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Review

Correlates of adjuvanticity: A review on adjuvants in licensed vaccines

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ABSTRACT

After decades of slow progress, the last years have seen a rapid acceleration of the development of adjuvanted vaccines which have lately been approved for human use. These adjuvants consist of different components, e.g. aluminium salts, emulsions such as MF59 and AS03, Toll-like receptor (TLR) agonists (CpG ormonophosphoryl lipid A (MPL) adsorbed on aluminium salts as in AS04) or combination of immunopotentiators (QS-21 and MPL in AS01). Despite their distinctive features, most of these adjuvants share some key characteristics. For example, they induce early activation (although at different levels) of innate immunity which then translates into higher antibody and cellular responses to the vaccine antigens. In addition, most of these adjuvants (e.g. MF59, AS03, AS04) clearly induce a wider breadth of adaptive responses able to confer protection against, for example, heterovariants of the influenza viruses (MF59, AS03) or against human papillomavirus strains not contained in the vaccine (AS04). Finally, the use of some of these adjuvants has contributed to significantly enhance the immune response and the efficacy and effectiveness of vaccines in the elderly who experience a waning of the immune responsiveness to infection and vaccination, as shown for MF59- or AS03-adjuvanted influenza vaccines and AS01-adjuvanted herpes zoster vaccine. These results, together with the track record of acceptable safety profiles of the adjuvanted vaccines, pave the way for the development of novel vaccines at the extremes of age and against infections with a high toll of morbidity and mortality. Here, we review the mechanisms associated with the performance of those adjuvanted vaccines in animal models and in humans through recent advances in systems vaccinology and biomarker discovery. We also provide some perspectives on remaining knowledge gaps but also on opportunities that could accelerate the development of new vaccines.

1. Introduction

Although adjuvants are used in the preparation of most of the inactivated vaccines, their development has been very slow. Up to now very few adjuvants have been approved for use in humans. Since the time aluminium salts (alum) started to be employed in the preparation of tetanus and diphtheria toxoids in the 1920's [1], we had to wait until the 1990's to see the approval of the first vaccine containing a "new" adjuvant, i.e. the oil-in-water MF59 as part of the influenza vaccine for the elderly [2]. Another wave of adjuvanted vaccines was approved in the new millennium, with the successive approval of vaccines protecting against avian influenza virus (with AS03) [3], hepatitis B virus (HBV) [4] and human papillomavirus (HPV), both with AS04 [5,6], and finally herpes zoster virus (with AS01) [7] and HBV (with CpG) [8] (Table 1).

This relatively slow development of new adjuvanted vaccines is due to several factors. The need for adjuvants has been mainly driven by the use of purer components (e.g. purified recombinant antigens) for safer vaccines, that, however, exhibit lower immunogenicity, in contrast to live attenuated or inactivated whole-cell vaccines, which contain inbuilt adjuvanticity mediated by various immunostimulatory components (e.g. bacterial components of the cell wall, genetic material). The demonstration of the added value of adjuvantation over plain antigen requires the generation of additional evidence to validate the use of adjuvant, hence increasing the time of vaccine development. In particular, adjuvanted vaccines were developed to reach a level and quality of immune response that was not achievable with more classical approaches or alum-based vaccines, as exemplified by the development of the malaria or HIV vaccine [9]. Since the vast majority of existing vaccines are targeting healthy subjects, the assessment of their safety is of paramount importance. This implies that for vaccines containing new adjuvants (i.e. adjuvants other than aluminium salts), it is necessary to create a large enough safety database of individuals of various ages before the adjuvanted vaccine receives approval from regulatory agencies. This requires large phase III studies with appropriate sample sizes and takes several years.

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Table 1
Components of the adjuvants discussed in this review.[a]

Adjuvants	Components	Vaccines registered
MF59	Squalene; polysorbate 80; sorbitan trioleate	Seasonal influenza; pandemic influenza; avian influenza
AS03	Squalene; α-tocopherol; polysorbate 80	Pandemic influenza; avian influenza
AF03	Squalene; polyoxyethylene cetostearyl ether; mannitol; sorbitan oleate	Pandemic influenza
AS01	MPL[b]; QS-21[c]; liposome	Herpes zoster
AS04	MPL[b]; aluminium hydroxide	Hepatitis B virus; human papillomavirus

[a] See text for relevant references.
[b] 3-*O*-desacyl-4′-monophosphoryl lipid A.
[c] *Quillaja saponaria* Molina, fraction 21.

Despite the discovery of the Toll-like receptors (TLR) in the 1990's and of more pattern recognition receptors of the innate immune system later on, several gaps remain mainly for those adjuvants that do not work via those receptors, such as aluminium salts and emulsions. An earlier understanding of the mechanisms of action of adjuvants would have likely been beneficial in expediting their development, as discovery of biomarkers of safety and correlates of protection against diseases would have facilitated evaluation of potency and safety of adjuvanted vaccines in development. New technologies, such as systems vaccinology, are now being applied earlier during the testing of vaccines formulated with novel adjuvants with the hope of accelerating their development and their eventual introduction into clinical practice (reviewed in [10])

In this review we will discuss the mechanisms of action and immunobiological effects of adjuvants that are used in current clinical practice. Other reviews in this monographic issue will cover other vaccine adjuvants which are still at various stages of development.

2. Aluminium salts-based adjuvants

Despite the fact that aluminium salts are commonly used in vaccines, most of what we know today comes from observational, and not dedicated mechanistic studies in humans.

It is widely accepted that, in mice, aluminium salts are potent inducers of Th2-type immune responses. It is much less clear whether such Th2 bias is also observed in humans. Overall, aluminium salts are poor inducer of T-cell responses when evaluated in humans. This may be due to the lack of potent stimulation of the innate immune system, in comparison to TLR stimulation, for example. In animals, aluminium salts have been shown to stimulate some degree of inflammation, although this largely depends on the site of injection and type of aluminium salts [11]. It is now clear that the adjuvanticity of alum cannot be simply explained by a depot effect, as shown by studies using radiolabeled antigens and following excision of the skin at the site of inoculation [12].

The signals leading to innate stimulation by aluminium salts remain a matter of debate. Their adjuvanticity does not take place via TLR-dependent signaling as antibody responses to T-cell-dependent antigens remained unchanged when alum-adjuvanted antigens were administered to MyD88 and to TRIF knock-out mice [13]. The exact molecular target of alum remained elusive until various groups reported that in the mouse this target could be the NOD-like receptor protein 3 (NLRP3). Indeed, antibody responses to alum-adjuvanted antigens (e.g. DT/TT, OVA, or HSA) was impaired in NLRP3- or in caspase 1-decifient mice [14]. This effect could not be reproduced by others, however [15]. More recently it has been hypothesized that the phagocytosis of aluminium crystals can be at the basis of the activation of NLRP3, via swelling and rupture of the phagolysosomes with subsequent release into the cytosol of cathepsin B, which is involved in activation of caspase 1 and release of IL-1β [16]. Although fascinating, these hypotheses need to be validated in humans. When healthy human volunteers were treated with canakinumab, a human monoclonal antibody (mAb) against IL-1β [17], and then immunized with a conjugated vaccine against meningococci adjuvanted with aluminium hydroxide, the antibody response to the vaccine did not differ from that observed in the controls who had not been treated with the mAb, arguing against a direct role of IL-1β [18]. Additional studies are necessary to unveil the actual molecular mechanisms behind the adjuvanticity of aluminium salts. It is possible that intrinsic stress signals, yet to be discovered, are responsible for the observed effect in mice.

Gene expression analysis in mice reveals that the pro-inflammatory pathways, including IL-1β, are also common to other adjuvants such as MF59 and CpG [19]. In that study only 24 out of the 312 up-regulated genes were specific to alum. In addition, the local expression of some cytokines and chemokines, including IL-1β, were delayed as compared to MF59 and CpG, as was the case for the expression of MHC class I and II. Finally, when compared with the other adjuvants, aluminium salts failed to induce detectable cytokines in the peripheral blood [19]. Although able to promote antigen uptake by antigen-presenting cells (APC), aluminium salts are unable to directly activate dendritic cells (DC) [20], suggesting that APC activation may be indirectly triggered by the local inflammation in vivo rather than by alum itself. This may explain why aluminium salts are not strong inducers of cellular immunity.

Recent studies in rhesus monkeys have confirmed and extended most of the observations in mice. Immunization of non-human primates (NHP) with alum-adjuvanted HIV Env vaccine induced an infiltration in the muscles of neutrophils, monocytes, and DC, comparable with the infiltration induced by other adjuvants [21]; however, this infiltration was less prominent in the draining lymph nodes (LN), in line with alum's described biodistribution [22]. In addition, alum induced up- or down-regulation of co-stimulatory molecules on DC and monocytes in the muscles and migration of antigen-positive (Env^+) myeloid and plasmacytoid DC to the draining LN. These effects however were not paralleled by the ability of alum-adjuvanted Env to induce T follicular helper (Tfh) $CD4^+$ cells ($CD3^+$ $CD4^+$ $CXCR5^{high}$, $PD\text{-}1^{high}$) and the formation of germinal centers (GC) in the draining LN, as compared with other adjuvants (see below) [21].

In another study in NHP receiving the HIV Env antigen formulated with different adjuvants, alum performed less well than other adjuvants in inducing high and persisting levels of anti-Env antibodies and did not induce Ag-specific IFN-γ or IL-4-producing cells by ELISPOT [23]. It should be noted that this difference between adjuvants observed in the NHP was less pronounced in similar experiments carried out in mice. In agreement with what was reported in mice [19], some inflammatory and myeloid-associated gene modules were up-regulated, while no increase in serum cytokines was detected [23]. It is worthy to note that the different adjuvants induced qualitatively different antibodies, with potentially different effector functions. For example, antibodies produced after vaccination with alum-adjuvanted Env induced the strongest production of IFN-γ by NK cells in vitro [23].

How these observations in animal models can be translated to humans is unclear. The HBV vaccine, composed of the hepatitis B surface antigen (HBsAg) adsorbed on aluminium hydroxide, triggered a very weak innate response (assessed by blood cytokines or selected gene expression) in young adults naïve to HBsAg, as compared to other adjuvants [24], and this was associated with lower HBsAg-antibody and T-cell responses. Similar findings were reported for other alum-based vaccines (GDG, personal communication). The absence of significant innate responses in the blood does not necessarily mean that aluminium salts do not trigger inflammatory signals at the site of injection but it shows at least that animal data should be interpreted with care. On the other side, as compared to animals, differences may be influenced in humans by other factors, independent of the adjuvant. For example, a study in humans showed that HBV vaccination up-regulates different

modules of genes in subjects of different ages: transcriptional modules involved in B-cell signaling, TCR signaling and antiviral responses in young subjects, and transcriptional modules involved in inflammatory responses, cell mobility, and type II IFN in older subjects [25]. This study suggests that the signatures found in response to the same vaccine are influenced by the age of the vaccinated subjects, more than by the adjuvant itself.

3. Aluminium salts as vehicles for TLR agonists

Aluminium salts have been used as a platform for the development of novel adjuvants, mainly consisting of various TLR agonists adsorbed on alum. One, referred to as adjuvant system AS04, is already in clinical practice as part of the registered HPV and HBV vaccines.

Specifically, AS04 consists of aluminium salt formulated with 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL), a detoxified form of lipopolysaccharide (LPS) extracted from *Salmonella minnesota*. Studies in mice have revealed that MPL retains its full immunostimulatory activity through TLR4 when adsorbed on aluminium salts [20]. TLR4 activation by AS04 leads to the rapid (within 3–6 h) production of cytokines and cell recruitment in the injected muscle and draining LN. An increase in activated antigen-loaded monocytes and dendritic cells is observed within the first day after injection, which then translates into activation of antigen-specific T and B cells and induction of strong and persistent antibody and cellular responses. Aluminium salt did not appear to synergize with MPL, but comparison of MPL and AS04 showed that the presence of aluminium salts prolonged the cytokine responses induced by MPL at the injection site. The immunostimulatory effect of AS04 is therefore mainly due to TLR4 on innate cells, since lymphocytes, that do not express TLR4 in humans, do not respond to AS04 directly [20].

Consistent with a transient and local inflammatory effect, AS04 has to be coadministered with the antigen or administered at the same site of injection within 1 day from the administration of the antigen. Recent studies using the HBV vaccine formulated with various adjuvants have confirmed that AS04, present in the HBV vaccine, is able to trigger innate immunity in humans, but at levels lower than those observed with other more potent adjuvants [24]. As compared to HBV vaccine adjuvanted with aluminium salts, a slight increase in C-reactive protein (CRP) and IL-6 was observed in serum after administration of the HBV vaccine containing AS04. Nevertheless, the levels of HBsAg-specific T cells and antibodies were higher than those induced by the HBV vaccine adjuvanted with aluminium salts [26].

Both HBV and HPV vaccines adjuvanted with AS04 induce high levels of antibodies as compared with the same vaccines adjuvanted with aluminium salts, demonstrating the added value of the TLR4 agonist MPL in humans [26,27]. This higher immunogenicity translates into a high and long-lasting efficacy of the HPV 16/18 vaccine against the development of pre-cancerous cervical lesions. Data available now show that the efficacy of the vaccine approaches or even reaches 100% more than 9 years after primary immunization [28]. Some data suggest that this vaccine can be efficacious also against HPV strains not contained in the vaccine [29]. This may be due to the generation of cross-neutralizing antibodies or through other, possibly cell-mediated mechanisms which may be triggered by AS04. This broad immunogenicity and efficacy resembles that observed with influenza vaccines formulated with oil-in-water adjuvants (see below). Further studies are required to understand the precise mechanisms behind this beneficial effect of the AS04-adjuvanted HPV vaccine.

4. Emulsion-based adjuvants: MF59 and AS03

Emulsions have been employed as vaccine adjuvants since long, but we had to wait until the final years of the last century to have them approved for human use. This happened because of the mineral oils used in the first generation adjuvanted vaccines were not metabolizable and, despite being strong potentiators of the antibody response, they caused aseptic abscesses which were not resorbed [30]. The development of oil-in-water emulsions such as MF59 and adjuvant systems based on emulsions such as AS03, using fully metabolizable oils, resolved this issue and first allowed the development of improved seasonal inactivated vaccines against influenza, followed by vaccines against avian influenza (H5N1 and other strains later) and eventually against the pandemic influenza (H1N1 in 2009). These vaccines have now been approved in Europe and in the USA (e.g. the MF59 adjuvanted seasonal inactivated vaccine for the elderly, and, in Canada, for children; the MF59- and the AS03-adjuvanted pandemic vaccines in Europe; the MF59- and the AS03-adjuvanted H5N1 avian influenza vaccines for stockpiling in the USA). They have also been used with a panoply of other antigens in a wide category of people, such as adults, elderly, children, even infants at birth, pregnant women, etc. [31–33]. Collectively, the benefit and safety profiles of emulsion-adjuvanted vaccines are now very well established, following their use in millions of people worldwide.

The common component of oil-in-water emulsions (MF59, AS03, AF03, see Table 1) is squalene, a fully metabolizable lipid synthesized by the human body along the pathway of the cholesterol synthesis [34]. In addition to well-defined emulsion stabilizers, one of these adjuvants (AS03) also contains an immunostimulant, α-tocopherol (vitamin E) and is therefore called adjuvant system.

Oil-in-water emulsions are stronger adjuvants as compared to aluminium salts and have different mechanisms of action. Nevertheless they have two things in common: (i) their mode of action is not via TLR binding; (ii) their development, as for alum, was empirical and based on an rather old technology used to formulate compounds, without a solid understanding of the mechanisms underlying their immunostimulatory properties. Thus, as for aluminium salts, the exact molecular mechanisms involved in the adjuvanticity of emulsions remain unknown.

As in many (if not all) cases, our limited knowledge of the mechanisms of action of emulsions comes from studies in mice, and only more recently from studies in NHP and humans. In mice, MF59 promotes local activation of cells at the site of injection, antigen uptake by DC, without inducing a depot effect, leading to recruitment of mononuclear CD11b and F4/80$^+$ cells. The emulsion is found after 2 days in CD80$^+$ CD86$^+$ MHC class II$^+$, CD11c$^+$, CD11b$^+$ cells in the subcapsular sinus of draining LN. In terms of magnitude of the inflammatory response, MF59 is more potent than CpG and aluminium salts in upregulating genes linked with the innate immune response, such as *IL-1b*, *caspase-1*, and *Ccr2* and its ligands (*Ccl2, Ccl7* and *Ccl8*) [19]. More recent data have shown that MF59, but not other adjuvants such as aluminium hydroxide or calcium phosphate, induced the release of extracellular ATP from the muscle that may serve as endogenous danger signal [35]. Overall, this suggests that sensing of the lipid droplets in emulsions triggers the release of endogenous stress signals leading to activation of innate immune pathways. Furthermore, MF59 promotes a rapid influx of CD11b$^+$ cells into the muscle compared to other adjuvants. MHC class II$^+$ cells were also recruited in the muscle at 4 days, suggesting that CD11b$^+$ cells differentiate into functional inflammatory DCs, expressing high levels of MHC class II [19]. In summary, although not capable of activating directly DCs in vitro, MF59 can generate a local immunostimulatory environment characterized by the expression of several cytokines, which may indirectly activate DCs through TLR-independent mechanisms. Similar effects have been reported for AS03 [36]. Therefore, monocyte-derived cells, rather than bona-fide DCs, seem to play an important role in the mode of action of emulsions. In addition to recruitment and activation of cells at the site of injection, emulsions also favor the uptake of the antigen by antigen-presenting cells and its transport to the draining LN [36,37]. The presence of the immunostimulant α-tocopherol in AS03 was associated with an increased uptake of antigen by monocytes, as well as increased expression of CCL2, CCL3, IL-6, CSF3 and CXCL1 and was associated with a higher antibody response [36].

In line with their transient and local effect, emulsions need to be co-

localized with the antigen and have an effect on antigen-specific response only during a limited time window of 1–2 days, as demonstrated for AS03 [36]. This is in line with biodistribution studies showing that the components of AS03 are rapidly cleared from the injection site [38]. Importantly, formation of lipid droplets in the emulsion is required for the adjuvant effect, as squalene alone has no effect on the immunogenicity of co-administered antigen [39]. Finally, the superiority of emulsions over aluminium salts in enhancing antibody response may also be linked to the ability to promote a potent Tfh response. Indeed, MF59 promotes a potent Tfh response controlling the magnitude of the germinal center (GC) B-cell response, which was fully functional already in 3-weeks old mice [40].

Many of these findings were confirmed recently in studies carried out in NHP after intramuscular immunization with the recombinant HIV Env protein formulated with MF59 [21]. The adjuvant induced a rapid infiltration of immune cells at the site of injection, with uptake of Env by neutrophils, monocytes and DC, and an increase of those cells in only the LN draining the site of injection. Monocytes, DC and to some extent neutrophils isolated from the draining LN were efficient at stimulating T cells in vitro. In addition, as in the mouse, MF59 promoted T-cell activation which translated into increased Tfh-cell differentiation and formation of GC [21].

In humans, MF59- or AS03-adjuvanted influenza vaccines have consistently shown superior immunogenicity as compared to non-adjuvanted (whether avian or pandemic vaccines) [34,41]. In addition, both of them exhibited enhanced effectiveness against hospitalization due to influenza and to influenza-associated diseases [42,43], and both MF59 and AS03 also contributed to the enhanced efficacy against seasonal or pandemic influenza in very young children [44,45] and against cytomegalovirus (CMV) in CMV-negative mothers [46].

The exact mechanisms through which these beneficial effects are exerted in humans are still poorly understood. However, the ability of emulsions to drive an early inflammation, as demonstrated in animal models, has also been shown in man. The effect of MF59 on innate immunity was investigated at the transcriptional level in children vaccinated with adjuvanted or non-adjuvanted influenza vaccine [47]. The presence of MF59 induced a rapid increase -on day 1- in expression of type I interferons which positively correlated with the enhanced antibody response after boosting, which was weaker and delayed (to day 7) in children receiving the non-adjuvanted influenza vaccine [47]. These data support the notion that these early events at the level of the innate immune system represent a pre-requisite for subsequent induction of adaptive, antigen-specific immunity via generation of Tfh cells and of GC [40]. This would then translate into activation of B cells able to produce high affinity antigen-specific antibodies and to differentiate into long-lived memory B cells (MBC). Accordingly, the generation of antigen-specific Tfh has also been shown in humans 7 days after vaccination with MF59-adjuvanted influenza vaccines [48,49]; the increase of Tfh cells was also associated with the enhanced antibody response to the vaccine [49].

Using high-throughput B-cell receptor sequencing of plasma cells 7 days post-vaccination with AS03-adjuvanted A/H1N1 pandemic influenza vaccine, it was possible to distinguish sequences from cells recently activated from naïve B cells from those activated by memory recall. This suggests a dual action of the adjuvant: through increased activation of naïve B cells and through expansion of pre-existing BMC pools [50]. Indeed, existing data support the notion that antigen-specific IgG BMC are much higher after immunization with MF59-adjuvanted seasonal, pandemic, or avian influenza vaccines, and can very rapidly expand upon boosting even up till 6–8 years after priming [51]. Interestingly enough, the post-boosting expansion is much stronger in the adults who had received the H5N1 vaccine adjuvanted with MF59 or with AS02 (an oil-in water emulsion containing MPL and QS-21) at the priming [52,53]. In addition, the presence of MF59 with seasonal influenza vaccines significantly enhanced the production of antibodies cross-neutralizing drifted strains of A/H3N2 influenza viruses [54,55] as well as drifted strains of A/H5N1 influenza virus clades [56], with a concomitant significant increase in the affinity of these antibodies. This was more evident in infants and adolescents as compared to adults immunized with the pandemic A/H1N1 vaccine [57] and also more evident after priming-boosting with A/H5N1 vaccines adjuvanted with MF59 [58]. A peculiar finding in these studies was that as compared to plain H5N1 and to alum-adjuvanted H5N1 vaccines, the presence of oil-in-water adjuvants induced IgG antibodies mainly directed against the HA1 region of the globular head of the HA, containing the binding site. Pre-vaccination antibodies and those induced by plain or alum-adjuvanted vaccines were mainly directed against the stem region (HA2) of the HA [56,57]. Similar results were obtained with avian influenza vaccines adjuvanted with AS03 [59]; and GDG, personal communication. Studying monoclonal antibodies originating from subjects vaccinated with MF59-adjuvanted H1N1 pandemic vaccine made it possible to show that these antibodies recognized the wild-type virus as compared to the non-adjuvanted vaccine which induced antibodies directed mainly to the egg-adapted virus strains [60]. This suggests the propensity of oil-in-water adjuvanted vaccines to be more efficacious than non-adjuvanted vaccines, in line with the data from phase III efficacy trials in children [45] and from effectiveness studies in the elderly [42,43].

Common to all adjuvanted vaccines, an increase in short-lived local and systemic symptoms (often referred to as reactogenicity) is observed after vaccination when emulsions are used. The mechanisms of reactogenicity have not been studied in detail and not surprisingly, there are no established biomarkers of reactogenicity of vaccines in general, including adjuvanted vaccines. A study in adults in the UK vaccinated with the AS03-adjuvanted A/H1N1 pandemic vaccine reported a higher expression of a small set of genes in those individuals who had reported adverse events of medium/high intensity [61], although it is not clear whether these genes directly related to clinical symptoms. Interestingly, the same subjects with medium/high adverse events overexpressed many B-cell genes before and after vaccination, a signature which correlated with a pre-vaccination overexpression of $CD27^{-}CD38^{high}CD24^{high}$ transitional B cells [61]. B-cell signatures appear evident very early after vaccination with other oil-in-water adjuvants (GDG, unpublished). It needs, then, to be determined whether these signatures are specific for one particular adjuvant, for a family of adjuvants, or could represent a common signature of a propensity to reactogenicity which becomes more apparent in individuals with specific pre-vaccination phenotypes or with defined, still unspecified genetic signatures.

5. Combination of immunostimulants: the example of AS01

AS01 is unique amongst other adjuvants as it contains two discrete immunostimulatory molecules known to have adjuvant properties on their own, i.e. MPL and the saponin QS-21. QS-21 is a triterpene glycoside purified from the bark extract of *Quillaja saponaria* Molina (fraction 21), known in animal studies to enhance antibody responses and to promote specific T-cell responses [62]. MPL and QS-21 are formulated together in liposomes in the presence of cholesterol in order to abrogate the hemolytic activity of QS-21. While the mode of action of MPL is well defined, it is since only recently that some molecular mechanisms of QS-21 adjuvanticity have been proposed. Upon intramuscular injection, QS-21 targets subcapsular macrophages in the lymph node draining the injection site where it activates caspase-1 [63]. Although capsase-1 activation is NLRP3-dependent in vitro, NRLP3 does not seem to play a role in adjuvanticity in vivo [63,64]. When formulated in liposomes, QS-21 signals through a cholesterol-dependent endocytosis followed by lysosomal destabilization and Syk kinase activation, similar to other adjuvants such as aluminium salts [65]. Collectively, the specific activation of innate pathways and cells by AS01 components is critical, as depletion of TLR4, caspase-1 or subcapsular macrophages individually has been observed to impact the

adjuvant effect of AS01 in mouse models.

AS01 was initially developed to promote sustained cellular responses, in addition to antibodies, in a quest to develop vaccines against pathogens for which cellular immunity was thought to be critical for protection [9]. AS01 has now been evaluated in several candidate vaccines and shown to consistently increase both antibody and T-cell responses, regardless of the antigen used, and age or specific immune conditions involved (reviewed in [66]). Importantly, this enhanced immunogenicity translated into clinical efficacy which allowed the development of the vaccine against *Plasmodium falciparum* malaria [67], and the vaccine against herpes zoster [7].

As in the case of other adjuvants, most of the knowledge on the mechanisms of action of AS01 derives from studies in animals -mice and NHP- in which this adjuvant was formulated with the different antigens (e.g. the VZV antigen gE contained in subunit herpes zoster vaccine, ovalbumin, HBsAg, etc). More recently, additional information has been acquired in clinical studies in which this adjuvant (either full dose of the immunostimulants, referred to as $AS01_B$, or half dose, referred to as $AS01_E$) was administered with HBsAg and compared with other adjuvants (alum, AS04, AS03, CpG) [24,26,68] and in studies in which volunteers vaccinated with RTS,S were challenged with *P. falciparum* [69,70].

In essence, data from mice show that AS01 induces a similar local activation of the innate immune system as previously described for emulsions or TLR ligand-based adjuvants. AS01 may nevertheless be more rapidly drained to the LN and be more potent at activating a broader repertoire of APC, comprised of resident and monocyte-derived dendritic cells. [71]. In addition, in contrast to alum-based adjuvants or emulsions, AS01 does not increase antigen uptake per se at the individual cell level but increases the number of cells carrying the antigen.

As for other adjuvants, AS01 must be delivered at the same site or within a 1–2 day time window in order to observe an enhanced antibody and T-cell response. What makes AS01 different from the other adjuvants described in this review is the synergistic effect observed between MPL and QS-21, a fundamental advantage obtained by combining two different immunostimulants. A striking feature is the ability of AS01 to induce novel pathways that are not triggered by either of the components alone [72]. One of these emergent pathways is IFN-γ-related. Blocking IFN-γ in vivo abrogates the synergistic effect of MPL and QS-21, characterized by the increase in polyfunctional $CD4^+$ T cells specific to the co-administered antigen (i.e. expressing IL-2, IFN-γ and TNF-α), independently of the antigen used [72,73]. A few hours after immunization with AS01-adjuvanted vaccines, a synergistic effect of MPL and QS-21, mediated by IL-12 and IL-18 and macrophages, triggers the rapid production of IFN-γ by cells resident in the draining LN (mainly NK cells). This early production of IFN-γ by NK cells is essential for the optimal activation of DC and for the induction of the Th1-type functional immunity by the AS01-adjuvanted vaccine. While the mechanism was described in mice, similar IFN-γ production is observed in the LN of AS01-injected macaques. An increase in serum IFN-γ at day 1 and in the frequency of cytokine-producing antigen-specific $CD4^+$ T cells was also evident in humans vaccinated with the RTS,S malaria vaccine [72].

In the comparative clinical study with HBsAg mentioned above, HBsAg formulated in AS01 and most notably with the full dose $AS01_B$ was the most potent inducer of innate responses, which was mainly detectable after two doses. A transient increase in CRP, limited number of cytokines (IL-6, IFN-γ and IP-10) as well as monocytes was observed as early as 24 h post-immunization, subsiding to baseline within 1–3 days. Increases in the expression of cytokines and selected genes associated with those cytokines were mainly observed after the second dose of the vaccine, in particular IFN-γ and IP-10 and corresponding IFN-inducible genes (e.g. *STAT1*, *IRF1*, *MX1*, *CXCL10*). Interestingly, this innate signature was similar between AS01 and AS03. However, AS01 adjuvant was the most potent adjuvant at promoting HBsAg-specific antibodies and $CD4^+$ T cells and was also associated with higher reporting of reactogenicity symptoms. In fact the intensity of the innate signature was associated with the level of antigen-specific responses on the one hand, and the level of reactogenicity symptoms on the other hand [24,26]. Of note, the only difference with AS03 was a slight up-regulation of the *STAT1* gene 1 day after the first dose of vaccine in more subjects who had received the HBsAg formulated with $AS01_B$ or $AS01_E$, as compared to HBsAg formulated with AS03, suggesting that an INF-γ response may be present already at dose 1 with AS01, in the absence of antigen-specific response (volunteers were naïve to HBV in this study) [24]. Surprisingly, despite a significant difference in the total frequency of antigen-specific $CD4^+$ T cells induced between the adjuvants, the proportion of polyfunctional $CD4^+$ T cells (i.e. positive for IL-2, IFN-γ and TNF-α) did not differ among the various adjuvants. This was somewhat counter-intuitive as strength of innate signals, in particular of Th1- polarizing nature, would be expected to lead to higher polyfunctionality.

A systems biology approach was also applied to studies in which RTS,S-vaccinated human volunteers were challenged with malaria parasites to try to identify potential markers associated with the vaccine-conferred protection. Circumsporozoite protein (CSP)-specific antibody titers before vaccination were associated with protection against malaria. In addition, protection was associated with molecular signatures of B cells and plasma cells. Intriguingly, NK-cell signatures in blood were negatively correlated with protection [69]. These data may seem contradictory to the observations in mice and in NHP where an early production of IFN-γ by NK cells was seen [72], and to the early IFN-γ production in humans as potential predictor of protection against malaria challenge [74]. However, a reduction in the number of NK cells in blood – which could potentially have been recruited locally at the site of vaccination – may explain the inverse correlation observed in the human challenge model. In a similar study, the expression of the IFN-γ signaling pathway, among others, represented the best model of prediction of the vaccine-conferred protection against *P. falciparum* malaria [70].

It remains to be understood mechanistically what the role of AS01 exactly is in the high efficacy provided by the vaccine against herpes zoster and post-herpetic neuralgia in the older adults, observed even at an age > 80 years [75–77]. It is clear that the vaccine somehow overcomes the limitations of a declining or compromised immune system and it is tempting to hypothesize that AS01 plays a major part in this process. Although restoring protective levels of cellular immunity to VZV is one of the proposed mechanisms, other mechanisms may be involved. Understanding this mechanism of protection will need to be accomplished in appropriately designed clinical studies by applying the methodologies of systems vaccinology and by integrating knowledge about age-related waning of the immune responsiveness [78]. Attention should be paid, however, to the role that factors different from age (e.g. environmental factors, co-morbidities, frailty, pharmacological treatments, immunological history) might have on the waning of the immune responsiveness against infections and following vaccination [79,80].

6. Conclusions

The past two decades have seen a tremendous progress towards the development of novel vaccine adjuvants which have now entered clinical practice and have shown very good performance in the prevention of infectious diseases, such as influenza and cervical cancer due to HPV in real-life settings and more recently in clinical settings with malaria and herpes zoster. In addition, the application of new methodologies, collectively referred to as systems vaccinology, has started to unveil the mechanisms underlying the enhancement of the protective immune response in adults, children, and the elderly. This knowledge is particularly important to anticipate any potential safety issues specifically linked to adjuvanted vaccines and possibly to better predict

efficacy. In this respect, the correlates of immune responsiveness and of safety and efficacy become of paramount importance.

An important point to consider is that most of the mechanistic studies on adjuvants, including aluminium salts, have been done in mice. The choice of the mouse model is reasonable because of the easy access to lymphoid organs and the possibility to finely dissect the various facets of the immune response. Nevertheless, most of the results obtained so far need to be confirmed and validated in human beings. Data available now in humans underline the fact that, if there are commonalities between animals (mice and NHP) and humans, there are also peculiarities that should not be disregarded. For example, immunization of mice with oil-in-water (e.g. MF59) adjuvanted vaccine induces a strong Th2 type antibody and cellular response which is not observed in humans at any age, where instead a Th0-Th1 profile tends to prevail. Also gene transcripts induced by these adjuvants can differ in mice and in NHP [19,23]. Hence, while models are useful to address specific questions, those same questions should be validated in humans. It will also be important to find ways to investigate the immune response in humans not only in the bloodstream, as it has been commonly done so far, but also in lymphoid organs at the most appropriate time points, for example in the bone marrow and in draining LN, which has started to be addressed. Even more importantly, a better understanding of the immune effectors at the priming site (site of injection) and at the site where the protective effect is expected will be critical to ensure that assessment of immune response parameters in the blood provides a truthful representation of the immune response [81].

Based on what is discussed above, it is striking to observe that, despite the clear differences in the composition of the different adjuvants in clinical use (aluminium salts, oil-in-water liposome- based adjuvants with various components, etc.), they exhibit a common inflammatory signature in the first few hours after vaccination. It seems that the differences are more quantitative than qualitative, and more linked to the kinetics of the events triggered. For example, subjects immunized with the avian H5N1 split vaccine adjuvanted with AS03 exhibited gene transcripts on day 1 post-vaccination related to IFN signaling (*STAT1, IRF1, GP1*), increased frequencies of monocytes, neutrophils, DC, NK cells, and serum cytokines IL-6 and IP-10 [82], not very different from the findings following vaccination with AS01-adjuvanted HBs Ag [24]. In addition, a similar peak of serum CRP, remaining within the physiological range, was observed one day post-vaccination in the study by Burny et al and in another study investigating MF59-adjuvanted seasonal trivalent vaccine, with levels peaking around day 2–3 post-vaccination (GDG, unpublished). As further studies will become available, it is possible that more subtle differences in innate responses become apparent that may explain the differences in the quantity and quality of the immune response triggered by vaccines with different adjuvants. These differences may also depend on the vaccine antigen, therefore the conclusions of such comparisons of adjuvants may not necessarily hold true for all antigens.

Another common behavior of the adjuvants is their ability to increase the number of antigen-loaded cells in the draining LN. However, all what we know on the effects of adjuvants on APCs strictly derives from studies carried out in mice, and more recently in NHP [23]. Very little, if any, information is available from humans. This is clearly an area that deserves careful attention and that will be better deciphered when methodologies to have access to LN material will be more broadly available and applicable to clinical studies. In particular, it will be important to understand the relative contribution of monocyte-derived versus bona-fide dendritic cells in the adjuvant-mediated increase in T-cell stimulation in humans.

Access to LN will also help to validate hypotheses related to Tfh and GC formation, in particular in the case of emulsions where effects on Tfh have been demonstrated. We know that the oil-in-water adjuvant MF59 is able to induce Tfh and formation of GC in the LN of mice [40], however this effect is still unknown in humans, although some preliminary studies based on the frequency of Tfh in the peripheral blood suggest that this can well be the case [48,49]. Definite differences exist among adjuvants, mainly those based on TLR agonists, based on the presence or absence of the relevant receptors on some cell populations. For example, AS04 is unable to act directly on B cells since these cells lack TLR4, suggesting that their immune potentiation of the B-cell response has to be mediated via activation of T cells [20]. The situation can be different for the oil-in-water adjuvants as MF59 is known to activate both naïve and MBC [83] and to be able to significantly expand pools of pre-existing MBC specific for avian H5N1 or for pandemic H1N1 influenza viruses [52,83].

Most of the parameters or correlates defined up to now following vaccination with adjuvanted vaccines relate to populations, and much less to individuals. For example, correlates of protection apply to individuals because they allow to determine whether or not a specific person can -or cannot- be considered protected against a given disease. This applies to well established vaccines such as those against tetanus, diphtheria, HBV, Hib, etc. [84]. Markers undeniably associated with protection or with reactogenicity at the individual level have not been defined for the moment for many other adjuvanted vaccines. Indeed, in most of the studies carried out so far using gene profiling or other systems biology approaches important variability has been consistently shown at the inter-individual level. This variability renders it very difficult to translate this information directly to establish predictors of efficacy and/or of safety of novel adjuvanted vaccines. These aspects are all very important to better understand mechanistically, and will have to be taken into consideration during the development of new adjuvants and adjuvanted vaccines.

Disclosures

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