonists resulted in synergistic production of IL-12p70 in neonatal DCs, suggesting this strategy could prove beneficial in driving a Th1 response in response to pathogens and vaccine antigens [44]. Importantly, the TLR7 agonist imiquimod is licensed as a topical therapy for malignant and non-malignant skin cell disorders, demonstrating a safety and efficacy track record for this class of TLR agonists as therapeutics.

Incorporation of pathogens associated molecular patterns in vaccine formulations as a means to improve vaccine efficacy is further highlighted by the generation of a recombinant fusion protein consisting of a TLR5 agonist (flagellin) and four tandem copies of the ectodomain of the conserved influenza matrix protein M2e (called STF2.4xM2e). Immunization of mice with STF2.4xM2e protects them from a lethal challenge with influenza A virus [45]. Using a similar strategy, the same group demonstrated that immunization of mice with the recombinant-flagellin-ovalbumin (OVA) fusion protein resulted in potent antigen-specific T and B cell responses that were equal to or better than responses induced by OVA emulsified in Complete Freund's adjuvant. Importantly, the enhanced immunogenicity to OVA is dependant on the direct fusion to flagellin, as co-delivery of OVA with flagellin unlinked failed to augment antigen-specific responses *in vivo* [46].

Novel adjuvant combinations

Extensive pre-clinical and a growing body of clinical data demonstrate that TLR agonists are potent vaccine adjuvants. However, for some difficult vaccine targets a single adjuvant may not be sufficient to achieve a protective immune response. For this reason several combinations of TLR agonists with other classes of adjuvants have been tested in pre-clinical and clinical settings. Notably, 37% reduction of malaria infection has been achieved in a clinical trial using the *Plasmodium falciparum* recombinant antigen RTS,S formulated with the adjuvant AS02, composed of MPL, QS21 (a natural saponin adjuvant) and an oil in water emulsion [47]. Recently the RTS,S vaccine has been further optimized by substituting the oil in water emulsion with liposomes [48,49]. The AS02 combination has been co-administered also with the non-small lung cancer recombinant antigen MAGE-A3 allowing for superior antibody and CD4⁺ T cell responses. Despite the fact that both MPL and QS21 are known to enhance cross-presentation in pre-clinical models only two patients out of seven developed a measurable CD8⁺ T cell response to MAGE-A3 [50]. In the future adjuvant combinations may be further improved by using different TLR agonists in the same formulations. *In vitro* profiling on human and mouse immune cells has demonstrated that TRIF-dependent TLR pathways (TLR3 and 4) synergized with TLR7, 8 and 9 for the production of a select subset of genes including IL12 and IL23, increasing the DC potential to prime Th1 CD4⁺ T cells [51]. Pre-clinical data also show that combining TLR3 and TLR9 or TLR2 agonists results in a synergistic effect on the development of antigen-specific CD8 T cells [52].

Future adjuvant targets

In addition to the TLR pathway, there are other innate pathways that could be exploited to design effective and potentially safe vaccine adjuvants. Intracellular innate receptors such as retinoic acid inducible gene I (RIG-I)-like receptors (RLR), nucleotide binding oligomerization domain (NOD)-like receptors (NLR) as well as c-type lectins receptors modulate to varying degree recognition of microbial motifs (e.g. nucleic acids and carbohydrates) [28]. An important recent finding has linked RIG-I to the recognition of the RNA of hepatitis C virus, resulting in the expression of interferon-alpha/beta and antiviral/interferon-stimulated genes (ISGs) that limit infection. The HCV PAMP RNA stimulated RIG-I-dependent signalling thus induces a hepatic innate immune response *in vivo*, and triggers interferon and ISG expression to suppress HCV infection *in vitro*, suggesting that RIG-I agonist could potentially be developed as vaccine adjuvant and/or immunotherapies against infectious diseases [53]. Likewise, the C-type lectin dectin-1 was shown to drive maturation of DC and secretion of inflammatory cytokines including IL-6, TNF and IL-23 or IL12. Moreover dectin-1 agonist elicited an adjuvant activity *in vivo* by promoting the differentiation of Th-17 and Th-1 cells *in vivo* [54].

These receptors and others to be discovered, however, require further study and validation before their utility as vaccine adjuvant targets can be realized.

Conclusion

Clinical data have highlighted the importance of adjuvants for effective subunit vaccines. The experience with oil in water emulsions in pandemic flu and of MPL with HPV and HBV vaccines will pave the way for the clinical development of next generation adjuvants. In addition, recent advances in the immune pathways involved in modulating the host protective immune response have opened new avenues to design improved vaccine adjuvants. It is predictable that new combinations of existing adjuvants and novel classes of adjuvants targeting emerging molecular targets will improve the efficacy of novel preventive vaccines directed against infectious diseases. The real challenge is whether adjuvants will overcome the limitations of existing vaccines, allowing safe and effective immunotherapy against chronic infections and cancer.

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6 Vaccines

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