WHO White Paper on Vaccine Research and Development:

State of the Art of Generic Vaccine/Vaccination Technologies

September 2013

Contents

Acronyms

Acronym	
TPP	Target Product Profile
EMA	European Agency for the Evaluation of Medicinal Products
FDA	Food and Drug Adminstration
GLA	glucopyranosyl lipid adjuvant
HCV	Hepatitis C Vaccine
HIC	High Income Country
HIV	Human Immunodeficiency virus
LBV	Live bacterial vectors
LMIC	Low and Middle Income Countries
LPS	lipopolysaccahride
MPL	Monophosphoryl lipid
ORF	Open Reading Frame
PAMPs	pathogen associated molecular patterns
PRRs	Pathogen recognition receptors
RVVC	Recurrent Vulvo Vaginal Candidiasis
TB	Tuberculosis
TPP	Target Product Profile
VLPs	Virus like particles

1.0 Executive Summary

Many vaccines technologies that could facilitate improved safety, effectiveness and delivery are in development, including needle free administration, increasing the magnitude and longevity of protective immune responses induced by vaccines whilst reducing the dose, and improved vaccine stabilization. Whilst there are success stories in each of these areas, the new technologies may not always be easy to implement or may render the vaccines unaffordable to the populations that need them. This review considers the requirements for an ideal vaccine and reviews the status of current vaccine technologies in development. The key messages are summarized below:

- The need for improved vaccines is driving the development of subunit vaccines that are sufficiently immunogenic, possible to characterize and practical to deliver.
- Modern vaccine candidates are likely to contain multiple antigens and immunopotentiators and/or delivery systems in order to generate a robust and sufficiently broad immune response, prioritizing need for continued development of these components.
- Vaccines containing multiple components (adjuvants, devices etc.) will have a complex development pathway due to the need for partnerships/consortiums to ensure access, and the requirement from the regulator to demonstrate the risk/benefit of each component.
- The target product profile (TPP) for a vaccine is determined by end-user requirements and provides the roadmap to its development. The TPPs for low and middle income countries (LMICs) often vary to those for high income countries (HICs), likely resulting in different considerations in development of vaccines for the same disease depending on the intended target population. While financial incentives tend to drive HIC-based TPPs, the greatest public health impact and need is often in LMICs.
- The number of vaccines that are recommended globally has increased with the launch of several new vaccines in many high and low income countries. This expansion is driving the need for the development of combination vaccines, introducing additional challenges of potential antigenic interference and formulation compatibility. However, if successfully developed, greater access to broader combination vaccines could result in significant uptake.
- Certain technologies that do not require changes to the vaccine formulation but improve ease of administration, such as needle free administration may facilitate immunization logistics, which have the potential to increase vaccine coverage. However, second generation candidates, or alternative delivery routes for a vaccine that is established within the existing vaccination schedule may be challenging due to the costs of demonstrating equivalence and non-interference, particularly in the case where a correlate of protection is not available. The availability of established biomarker may facilitate the transition from a regulatory perspective.
- It is clear that, in addition to safety, efficacy and cost, one of the major criteria for vaccines against diseases such as TB, malaria and HIV is the ease of use in low and middle income countries. With this is mind, the research community should include a focus on developing technologies that result in thermostable, appropriately presented and packaged vaccines that are administered safely and without the need for extensive training of healthcare workers for delivery. These advancements would reduce the cost of delivering and implementing the vaccine and be attractive to LMIC markets.

2.0 Introduction

Worldwide, immunisation programs have had a tremendous impact on the prevalence of many life-threatening diseases. Twenty seven infectious diseases are now preventable by vaccination, and according to the WHO, each year vaccines prevent up to three million deaths [1] . Smallpox has been eradicated, resulting in global savings of over US$1 billion each year [2]. These successes provide the rationale for continued development of vaccines as one of the most cost-effective public heath investments and there are more gains to be made since effective vaccines for diseases such as tetanus, poliomyelitis, measles, rubella, *Haemophilus influenzae* type b, *S. pneumoniae* and rotavirus do now exist but have yet to be fully implemented in many cases. In 2008, the WHO estimated that 1.5 million deaths among children under 5 years were due to diseases that could have been prevented by routine vaccination [3], underlining the need for approaches to increase access and coverage with available vaccines.

Despite the successes, there are still numerous viral, bacterial and parasitic diseases such as HIV, malaria, tuberculosis, and dengue that remain prevalent or are re-emerging, predominantly afflicting low and middle income populations [4]. Relatively recently, diseases that that have large markets in high income countries such as cancer and certain autoimmune disorders have received attention as targets for both prophylactic and therapeutic vaccines, and the cancer field has been at the forefront of many experimental strategies, such as the development of novel adjuvants, to induce immune responses in aging or immune-suppressed populations [5]. While many aspects of the TPP for cancer vaccines are different to those for infectious disease vaccines, it will be important that technological advances are translated between vaccine R&D areas.

In contemplating the design of any vaccine, there are common challenges such as understanding the mechanism of disease and required immune response to enable identification of optimal antigens and delivery methods, and generating a sufficient and appropriate, long term immune response safely, in an often immuno-compromised population. Beyond these fundamental issues, the TPP of the ideal candidate for a LMIC vaccine is more constrained than a vaccine for high income countries, and the required attributes need to be considered in the earliest stages of development [6-8]. Other than the cost of the vaccine, challenges specific to developing and implementing a vaccine for low and middle income populations include:

- Developing an immunization schedule that has the lowest possible number of immunizations through the easiest route of administration
- Adherence to the EPI schedule for infants, where possible
- Delivery of the vaccine to the population without loss of activity, in an economical, easy to use format
- Amenability to combination vaccines and non-interference with other antigens
- Training of health care workers within endemic populations to administer the vaccines safely and adherence to the full schedule of immunizations.

Technologies which minimize or overcome these barriers could facilitate immunization and expedite implementation. For example, simplified delivery systems such as those used for smallpox (dermal scarification) or oral polio vaccine (oral drops) are considered to have contributed significantly to the success of these vaccines.

Whilst acknowledging the successes in the vaccine field, there is acknowledgement that many existing vaccines are not ideal, either because of lack of thermostability, sub-optimal immunogenicity (requiring multiple doses and often boosters), needle administration (generating biowaste and injection safety considerations) etc., but it is often considered uneconomical to reformulate existing vaccines because of the expense and regulatory complexity of bridging to an established vaccine, especially when a correlate of protection is not available as a surrogate maker to demonstrate non-inferiority. For this reason, it is imperative that the 'preferred' parameters for a target product profile are considered early in process and clinical development of a vaccine. The desire to move out of the cold chain in the next timeframe is an example of an achievable technical advance which early stage vaccine researchers are highly encouraged to take into account as cold chain requirements become increasingly onerous with the numbers of available vaccines.

3.0 The Need for 'Better' Vaccines

Advances in the quality of vaccine candidates in accordance with regulatory requirements, and a more rational approach to targeting what are believed to be protective antigens mean that current approaches often utilise single or combinations of highly purified and therefore less immunogenic recombinant proteins or vectors. Whilst these formulations may be easier to characterise both biochemically and immunologically, they are invariably less immunogenic than cruder preparations or attenuated or whole inactivated microbes which present multiple antigens as well as native immunostimulatory molecules. Moreover, these simpler vaccines lose the ability to induce broad immune responses that activate both humoral and cell mediated immunity. It is primarily for these reasons, in addition to the fact that a more efficient immune response may enable dose sparing that the development of adjuvants and delivery systems remains urgently needed.

Up until 2009, when GSK's Cervarix was approved, Alum was the only licensed adjuvant in the USA, having been introduced in the 1920s. Cervarix contains the adjuvant system AS04, which contains monophosphoryl lipid A (MPL), a Toll-Like Receptor ligand adsorbed to Alum. This was significant because it was the first novel adjuvant approved by the FDA in over 80 years, and sets precedence for the approval of additional novel adjuvant systems. Recently three additional adjuvants have been licensed in Europe: GSK's AS03, Novartis' MF59, and Berna Biotech's Virosomes ([4], table 1). Whilst Alum is effective at generating humoral immune responses, it is inadequately immunogenic for intracellular pathogens where a cytotoxic T-cell mediated response is thought to be required, and cannot be applied to the mucosal route of administration which may be optimal for targeting these disease indications [9]

Adjuvant name (year licensed)	Adjuvant class	Components	Vaccines (disease)
Adjuvants licensed for use in human vaccines			
Alum* (1924)	Mineral salts	Aluminium phosphate or aluminium hydroxide	Various
MF59 (Novartis; 1997)	Oil-in-water emulsion	Squalene, polysorbate 80 (Tween 80; ICI Americas), sorbitan trioleate (Span 85; Croda International)	Fluad (seasonal influenza), Focetria (pandemic influenza), Aflunov (pre-pandemic influenza)
AS03 (GlaxoSmithKline; 2009)	Oil-in-water emulsion	Squalene, Tween 80, α-tocopherol	Pandremix (pandemic influenza), Prepandrix (pre-pandemic influenza)
Virosomes (Berna Biotech; 2000)	Liposomes	Lipids, hemagglutinin	Inflexal (seasonal influenza), Epaxal (hepatitis A)
AS04* (GlaxoSmithKline; 2005)	Alum-absorbed TLR4 agonist	Aluminium hydroxide, MPL	Fendrix (hepatitis B), Cervarix (human papilloma virus)

Table 1 Adjuvants included in currently approved vaccines, taken from [4]. Asterisk denotes adjuvants that are licensed in the USA only.

Due to the limitations of alum, there is broad agreement in the scientific community that a toolbox of adjuvants is needed, however the field is frustrated by the lack of a programmatic approach, funding constraints, and the need for a better defined regulatory strategy for licensure. The lack of co-ordinated, and published, animal studies to compare multiple adjuvants is often cited as the missing link in adjuvant development [10]. However, preclinical head to head studies are limited in that adjuvant effects vary between species and are most often not predictive of what is seen in humans; it is well documented that TLRs on the surface of immune cells which control downstream activation vary between mice and humans [11]. In addition, the need for well characterized, stable and reproducible formulations early in preclinical evaluation is imperative, so that material used to generate data in pivotal animal studies to justify development is reflective of data generated with clinical material. Point-of-use admixing of antigen with adjuvant can be helpful in this regard.

Vaccination programmes, especially for children have grown in the number of vaccines they include over the last several years [12] . This development has increased the need for combination vaccines, with fewer shots per regimen as well as non-invasive, simpler immunization techniques requiring minimal supervision to ease administration and increase adherence. As a result, significant effort has been made in the development of alternative delivery mechanisms, such as mucosal and transcutaneous routes [13, 14]. For example, needle free vaccines have the potential advantage of being easy to use, with a lower requirement for trained health workers, applicable to large scale immunization programmes and relatively cheap to implement assuming a cold chain is not needed.

Infants, young children and the elderly need to be regarded as immunologically and physiologically distinct from healthy adults, and this has implications on the delivery strategy [4]. For example, mucosal administration via the oral rather than nasal route would favour young children, and pulmonary delivery in children requires the need of a spacer in combination with an inhaler. In addition, these age populations are generally less efficient at developing an immune response requiring stronger adjuvants or additional shots.

As well as considering the 'preferred' target product presentation, it is necessary to ensure that several other attributes are considered in the selection of adjuvants for human trials, such as formulation robustness and presentation – the adjuvants need to be formulated in such a way that the vaccine is stable and practical to implement in the developing world, as well as being accessible, affordable and possible to manufacture at scale, etc. [10].

4.0 Components of a vaccine

As we transition from empirical vaccine design involving attenuated or killed whole organisms to a more rational approach, we need to be able to select the optimal antigens, adjuvant and delivery system for any given disease. This requires an in depth understanding of the disease mechanism, as well as how the vaccine formulation can be designed to deliver the appropriate response. Depending on the 'preferred' target product profile, selection of an adjuvant and/or delivery system is needed to support the requirement of the formulation and presentation (considerations such as storage, shelf life and delivery device), and this is driven to some extent by the desired route of administration and target population ([7]).

Figure 1 below depicts how these components interface and the considerations for each. The immunogenicity of an antigen can be increased, or 'adjuvanted', by combining it with an immunopotentiator which directly activates the immune system, i.e. through binding to receptors on cells to elicit a response, or by presenting it using a delivery system which promotes the uptake of antigen into the cells for processing [15]. Many approaches combine the use of immunopotentiators and delivery systems to form 'adjuvant systems', to which the antigen/s are added. In any of these cases, the antigen/adjuvant formulation must be stable and preserve the integrity of the antigen through the addition of formulation buffers, antimicrobials, cryopreservatives etc. The vaccine formulation must then be filled to produce the final drug product, which may be lyophilised, encapsulated or filled as a liquid into vials, possibly along with a delivery device.

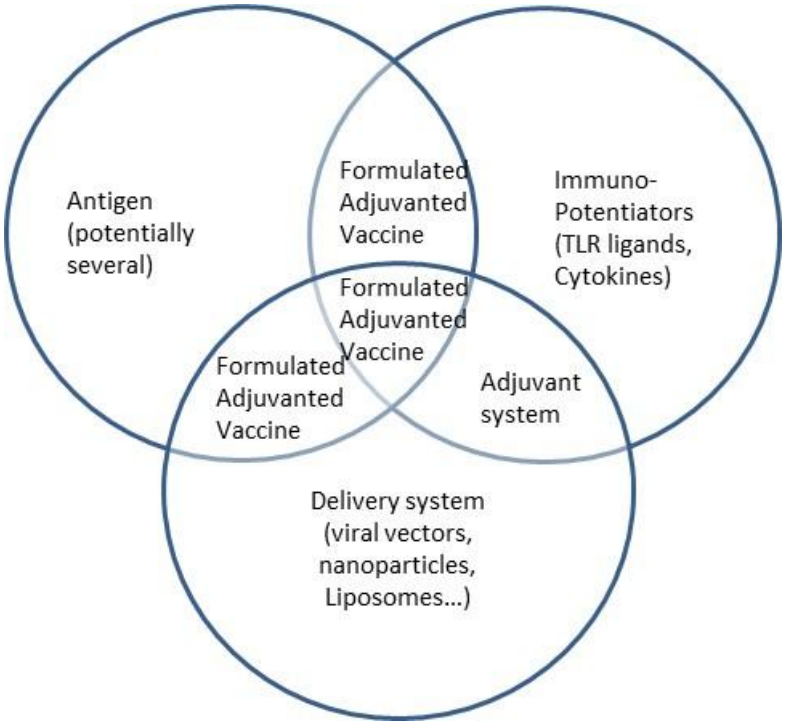

Figure 1: Various components of a vaccine

Given the regulatory complexity and likely accessibility hurdles for candidates that require the combination of multiple antigens, immunopotentiation and a delivery device, it is logical to strive for simplicity in the first instance by adopting the following rational approach:

i) ensure that the immunogenicity of the target antigen is optimised and presented in the most immunogenic format, such as a multimeric VLP or as a chimera, fused to a carrier protein such as a bacterial toxin [16]. If such optimisation avoids the need for adjuvants or allows use of adjuvants that widely used in available vaccines this is a great advantage.

ii) evaluate the antigen(s) in combination with previously licensed and open access adjuvants such as alum by the injectable route to establish proof of principle

iii) if the immunogenicity observed with alum is not sufficient, or an adjuvant/delivery systems that elicits effector T-cell responses is required, attempt to evaluate in combination with alternative licensed adjuvants such as AS04, MF59 or licensed virosomes

iv) in the event that access to these adjuvants is not available, investigate responses with adjuvants with similar properties or modes of actions, or by alternative methods of delivery such as the mucosal route, assuming GMP material is available and the formulation meets the TPP requirements.

Ensure that the formulation is optimized, well characterised and stable prior to initiating any preclinical studies. Candidates which are able to meet these criteria, and perform favourably in comparative preclinical studies with a number of antigens present strong justification to assess and rank these in Phase I safety and immunogenicity studies to down select those that are worthy of future clinical development.

5.0 Current Development Efforts

There are several areas of focus for the optimization of vaccine design, manufacture and delivery.

5.1 Identification of better antigenic targets

Reverse vaccinology was developed to identify genes that express surface or secreted proteins that may become antigens of interest for vaccine candidates [17]. First, the genome is sequenced and the ORFs identified computationally. These DNA ORFs are then inserted into E.coli vectors for expression and the expressed proteins are used to immunise mice. The serum samples are then tested for cytotoxic activity, and those that are most effective and conserved represent the best vaccine candidates. This approach was used to identify the five candidate antigens from group B *Neisseria meningitides* that are contained within Novartis's recently licensed Men B vaccine, Baxsero®.

5.2 Improved design of recombinant antigens

Once an antigen of interest has been identified, consideration should be given as to if and how the presentation of the antigen can be optimized, as this may obviate the need for addition of adjuvants or delivery systems. To date, five carrier proteins have been used in licensed conjugate vaccines: a genetically modified cross-reacting material (CRM) of diphtheria toxin, tetanus toxoid (T), meningococcal outer membrane protein complex (OMPC), diphtheria toxoid (D), and *H. influenzae*

protein D (HiD) [18]. Recently the immunogenicity to the malaria antigens AMA1 and Pfs25 was shown to be increased by chemical conjugation to the mutant, nontoxic Pseudomonas aeruginosa ExoProtein A (rEPA) [19] and these conjugates have since passed beyond the phase 1 clinical trial stage. Novartis Vaccines Institute for Global Health (NVGH) is developing a conjugate vaccine against *Salmonella* Typhi and *Salmonella* Paratyphi A, which combines the Vi antigen to the carrier protein CRM_{197} [20]. In addition, the literature cites several approaches to couple Toll-Like Receptor (TLR) ligands directly to antigens, for example as recombinant proteins as with an approach in which antigens are fused to flagellin. Alternatively, presentation of the antigen in a multimeric system such as a virus like particle (VLP) results in increased immunogenicity. VLPs are non-infectious but mimic the morphology of the virus, presenting the antigen in the correct conformation often in the context of the virus's pathogen associated molecular patterns (PAMPs) that act as inherent adjuvants. VLPs are the most successful recombinant vaccine technology to date, with licensure of the first Hepatitis B (HepB) vaccine in 1985 (GSK's Engerix, followed by Merck's Recombivax), Human Papilloma Virus (Merck's Gardisil and GSK's Cervarix) in 2009, and Malaria (GSK's RTS,S) currently in Phase III [16].

5.3 New strategies for safer live vaccines

Historically vaccines have been based on whole microorganisms that have been killed, or adapted to have attenuated virulence, or more recently pathogen purified antigens and/or polysaccaharides. Live attenuated vaccines are the most successful as they mimic natural infection and effectively elicit both humoral and cellular responses, however they carry the risk of genetic instability and residual virulence [21]. A number of improved attenuation techniques have been developed over the recent decades that reduce the safety risk of attenuated vaccines, such as i) reassortment, as with influenza where live attenuated viruses express multiple hemaglutinin antigens, ii) reverse genetics, where viral RNA is substituted with altered cDNA and the virus reconstituted, iii) recombination, where the gene of one microbe is inserted into the gene of another, iv) deletion mutants, where replication essential open reading frames are deleted v) codon deoptimization, which modifies the viral genome to encode for rarer codons, vi) control of replication by enhancing replication of error-prone RNA polymerases thereby reducing the reversion from attenuation to virulence.

5.4 Adjuvants and delivery systems capable of inducing robust, broad, long-lived responses

The fact that three additional adjuvant systems have been licensed as components of marketed vaccines recently supports the notion that multiple adjuvants, with various mechanisms of action, will be needed for the development of vaccines against the remaining preventable diseases. Undoubtedly the development and licensure of additional adjuvants will depend on our ability to elucidate their mechanism of action in order to justify their inclusion and demonstrate safety, particularly as we develop more complex adjuvants with multiple components. It is likely that future vaccines will contain multiple adjuvants, or combinations of adjuvants and delivery systems in order to generate a robust response involving both antibody and cell mediated responses [22]. The regulatory guidelines stipulate that the developer must demonstrate a clear need for the adjuvant to be present in terms of a benefit to the immune response, and that the inclusion of an additional adjuvanting component must have a positive impact on the risk benefit analysis of the vaccine [23]. TLR ligand combinations are already in use: the live attenuated yellow fever vaccine (YF-17D) activates via TLR2, TLR7/8 and TLR9 generating a mixed Th1/Th2 response [24]. BCG contains ligands

for TLR2 and TLR4 [25]. Table 2 contains examples of multi-adjuvanted vaccines that have been licensed or are in development.

Adjuvant combination	Applications	Effect on immune system
AS01 (MPL + QS21, liposome)	Malaria (Phase III), TB (Phase I/II), HIV (Phase II)	↑ $CD8^+$ T-cell responses, large IgG boost for RTS,S (malaria);
AS02 (MPL + QS21, oil in water)	Malaria (Phase II), TB (Phase II), HBV (Phase I), HIV (Phase I/II), but no recent development I(replaced by AS01)	↑ antibody titers, ↑ $CD4^+$ T-cell responses
AS04 (Alum + MPL)*	HBV (Fendrix®), HPV (Cerverix®), HSV2 (Phase III), EBV (Phase II)	↑ $CD4^+$ T cell, ↑ antibody titers
AS15 (MPL + QS21 + CpG, liposome)	NSCLC (Phase III), melanoma (Phase III)	↑ $CD8^+$ T cell
Montanide 720 + CpG	Melanoma (Phase I/II)	↑ $CD8^+$ T cell
Montanide + resiquimod	Melanoma (Phase I)	↑ Th1, ↑ effector + memory $CD8^+$ T cell
Montanide 51 VG + Poly-ICLC	Melanoma (Phase I/II), ovarian cancer (Phase I)	↑ $CD8^+$ T cell, ↑ $CD4^+$ T cell, ↑ antibody titers
BCG*	Bladder cancer (clinic), colon cancer (Phase III), melanoma (Phase III)	↑ innate response, DTH, ↑ antibody titers
Cadi-05 (MIP)	Leprosy (clinic), bladder cancer (Phase I/II), melanoma (Phase I, terminated)	↑ IFN-γ, ↑ $CD8^+$ T cell, ↑ $CD4^+$ T cell

Table 2: Examples of multi-adjuvanted vaccines, * denotes those systems that are components of licensed vaccines [22].

There is clear rationale to support the synergistic combination of adjuvants, however it should be noted that negative cross regulation of pathogen recognition receptor (PRR) pathways has been reported [26]. A vaccine that contains a TLR ligand may simultaneously target other receptors with resulting safety concerns, therefore it is preferable that immune mechanism of candidates that contain multiple adjuvants be thoroughly characterized, which is complicated by the fact that mice express TLRs differently to humans. In addition to these hurdles, are the requirements for manufacturing, characterization and stability to support multi-adjuvanted formulations.

5.5 Formulation stabilization

Vaccines are usually administered as injectable aqueous liquids which are often prone to degradation and/or aggregation. In order to overcome this, vaccines are frequently stored and transported under refrigerated conditions to extend their shelf life long enough to reach the intended recipient. This presents logistical challenges, particularly for a vaccine requiring multiple immunizations in a remote location. Various excipients have been identified that can be used as stabilisers of liquid vaccines, or the formulation can be dried to reduce the likelihood of degradation providing suitable dessico-protectants can be identified [27, 28]. Several companies such as

Stabilitech, Cambridge Biostability (now Nova Laboratories Ltd) and Indian Immunologicals are developing technologies aimed at thermostabilization, however impact on COGs and the capacity to finish these vaccines at commercial scale will need to be considered.

5.6 Vaccine presentations tailored to route of administration and delivery devices

The gold standard for vaccine delivery is injection of an aqueous liquid, usually by the intramuscular route of administration due to ease of use, cost and proximity of local lymph nodes to mount an immune response. However, as discussed, there are disadvantages to this route as well as the real health risk from needle stick injuries, and this risk is larger in LMIC countries. The WHO estimates that sharps injuries cause about 66,000 HBV, 16,000 HCV and 200-500 HIV infections among healthcare workers each year [29]. From a commercial perspective, needle free administration technologies could provide an advantage in LMIC populations because of ease of administration but also offer competitive benefits in the highly competitive sectors of the high income countries market, such as Influenza.

5.7 Vaccine manufacture

Investigation into new production substrates and technologies, such as plant based production, is driven by the need for cheaper cost of goods and a larger number of doses. Improved techniques for vaccine manufacture have been constrained by the regulatory acceptability of new substrates since traditionally vaccines have been derived from primary and diploid cell culture systems. However Baxter's Vero cell line (lineage isolated from kidney epithelial cells extracted from an African green monkey) has been approved for the last 30 years for the production of the inactivated polio, rabies and recently smallpox (ACAM2000) vaccines, and the use of primary and diploid cell culture systems will be replaced by the use of continuous cell lines (CCLs) [30]. These substrates are gaining increasing acceptance from regulatory authorities as improved screening technologies remove fears regarding their potential oncogenic properties. Several cell lines are currently under development focusing on a range of new viral vaccines, particularly H5N1 pandemic influenza [31].

The remainder of this paper will focus on a review of most promising adjuvants, delivery systems and delivery devices.

6.0 Immunopotentiators

The immune system recognizes pathogen-associated molecular patterns (PAMPs) by means of pathogen recognition receptors (PRRs) which are composed of the Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-I-like receptors (RLRs). A table of the currently identified pathogen recognition receptors (PRRs) and their ligands are shown in table 3. The ligands that bind to PRRs comprise a wide array of molecules including oligonucleotides, small molecules and phospholipids which are derivatives of PAMPs that appear on the surfaces of pathogens, and the current status of research and development has been reviewed comprehensively elsewhere [15, 32, 33]. These molecules are known to activate the innate immune system through Toll-Like-Receptors (TLRs), and are usually included in vaccine formulations along with delivery systems to co-present the TLR agonist with the antigen for maximal potency. Association of the agonist with a delivery system also ensures that the immune activation signals are only being delivered to the cells that recognise the antigen, removing non-specific bystander effects and potential toxicity. First generation vaccines,

including those consisting of inactivated or attenuated virus and bacteria, inadvertently contained inherent adjuvant activity due the presence of bacterial cell walls, bacterial DNA, and viral RNA in their preparations. The quality and mode of action of modern vaccine adjuvants however, needs to be well characterized in order to rationalise their inclusion [23].

Pattern-recognition receptors	Ligands
TLR1	Bacterial lipoproteins from Mycobacteria, Neisseria
TLR2	Zymosan yeast particles, peptidoglycan, lipoproteins, glycolipids, lipopolysaccharide
TLR3	Viral double-stranded RNA, poly:IC
TLR4	Bacterial lipopolysaccharides, plant product toxol
TLR5	Bacterial flagellins
TLR6	Yeast zymosan particles, lipotechoic acid, lipopeptides from mycoplasma
TLR7	Single-stranded RNA, R-837 and R848
TLR8	Single-stranded RNA, R-837 and R848
TLR9	CpG oligonucleotides
TLR10	Unknown
TLR11	Bacterial components from uropathogenic bacteria
NOD1, NOD2	Peptidoglycans
Macrophage mannose receptors and other c-type lectin receptors	Sulfated sugars, mannose-, fucose- and galactose-modified polysaccharides and proteins
Type 3 complement receptors and dectin type receptors	Zymogen particles, β-glycan

Table 3 Pattern-recognition receptors, taken from Pashine et al [15]

6.1 Bacterial lipoproteins (TLR2)

Activation of toll-like receptor 2 (TLR2) by bacterial lipoproteins induces fast non-specific immune responses against pathogens followed by slow but specific adaptive immune responses. Development of synthetic TLR2 agonists/antagonists is currently in preclinical development but have the advantage of being easy to characterize [34].

6.2 Lipopolysaccharide (LPS) (TLR4)

The most advanced TLR ligand is monophospholipid (MPL), a TLR4 agonist which is included in the GSK adjuvant AS04 that has been licensed for HPV (Cervarix) and HepB (Fendrix) vaccines. In AS04, the MPL is located on the surface of alum particles by adsorption, in the same way as the antigen. MPL is under evaluation with other delivery systems including emulsions, nanoparticles and liposomes, including as part of the AS01 adjuvant with GSK's malaria vaccine candidate RTS,S and herpes zoster candidate in Phase III testing.

MPL is derived from Lipid A, which is a biologically active component of LPS, present in Gram-negative bacterial cell walls. Since Lipid A is too toxic for use in human vaccines, there has been significant effort to modify LPS to remove the toxicity associated with Lipid A from its immunostimulatory activity [35]. MPL is 1000 fold less toxic than LPS and has now been administered to over 100,000 patients worldwide.

That said, the use of MPL still faces several challenges. The product is that is naturally isolated from *Salmonella minnesota* is significantly less potent than the product from *E.coli*. Differences in acylation affect the immunostimulatory properties of MPL, therefore accurate characterization of its composition is needed to ensure a consistent end product. Reproducibility has been addressed somewhat through the use of synthetic lipid A, however due to difficulties in the synthesis process, expense and insufficient scalability none of these compounds have yet entered the clinic as vaccine adjuvants. Companies that are developing synthetic Lipid-A based molecules include Biomura, OM Pharma, ONO Pharmaceutical, and Eisai Chemical.

The Infectious Disease research Institiute (IDRI) has recently developed a synthetic lipid A derivative known as GLA (glucopyranosyl lipid adjuvant, trade name PHAD™) that is reproducible to manufacture and has been evaluated in Phase 1 for Influenza and schistosomiasis. It is also undergoing phase I/II clinical development with the Walter Reed Army Institute's malarial antigen CelTos, as well as preclinical evaluation as an adjuvant for vaccines against TB, leishmaniasis and hookworm.

6.3 Flagellin (TLR5)

Vaxinnate's flagellin type 2, or fljB (STF2) ligand derived from *Salmonella typhimurium* is the most advanced TLR5 agonist, with universal (VAX102, expressing the conserved antigen M2e), seasonal (VAX125 expressing H1HA) and pandemic (VAX161 expressing H5HA) influenza candidates having completed Phase I in the clinic. The company was recently awarded a grant from NIAID to develop a trivalent Dengue vaccine with its technology. The flagellin sequence is expressed in tandem with the target protein as a single recombinant antigen and can be produced and purified at low cost, in high yields from *E.coli,* potentially removing the dependence on eggs for the manufacture of flu vaccines.

6.4 Single stranded RNA analogues (TLR7 and TLR8)

The natural ligands for TLR7 and 8 are ssRNA but these receptors can also be activated by synthetic small molecule agonists of approximately 200-300Da such as the imidazoquinolones imiquimod (R-837 for TLR7) and resiquimod (R-848 for TLR8).

Imiquimod is commercially available in a topical cream approved for the treatment of HPV-mediated external genital warts and superficial basal cell carcinoma [36]. Both imiquimod and resiquimod have been evaluated in the clinic as drugs for viral infection and cancer because of their ability to induce cellular immunity, and a number of companies such as Novartis, Telormedix and VenttiRx are evaluating them as vaccine adjuvants. All are currently in the preclinical stage of development and have demonstrated that these molecules effectively induce a Th1 response when applied as an ointment or co-delivered with antigen by microneedles, or injection.

One of the challenges in the development of these small molecules as adjuvants has been that they rapidly disseminate from the injection site. New classes of TLR7/8 agonists are being designed to conjugate directly to antigens or to promote retention of the TLR ligand by association with the antigen at the injection site (3M-051 and 3M-052 respectively) resulting in greater efficacy [37]. It has also been shown that it is possible to induce CD8 responses in mice by covalent conjugation of imidazoquinoline to HIV Gag protein [38].

6.5 Unmethylated CpG motif-based oligodeoxynucleotide (TLR9)

Unmethylated CpG motifs are regions of genomic DNA containing the cytosine-guanine dinucleotide in which cytosine remains unmethylated, especially in prokaryotic DNA. These sequences engage TLR9 and enhance T-cell immune responses to co-delivered antigens in animal models and have been developed for clinical use as either vaccine adjuvants or immune therapeutics by Coley Pharmaceuticals (Pfizer) and Dynavax Technologies, among others. When included as an adjuvant in a pneumococcal conjugate vaccine, Coley's CpG 7909 significantly increased the proportion of vaccine high responders among HIV-infected individuals [39]. This effect was also observed in HIV infected Hepatitis B patients [40]. As such, TLR9 agonists demonstrate significant promise for inclusion within vaccines intended for immune suppressed individuals such as HIV patients or the elderly. Dynavax's HepB vaccine candidate, Hepislav has just completed Phase III testing in 18-70 year olds where it was shown to induce a better immune response than GSK's Engerix, with one less shot [41]. An additional recent Phase III trial has demonstrated immunogenicity and safety of Heplisav compared to Engerix in patients suffering from chronic kidney disease (CKD); three doses of Heplisav provided significantly higher and persistent seroprotection rates as compared to the control licensed hepatitis B vaccine [42]. However despite its promising immunogenicity, in June of 2013 the FDA has requested additional safety data due to the fact that this vaccine contains a novel adjuvant, and the presence of one autoimmune adverse event in the Hepislav arm.

6.6 Carbohydrate based adjuvants

Carbohydrates have the advantage of being native signalling molecules within the immune system and usually demonstrate high biocomparability and low toxicity. Several compounds have been evaluated in human clinical studies (with MPL as a component in GSK's licensed AS04 adjuvant) and some such as cord factor (Trehalose-6-6-dimycolate) and dextran have been licensed in veterinary vaccines [43]. Carbohydrate manufacturing can be challenging, for example MPL and QS 21 are complex molecules that co-purify with heterogeneous or partial homogenous molecules from natural sources, which in turn presents challenges for characterization and release testing. In many instances this has been overcome by developing synthetic alternatives, for example synthetic analogues of MPL such as GLA-SE are now available. QS21 is a saponin that is derived from the bark of the South American soap bark tree, Quillaja saponaria that has been shown to induces Th1 responses. Numerous clinical trials have been conducted using QS21 alone or as a component of adjuvants systems such as ISCOMs and the GSK adjuvants AS02 and AS01. Synthetic analogues have been developed. Deacylated QS21, known as GPI-0100, may be less reactogenic [44].

7.0 Delivery Systems

7.1 Nanoparticles

Over the past few decades, it's become possible to produce nanoscale size (<1000nm) particles that are able to mimic natural pathogens in terms of presentation to the immune systems. The antigen is either encapsulated within or presented on the surface of the particle, in many cases stabilising the antigen/s and enabling delivery through mucosal routes and intradermal administration. Emulsions, VLPs, ISCOMs, liposomes and polymeric nanospheres have been shown to increase immunogenicity of antigens by interacting with or entering antigen presenting cells through different pathways thereby modulating the immune response, and successfully inducing cellular immunity.

Biodegradable nanoparticles produced from polymers such as poly(lactide-co-glycoside) (PGLA) have been investigated for their applicability to deliver vaccines, as their rate of degradation within the body can be controlled, thereby releasing antigen over period of days or weeks. Immunopotentiators, such as TLR7 can be incorporated to boost the immune response [45, 46]and PGLA nanoparticles coated with muco-adhesives have been used to facilitate mucosal delivery [46]. Vaccine antigens encapsulated into PLGA nanoparticles were shown to induce broad and potent immune responses. Preclinical studies in which hepatitis B core antigen (HBcAg) was loaded into PLGA nanoparticles (300 nm) with or without monophospholipid A (MPLA) adjuvant were evaluated in mice [47]. A single immunization with HBcAg- encapsulating PLGA nanoparticles containing MPLA induced a stronger cellular immune response than those induced by HBcAg alone or by HBcAg mixed with MPLA in a murine model. Recently, Novavax completed a Phase I study to evaluate the safety and immunogenicity of a nanoparticle-based RSV candidate in healthy elderly adults, and companies such as Selecta Biosciences are developing synthetic nanoparticles that can be tailored to deliver specific antigens and immunopotentiators.

Stability of the particles, retaining conformation of antigens, sterility during manufacture, scale up and reproducibility and validation of particle size are all areas of ongoing work. Several comprehensive reviews of these delivery systems have been published [48, 49]

i) Emulsions

Emulsions consist of 2 immiscible phases and are either oil-in-water (o/w) where oil droplets are emulsified in in a bulk aqueous phase or water-in-oil (w/o) where water droplets are emulsified in a bulk oil phase. In 1997, MF59 became the first o/w emulsion to be licensed as a component of the Novartis Influenza vaccine Fluad, closely followed by GSKs AS03 and Sanofi's AF03 (all three in the EU) so that nearly 200 million doses of w/o emulsions have now been safely administered to humans. Others, such as GSKs AS02 are in late stage clinical development.

Table 3 shows the composition of clinical emulsion-based adjuvant formulations. Most are squalene based o/w and have been reviewed recently [50]. The challenge with emulsion based adjuvants remains the production of reproducible, uniform particles sizes as this effects the immunogenicity and stability of these formulations, and most have a shelf life of 1-2 years as well as require a cold chain. One strategy to extend the shelf life and ease stability constraints would be to develop a two vial approach, where the antigen is mixed with the adjuvant at the point of use, requiring short (24 hour) stability and allowing the adjuvant to be stockpiled separately. This is particularly useful for

pandemic vaccines where the disease causing strain is unknown until the pandemic has arrived. However this approach requires that the emulsion is easy to prepare and reproducible at the bedside, requiring little or no testing prior to administration, but has been employed by GSK for their Pandemrix pandemic flu vaccine adjuvanted with AS03.

Both MF59 and AS03 have been licensed for pandemic Influenza and have been shown to enhance antibody responses in all age groups, enabling dose sparing and to broaden the immune response to provide coverage over heterotypic strains. Similar responses have been seen with AF03 and IDRI's GLA-SE. However, a MF59-adjuvanted HSV-2 vaccine candidate was discontinued post phase III due to poor efficacy, despite its ability to induce strong antibody and CD4 T-cell responses. More difficult vaccine targets will likely require the addition of immunopotentiators to induce an adequate response. For example, GSK's malaria candidate RTS,S which is currently in Phase III was initially evaluated with AS03, but protection in a human challenge model was found to be significantly increased with the addition of MPL and QS21 in their AS02 formulation, and yet better still when presented as a liposomal formulation (including MPL and QS21) with AS01 [51].

Unfortunately for Pandemrix an association with narcolepsy in children and adolescents has been reported, causing the EMA to restrict the use of the vaccine in persons less than 20 years of age. Investigations into this association are ongoing, and the CDC is currently sponsoring an international study on the associations between adjuvanted monovalent 2009 H1N1 influenza vaccines and narcolepsy. That study is expected to be completed in 2014 [52].

W/o emulsions have been extensively evaluated in humans but are associated with greater reactogenicity than o/w emulsions, as well as challenges with formulation consistency, which have limited their clinical advancement. That said, ISA51 is a component of a therapeutic lung cancer vaccine that has recently been licensed in Cuba [53]. Review of the clinical data with ISA 720 and ISA 51 suggests that reactogenicity may vary depending on antigen, dose and number of immunizations.

Name	Company	Components	Particle size (nm)	Manufacturing process	Admin route	Clinical phase
MF59*®	Novartis	Squalene, polysorbate 80, sorbitan trioleate, citrate buffer	165	Microfluidization	Intramuscular	Licensed
AS03*	GSK	Squalene, α-tocopherol, polysorbate 80, phosphate-buffered saline	150	Microfluidization	Intramuscular	Licensed
AS02	GSK	AS03 plus MPL and QS21	150	Microfluidization	Intramuscular	Phase III
AF03	Sanofi Pasteur	Squalene, polyoxyethylene cetyl-stearylether, sorbitan oleate, mannitol, phosphate-buffered saline	100	Phase inversion temperature	Intramuscular	Phase III
SE, MPL-SE, GLA-SE	IDRI/ Immune Design	Squalene, phosphatidylcholine, poloxamer 188, glycerol, ammonium phosphate buffer, MPL or GLA may be incorporated	120	Microfluidization	Intramuscular	Phase I/II
W805EC	NanoBio	Soybean oil, polysorbate 80, cethylpyridinium chloride, ethanol	~400	High-speed emulsification	Intranasal	Phase I
CoVaccine HT™	BTG	Squalane, polysorbate 80, phosphate-buffered saline, sucrose fatty acid sulfate esters	135	Microfluidization	Intramuscular	Phase II
Montanide® ISA 720	Seppic	Squalene, mannide monooleate	~1000–1500	Two-syringe emulsification	Intramuscular/subcutaneous	Phase I
Montanide® ISA 51*	Seppic	Mineral oil, mannide monooleate	~1000–1500	Two-syringe emulsification	Intramuscular/subcutaneous	Licensed
NH_2	Kurume University (Japan)	Mineral oil, sorbitan monooleate	~1000	Two-syringe emulsification	Subcutaneous	Phase I

Table 4: the composition of emulsion-based adjuvant formulations that are included in licensed vaccines * or in clinical development, modified from Fox et al [50]

ii) Liposomes

Liposomes are spherical vesicles composed of phospholipid bilayers surrounding an aqueous environment with antigens and often immunopotentiators encapsulated within the lumen, linked to the surface of embedded in the bilayer, whilst protecting the antigen from clearance [54]. Cationic liposomes appear to be particularly immunogenic, and it is believed that the cationic charge plays a role in retention of the vaccine at the injection site, enabling prolonged presentation to the immune system. Whilst there are encouraging results from several candidates have entered the clinic, challenges with respect to implementation remain; reduction of cost, increased stability and development of a thermostable formulation are areas of focus at this early stage of development [55].

iii) VLPs and Virosomes

Virus like particles are virus caspids which inherently self-assemble when structural viral proteins are expressed in bacteria or eukaryotic cells. They retain the particulate nature of the parent particle and are processed as native viruses by the host immune system whilst displaying antigenic epitopes in the correct conformation, but are unable to replicate. VLPs can be produced from a number of recombinant systems, and antigens can be expressed as genetic fusions or chemical conjugates to viral structural proteins resulting in chimeric VLPs. The particulate structure and the conformational integrity support the immunogenicity of this platform, however these particles are often co-purified with other host cell components such as lipids, nucleic acids and proteins that stimulate innate immunity. These may be considered to be a potential safety concern, requiring thorough characterization of candidates based on this approach. An overview of all licensed VLP based vaccines and those in development is reviewed in Kushnir et al [16].

Virosomes are viral envelopes assembled in vitro from lipids and viral envelope proteins purified from the parental virus, thereby circumventing the issue of host derived contaminants. As such, virosomes are considered to be VLPs but do not contain any genes or internal proteins. The virosomes principle can be applied to any enveloped virus, however only influenza virosomes have been used in clinical development and licensed products [56]. Berna biotech's Expaxal and Inflexal were launched in 1994 and 1997 respectively and are now manufactured and marketed by Crucell/J&J with over 80 million doses administered and a robust safety database. Nasalflu (Berna Biotech AG) a nasal application of the seasonal influenza vaccine was launched in 2000 but withdrawn due to association with Bell's palsy, believed to be related to the adjuvant heat-labile enterotoxin [57].

Virosomes are currently under investigation as antigenic delivery platforms for several disease indications. The heterologous antigen needs to be associated or covalently linked to the viral particles but can be exposed on the surface of the virosome to generate an antibody response or encapsulated in within the virosome for cytoplasmic delivery and CD8 induction [58]. Several clinical studies with a number of antigens have been performed with encouraging data for the antibody targets, however the encapsulation approach did not induce sufficient responses in a Phase 1 study with HCV derived peptides [59]. The mucosal route of administration is still under investigation as this appears to elicit superior immunogenicity. Table 5 lists the virosomal candidates in clinical development.

Disease, target, effector	Antigen configuration	Administration route	Clinical development and observation
RVVC, Candida albicans Sap2, antibody	One membrane-anchored recombinant protein	Intramuscular or mucosal	One Phase I trial completed (ClinicalTrials.gov identifier: NCT01067131): safe and well-tolerated immunogenicity shown for intramuscular and under investigation for mucosal
HIV, gp41, antibody	One membrane-anchored peptide	Intramuscular prime and intranasal boost	One Phase I trial completed (ClinicalTrials.gov identifier: NCT01084343): safe, well tolerated and immunogenic
Malaria, Plasmodium falciparum AMA-1 and CSP, antibody	Two membrane-anchored peptides	Intramuscular	Three trials Phase I/IIa completed (ClinicalTrials.gov identifiers: NCT00408668, NCT00513669): safe, well tolerated and immunogenic; indication of efficacy
Chronic HCV, HCV core 132, T cells	Two encapsulated peptides and one membrane-anchored peptide	Intramuscular	One Phase I trial completed (ClinicalTrials.gov identifier: NCT00445419): safe, well tolerated but insufficiently immunogenic
Breast cancer, Her2/neu, antibody	Three membrane-anchored peptides	Intramuscular	One Phase I trial completed: safe, well tolerated and immunogenic

Table 5 Virosomal vaccine candidates in clinical development, taken from Moser et al [56].

iv) ISCOMATRIX

CSL's ISCOMATRIX adjuvant contains purified fractions of QS21, phospholipid and cholesterol which combine under controlled conditions to form cage-like structures. All of the components are synthetic or plant derived. As reviewed in Morelli et al [60], the adjuvant is effective at both antigen delivery and immune stimulation, resulting in both Th2 and Th1 responses, in particular inducing cytotoxic CD8 responses. ISCOMATRIX has been combined with TLR agonists, specifically CpG ODN to increase CD8 responses, as well as alum to increase antibody responses. CSL has completed 6 clinical studies for therapeutic HPV, therapeutic HCV and prophylactic influenza vaccines in which safety and tolerability was demonstrated in a total of 1600 individuals for all populations tested, with encouraging data in the elderly for the influenza vaccine.

Sweden-based Isconova have developed a 40 nanometer particle that is formed by mixing Quillaja saponins, cholesterol and phospholipids. Matrix-M™ particles have a shelf-life of several years in aqueous solution at +2-8°C, and are formulated by mixing with the vaccine antigen prior to administration. The company has licensed several veterinary vaccines and has completed a phase I study for Influenza in which good safety and humoral and T-cell immunity was observed. Several organizations (Crucell/J&J, Genocea, Jenner institute) have partnered with Isconova to develop candidates for a range of indications, including malaria, Chlamydia, HIV and Herpes Simplex Virus 2.

7.2 Vectors

Vectors are non-pathogenic delivery vehicles into which genes from pathogens are inserted and subsequently expressed, offering the advantage of providing protection against both itself, as well as the heterologous genes of interest. This has become the most frequently used approach in experimental vaccinology based on a range of viruses and bacteria. For example, the most advanced candidate for Dengue fever utilises the attenuated 17D yellow fever virus as a vector. The major surface antigens of this virus are encoded by the PrM and E genes which have been removed and replaced by the Dengue derived genes of serotypes 1 to 4, generating Sanofi's quadravalent vaccine that has been shown to be immunogenic in early clinical studies [61]. Efficacy studies are ongoing with results from a Thai Phase 2b study neither proving nor disproving efficacy of the vaccine. Results from large Phase 3 trials are anticipated in the next few years. The yellow fever 17D vector has also been used to improve the Japanese encephalitis virus and develop a candidate for West Nile Virus [62].

There are several safety and regulatory considerations for development of vector based vaccines [63, 64]. The attenuation of the carrier strains need to be stable to ensure against reversion to pathogenicity. The therapeutic window may be different in endemic vs non-endemic settings, for example if the population has been previously exposed to the vector there may be an issue of pre-existing immunity that affects the immune response, and the optimal dosage [65]. The possibility of horizontal gene transfer (transmissibility) to host flora or environmental microorganisms may represent an environmental risk and this needs to be evaluated.

i) Bacterial vectors

Most pathogens infect through the mucosal route, therefore elicitation of an efficient response at this site, as well as at the systemic level is desirable. A number of live, attenuated strains of bacteria are being utilised to deliver foreign antigens via mucosal surfaces to antigen presenting cells. Once phagocytosed, these bacterial vectors can survive in the cell and express their DNA either in the cytosol or via the nucleus and as such are effective at generating both T-cell and humoral mediated responses. Bacterial vectors have the advantage of low cost of production, easy scalability with absence of animal derived products and are ideally targeted to mucosal routes as these are their natural sites of entry.

Attenuated mucosal pathogens	Commensal strains
L. monocytogenes	S. gordonii
Salmonella spp.	Lactobacillus spp.
V. cholerae	Sapylococcus spp.
E.coli	Yersinia spp.
Shigella spp.	
M. bovis BCG	
Y. enterocolitica	
B. anthracis	

Table 6: live bacterial vectors that have been successfully used as antigen delivery vectors [63]

Three strains of attenuated live bacterial vaccines (LBV) are currently licensed for human use: *Mycobacterium bovis* strain Bacille-Calmette–Guerin (BCG against tuberculosis), *Vibrio cholera* CVD103-HgR (oral vaccine against cholera), and *Salmonella typhi* Ty21a (orally administered against

typhoid fever). These LBV have been used for the safe and protective immunization of virtually billions of people, with Ty21a and CVD103-HgR by the oral mucosal route, and as such are obvious candidates to express heterologous antigens as carriers.

The registered vaccine strain Ty21a is an attenuated mutant strain of *S. enterica* serovar Typhi Ty2. The multiple chemical mutations render it genetically stable, however they have significantly reduced the immunogenicity of the bacterium. As a result, several strains have recently been engineered from the Ty2 serovar in order to develop a vaccine that delivers optimal efficacy after a single oral dose, such as strains CVD908 and CVD-908 *htrA,* as shown in table 7. Attenuated mutant strains of Salmonella serotypes such as *S. enterica* serovar Typhi as well as serovar Typhimurium have been shown to successfully deliver heterologous antigens in preclinical models for a range of antigens [66, 67] and recently in a phase I study evaluating recombinant vectors expressing *Streptococcus pneumoniae* surface protein antigen PspA. The immunogenicity in this study performed in healthy adults was found to be disappointing, possibly due to pre-existing antibodies [68].

ii) Viral Vectors

Viruses have evolved highly efficient mechanisms for infecting cells and utilizing the cellular machinery for production of virally encoded proteins. As such, attenuated live virus vaccines, whether representing the virus itself (e.g., measles) or a recombinant organism based on the vector (e.g. measles with heterologous gene inserts from other pathogens), viral vectors have become preferred vehicles for heterologous gene delivery because they mimic real-life transmission and co-present pathogen-associated-molecular-patterns to the immune system as they infect. The most comprehensively studied are the poxviridae family, which include derivatives of vaccinia, as well as fowlpox and canary pox. Vaccinia was used to vaccinate over 1 billion people against smallpox in the 1970s, leading to eradication of the disease. Certain pathogens, however, such as HIV, will never be utilised as delivery systems as the vaccine gene(s) may integrate into the host genome or the vaccine may revert to wild-type.

As discussed previously, there are several methods for increasing the immunogenicity of a vaccine such as route and method of delivery, and in the case of vectors, it has been shown that priming with one type of immunogen and boosting with one or two different recombinant vectors (or proteins) successfully boosts cellular and humoral immunity immunogenicity in both preclinical and human studies [69].

The intrinsic properties of each virus determines its applicability to the vaccine in question, such as induction of innate immune responses which modulates the immune response, potential safety concerns, and the fact that humans have pre-existing immunity against many of the viruses that are used as vectors.

Table 8 reviews the various advantages and disadvantages of the various viral vectors in development.

Vaccine strain	Indication	Development phase	Developer
S.enterica serovar Typhi			
Ty21a	Typhoid fever	licensed	Berna Biotech (Crucell)
ZH9	Typhoid fever	Phase I	Microscience (Emergent Biosolutions)
CVD908	Typhoid fever	Phase I	University of Baltimore
CVD908-htrA	Typhoid fever	Phase II	University of Baltimore
PBCC211	Typhoid fever	Phase I	Wyeth
PBCC222	Typhoid fever	Phase I	Wyeth
Ty800	Typhoid fever	Phase I	Massachusetts General Hospital
X3927	Typhoid fever	Phase I	University of Baltimore
X4073	Typhoid fever	Phase I	University of Baltimore
ISP 1820	Streptococcus pneumoniae	Phase I	St Louis University
Ty2 $RpoS^{-}$	Streptococcus pneumoniae	Phase I	St Louis University
Ty2 $RpoS^{+}$	Streptococcus pneumoniae	Phase I	St Louis University
S. enterica serovar Typhimurium			
LH1160	Expressed H. pylori urease	Phase I	Massachusetts General Hospital
WT05	Typhoid fever	Phase I	Microscience (Emergent Biosolutions)
Shigella flexneri			
SFL124	shigellosis	Phase I	Karolinska Institute
SFL1070	shigellosis	Phase I	Karolinska Institute
SC602	shigellosis	Phase 1	USAMRID
CVD1207	shigellosis	Phase I	University of Baltimore
CVD1203	shigellosis	Phase I	University of Baltimore
CVD1208	shigellosis	Phase I	University of Baltimore
CVD1204	shigellosis	Phase I	University of Baltimore
Shigella sonnei			
WRSS1		Phase I	University of Baltimore
Listeria monocytogenes			
LH1169		Phase I	Massachusetts General Hospital
Lm-LLO-E7	Cancer	Phase I	Advaxis
CRS-207	Cancer	Phase II	Aduro

Table 7 Live attenuated bacterial vectors that have been tested in clinical studies in humans, adapted from Daudel et al [64]

Viral Vectors	Candidates	Developer	Indications	Advantages	Disadvantages
Pox viruses					
Fowlpox	FP9-ME-TRAP	Oxford University	Malaria	Can accommodate large and/or several antigens, virus does not replicate in cytoplasm therefore no risk of insertional mutagenesis, large human safety data base, often no pre-existing immunity but neutralizing antibodies develop with subsequent vaccinations	Complex and time consuming construction therefore costly, constructions not always stable through production cycle, Replication-competent virus (VV), not appropriate for use in immunocompromised patients
	FP9-85A	Oxford University	Tuberculosis		
	rFPV-HIV-AE	Thai Red Cross	HIV		
	Cancer	NIAID	various		
Canarypox (ALVAC)	RV144 (ALVAC+HIV gp120)	Sanofi/ Genentech	HIV		
MVA	TROVAX™ (MVA-5T4)	Oxford Biomedica	Renal cancer, Colon, Prostate, NSCLC		
	TG4010 (MVA-MUC1-IL2)	Oxford Biomedica	Renal cancer		
	MVA ME-TRAP/AMA1/MSP1	Oxford University	Malaria		
NYVAC	NYVAC-C	University of Lausanne	HIV		
Adenovirus	rAd-p53	Shenzhen SiBiono GeneTech	cancer	Effective cellular uptake and protein expression, rapid indiction of immunity, stable constructs, No risk of insertional mutagenesis; Replication-deficient strains used limiting pathogenicity, many serotypes available to overcome pre-existing immunity, recent experience in humans with recombinant and chimp adenoviruses	Infection of target cells dependant on expression of Ad receptor (e.g. CAR), pre-existing immunity to some strains, perceived safety concerns of Adenovirus 5 following HIV STEP study in which vaccine significantly increased the risk of HIV acquisition and did not significantly lower viral load or preserve CD4 T cell counts in participants who became infected
	Ad5 with gag, pol, and nef	Merck	HIV		
	Ad26, Ad35, Ad5	various	Malaria		
	ChAd63 ME-TRAP, ChAd63 AMA1/ChAd63 MSP1, ChAd63 Pfs25	Oxford University, Okairos	Malaria		
	ChAd63 SIV Gag	NIAID	HIV		
	ChAdY25	Oxford University			
	AdCh3NSmut	Okairos	HCV		
Alphavirus	Sindbis virus (SIN), Venezuelan Equine Encephalitis (VEE), Semliki Forest Virus (SFV)	Harris vaccines, NIAID	Numerous preclinical studies (Lundstrom, 2012)	Naturally immunogenic, multiple vaccinations possible; no neutralizing antibodies develop,high expression capacity,	Limited duration of expression of transgene due to induction of apoptosis in infected cell, little experience in humans, limited antigen load

	VSV-HA	Yale University	H5N1		
	VSV-GagEnv	NIAID	HIV		
Viral Vectors	**Candidates**	**Developer**	**Indications**	**Advantages**	**Disadvantages**
Measles Virus (MV)	Paramyxovirus vaccine	Crucell	Paramyxovirus	Vaccine strain non-pathogenic, noncontagious; RNA virus therefore no risk of insertional mutagenesis, mucosal receptors, immunity against both measles and heterologous gene, long lived immunity incl.CD8, approved strains of the vaccine already exist	Specificity for tumor cells, Contraindicated in severely immunocompromised patients, Pre-existing immunity to MV, Viral transgene expression limited by lysis of target cell
Vesicular stomatitis virus (VSV)	VSV-HA	Yale University	H5N1	No risk of insertional mutagenesis, vaccine strain non-pathogenic and noncontagious, no pre-existing immunity	safety concerns due to neural tissue receptor
	VSV-GagEnv	NIAID	HIV		
	VSV-RSV VSV HIV1 gag	Yale University Profectus	RSV HIV		

Table 8: Overview of advantages and disadvantages of major viral vectors and their clinical application

iii) DNA vaccines

Vaccines based on plasmid DNA have been shown to elicit strong antibody and T-cell responses in animal models however there have been several unsuccessful in human studies with a range of antigens, believed to be due to low levels of expression, inefficient transfection into the nucleus of host cells and inadequate stimulation of the immune system (Li et al 2012). A range of strategies have been developed to overcome the poor immunogenicity observed, including codon and promoter optimization, addition of adjuvants such as inclusion of TLRs or cytokines, electroporation and prime boost regimens with protein antigens or viral vectors. These approaches have resulted in some improvement in vaccine efficacy; the immunogenicity of a HIV-1 DNA vaccine candidate PENNVAX® co-administered with an interleukin 12 (IL-12) DNA plasmid expressing human IL-12 proteins p35 and p40, delivered by electroporation resulted in 89% of vaccines developing a CD4(+) or CD8(+) T-cell response after the third vaccination [70, 71] Biotech company Inovio have developed DNA vaccine based candidates for prostate cancer, hepatitis C virus, HIV, malaria and influenza using their SynCon® technology with a candidate for cervical dysplasia in a Phase II clinical study [72]. The approach focuses on identifying consensus sequences for antigens of interest, coupling these with patentable sequences that facilitate transfection and expression and delivery by electroporation, resulting in favourable effector T-cell responses. The DNA delivery approach potentially has renewed potential when coupled with improvements in delivery technology, such as Ichor's TriGrid™ Delivery System, which is currently being evaluated in a number of clinical studies for a range of indications [73, 74].

iv) RNA vaccines

An alternative to DNA delivery is the utilization of RNA as a vector for presentation of antigens. RNA vectors have potential advantages over DNA, such as there being no possibility for integration into the host genome, no pre-existing immunity and no need to traverse to the nucleus as the RNA is translated in the cytoplasm [75]. Novartis have developed a self-amplifying RNA vaccine and shown that systemic delivery of short interfering RNA (siRNA) using lipid nanoparticles (LNPs) increased immunogenicity for an RSV F-subunit candidate compared with delivery of unformulated RNA in preclinical studies [76]. CureVac is combining both the antigenic and self-adjuvanting properties (TLR7) of modified, non-coding, long and complexed mRNAs to develop novel mRNA vaccines [77]. In preclinical studies the platform has been shown to be able to protect against influenza challenge and have anti-tumour properties and the company has therapeutic mRNA vaccines in oncology and therapeutic/prophylactic vaccines for infectious diseases under development in collaboration with Sanofi Pasteur and the Defense Advanced Research Projects Agency (DARPA).

8.0 Novel vaccine administration technologies

Vaccine administration is an important issue in both low and high income countries, however there are different unmet needs in the two settings: in high income countries, needle free vaccine administration, or administration by alternative routes would result in greater compliance and uptake because of painless, easier administration, whereas in low income countries, factors such as elimination of the cold chain and biowaste, as well as removal of the need for trained personnel to administer vaccines are additional key factors. In both scenarios, the switch to alternative non-parenteral routes of administration may be advantageous as both the systemic and mucosal arms of the immune response are stimulated [9, 78]. In addition, the biodefense or pandemic vaccine sector would benefit from technologies that facilitate mass injection campaigns, for example that obviate the need for trained personnel and enable quick, efficient immunization [79].

However, novel vaccine administration technologies will only present a viable market opportunity if they show at least equivalent efficacy to current methods, whilst reducing health risk and taking cost considerations into account [80]. Some novel delivery devices can be approved as stand-alone products under the FDA's 510(k) regulation, under which equivalence to another US marketed device has been demonstrated i.e. safety, lack of potential for cross-contamination, however other factors such as bioavailability of the antigen and vaccine denaturation require the combined development of a candidate and device. The implication of this is that vaccine developers need to partner with or in-license from delivery device developers, potentially restricting freedom to operate and reducing access to benefit addition applications.

Eager to capitalise on the market for alternative vaccine delivery mechanisms, several companies have established development programmes to address this need. These can be separated into two categories: dermal vaccine administration, and mucosal administration via the nasal, oral and pulmonary routes.

8.1 Needle free dermal administration

Vaccination through the skin can be by either the transdermal or transcutaneous route where the vaccine is applied directly to the surface of the skin to deliver the vaccine to antigen presenting cells in the epidermis, to generate systemic and mucosal immune responses. There are various strategies in development, the most advanced of which are transdermal patches and microneedles [78].

i) Transdermal patches

This technique usually involves disruption of the skin's upper layer, the stratum corneum, followed by the application of a patch to the surface of the skin.

ii) Microneedles

These are arranged in arrays of various lengths from 25uM to 1mm depending on the targeted skin layer. Sanofi Pasteur/Becton Dickinson have successfully marketed this type of device in Europe in their Intanza influenza vaccine which utilises Becton Dickenson's prefilled microinjection system BD Soluvia [81].

iii) Needle-free solid, liquid or powder injection technologies

Needle-free injection uses a gas to force the vaccine through the skin delivering it intradermally, subcutaneously or intramuscularly, depending on the force of the injection. There are a wide range of these devices in development as they have the potential for high throughput (up to 1000 people per hour) however not without safety concerns following the outbreak of Hepatitis B that was linked to the use of multi-use jet injectors [14]. Table 9 presents an overview of dermal administration technologies in development

	Company	Product	Indication	Advantages	Disadvantages
Dermal vaccine delivery				Large target organ, non-invasive delivery, no risk of cross contamination, relatively painless, self-administration possible removing the need for trained personnel, ideal for mass vaccination campaigns	Disruption of the stratum corneum required, addition of adjuvants likely as well as larger doses, regulatory uncertainty especially if device is required. May increase cost, need for reformulation of existing vaccines
Needle-free jet injectors	Avant Medical	Guardian 101 Pulse Injection System	N/A, has FDA 501(k) clearance	Most likely will utilise the same formulation as conventional vaccines, enable delivery to different layers of skin, 'fast' vaccination so applicable to mass immunization campaigns	Pain, risk of transmission of blood borne diseases. Same stability/storage issue as current vaccines
	Bioject	Biojector 2000	Various, has FDA 501(k) clearance		
	EuroJet Medical	E-Jet 500	Various		
	INJEX	Injex	Various, has FDA 501(k) clearance		
	Pfizer	Powder injection	Influenza, cancer		
	PharmaJet	PharmaJet System	FDA 501(k) clearance		
Transdermal patches	DBV Technologies	VIASKIN	Hepatitis A and B, meningitis, influenza, typhoid, shigella	Painless, many are stable at room temperature, add-on adjuvant patches could be developed.	Dosing inaccuracy
	Intercell	Transdermal patch containing LT	Travellers' diarrhoea, pandemic influenza		
	Genetic immunity	DermaVir	HIV therapy		
	Vaxin	Patch containing bacterial vectors	Anthrax		
	Zosano Pharma	ZP patch	Influenza		
Microneedles	Becton Dickinson	BD Soluvia pre-filled system	Approved for use in EU (Sanofi Pasteur)	Ease of administration, reliably disrupts stratum corneum, painless, does not necessarily require reformulation, may be stable at room temp.	Dosing accuracy challenging
	Cyto Pulse	Derma Vax, Easy Vax	Caner, HIV		
	3M	sMTS	Influenza (Vaxinnate)		
	TheraJect	Vax MAT	HIV (BMFG) Influenza, hep B		

Table 9 Overview of dermal vaccines in development

8.2 Mucosal Vaccine Administration

Most infectious pathogens infect their host through a mucosal route, therefore elicitation of an efficient mucosal immune response as a first line of defence is highly desirable. Mucosally administered vaccines against various indications have been available for decades however most still use live attenuated pathogens in order to ensure stability and generate a sufficiently high immune response. Compared to the injectable route, mucosal administration has numerous advantages, such as being non-invasive, removing the risk of needle stick injury and disease transmission, elicits systemic and mucosal immune responses an is often easier to administer [82]. However there are also numerous challenges, such as the safety concerns associated with mucosal delivery of live attenuated delivery, for example, live attenuated polio virus in the Sabin vaccine have been shown to mutate back to the infectious virus, causing some high income countries to convert to an injectable form of the vaccine. Transition to safer approaches using subunit vaccines requires sophisticated formulation (encapsulation) and/or delivery devices to protect them from proteolytic degradation in the stomach or mucociliary clearance from the nasal mucosa, amounting to a more complex regulatory pathway.

Over the past few decades all mucosal surfaces including oral, nasal, pulmonary, rectal, conjuctival and vaginal have been considered potential delivery routes, however many of these are not viable due to practical constraints, unlikely acceptance resulting in low compliance [83]. For this reason, the majority of R&D effort has focused on oral, nasal and pulmonary vaccine delivery. Table 10 lists the oral vaccines that are either marketed or in clinical development. There are a number of candidates also in preclinical development, too numerous to mention, but the majority of these focus on non-live attenuated vaccine strategies.

i) Oral delivery

Oral delivery targets the largest mucosal surface in the gut. The convenience of oral vaccine delivery means that trained personnel is unlikely to be required, and the vaccine can be administered, safely and quickly with reliable dosing. The most successful oral vaccines are the Sabin polio vaccine (approved in 1961) and the two recent rotavirus vaccines RotaTeq (Merck) and Rotarix (GSK), both VLP based vaccines approved in 2006.

ii) Intranasal delivery

Nasal delivery is attractive because it mimics the route by which many pathogens naturally infect the body, stimulating both systemic and mucosal immunity. Like oral delivery, it has the advantage of being painless, and relatively non-invasive obviating the need for administration by trained personnel. Nasal vaccines have the advantage that there is less degradation than via the oral route, but need to be designed to be rapidly taken up by nasal mucosa before they are cleared. Strategies to overcome the short residence time in the nasal cavity include inclusion of mucoadhesives and adjuvants in the formulation, targeted delivery systems and the use of delivery devices. Candidates that contain both adjuvants and/or delivery systems, as well as require a delivery device are likely to be costly involving complex development partnerships. The most advanced targets for the development of nasally delivered vaccines are against respiratory pathogens that infect through the respiratory tract, such as influenza, RSV and parainfluenza virus, however the only intranasal vaccine

that is currently marketed is Astra Zeneca's live attenuated Flumist influenza vaccine which has had disappointing market performance to date.

As with oral vaccines, most candidates are still based on live attenuated pathogens, which are associated with safety risks. The first intranasal vaccine to reach the market was Berna Biotech nasal flu vaccine Nasalflu, launched in Switzerland and Germany in 2000, however it was withdrawn in late 2001 due to association with incidence of Bell's Palsy in vaccine recipients and believed to be caused by the LT adjuvant included in the vaccine. More recent approaches are focusing on development of adjuvanted split or sub-unit vaccines, as shown in table 10.

iii) Pulmonary delivery

This route is most applicable to pathogens that cause infection through the lungs, such as tuberculosis, one of the most prevalent and deadly diseases. Although painless and needle free, the pulmonary route requires a delivery device to aerosolise the vaccine, of which there are three main types: nebulisers, metered dose inhalers (MDIs) and dry powder inhalers (DPIs) [84]. All of these are already available for delivery of drugs for treatment of pulmonary illnesses for example asthma, and optimal device depends on type of antigen, the formulation and the desired immune response which can be influenced by the aerosol particle size. The target patient group is also of importance as nebulisers and MDIs to not require the individual's breathing efforts, but the dry powder inhalers does rely on the strength of inhalation rendering this device unsuitable for young children or the elderly. Specific inhalation techniques to reproducibly deliver the specified dose remains one of the major hurdles for this route of delivery. In addition, pulmonary delivery is associated with significant safety concerns since the delivery site is a vital organ, particularly for a vaccine that contains an adjuvant.

	Company	Product	Indication	Presentation of antigen	Advantages	Disadvantages
Mucosally administered vaccines					Non-invasive, needle free, potential to elicit both systemic and mucosal immunity, ease of administration,	Live attenuated products are associated with safety concerns, subunit candidates suffer from stability issues and low immunogenicity requiring adjuvants
Oral	Various	Oral Sabin polio vaccine*	Polio	Live attenuated virus	Painless and easy to administer and therefore greater compliance, no need for trained health care personnel resulting in lower cost, potential for mass administration in endemic and developing world regions	Need for sophisticated formulation to protect against proteolytic degradation, such as encapsulation Poor understanding of the mechanism of generation of mucosal immunity Large quantities of antigen required, likely requiring adjuvant Safety concerns with live attenuated vaccines
	Crucell (J&J)	Dukoral*	Cholera, enterogenic E.coli	Live attenuated bacterium		
	Crucell (J&J)	Vivotif*	Typhoid fever	Live attenuated bacterium		
	Crucell (J&J)	Orochol*	Cholera	Live attenuated bacterium		
	GSK	Rotarix*	Rotavirus	Live attenuated virus		
	Merck	RotaTeq*	Rotavirus	Live attenuated virus		
	Vaxart	Adenovirus 4 & 7	Adenovirus	Live attenuated virus + TLR3		
	Barr Laboratories	Adenovirus 4 & 7	Adenovirus	Live attenuated virus		
	Allergy Therapeutics	Oral allergy vaccine	Grass allergy	Allergen extracts, MPL		
	Bharat Biotech	Rotavirus vaccine (Ph II)	Rotavirus	Live attenuated virus		
	ACE Biosciences	ACE 527, 537, 920	Entero. E.coli	Live attenuated bacterium		
Intranasal	AstraZeneca	FluMist*	Influenza	Live-attenuated virus	Potential to elicit systemic and mucosal administration; highly immunogenic route Antigens encounter only minor degradation in the nose, painless administration potentially without trained personnel	Mucociliary clearance, may require adjuvants and application devices resulting in complex development partnerships and regulatory pathways, concerns about safety following NasalFlu, may require large antigen doses
	AstraZeneca	MEDI560 and 534	PIV and RSV	Live-attenuated virus		
	BioDiem	Live attenuated vaccine	Influenza	Live-attenuated virus		
	Avir Green Hills	FluVacc	Influenza	Live-attenuated virus		
	Ligocyte (now Takeda)	Norovirus vaccine	Norovirus	VLP, MPL, chitosan		
	NasVax	InflusomeVac	Influenza	liposome		
	Polymun Scientific	Vero-Vac	Influenza	Live-attenuated virus		
	Vaxin	Influenza vaccine	Influenza	adenovirus		
Pulmonary	WHO	Measles vaccine	Measles	Live-attenuated virus	Long persistence of antigen in the lungs may boost immune response, applicable to mass immunization campaigns	Challenges in developing reproducible dose delivery, requirement for a device and therefore business partnerships, safety of vaccine in pulmonary delivery, increased risk of product failure as vaccine consists of three components (antigen, adjuvant, device)
	NIAID	Influenza (H7N3)	Influenza	Live-attenuated virus		
	Havard University/MEND	Diptheria and TB	Diptheria and TB	Live-attenuated virus		

Table 10 Overview of mucosal vaccines in development

9.0 General regulatory considerations

Safety, demonstration of sufficiently high efficacy and lack of a consistent evaluation/development pathway are major stumbling blocks for development of new vaccines, together with the fact that we do not always understand the mechanism of adjuvanticity or the possible ramifications on other arms of the immune response. No adjuvant is licensed as a medicinal product in its own right; it's a component of the vaccine and therefore characterization, preclinical and toxicology studies need to be designed on a case by case basis to demonstrate quality, scientific rationale and safety [85]. Safety is a primary concern in vaccine development as vaccines are largely tested and administered to healthy populations and children, requiring extensive preclinical safety studies of adjuvants and adjuvanted vaccines, including local reactogenicity and systemic toxicity testing to establish the risk:benefit ratio [86, 87]. Vaccines that contain novel adjuvants to be introduced into LMIC and that do not have a significant market in HIC, for example the malarial vaccine RTS,S face particular challenges in that the vaccine (and in this case the adjuvant) has not accumulated safety data in comparatively resource rich HIC. All safety data, pre- and post approval will need to be collected in LMIC where the healthcare systems required for long term follow up and pharmacovigilance are less robust [88]. This highlights the need for vaccines specifically for LMIC to utilise technologies for which safety databases already exist, where possible. As this may often not be possible, while retaining efficacy, strengthening of pharmacovigilance systems in LMIC is also highlighted as a high priority.

The EMA's Committee for Proprietary Medicinal Products (CPMP) has established a Vaccine Expert Group (VEG). This group oversees some of vaccine product-related issues including the development of novel adjuvants and published their own guideline [89].

The CHMP has established a number of working parties to consult on regulatory matters. In cases where more than one antigen or adjuvant are present in a vaccine, each adjuvant must be investigated with each of the antigens. Adjuvants that are covered by the guidelines include:

- Mineral salts (aluminium hydroxide, aluminium or calcium phosphate)
- Water-in-Oil or O/W emulsions, i.e. MF59, AS03 and Montanides
- Particulates such as nanoparticles, virosomes, liposomes, ISCOMs
- Natural and synthetic microbial derivatives such as CpG, MPL, toxoid mutants
- Endogenous human immunodulators such as GM-CSF and interleukins

In summary, for a new adjuvant a thorough physico-chemical characterization package is expected, including identification, control and validation of manufacturing process parameters and any bridging to the compound used during the development stage. In terms of clinical studies, trials should be designed to generate information to support:

- Inclusion of the adjuvant
- Enhancement of the immune response without undue local and systemic reactions
- Non-interference in the case of a multi-antigen vaccine
- Risk-benefit analysis of the new product should be at least as favourable as the existing
- intervention, if one exists

With this in mind, it is important to contact the regulatory agencies early on in development (prior to Phase I) to understand the quality and characterization requirements for a new adjuvant, particularly if the vaccine contains multiple components.

10 Concluding remarks

Immunization is one of the highest impact and most cost effective methods of preventing infectious diseases, particularly in low and middle income countries with minimal healthcare infrastructure in remote locations. Despite its unprecedented impact, there are a number of challenges still remaining to be tackled, such as development of effective vaccines for the diseases that cause the most morbidity and the need to urgently improve immunization coverage for diseases for which effective vaccines are available. This requires focus on improvement of delivery methodology, and goes hand in hand with developing practical end user requirements and strengthening healthcare infrastructure.

Development of needle free technology would be advantageous for both low and middle income countries which would benefit from a reduction in the need for healthcare workers, as well as high income countries, which would observe higher uptake and compliance for those vaccines that are less painful or do not require a doctor's visit.

Thermostability of vaccines is critical for improving vaccine coverage in low and middle income countries for which continuous cold storage is a logistical constraint but is less of a concern for high income countries. Accidental exposure to heat, or to freezing leads to reduction of vaccine efficacy in some cases, requiring disposal and sometimes associated with administration of sub-optimal vaccines.

Any development that results in a reduction in the number of doses for a vaccine is likely to observe greater compliance and higher coverage. This may be permitted by the identification of more immunogenic or effective antigenic targets as well as the potential addition of adjuvants or delivery systems. This benefits both high and low income countries alike. In all cases developers should seek to test their candidates with adjuvants or delivery systems where regulatory precedence exists, especially for indications that are intended only for LMIC, such as malaria, where there are fewer market incentives for development of new adjuvant platforms. Only where it is clear that adjuvants or delivery systems with regulatory precedence are inadequate to achieve immunogenicity and efficacy for a given indication, should development with novel adjuvants be prioritised.

Due to safety and regulatory concerns, and the need for better characterization of vaccines from the regulatory authorities, there is an increasing trend towards development of sub-unit vaccines rather than live attenuated or heat killed micro-organisms. These new candidates are likely to be vectors that are able to express multiple antigens, or multi-meric VLPs and are likely to require an adjuvant and/or delivery system. Since the majority of diseases for which there is not yet an effective vaccine contain intracellular life-cycle stages, we need better understand how to target a cell mediated response, ideally at the mucosal point of entry in order to tackle diseases such as HIV and TB. Some 'old' technologies are seeing a revival, for example RNA vaccines, as our understanding of immunological targeting and methods to achieve more efficient delivery improves.

What is clear is that development of future vaccines, regardless of the population or market, will require multiple components (whether through subunit or whole pathogen approaches) in order to generate a tailored immunogenic response. This is driving the field towards better co-ordinated multi—disciplinary and collaborative approaches, and a greater programmatic approach from the funding bodies which are partnering in development.

Acknowledgements

The funding source is acknowledged as the ADITEC Project, which is funded by the European Union, Seventh Framework Programme, Grant Agreement 280873

11.0 References

1. WHO. *World Immunization Week 2012*. 2012.
2. WHO. *Statue commemorates smallpox eradication*. 2010.
3. WHO. *Vaccine-preventable diseases*. 2013.
4. Rappuoli, R., et al., *Vaccines for the twenty-first century society.* Nat Rev Immunol, 2011. **11**(12): p. 865-72.
5. Finn, O.J., *Cancer vaccines: between the idea and the reality.* Nat Rev Immunol, 2003. **3**(8): p. 630-41.
6. Levine, M.M., *"IDEAL" vaccines for resource poor settings.* Vaccine, 2011. **29 Suppl 4**: p. D116-25.
7. Amorij, J.P., et al., *Towards tailored vaccine delivery: needs, challenges and perspectives.* J Control Release, 2012. **161**(2): p. 363-76.
8. Oyston, P. and K. Robinson, *The current challenges for vaccine development.* J Med Microbiol, 2012. **61**(Pt 7): p. 889-94.
9. Lycke, N., *Recent progress in mucosal vaccine development: potential and limitations.* Nat Rev Immunol, 2012. **12**(8): p. 592-605.
10. Brito, L.A., P. Malyala, and D.T. O'Hagan, *Vaccine adjuvant formulations: A pharmaceutical perspective.* Semin Immunol, 2013. **25**(2): p. 130-45.
11. Pasare, C. and R. Medzhitov, *Control of B-cell responses by Toll-like receptors.* Nature, 2005. **438**(7066): p. 364-8.
12. *The Right Shot: Extending the Reach of Affordable and Adpated Vaccines*. 2012, Access Campaign Medicins Sans Frontieres: http://www.msfaccess.org/content/rightshot.
13. Moingeon, P., C. de Taisne, and J. Almond, *Delivery technologies for human vaccines.* Br Med Bull, 2002. **62**: p. 29-44.
14. Giudice, E.L. and J.D. Campbell, *Needle-free vaccine delivery.* Adv Drug Deliv Rev, 2006. **58**(1): p. 68-89.
15. Pashine, A., N.M. Valiante, and J.B. Ulmer, *Targeting the innate immune response with improved vaccine adjuvants.* Nat Med, 2005. **11**(4 Suppl): p. S63-8.
16. Kushnir, N., S.J. Streatfield, and V. Yusibov, *Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development.* Vaccine, 2012. **31**(1): p. 58-83.
17. Donati, C. and R. Rappuoli, *Reverse vaccinology in the 21st century: improvements over the original design.* Ann N Y Acad Sci, 2013. **1285**: p. 115-32.
18. Pichichero, M.E., *Protein carriers of conjugate vaccines: Characteristics, development and clinical trials.* Hum Vaccin Immunother, 2013. **9**(12).
19. Qian, F., et al., *Conjugating recombinant proteins to Pseudomonas aeruginosa ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates.* Vaccine, 2007. **25**(20): p. 3923-33.
20. Micoli, F., et al., *Vi-CRM 197 as a new conjugate vaccine against Salmonella Typhi.* Vaccine, 2011. **29**(4): p. 712-20.
21. Plotkin, S.A., *Vaccines: the fourth century.* Clin Vaccine Immunol, 2009. **16**(12): p. 1709-19.
22. Mount, A., et al., *Combination of adjuvants: the future of vaccine design.* Expert Rev Vaccines, 2013. **12**(7): p. 733-46.
23. *Guideline on Adjuvants in Vaccines*, in *Committee for Proprietary Medicinal Products* 2004.
24. Querec, T., et al., *Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity.* J Exp Med, 2006. **203**(2): p. 413-24.
25. Heldwein, K.A., et al., *TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG.* J Leukoc Biol, 2003. **74**(2): p. 277-86.
26. Simmons, D.P., et al., *Mycobacterium tuberculosis and TLR2 agonists inhibit induction of type I IFN and class I MHC antigen cross processing by TLR9.* J Immunol, 2010. **185**(4): p. 2405-15.

27. McAdams, D., D. Chen, and D. Kristensen, *Spray drying and vaccine stabilization.* Expert Rev Vaccines, 2012. **11**(10): p. 1211-9.
28. Sen, A., et al., *Role of heavy water in biological sciences with an emphasis on thermostabilization of vaccines.* Expert Rev Vaccines, 2009. **8**(11): p. 1587-602.
29. Simonsen, L., et al., *Unsafe injections in the developing world and transmission of bloodborne pathogens: a review.* Bull World Health Organ, 1999. **77**(10): p. 789-800.
30. Pro, V.a.R.B. and d.A. Committe, *Cell Lines Derived from Human Tumors for Vaccine Manufacture*.2012: http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/VaccinesandRelatedBiologicalProductsAdvisoryCommittee/UCM319573.pdf.
31. Montomoli, E., et al., *Cell culture-derived influenza vaccines from Vero cells: a new horizon for vaccine production.* Expert Rev Vaccines, 2012. **11**(5): p. 587-94.
32. Sasai, M. and M. Yamamoto, *Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors.* Int Rev Immunol, 2013. **32**(2): p. 116-33.
33. Pichichero, M.E., *Improving vaccine delivery using novel adjuvant systems.* Hum Vaccin, 2008. **4**(4): p. 262-70.
34. Abdel-Aal, A.B., et al., *Simple synthetic toll-like receptor 2 ligands.* Bioorg Med Chem Lett, 2011. **21**(19): p. 5863-5.
35. Ireton, G.C. and S.G. Reed, *Adjuvants containing natural and synthetic Toll-like receptor 4 ligands.* Expert Rev Vaccines, 2013. **12**(7): p. 793-807.
36. Vasilakos, J.P. and M.A. Tomai, *The use of Toll-like receptor 7/8 agonists as vaccine adjuvants.* Expert Rev Vaccines, 2013. **12**(7): p. 809-19.
37. Smirnov, D., et al., *Vaccine adjuvant activity of 3M-052: an imidazoquinoline designed for local activity without systemic cytokine induction.* Vaccine, 2011. **29**(33): p. 5434-42.
38. Wille-Reece, U., et al., *HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates.* Proc Natl Acad Sci U S A, 2005. **102**(42): p. 15190-4.
39. Offersen, R., et al., *TLR9-adjuvanted pneumococcal conjugate vaccine induces antibody-independent memory responses in HIV-infected adults.* Hum Vaccin Immunother, 2012. **8**(8): p. 1042-7.
40. Angel, J.B., et al., *CpG increases vaccine antigen-specific cell-mediated immunity when administered with hepatitis B vaccine in HIV infection.* J Immune Based Ther Vaccines, 2008. **6**: p. 4.
41. Heyward, W.L., et al., *Immunogenicity and safety of an investigational hepatitis B vaccine with a Toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared to a licensed hepatitis B vaccine in healthy adults 40-70 years of age.* Vaccine, 2013.
42. Janssen, R.S., et al., *Immunogenicity and safety of an investigational hepatitis B vaccine with a toll-like receptor 9 agonist adjuvant (HBsAg-1018) compared with a licensed hepatitis B vaccine in patients with chronic kidney disease.* Vaccine, 2013.
43. Petrovsky, N. and P.D. Cooper, *Carbohydrate-based immune adjuvants.* Expert Rev Vaccines, 2011. **10**(4): p. 523-37.
44. Marciani, D.J., et al., *Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity.* Vaccine, 2000. **18**(27): p. 3141-51.
45. Sudhir Pai Kasturi, I.S., Randy A. Albrecht, Dimitrios Koutsonanos, Tang Hua, Helder I. Nakaya, Rajesh Ravindran, Shelley Stewart, Munir Alam, Marcin Kwissa, Francois Villinger, Niren Murthy, John Steel, Joshy Jacob, Robert J. Hogan, Adolfo García-Sastre, Richard Compans & Bali Pulendran, *Programming the magnitude and persistence of antibody responses with innate immunity.* Nature, 2011. **470**: p. 543–547.

46. Pawar, D., et al., *Development and characterization of surface modified PLGA nanoparticles for nasal vaccine delivery: Effect of mucoadhesive coating on antigen uptake and immune adjuvant activity.* Eur J Pharm Biopharm, 2013.
47. Chong, C.S., et al., *Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery.* J Control Release, 2005. **102**(1): p. 85-99.
48. Gregory, A.E., R. Titball, and D. Williamson, *Vaccine delivery using nanoparticles.* Front Cell Infect Microbiol, 2013. **3**: p. 13.
49. Takami Akagi, M.B., and Mitsuru Akashi, *Biodegradable Nanoparticles as Vaccine Adjuvants and Delivery Systems: Regulation of Immune Responses by Nanoparticle-Based Vaccine.* Adv Polym Sci 2012. **247** p. 31–64
50. Fox, C.B. and J. Haensler, *An update on safety and immunogenicity of vaccines containing emulsion-based adjuvants.* Expert Rev Vaccines, 2013. **12**(7): p. 747-58.
51. Garcon, N., P. Chomez, and M. Van Mechelen, *GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives.* Expert Rev Vaccines, 2007. **6**(5): p. 723-39.
52. Control, C.f.D., *CDC statement on narcolepsy following Pandemrix influenza vaccination in Europe*.2013: http://www.cdc.gov/vaccinesafety/Concerns/h1n1_narcolepsy_pandemrix.html.
53. CIMAVAX-EGF website.
54. Korsholm, K.S., P.L. Andersen, and D. Christensen, *Cationic liposomal vaccine adjuvants in animal challenge models: overview and current clinical status.* Expert Rev Vaccines, 2012. **11**(5): p. 561-77.
55. Christensen, D., et al., *Cationic liposomes as vaccine adjuvants.* Expert Rev Vaccines, 2011. **10**(4): p. 513-21.
56. Moser, C., et al., *Influenza virosomes as vaccine adjuvant and carrier system.* Expert Rev Vaccines, 2013. **12**(7): p. 779-91.
57. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.
58. Bungener, L., et al., *Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity.* Vaccine, 2005. **23**(10): p. 1232-41.
59. Hunziker, I.P., et al., *Perspectives: towards a peptide-based vaccine against hepatitis C virus.* Mol Immunol, 2001. **38**(6): p. 475-84.
60. Morelli, A.B., et al., *ISCOMATRIX: a novel adjuvant for use in prophylactic and therapeutic vaccines against infectious diseases.* J Med Microbiol, 2012. **61**(Pt 7): p. 935-43.
61. Guy, B., M. Saville, and J. Lang, *Development of Sanofi Pasteur tetravalent dengue vaccine.* Hum Vaccin, 2010. **6**(9).
62. Guy, B., et al., *Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses.* Vaccine, 2010. **28**(3): p. 632-49.
63. Medina, E. and C.A. Guzman, *Use of live bacterial vaccine vectors for antigen delivery: potential and limitations.* Vaccine, 2001. **19**(13-14): p. 1573-80.
64. Daudel, D., G. Weidinger, and S. Spreng, *Use of attenuated bacteria as delivery vectors for DNA vaccines.* Expert Rev Vaccines, 2007. **6**(1): p. 97-110.
65. Saxena, M., et al., *Pre-existing immunity against vaccine vectors--friend or foe?* Microbiology, 2013. **159**(Pt 1): p. 1-11.
66. Spreng, S., G. Dietrich, and G. Weidinger, *Rational design of Salmonella-based vaccination strategies.* Methods, 2006. **38**(2): p. 133-43.
67. Zhang, S., et al., *Recombinant Salmonella vaccination technology and its application to human bacterial pathogens.* Curr Pharm Biotechnol, 2013. **14**(2): p. 209-19.

68. Frey, S.E., et al., *A Phase I, dose-escalation trial in adults of three recombinant attenuated Salmonella Typhi vaccine vectors producing Streptococcus pneumoniae surface protein antigen PspA.* Vaccine, 2013.
69. Radosevic, K., et al., *Heterologous prime-boost vaccinations for poverty-related diseases: advantages and future prospects.* Expert Rev Vaccines, 2009. **8**(5): p. 577-92.
70. Kalams, S.A., et al., *Safety and immunogenicity of an HIV-1 gag DNA vaccine with or without IL-12 and/or IL-15 plasmid cytokine adjuvant in healthy, HIV-1 uninfected adults.* PLoS One, 2012. **7**(1): p. e29231.
71. Kalams, S.A., et al., *Safety and comparative immunogenicity of an HIV-1 DNA vaccine in combination with plasmid interleukin 12 and impact of intramuscular electroporation for delivery.* J Infect Dis, 2013. **208**(5): p. 818-29.
72. Bagarazzi, M.L., et al., *Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses.* Sci Transl Med, 2012. **4**(155): p. 155ra138.
73. Ohlschlager, P., et al., *The combination of TLR-9 adjuvantation and electroporation-mediated delivery enhances in vivo antitumor responses after vaccination with HPV-16 E7 encoding DNA.* Int J Cancer, 2011. **128**(2): p. 473-81.
74. Vasan, S., et al., *In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers.* PLoS One, 2011. **6**(5): p. e19252.
75. Ulmer, J.B., et al., *RNA-based vaccines.* Vaccine, 2012. **30**(30): p. 4414-8.
76. Geall, A.J., et al., *Nonviral delivery of self-amplifying RNA vaccines.* Proc Natl Acad Sci U S A, 2012. **109**(36): p. 14604-9.
77. Kallen, K.J., et al., *A novel, disruptive vaccination technology: Self-adjuvanted RNActive ((R)) vaccines.* Hum Vaccin Immunother, 2013. **9**(10).
78. Hirobe, S., N. Okada, and S. Nakagawa, *Transcutaneous vaccines--current and emerging strategies.* Expert Opin Drug Deliv, 2013. **10**(4): p. 485-98.
79. Wolfe, D.N., W. Florence, and P. Bryant, *Current biodefense vaccine programs and challenges.* Hum Vaccin Immunother, 2013. **9**(7).
80. website, F.
81. Holland, D., et al., *Intradermal influenza vaccine administered using a new microinjection system produces superior immunogenicity in elderly adults: a randomized controlled trial.* J Infect Dis, 2008. **198**(5): p. 650-8.
82. Bouvet, J.P., N. Decroix, and P. Pamonsinlapatham, *Stimulation of local antibody production: parenteral or mucosal vaccination?* Trends Immunol, 2002. **23**(4): p. 209-13.
83. Yuki, Y. and H. Kiyono, *Mucosal vaccines: novel advances in technology and delivery.* Expert Rev Vaccines, 2009. **8**(8): p. 1083-97.
84. Dolovich, M.B. and R. Dhand, *Aerosol drug delivery: developments in device design and clinical use.* Lancet, 2011. **377**(9770): p. 1032-45.
85. Sesardic, D., S. Rijpkema, and B.P. Patel, *New adjuvants: EU regulatory developments.* Expert Rev Vaccines, 2007. **6**(5): p. 849-61.
86. WHO. *WHO GUIDELINES ON NONCLINICAL EVALUATION OF OF VACCINES*. 2004.
87. *Note for Preclinical guidance on preclinical pharmacological and toxicologial testing of vaccines*. 1997.
88. Birkett, A.J., et al., *Malaria vaccine R&D in the Decade of Vaccines: breakthroughs, challenges and opportunities.* Vaccine, 2013. **31 Suppl 2**: p. B233-43.
89. *GUIDELINE ON ADJUVANTS IN VACCINES FOR HUMAN USE*. 2005, EMA COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE Vaccine Expert Group.