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One important lesson learned is to apply the KISS principle whenever possible, KISS is an acronym for Keep It Simple Stupid! To encourage success in adjuvant development, unnecessary complexity must be avoided.

The path to a successful vaccine adjuvant – 'The long and winding road'

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New generation vaccines will increasingly comprise highly purified recombinant proteins. Unfortunately, these antigens are often poorly immunogenic. Therefore, adjuvants will be required to enable these proteins to become effective vaccines. Although several novel adjuvants have recently emerged, including formulations comprising more than one adjuvant, the approval of vaccines containing novel adjuvants has been slow, particularly in the US. However, despite significant ongoing concerns, the necessary safety data is now emerging to show that new generation adjuvants can be safely used in diverse human populations. In combination with data showing the positive contributions of the adjuvants to the immune response, this safety data should allow several vaccines containing novel adjuvants to obtain licensure within the next few years.

What are adjuvants, which ones are currently available?

Vaccine adjuvants are defined by the effects that they achieve, so they tend to defy easy descriptions of what they actually are. The earliest definition of vaccine adjuvants describes them as components that are added to vaccine antigens to make them more immunogenic [1]. Because adjuvants are defined so imprecisely, there are many candidates available and new ones are regularly described in the literature. To add to the lack of clarity around adjuvants, many of the 'new' ones are actually variations on old themes, often repackaged to make them more potent. Moreover, as the signaling pathways involved in immune activation are becoming increasingly better defined, many more 'new' adjuvants will emerge, or be rediscovered. This will inevitably add to the complexity. Attempts have been made to group adjuvants into classes, to try better to define how they work. For example, a broad range of adjuvants have been grouped as 'delivery systems', which means that their predominant mechanism of action was thought to be the delivery of antigens to immune cells [2]. In addition, an alternative range of adjuvants have been described as 'immune potentiators', because they exert direct effects on immune cells, leading to their activation [2]. However, this classification system, which was always simplistic, has been largely superceded by recent observations, that highlighted that delivery systems are also immune potentiators [3,4]. An alternative way to define adjuvants is in terms of the signals

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TABLE 1

Adjuvant formulations tested in humans

Name	Company	Class	Indications	Stage
Generation 1 adjuvants				
Alum	Various	Mineral salt	Various	Licensed
MF59	Novartis	O/W emulsion	Influenza(Fluad)/pandemic flu	Licensed (EU)
Liposomes	Crucell	Lipid vesicles	HAV, Flu	Licensed (EU)
Montanide	Various	W/O emulsion	Malaria, cancer	Phase III
PLG	Novartis	Polymeric microparticle	DNA vaccine (HIV)	Phase I
Flagellin	Vaxinnate	Flagellin linked to antigen	Flu	Phase I
QS21	Antigenics	Saponin	Various	Phase I
Combination adjuvants – generation 2				
AS01	GSK	MPL + liposomes + QS21	Malaria, TB	Phase II
AS02	GSK	MPL + O/W emulsion + QS21	Malaria	Phase II
AS03	GSK	O/W emulsion + α tocopherol	Pandemic flu (Pandemrix)	Licensed (EU)
AS04	GSK	MPL + Alum	HBV (Fendrix), HPV (Cervarix)	Licensed (EU)
RC-529	Dynavax	Synthetic MPL + Alum	HBV	Phase II
Iscom	CSL, Isconova	Saponins + cholesterol + phospholipids	Various	Phase I
IC31	Intercell	Peptide + oligonucleotides	TB	Phase 1
CpG 7909	Coley/Pfitzer Novartis	Oligonucleotide + Alum, oligonucleotide + MF59	HBV, malaria, HCV	
ISS	Dynavax	Oligonucleotide Alum	HBV	Phase II
MF59 + MTP-PE	Chiron/Novartis	Lipidated MDP + O/W emulsion	HIV, Flu	Phase I

that they provide to the immune system [5,6], but this does not really aid in understanding what they are, although it says a little more about what they do. Unfortunately, this classification system will inevitably be subject to regular redefinitions as more is understood about the signaling pathways involved in the activation of innate immunity. For a long time, there was very little progress in understanding the mechanism of action of adjuvants, but there has been a recent renaissance in this area. Currently, the danger is that so much information is emerging, if we try to use the signaling pathways involved to define the adjuvant, many different groups will emerge, which will regularly need to be reclassified. However, we believe that it is possible to gain a clearer understanding of adjuvants, if we think of them simply in terms of different generations. This offers an opportunity to find commonality among the diverse approaches under evaluation, while not being prone to regular reassessments.

The canonical member of the first generation of vaccine adjuvants is represented by insoluble aluminum salts, which have been generically called Alum (Table 1). Alum was originally identified in the 1920s [7] and is now used in licensed vaccines all over the world [8]. Emulsion adjuvants were first introduced only a decade later by Freund [9] and also belong to the first generation of adjuvants. Water in oil (W/O) emulsions were originally thought to work similarly to Alum, because they released antigen over an extended time period from the entrapped water in heavy mineral oils [10]. Hence, the two most successful generation 1 adjuvants are linked by their physical structure and dimensions; they are both particulate dispersions (Alum aggregates or emulsion droplets), to which antigen is bound or associated. Moreover, both of them were designed to extend the duration of antigen persistence at the injection site. It was also thought to be important that the adjuvant induced a degree of local inflammation, to help recruit antigen-presenting cells (APCs) to the injection site. In addition, several alternative particulate carriers were also evaluated as adjuvants in the 1970s, including polymeric particles [11] and liposomes [12]. Like Alum, these alternative approaches had the appropriate dimensions for uptake into immune cells and they were able to adsorb or encapsulate antigen to enhance persistency and improve delivery. On the basis of their particulate structure and dimensions, polymeric particles (or microparticles) and liposomes also belong to the first generation of adjuvants.

Generation 2 adjuvants were initiated in the 1970s when additional components were added to generation 1 adjuvants to enhance their potency. The development of generation 2 adjuvants was really followed from the discovery of synthetic components which activated the immune system, including muramyl dipeptide (MDP). MDP was originally identified as the smallest water-soluble component of mycobacterial cell wall with adjuvant activity [13]. It was quickly realized, however, that components extracted from microorganisms would not be optimally effective as single components, so they were coupled to, or linked to the existing generation 1 adjuvants, including liposomes [14]. Even before MDP had been identified, synthetic double-stranded RNA had been added to an emulsion adjuvant in 1969 to improve its potency for flu vaccines [15]. Hence, generation 2 adjuvants, which comprise more than one adjuvant component, have been around for almost 40 years (Table 1). Only now, however, are generation 2 adjuvants reaching licensure in approved vaccine products. Hence, adjuvant development is clearly a slow and difficult process and there have been many failures along the way. This article will not only discuss some of the failures, but also highlight successes, while speculating on what might have made the difference. In addition, we will look forward and define what we believe will represent the third generation of vaccine adjuvants.

Success in adjuvant development is limited and will continue to be elusive

Although there are an increasingly large number of adjuvant candidates available [16], if we define success as inclusion in a licensed vaccine product, there are very few 'successful' adjuvants (Fig. 1). The most successful adjuvant is Alum, because this has been included in licensed products all over the world for more than 50 years [8]. A second adjuvant that could be considered a success is the MF59 oil in water emulsion (O/W), which has been

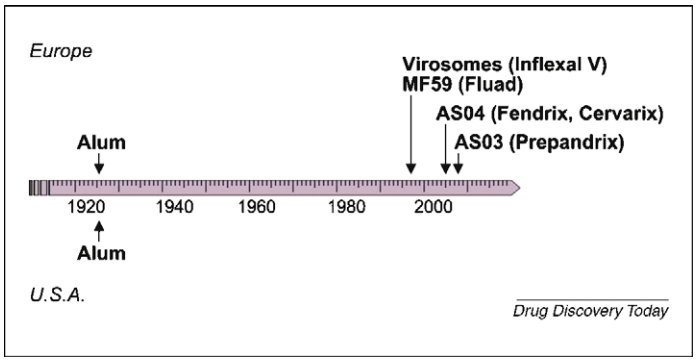

FIGURE 1

Alum was licensed in the 1920s and is still the only adjuvant included in vaccines approved for human use in the US. MF59 and virosomes were licensed for inclusion in flu vaccines (Fluad and Inflexal V) in 1997. The LPS analog monophosphoryl lipid A (MPL) formulated with Alum (AS04) was included in a licensed vaccine for a HBV (Fendrix) in 2005 and for a HPV vaccine (Cervarix) in 2007. The oil-in-water emulsion AS03 was approved for a pandemic flu vaccine (Prepandrix) in 2008.

licensed for more than a decade in a significant number (>20) of countries [17]. An additional adjuvant technology that has gained approval is virosomes, which, like MF59, have been licensed as a component of an influenza vaccine [18]. It is not clear, however, whether virosomes significantly enhance immune responses over the response achieved with the vaccine alone [19]. Therefore, it is not clear if virosomes are actually adjuvants, because they may fail to meet the primary definition [1]. Nevertheless, if we consider Alum, MF59 and virosomes as successful adjuvants, on the basis of licensure in a vaccine and an acceptable safety profile, it may be useful to consider their shared features and similarities. As a consequence of their size, Alum, MF59 and virosomes are efficient at delivering coadministered antigens to immune cells, which have evolved to take up, degrade and process microorganisms. More practical considerations under-pinning the success of these technologies include their ability to be scaled and reproducibly manufactured, within parameters that can be easily quantified. To optimize their chances of success, adjuvants should ideally be composed of cheap and easily obtainable components that are not inherently variable and should be biodegradable, or at least biocompatible and well tolerated. Although it might be argued that only Alum has really achieved broad success as an adjuvant, because it is used in a range of vaccines produced by different manufacturers, preclinical data show that MF59 is a more potent adjuvant for a broader range of vaccines than Alum [20]. Moreover, additional manufacturers are also bringing forward O/W emulsion-based adjuvant technologies similar to MF59, although sometimes additional components are added, which are claimed to be immune potentiating [21,22]. Our own experience teaches us to be very cautious in adding additional components to emulsion adjuvants and developers will need to show that added components genuinely enhance immune responses over the emulsion alone, while not contributing negatively to the safety profile.

The most advanced second generation adjuvant is Alum/MPL (AS04), which has been licensed in a significant number of countries as a component of a vaccine against human papilloma virus [23] and for an improved vaccine against Hepatitis B virus [24]. GSK have led the field in the development of second generation adjuvants, with their so-called Adjuvant Systems (AS) [25]. Although it is often not clear which components are actually contained in the adjuvant compositions, the AS series are generally combinations of Alum, or emulsion, or liposomes, combined with the immune potentiators MPL and/or QS21. Hence most of these systems are actually multi-component adjuvants. The immune potentiator components are added to increase antibody titers, or to induce a more potent and focused T cell response. The more complex formulations, comprising three or more adjuvant components are particularly designed to induce more potent Th1 cellular immune responses, as defined by IFN-γ secreting CD4+ T cells [26]. AS02, a multicomponent adjuvant formulation is showing particular promise as a malaria vaccine candidate [27]. Less complex formulations, including AS04, which contains only two components, have already achieved significant success in terms of inclusion in licensed products all over the world, excluding the US. In addition to the GSK AS, there are several alternative generation 2 adjuvants in various phases of development (Table 1). However, there is already a long and largely unsuccessful history of previous attempts to include immune potentiators in vaccine adjuvants, so it is difficult to predict which of these approaches will ultimately prove successful. Hence, the decision on which immune potentiator to include in second generation adjuvant formulations will continue to be a difficult one, until more success is achieved and the path to success becomes clearer. Moreover, given this challenging environment, a key early question in any vaccine development program is to consider the need for a more potent adjuvant beyond generation 1 approaches. Clearly, the path to vaccine licensure will be longer if a second generation adjuvant is required to enable vaccine efficacy. It will be necessary to show that the second generation adjuvant is required to achieve the desired response and that it provides a clear and consistent benefit over first generation alternatives, to justify inclusion. The challenges that arise as a consequence of including a second generation adjuvant in a vaccine product are very demanding from a regulatory perspective, and this needs to be fully appreciated by the group developing the vaccine candidate (Box 1).

Why do we need new adjuvants, why do we keep trying?

In light of the enormous difficulties encountered in developing new vaccine adjuvants and the limited success achieved so far,

BOX 1

Some of the hurdles for adjuvant development

1. Risk/benefit analysis for the adjuvanted vaccine must be favorable in relation to pathogen threat, incidence of disease and consequences of infection.
2. Vaccines are used in healthy people, often children, risks must be minimal.
3. Must show a clear need for the adjuvant to be present.
4. The presence of second adjuvant (generation 2 adjuvants) must have clear impact on potency, while not significantly changing risk/benefit analysis.
5. Large safety database will be needed, probably including an analysis of potential impact of adjuvant on auto immune diseases.
6. Preclinical toxicology studies may not be predictive of adverse events in humans.

BOX 2

Why adjuvants are included in vaccines

1. Lower antigen dose in vaccine.
2. Increase breadth of response – heterologous activity.
3. Enable complex combination vaccines, overcome antigenic competition.
4. Overcome limited immune response in some populations – such as the elderly, young children, chronic diseases and immunocompromised.
5. Increase effector T cell response and antibody titers.
6. Induce protective responses more rapidly.
7. Extend the duration of response by enhancing memory B and T cell responses.

why do we continue to invest significant resources in this area? This question is, in fact, easy to answer. The bottomline is that adjuvants are, and will become increasingly necessary to enable vaccine development. Alum adjuvants have contributed significantly to the currently available vaccines, which could not otherwise have been developed. Moreover, a simplistic view of the status of vaccine development is that the relatively 'easy' vaccines are already done, using generation 1 adjuvants. The remaining vaccine targets will be more difficult to develop and will require more potent immune responses to enable success [28]. Moreover, there are still a significant number of important pathogens for which we do not yet have effective vaccines and these targets are very challenging [28]. To enable vaccine development against these targets, more potent adjuvants will be necessary. Such new adjuvants will need to offer advantages, including more heterologous antibody responses, to cover pathogen diversity, the induction of potent functional antibody responses, to ensure pathogen killing or neutralization and the induction of more effective T cell responses, for direct and indirect pathogen killing. In addition, adjuvants may be necessary to achieve more pragmatic effects, including antigen dose reduction and overcoming antigen competition in combination vaccines (Box 2).

Because new generation vaccine candidates will increasingly comprise highly purified recombinant proteins, which, although very safe, are poorly immunogenic, adjuvants will become increasingly necessary. Alum is known to be a relatively weak adjuvant for recombinant proteins, but works well for traditional bacterial toxoids. In addition to the established pathogens, which have eluded vaccine control, there are also new and emerging problems to consider. These include new and re-emerging infectious disease, including the threat of pandemic flu [29]. Moreover, with an aging population, which is increasingly susceptible to infectious diseases, new adjuvants will be necessary to overcome the natural deterioration of the immune response with age (Table 2). While the regulatory hurdles for the approval of vaccines containing new adjuvants will remain understandably high, this must be considered in relation to the likely approval times for alternative technologies. While alternative approaches are also able to achieve potent immune responses, for example live replicating vectors, single cycle inactivated vectors, DNA/RNA vaccines, the path to approval for these technologies is not more simple than for adjuvants and probably will prove more complex.

What are the reasons for the failure of most adjuvant candidates?

We do not lack effective adjuvants, because many approaches have been available for a quite some time [16], what we lack are successful adjuvants, those that have gained approval in licensed products. There are many different reasons why adjuvants have failed and some of these are highlighted in Table 2. The main problem is safety, or perceptions surrounding safety, and this represents a complex and evolving challenge. Safety really needs to be considered as an informed risk/benefit analysis. Some 'safety' issues are immediate, but these may really be issues of tolerability, which may or may not reflect a longer term issue. Nevertheless, if an adjuvant induces a significant degree of local reactions, then almost certainly the adjuvant would be considered unsuitable for further development. The 'acceptability' of the tolerability profile observed needs, however, to be defined in the context of the vaccine target indication, taking into consideration for example, the incidence of infection and the severity of the disease. In a simple illustration, the acceptable tolerability profile for an adjuvant to be used in a therapeutic cancer vaccine would probably be very different from a prophylactic vaccine to be used in infants. Hence, important questions in adjuvant selection, include: what is the indication; what is the disease incidence and probable outcomes, what are the alternatives, are effective drugs likely to be available in time, who are the target population and what is the acceptable balance of risk to benefit in this population. The tolerance for even a perception of 'risk' for a vaccine to be used in a large numbers of healthy children is understandably very low.

Even though short-term safety issues have been mainly responsible for limiting adjuvant development, intermediate and parti-

TABLE 2

Characteristics of an optimal adjuvant candidate

Successful adjuvants	Unsuccessful adjuvants
Safe, not associated with any long-term effects	Unacceptable tolerability profile
Well tolerated	Significant local reactions
Simple synthetic pathway	Complex, difficult to scale up, lack of reproducibility in formulation
Simple inexpensive components	Raw materials expensive or not available of suitable purity from reliable source
Biodegradable	Nondegradable, leaves long-term residue at injection sites
Compatible with many different kinds of vaccine antigens	Difficult to formulate with diverse antigens, negative impact on antigen stability
Capable of codelivery of antigen and immune potentiator	Inflexible, not easy to combine with additional formulation components

cularly long-term safety issues are actually much more challenging. Obviously, they not only take more time to manifest, but may also cover a range of theoretical concerns, for which the actual risk is difficult to define and quantify. In this context, the clinical experience with W/O emulsion adjuvants, including incomplete Freund's adjuvant (IFA) [9], which is based on mineral oil, is an interesting story. IFA was used in humans for virus vaccines for which Alum was ineffective, including influenza vaccines [30]. Although the original IFA was poorly tolerated, it was improved by Salk, who used Arlacel A as a less toxic emulsifier [31]. Nevertheless, after an estimated 900,000 doses of IFA were used in the elderly in the UK in the early 1960s, the adjuvant was voluntarily withdrawn, owing to the incidence of occasionally severe local reactions [32]. The adjuvant induced sterile abscesses in 3–17 subjects per 100,000 immunizations. Similar oil adjuvants were also used in the US military, and showed a similar incidence of local reactions. Importantly, follow-up in the US showed that there were no long-term consequences from the use of the adjuvant [33]. Hence, although the short-term reactions were initially considered acceptable, even though it is improbable that they would be considered acceptable today, the vaccine was withdrawn, when it became clear that the short-term reactions occasionally developed into intermediate problems. Nevertheless, extensive follow-up over 35 years has shown that the intermediate problems did not develop into longer term problems. Hence, reactions in the short to intermediate term may not necessarily reflect a long-term problem. However, the converse could also conceivably be true, which is a more difficult issue, because a long-term problem may not be predicted by the short-term response. Although W/O adjuvants continue to be used today in therapeutic vaccines [34] and for veterinary vaccines [35], they are unlikely to be included in prophylactic vaccines. In an attempt to overcome the safety concerns surrounding the use of mineral oil adjuvants, vegetable oil adjuvants were developed, using oils that were completely biodegradable [36]. The use of the Arlacel A emulsifier was, however, finally curtailed when this agent was shown to be carcinogenic in preclinical mouse models [37].

Overall, the early use of W/O emulsions as adjuvants allowed some important lessons to be learned. The oils needed to be biodegradable, while the surfactants needed to have an established history of safe use in humans. Moreover, the tolerability profile for emulsions could be improved significantly if the oil content was lowered, which could be achieved by reversing the phases and using a low content of oil dispersed in an aqueous phase, an O/W emulsion. Changing the formulation to an O/W also significantly improved ease of use, through easier administration and preparation, because of reduced viscosity. Importantly, emulsions are a well-established dosage form approach within the pharmaceutical industry. Therefore, sourcing of raw materials and scale up and control of manufacturing processes have been in place for quite some considerable time. Early work had highlighted that emulsions were very effective adjuvants for influenza vaccine, allowing enhanced responses and a significant dose reduction, while Alum was largely ineffective. Unfortunately, this experience was forgotten and needed to be learned again in the recent past, when the world became aware of the threats of a potential flu pandemic.

For the second generation adjuvant candidates, it is clearly the long-term safety questions that will be the most difficult ones to deal with, particularly because vaccines are often focused on young individuals who will live for many decades after vaccine administration. Although theoretical safety concerns and issues are relatively easy to highlight, they are often difficult to disprove. It is difficult to prove a negative, even with large numbers of subjects, when the possible risk is very low. The adjuvant field is currently keenly awaiting the outcome of the FDA review of the first second generation adjuvant to be considered for inclusion in a licensed vaccine in the US. Alum/MPL (AS04), is a key component of an HPV vaccine (Cervarix), which was first filed for approval in the US in March 2007, and additional data were filed in 2008. The company does not expect a final response until late 2009 (GSK website http://www.gsk.com/index.html – accessed December 2008). This is a complex situation, however, in which a product with a two-component adjuvant is being brought forward when there is already a competing licensed product which contains only Alum (Gardasil). Therefore, presumably it will be necessary to show a clear impact for the second adjuvant component, while not significantly changing the safety profile of the product. Moreover, the products have additional differences beyond the adjuvant compositions, while Cervarix is divalent, containing two types of VLP, Gardasil contains four [38]. In addition, Gardasil contains antigens expressed in yeast, while those in Cervarix are expressed in a baculo system. Hence, any suggestion that the delay in Cervarix approval is due to the inclusion of a second adjuvant is merely speculation, which may be entirely ungrounded. Overall, the available data suggest that both HPV vaccines (Gardasil and Cervarix) are safe, immunogenic and highly efficacious, offering a high degree of protection against infection [38]. On a positive note, a recent study has highlighted the safety profile of the AS04 adjuvant in over 68,000 individuals [39]. Reassuringly for the field of novel adjuvant development, Novartis also recently made a public presentation on the safety profile of MF59, including data from 33,000 subjects and showed that there was no association with any increase in autoimmune disorders in vaccinated subjects (FDA Adjuvant Meeting, Bethesda, December 2008).

Some of the reasons that have ensured that adjuvants have proven difficult to develop are highlighted in Table 2. One of the problems for current candidates is the origin of some of the materials being used. The adjuvants often comprise natural products with inherent variability, some of which have proven impossible to synthesize. Sometimes, the extraction of sufficient quantities of fully characterized and reproducible material becomes an issue, as does the expense involved. Synthetic analogs of some of the natural compounds are, however, now becoming available [40]. Moreover, some new generation adjuvants are highly amenable to rapid, efficient and inexpensive synthetic approaches [2]. In this context, small-molecule immune potentiators, or SMIPs, will be discussed in more detail later.

Serendipity is the mother of invention, what did we learn during the development of MF59 adjuvant?

Because MF59 has been included in a licensed influenza vaccine for more than ten years, has been administered to more than 50 million people and has accumulated a significant safety database [17,41], it could reasonably be considered to be a success. Before MF59 became a success, however, it was deemed a failure. Moreover, it was a failure on more than one occasion, although it never

failed to do what it was supposed to do! Importantly, the failures of MF59 were never because of safety concerns, although a safety problem with an early emulsion formulation was an important signal which drove the later development of MF59. Hence, perhaps the most important lesson learnt with MF59 was do not give up too easily! A second lesson was not to declare success or failure too early in a program, while there was still much to learn. Originally, Chiron Vaccines developed O/W emulsions as delivery systems for an immune potentiator called MTP-PE, which was able to activate the immune system by a then poorly understood mechanism. MTP-PE was a synthetic version of MDP, a naturally occurring molecule, identified as the minimal component of mycobacterial cell wall that was able to activate the immune system [13]. The emulsion had a low content of the oil squalene, which is biodegradable and biocompatible, and was stabilized by two nonionic surfactants, which had been used previously for alternative biomedical purposes. In early clinical testing, however, the adjuvant formulation of MTP-PE, proved to be poorly tolerated, and was too reactogenic for routine clinical use [42,43]. Hence, at that time, the MTP-PE formulation looked like another failed adjuvant approach, with failure owing to short-term safety problems. Additional work, however, highlighted that the emulsion delivery vehicle alone, without the additional immune potentiator, was a surprisingly effective adjuvant. Moreover, for flu vaccine, the emulsion alone (MF59) was very effective and the MTP-PE did not add to the potency [44]. Hence, this was a clear situation in which an immune potentiator did not justify inclusion, because it did not add potency, while inducing significant and unacceptable local and systemic reactions [42]. These observations encouraged moving forward and developing MF59 as a standalone adjuvant for flu vaccines [45]. The clinical use of MF59 adjuvant has allowed us to learn a significant amount about the value of adjuvants for flu vaccines, including which populations most require an adjuvant, the requirement for an adjuvant to be included in pandemic vaccines, and the ability of adjuvants to induce heterologous activity against divergent strains, which is important both for pandemic and seasonal vaccines [17]. Recently we have extended the use of MF59 into pediatric populations, who otherwise respond poorly to influenza vaccines, due both to immunological naivety and lack of full immunological development (T. Vesikari *et al.*, PIDJ, in press). We believe that we learned many things in the development of MF59 as an adjuvant [46], but perhaps the most important lesson was to be very careful about which immune potentiators to combine with first generation adjuvants.

There have been several areas in which the MF59 adjuvant might be perceived to have 'failed', but what really failed were the assumptions on which the vaccine candidate was based. For example, MF59 was evaluated in a phase III efficacy trial for protection against genital herpes simplex infection, but the vaccine failed to offer sustained protective efficacy [47]. In a previous study, the same vaccine formulation had also failed to show efficacy as a therapeutic vaccine candidate [48]. The real failure here, however, was of trial design and concept. The adjuvant was originally chosen because it induced high levels of neutralizing antibodies and potent T cell responses [49]. By this simple analysis the adjuvant was successful, because it achieved what it was designed to do [47]. However, we learnt that serum-neutralizing antibodies did not offer protection against herpes simplex infection, which is a sexually transmitted disease, for which there is often repeated exposure of high viral load [50]. There are additional examples in which MF59 has done what was expected of it, but a vaccine did not emerge. For example, MF59 induced potent neutralizing antibody responses against HIV gp120 envelope [51] and high levels of protective titers against Hepatitis B surface antigen [52]. Nevertheless, the antibody responses induced against HIV gp120 were not able to neutralize relevant 'field' strains, a failing of each and every vaccine candidate evaluated so far.

So what are the key lessons for adjuvant development that can be learnt from MF59? Perhaps that a good adjuvant cannot fix a bad antigen, or cannot compensate for an incorrect assumption in trial design. Success can follow failure, but you must learn from the failure to focus your efforts. Success may follow if a safe and potent adjuvant is linked with a vaccine candidate for which there is a clear need for enhanced potency. From a failed second generation adjuvant candidate, a more potent first generation adjuvant was developed, MF59.

Understanding innate immunity – the path to developing better adjuvants

First generation adjuvants are believed to deliver antigens to the appropriate immune cells and to increase antigen persistency at the injection site. For example, antigens adsorbed onto Alum are more stable and persist for longer time periods at the injection site (reviewed in [53]) and are more efficiently internalized *in vitro* by DCs [54]. Similarly, MF59 has been associated with improved antigen uptake *in vivo* [55]. These effects in some cases have been sufficient to induce a long lasting protective immune response in humans. Poorly immunogenic antigens, including recombinant proteins, are, however, often unable to induce a protective immune response with generation 1 adjuvants and may require the addition of an immune potentiator. The mechanism of action of immune potentiators is through the activation of innate immune receptors on APCs, including macrophages and dendritic cells (DCs), which take up and present antigens to T cells. These receptors are used by APCs to detect pathogen-associated molecular patterns (PAMPs) and are called pathogen recognition receptors (PRRs) [56]. Indeed, most known immune potentiators, including LPS, flagellin or MDP, are microbial products. Others, including CpG and poly I:C oligonucleotides, are mimics of bacterial DNA and viral RNA. The activation of PRRs by immune potentiators induces the secretion of proinflammatory cytokines and type I interferon, the upregulation of costimulatory molecules and MHC class II. Importantly, PRRs also trigger the migration of APC from injection site to the T cell area of draining lymph nodes. All these events are required for naïve T cell activation, a primary step of the adaptive immunity required for both humoral and cellular immune responses [57]. In recent years, our knowledge of the molecular mechanisms of innate immune activation has expanded rapidly, thereby providing a large number of potential new targets for immune potentiators. Three classes of PRRs have been extensively studied: Toll-like receptors (TLRs); NOD-like receptors (NLRs) and RIG-I-like helicases (RLHs). The intracellular signaling events associated with each of these receptors are well characterized and information on the structures of PRR–ligand complexes is starting to accumulate. This rapid progress in molecular immunology is providing scientists with an increasing num-

TABLE 3

Toll-like receptors (TLRs) as vaccine adjuvant targets

TLR	Localization	Elicitor	Vaccine target
TLR1-2	Plasma membrane	Triacyl lipopeptides (PAM3CSK4)	Preclinical
TLR2-6	Plasma membrane	Diacyl lipopeptides (PAM2CSK4); MALP2	Preclinical
TLR3	Endosome	dsRNA (poly I:C)	Preclinical
TLR4	Plasma membrane	LPS (MPL; synthetic MPL)	MPL approved in EU for HBV and HPV vaccines
TLR5	Plasma membrane	Flagellin	Clinical trials for flu
TLR7	Endosome	ssRNA; imiquimod; resiquimod	Preclinical
TLR8	Endosome	ssRNA; resiquimod	Preclinical
TLR9	Endosome	DNA; CpG oligonucleotides, ISS	Clinical trials for various indications
TLR10	Plasma membrane	Unknown	N/A

ber of cellular and target-based assays to screen for new classes of small-molecule immune potentiators (SMIPs). Therefore, it is easy to predict that in the next few years an array of new molecules capable of eliciting distinct types of immune responses will emerge, which may be used as vaccine adjuvants adapted for different classes of pathogens.

The PRRs that have been most closely linked to vaccines are TLRs, which are expressed on immune cells, including macrophages, DC and B cells [58]. Two classes of TLRs can be defined, on the basis of their cellular localization: TLRs 1, 2, 4–6 are expressed on the plasma membrane and recognize bacterial components, while TLRs 3, 7, 8 and 9 are expressed in intracellular compartments and are nucleic acid sensors. Interestingly, in addition to PAMPs and PAMP-derived products, small molecules have also been identified which activate TLR7 and 8 (for a complete list of human TLRs and associated PAMPs see Table 3). Besides cellular localization, TLRs differ in their cellular distribution and in the intracellular signaling events that they trigger. For example, TLR9 is expressed on B cells and in a specific subset of DC called plasmacytoid DC and activation by CpG oligonucleotides induce the production of large amounts of interferon α. By contrast, TLR4 is expressed in a different subset of DC called myeloid DC, and in macrophages, but not in human B cells, and its engagement by MPL or LPS induces the production of proinflammatory cytokines such as TNFα and interferon β [58,59]. All TLRs trigger an intracellular signal transduction pathway initiated by the adaptor protein MyD88, with the exception of TLR3, which utilizes an alternative adaptor called TRIF. Interestingly, TLR4 activated both MyD88 and TRIF. Despite the differences in expression and intracellular signaling of TLRs, almost all TLR agonists evaluated so far have the potential to increase the immunogenicity of coadministered antigens in preclinical models [60]. CpG oligonucleotides have been evaluated as vaccine adjuvants in human clinical trials in combination with several antigens [61] and MPL has been licensed in vaccine products [25]. Furthermore, new candidate TLR-dependent adjuvants may emerge from the increased knowledge of TLR biology, and from the availability of TLR functional assays that can be used to screen libraries of small molecules or natural products. Recently, the structures of TLRs 1–4 complexed with their respective ligands have been solved [62–64]. These studies have demonstrated that in some cases, such as TLR3 activation by dsRNA and TLR1 and 2 activation by lipopeptides, a direct interaction of the PAMP induces a TLR dimerization-dependent activation. By contrast, an adaptor protein called MD2 is required for TLR4 activation and represents the real target of MPL. The structural information becoming available on TLRs will certainly boost the development of rationally designed immune potentiator molecules, which can be highly specific for their molecular targets. Nevertheless, although TLRs play a central role in innate immunity and are validated targets for adjuvants, they are not necessary for the adaptive immune response to vaccination. Using a mouse strain deficient for TLR signaling, it has been demonstrated that first generation adjuvants, such as Alum and Freund's incomplete adjuvant are actually TLR-independent [65,66].

Alternative PRRs that can be exploited as vaccine adjuvants to activate innate immunity include RLHs and NLRs. The most extensively studied RLHs are retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Both proteins, similar to TLRs 3, 7 and 8, recognize RNA structures produced by virus replication and trigger the expression of proinflammatory cytokines and type I IFN. While TLR nucleic acid sensors are expressed in endosomes, RLHs are cytoplasmic [67]. Although it is most probable that RLHs may be involved in the adjuvant effect of RNA-derived structures, more work needs to be done to dissect the differential contribution of RLHs and TLRs, and to validate RLHs as possible adjuvant targets. The prototypic NLRs are the cytoplasmic sensors NOD1 and NOD2, which recognize bacterial cell wall components derived from the peptidoglycan such as MDP [68]. Although the molecular details of MDP–NOD interaction have not yet been solved, new, safer and more potent elicitors of NOD proteins could be discovered, which have potential as vaccine adjuvants. Another NLR protein that has recently been linked to adjuvanticity is NLRP3, a component of the inflammasome complex. Unlike the other PRRs described below, NLRP3 does not induce the expression of cytokines and costimulatory molecules, but triggers the activation of a protease, caspase 1, required for the maturation of several proinflammatory cytokines, including IL-1β [69]. It has been demonstrated that MDP activates NLRP3 [70]. Recently, it has been shown that NLRP3 is required for Alum adjuvanticity, although others have reported conflicting results [71–73]. It is clear, however, that NLRP3 can play an important role in adjuvanticity and represents an additional validated target for adjuvant discovery [74].

In addition to the PRRs described, which are considered possible targets for improved immune potentiators, other receptors,

including C-type lectin receptors (CLRs) or scavenger receptors, which promote uptake by APC, could be considered as targets for improved antigen delivery systems. For example, it has been shown that targeting an antigen to the DC internalization receptor, DEC205, by a specific antibody in combination with maturation signals induces a strong immune response in mice [75]. Therefore, by using CLR ligands, it may be possible to achieve selective targeting of both antigen and immune potentiators to DC, increasing both safety and immunogenicity. Moreover, the combinations of immune potentiators targeting different TLRs or different PRRs may be evaluated to achieve more potent immune responses. Indeed examples of synergy and crosstalk between TLR agonists and between TLR and NOD agonists have already been reported. TLR3 and TLR4 agonists strongly synergize with agonists of TLR7, 8 and 9 leading to the development of DC with an enhanced Th1 polarizing activity [76]. In addition, TLR4 agonists synergize with NOD1 and NOD2 agonists to induce DC maturation [77]. Although the combinations of immune potentiators are very promising, they may increase regulatory concerns on safety, and will need to be formulated into a delivery system to limit systemic exposure.

The mechanism of action of generation 1 adjuvants

Recent work on the licensed adjuvants Alum and MF59 has suggested that particulate adjuvants are not simply antigen delivery systems but can also act as immune potentiators. Alum can activate human monocytes and macrophages *in vitro* [3,78,79] and synergize with TLR-dependent adjuvants for the secretion of IL-1β by human PBMCs through the activation of the inflammasome complex [80]. In addition, Alum elicits innate immune reactions at the injection site. Injection of Alum induced the recruitment of inflammatory monocytes, that were capable of migrating into lymph nodes, and the production of uric acid, which activates the immune system through the NLRP3 inflammasome complex [81]. Consistently, as already described above, NLRP3 has been implicated in Alum adjuvanticity [71]. Like Alum, MF59 has been associated with the stimulation of monocytes *in vitro* [3] and to cell recruitment events *in vivo* [82]. Recently, by monitoring the adjuvant expression profile at the injection site, it has been shown that both Alum and MF59 induce, in mouse muscle, the local activation of a large number of innate immunity-related genes, which promote the recruitment of APCs. Interestingly, MF59 is a more potent inducer of innate immunity pathways in muscle than Alum and, more surprisingly, is also more potent than the TLR9-dependent immune potentiator, CpG [83]. These data strongly suggest that the reason for the success of first generation adjuvants is that they possess a dual function, in promoting antigen uptake and in stimulating an immunocompetent environment at the injection site. Nevertheless, it is still possible to increase their efficacy by adding an additional immune potentiator, such as a TLR-dependent adjuvant [84–86]. The challenge for vaccine developers is to determine how best to use combination adjuvants, to achieve optimal immune activation and yet avoid unwanted toxic reactions. Finally, it is worth considering that unlike PRR-dependent immune potentiators, Alum and MF59 are unable to stimulate DC directly. Nevertheless, they can activate and recruit DC at the injection site indirectly, by acting on other cell types such as monocytes. In addition, MF59 was shown to activate in muscle fibers innate immune genes like pentraxin 3, that may in turn activate tissue resident or recruited DC [74]. These findings demonstrate that adjuvant mechanisms of action must be assessed *in vivo*, where signals from different cells and tissues cooperate to establish an immunocompetent environment.

Next generation adjuvants, what will generation 3 look like?

Accumulated lessons and setbacks have, hopefully, given us some insight on which to base our predictions of what a generation 3 adjuvant will look like. The characteristics of what we believe an optimal adjuvant formulation should look like are highlighted in Table 2. In essence, we propose that a delivery system should be used to ensure codelivery of antigens and immune potentiators to the key immune cells. Moreover, the delivery system should ensure that the delivery of the immune potentiator is specific to the cells of interest and not more generalized. The immune potentiator should be localized at the injection site, to minimize the impact on more diverse cell populations. It is already clear that the TLR and other PRR are expressed on a diverse range of cells and are not specialized only to immune cells. Therefore, for reasons of safety, the delivery of potent immune potentiators needs to be controlled and focused. The objective will be to deliver the right molecule or molecules to the right site, for the right amount of time, but unfortunately, we still know very little about what is really required, particularly in relation to the temporal issues. Nevertheless, we believe that the technology for delivery is in place, although the immunology is still far behind and we have much yet to learn. We propose that delivery can be achieved using biodegradable microparticles, which have already been successfully scaled up and manufacturing is in place, because polymeric microparticles have already been commercialized for drug delivery.

The adjuvant effect of linking antigens to synthetic microparticles was first described quite some time ago [87] and nondegradable particles have been used extensively since [88]. However, the development of biodegradable polymers to form the microparticles allowed this area to significantly advance. Microparticles prepared from biodegradable polymers were initially evaluated for systemic [89] and oral [90] vaccine delivery at the end of the 1980s. A subsequent development was the use of the more common biodegradable polymer, the polylactide co-glycolides (PLGs) for vaccine delivery [91]. Because this polymer had already been used for the development of drug delivery systems, it was an attractive approach. Although there were some significant challenges in the early stages of this technology, mostly owing to the degradation of entrapped antigens [92], an important advance was the use of preformed microparticles for the delivery of adsorbed antigen [93,94] The use of preformed microparticles allowed PLGs to be used more like the established Alum adjuvant, but with the advantages of a completely biodegradable polymer with the potential to codeliver additional adjuvant components. Second generation microparticles are able to codeliver adjuvants, which can be adsorbed [95], or entrapped [96]. Although microparticles worked better for traditional vaccines, if they were combined with Alum [97], this eliminated some of the advantages of the technology. A similar path has also been followed for nanoparticles, which were originally described as adjuvants in the 1970s [11], but have more recently been described using the PLG polymer [98]. Nanoparti-

cles, however, do not yet appear to have clear advantages in terms of potency over microparticles [99], but they may have other advantages, including ease of manufacture [98]. A recent paper has highlighted the mechanism of action of PLG microparticles and other particulate adjuvants, and has begun to explain how the particulates work in synergy with immune potentiators to activate more potent immune responses [100]. Although both microparticles and nanoparticles are capable of modifying the release of entrapped agents, it is not yet clear what is the desirable release profile for immune potentiators. This is likely to be a productive area of future research.

As discussed briefly earlier, new generation adjuvants include SMIPs, which have many inherent advantages over microbial-derived compounds. Small molecules can be easily and inexpensively synthesized and can be optimized to be highly specific for the preferred target, while avoiding undesired toxic effects. In addition, their potency may be modified relatively easily, as can their pharmacokinetic properties to avoid systemic exposure. Moreover, synthetic drugs can be modified by appropriate linkers to enable easier formulation and delivery. Despite these many potential advantages, so far only a few small molecules, mainly belonging to the imidazoquinoline class and targeting TLR7 and 8, have been used as vaccine adjuvants [101]. The future development of new classes of SMIPs targeting selective PRRs such as TLRs, NLRs or RLHs is, however, very promising. The development of new SMIPs, highly specific for their target, will also allow the investigation of which PRR is the best target for a given class of pathogens. For example, an intracellular RNA sensor such as TLR3, 7, 8 or RIG-I, could be the optimal target for vaccination against RNA viruses, while a plasma membrane microbial sensor such as TLR2 or 4 may be the optimal PRR to target to achieve protective immunity to extracellular bacteria.

Conclusions

This review should make it clear that vaccine adjuvants are difficult to develop. Moreover, with increasing concerns in relation to safety, it is becoming even more challenging. In this context, it is important to consider how we can enhance our chances of success. One important lesson learned is to apply the KISS principle whenever possible, KISS is an acronym for Keep It Simple Stupid! To encourage success in adjuvant development, unnecessary complexity must be avoided. A simple and scaleable formulation needs to be identified early, preferably using readily available and inexpensive materials, which have an established safety profile, ideally in an existing medical product. In interactions with regulators, it will be necessary to show a clear and compelling need for the inclusion of all active components in the formulation, particularly those which are added for an adjuvant effect. The impact of a second adjuvant must be clear and easily quantifiable, preferably improving clinical protection, or enhancing an immune correlate or surrogate of protective immunity. Although the approval of vaccines containing novel adjuvants will continue to be challenging, we believe that the path is becoming somewhat clearer, if not less challenging, but more clarity is urgently sought. Further clarity and perhaps clear guidelines may emerge from an adjuvant workshop which was organized by the FDA in December 2008. Overall, we believe that the MF59 adjuvant will attain additional success in the near future, gaining further approvals in licensed vaccines, because it has a well-established safety profile and has established a clear and compelling case for contributing to the potency of flu vaccines, particularly in the pre pandemic setting. However, the regulatory agencies will make decisions about vaccine licensure.

As discussed, AS04-containing vaccines have recently gained significant regulatory approvals, but it is difficult to predict how quickly approval might be gained in the US. The picture is made more complex by the knowledge that an unrelated product used for allergy therapy, which contains the same MPL adjuvant, has been on clinical hold since 2007, after a patient developed neurological symptoms (Biocentury Extra, July 24, 2007). Although the use of immune potentiators, including TLR agonists in second generation adjuvants holds huge promise, clinically this area is still in its infancy. Immune manipulation using TLR agonists and other innate activators is a poorly understood area, with many unanswered questions and concerns. Much of the work performed so far has been empirical and observational, mostly highlighting that one plus one may equal more than two. To make serious advances, we need to apply the established tools of drug discovery, delivery and evaluation to the field of vaccine adjuvants. We need to focus on the optimal molecules to deliver and enable them to selectively activate the appropriate targets, to induce only the desired protective immune responses. Advances in biomarkers can be applied through the use of translational medicine to better understand and quantify the immune activation achieved, while also evaluating more thoroughly the safety profile. The extent to which translational medicine can contribute to the safety evaluation of adjuvants, through the judicious application of novel biomarkers needs to be determined, but is an important area of research.

The safety issues surrounding adjuvants have been with us for a long time and were discussed knowledgeably back in 1980 [102]. Most of the concerns raised almost 30 years ago still remain valid today, although perhaps we now know a little more about how the adjuvants work. Even back in 1980, it was highlighted that there were concerns that potent immune stimulators could potentially trigger autoimmune diseases, because this had been seen with Freund's adjuvants in animal models. Recently, this has been discussed in the literature as a concern for TLR agonists [103]. Unfortunately, this will remain a challenging issue, particularly because the available animal models are unlikely to be predictive. The models are not helped by the presence of TLR differences between species, along with immunological differences, which further limit their probable predictability. The development of improved preclinical models to better predict the safety of vaccine adjuvants is obviously an area requiring further research. Encouragingly, although infections have been associated with triggering autoimmune disease, vaccination has not been directly linked with autoimmune disease [104]. Moreover, with significant experience worldwide, autoimmune disease does not seem to be a problem for generation 1 adjuvants, including Alum and MF59. The use of Alum as a vaccine adjuvant was re evaluated at an international workshop in 2002 and it was concluded that Alum had an excellent safety record, with a low incidence of adverse events [105]. However, the safety and acceptability of generation 2 adjuvants, including TLR agonists, will need to be established in the clinic, with extensive monitoring of patients likely to be

necessary in early stages of clinical evaluation. Although concerns have been raised about potential safety issues of using TLR agonists in humans, it should be remembered that such an approach actually has a long and established safety record. Whole bacterial vaccines have been used worldwide for many decades in hundreds of millions of people, without showing significant safety signals, even when monitored very closely. Bacterial cells contain significant amounts of bacterial cell wall components, such as LPS and bacterial DNA, which are the natural components on which new generation synthetic adjuvants have been based.

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