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Key Steps in Vaccine Development

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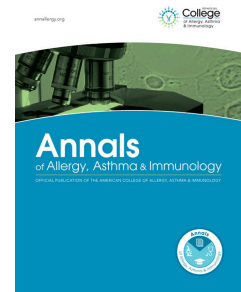
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Title: Key Steps in Vaccine Development

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Abbreviations: Antigen presenting cells, APCs; pattern recognition receptors, PRRs;
pathogen- or damage-associated molecular patterns, PAMPs or DAMPs; tumour necrosis
factor, TNF; interferon gamma, IFN γ ; human immunodeficiency virus, HIV; measles-
mumps-rubella, MMR; Bacillus Calmette Guerin, BCG; diphtheria-tetanus-acellular
pertussis, DTaP; Adjuvant System, AS; Quillaja saponaria Molina: fraction 21, QS-21; 3-
deacylated monophosphoryl lipid, MPL; Lipopolyaccharide, LPS; Toll-like receptor, TLR;
Human papillomavirus, HPV; major histocompatibility complex, MHC; Hepatitis B virus,
HBV; Complement, C; deoxycytidyl-phosphate-deoxyguanosine, CpG; Virus like particle,
VLP; Cervical intraepithelial neoplasia 3, CIN3; Genital warts, GW; neutralizing antibody,
nAb; double-stranded, DS; bivalent-, quadrivalent-, nonavalent vaccine, bV, qV, nV.

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Objective: The goal of a vaccine is to prime the immune response so the immune memory can facilitate a rapid response to adequately control the pathogen on natural infection and prevent disease manifestation. This article reviews the main elements that provide for the development of safe and effective vaccines. **Data Sources:** Literature covering target pathogen epidemiology, the key aspects of the functioning immune response underwriting target antigen selection, optimal vaccine formulation, preclinical and clinical trial studies necessary to deliver safe and efficacious immunization.

Study Selections: Whole live, inactivated, attenuated or partial fractionated organism based vaccines are discussed in respect of the balance of reactogenicity and immunogenicity. The use of adjuvants to compensate for reduced immunogenicity is described. The requirements from preclinical studies, including establishing a proof of principle in animal models, the design of clinical trials with healthy volunteers that lead to licensure and beyond are reviewed.

Results . The three vaccine development phases, preclinical, clinical and post licensure integrate the requirements to ensure safety, immunogenicity and efficacy in the final licensed product. Continuing monitoring of efficacy and safety in the immunized populations is essential to sustain confidence in vaccination programs. **Conclusion:** In an era of increasing vaccine hesitancy the need for a better and widespread understanding of how immunization acts to counteract the continuing and changing risks from the pathogenic world is required. This demands a societal responsibility for obligate education on the benefits of vaccination, which as a medical intervention has saved more lives than any other procedure.

Introduction

Successful vaccination strategies have already provided significant protection against at least 31 human diseases, an extraordinary impact on human health.¹ In this article, the means for the continuing development of safe and effective vaccines will be outlined. Firstly, key aspects of our understanding of the molecular mechanisms underlying natural immunity will be considered. Secondly, how integrating the latter with a specific knowledge of the biology and epidemiology of the pathogenic threat can provide for an appropriate antigen selection, optimal vaccine formulation and an effective delivery strategy are discussed. The path to vaccine licensure is then described, involving extensive preclinical studies to test immunogenicity, followed by different stage human clinical trials to rigorously ensure safety and provide early evidence of efficacy. The design of vaccination strategies including sufficiency of population immunization coverage to deliver useful and sustained protection is discussed. Lastly, the importance of post vaccine licensure aspects, including monitoring of efficacy and safety in the immunized populations are discussed in the context of sustaining confidence in vaccination programs in an era of increased vaccine hesitancy²

Natural immune control of infection

Innate immunity is the first line of defense and is non-specific while the adaptive immune response is characterized by the expansion of secreted and cellular effectors with specificity and the generation of immune memory. The sequential activation of innate and adaptive components is bridged by specialized antigen-presenting cells (APCs) (e.g. dendritic cells) providing for integration of an optimal immune response to the specific threat.³ Within 4-96 hours of an infection, the activation of local APCs occurs through one or more of their pattern recognition receptors (PRRs) that recognize different pathogen- or damage- associated molecular patterns (PAMPs, DAMPs).⁴ PAMPs are molecules shared by groups of related

microbes that are essential for the survival of those organisms and are not found associated with mammalian cells. Examples of PAMPs (sources) and their PRRs including their principle mode of action are summarized in Table 1. DAMPs are unique molecules displayed on stressed, injured, infected, or transformed human cells which can also be recognized as a part of innate immunity. Examples include heat-shock proteins and altered membrane phospholipids. The infection delivered/induced PAMPs and/or DAMPs transduce signals through their specific PRRs that activate the APCs with a flavour which provides for the expansion of a suitable combination of cells of adaptive immunity targeted to the particular pathogen through antigen specific T cells and antibodies. Antibodies can neutralize pathogens directly (receptor blocking, toxin neutralization) or indirectly (activation of complement and granulocytes; opsonisation to aid phagocytosis) while cellular effectors can target infected cells directly by cytotoxicity or through the actions of cytokines like tumour necrosis factor (TNF) or interferon gamma (IFN γ). Thus the integrated combinations of innate and specific immune effector mechanisms develop sufficiently rapidly to provide for control and elimination of the infection. The consolidation of this gain is the provision of a legacy of immune specific memory cells. This enables a more rapid (amnesic) adaptive immune response, through specific antibody and T cells, on reencounter with the same pathogenic threat. Figure 1 summarizes the key components and Figure 2 the general kinetics leading to the immune control of an infection or in some circumstances other outcomes.^{5, 6}

Pathogen biology and epidemiology

A detailed knowledge of the pathogen structure, biology, associated disease epidemiology and its clinical characteristics is highly influential to vaccine requirements. This information can also help select the antigen component(s) of any potential vaccines. Challenges include being able to distinguish commensal and pathogenic forms of a microorganism and the need

to generate cross reactive and/or individual type specific immunity to recognize the most medically important strains or serotypes of a pathogen. Serotypes may also vary in geographic or temporal distribution and this consideration can significantly influence the composition of vaccines, the cost effectiveness of their production and the delivery strategy (e.g. meningococcal vaccines⁷). When variation of the target antigens is inevitable as with seasonal influenza, a new vaccine is required annually.⁸ Vaccination approaches also need to consider the route of entry and subsequent replication locations of a pathogen (e.g. respiratory: influenza; pneumococcus; gastrointestinal: salmonella; genital tract: herpes simplex; blood stream via damage human immunodeficiency virus or malaria via mosquito bite). The demographics of infection (e.g. poverty), specific risk groups and age specific infection rates all determine the populations to immunize and at what age. To determine the impact of any vaccination program it is necessary to be able to accurately diagnose the infection including the specific types. Equally important is the value of a clear immune correlate of protection⁹ such as is observed for measles infection where specific antibody levels are correlated with clinical outcome.¹⁰

In war the number one rule is deception (Sun Tzu) and different pathogens use a plethora of such strategies that can allow for an infection to proceed relatively unhindered by the immune system increasing the likelihood of overt disease.^{11,12} This includes some viruses and bacteria that manage to infect individuals without any significant alert of the innate immune response through their stealthy life cycles which cause little tissue damage. The often considerable genetic diversity of a pathogen species can derive from the selection of advantaged subtypes driven partly by the need for immune evasion. Such virulence factors contribute not only to immune avoidance but can also facilitate infectivity, transmission and structural changes to promote intracellular sequestration. Many pathogens produce proteins

(e.g. enzymes) or nucleic acids (e.g. microRNAs) that can directly inhibit host-pathogen recognition mechanisms which otherwise would activate innate immunity. Other evasion strategies include production of toxins that lead to tissue destruction, deployment of mimics of host proteins or life cycles that include latent phases where the microorganisms remain undetected by the immune system. The very high mutation rates of RNA viruses (Influenza, human immunodeficiency virus (HIV) and Hepatitis C) result from their rapid replication with relatively poor fidelity. This generates many variants allowing for selection of strains with immune advantage and thus continuously compromising effective immune control.¹³ Understanding the impact of such immune escape mechanisms is central to the design of effective vaccines to prevent acute or chronic and possibly latent infection. It is worth pointing out that most vaccines do not necessarily protect against infection per se but rather protect against the consequences of infection. The goal of a vaccine is to prime the immune response so that this immune memory can be utilized to facilitate a rapid response to adequately control the pathogen on natural infection and prevent disease manifestation. The kinetics of the immune response and how vaccination essentially provides the means to prevent an infection running out of control and leading to disease is illustrated in Figure 2.

Antigen selection and vaccine formulation

Vaccine evolution has progressed from a largely empirical approach exemplified by the use of variolation with live smallpox in the 18th century (a protection with significant risk but ten fold reduced compared to the consequences of a natural smallpox infection), through the development of procedures for attenuation and inactivation of whole pathogens for use as vaccine antigens. However, whole organism based vaccines may not always be practical (e.g. pathogen production) or reversion of infectivity (after attenuation) or the presence of reactogenic components may compromise their safety or tolerability. Examples of vaccines

based on whole live and attenuated pathogens include oral poliovirus, measles-mumps-rubella (MMR), varicella, influenza and Bacillus Calmette Guerin (BCG).¹⁴ These mimic natural infection and effectively prime durable immunity. They can induce mild symptoms and, albeit rarely, there can be virulence reversion, which prevents their use in immunosuppressed or pregnant women. In addition, a need for consistency of live vaccine stocks can present production hurdles. This approach is also unavailable if the pathogen cannot grown in culture or where the pathogen has latent stages or variable characteristics in its natural lifecycle (e.g. parasites) as well as when there are effective immune evasion mechanisms.

Whole killed pathogen vaccines include inactivated poliovirus, hepatitis A and whole-cell pertussis and these induce broad immune responses to multiple antigens. They can be reactogenic, some important epitopes may be destroyed and multiple doses are often required. For example, acellular pertussis vaccine is based on partially purified protein components. This is not infectious, shows low reactogenicity and although it can induce a highly specific response it is of lower immunogenicity.¹⁵ In other cases, the 3D structural integrity of some important epitopes may be destroyed, or multiple subunits may be are required, with the consequence that any induced immunity may show little cross reactivity and thus the potential for immune escape. Clearly this approach is not appropriate for pathogens with changing characteristics across their life cycle or some chronic and latent infections.

While partial fractionation can reduce the reactogenicity but there can also be a loss of immunogenicity potentially significantly reducing vaccine effectiveness. This is now recognized as consequence of the removal of natural components of whole organisms such as

PAMPs. It is also possible to utilize recombinant nucleic acid and/or biochemical purification approaches to produce subunit vaccine antigens. Clearly there is a requirement to know that such purified targets are immunologically relevant. Importantly, reduced immunogenicity can be improved by the addition of adjuvants that act to optimise the adaptive immune response. For example, alum (aluminium hydroxide or phosphate) is an adjuvant used by multiple vaccines including combined diphtheria-tetanus-acellular pertussis (DTaP).¹⁶ It is believed to act through a depot effect, directing responses favouring T cell help for antibody production. The adjuvant MF59 is an oil-in water emulsion with squalene that is used with influenza (seasonal & pandemic) vaccines and promotes antigen uptake by APC, cytokine and chemotactic responses stimulating local immunity increasing the magnitude and breadth of the antibody response.¹⁷ Adjuvant system (AS) 01, used in malaria and zoster vaccines, combines Quillaja saponaria Molina fraction (QS)-21 and deacylated monophosphoryl lipid (MPL) with liposomes. This induces activation of a broad population APCs through the synergistic action of QS-21 and MPL that enhances the adaptive immune response.¹⁸ In particular MPL is a detoxified form of bacterial lipopolysaccharide (LPS) which binds to toll-like receptor (TLR)4. Likewise, in AS04, (used in human papillomavirus (HPV), a particular Hepatitis B and some influenza vaccines), MPL is adsorbed onto aluminium hydroxide or phosphate and this leads to transient NF- κ B, cytokine and chemokine responses stimulating increased numbers of activated APC enhancing antibody responses.¹⁹ AS03 is composed of α -tocopherol, squalene and polysorbate 80 in an oil-in-water emulsion. It has been used to boost the immunogenicity of an inactivated split-virion pandemic influenza vaccine.²⁰

Polysaccharide protein conjugates are used to trigger T cell dependent mechanisms that enhance immune memory to target epitopes in vaccines against *Neisseria meningitidis*,

Streptococcus pneumoniae and Haemophilus influenzae type b.²¹ The encapsulated strains of these bacteria are a major virulence factor and define distinct serotypes within each species. Key victims of such infections are young children who cannot mount an effective immune response and are at high risk of death or serious consequences if not promptly treated by antibiotics. Vaccines against the purified polysaccharides components have only a limited short-lived protective affect and are only poorly immunogenic in young children. With vaccine conjugates, specific B cells bind the carbohydrate part leading to internalization, processing and presentation in the context of major histocompatibility complex (MHC) molecules of the protein carriers like tetanus or diphtheria toxoids. The B cells can then act as APC to activate specific T cells which subsequently provide T cell help for the polysaccharide specific B cells leading to immunoglobulin class switching yielding improved antibody responses and provision of long lived memory B cells. However, there are both technical and cost limitations in their production with only a finite number of strains possible in one vaccine. This is because with additional distinct serotypes in the vaccine there can be antigenic competition where increasing the concentrations of any disadvantaged components may still not be sufficient to provide adequate immunogenicity and can significantly increase the vaccine price.

Nucleic acid recombinant technologies for efficient manufacture of subunit antigens are particularly appropriate where the growth of the pathogen is difficult in culture: Hepatitis B virus (HBV), HPV and Herpes zoster. When such subunit vaccines utilise pathogen like particles such as for Hepatitis B²² and the virus-like particles (VLP) of HPV²³ supra-normal immune responses are induced compared to natural immunity. Using RNA replicons for target antigen expression without infectivity is another strategy in development although antigen spread may be limited and there is theoretically a possibility of recombinant events

leading infectivity. Various safe viral and bacterial vectors can also be used as an alternative means for target antigen delivery but pre-existing antibodies to the vehicle may limit responses and different vectors for prime and boost may be required.²⁴

Vaccine pre-clinical testing

The principle hurdle for any new vaccine design is how to measure the effectiveness in the target populations. The use of animal models that accurately mimic human disease can establish proof of principle but do not easily translate into useful immune correlates of protection.^{25, 26} Unfortunately in many cases such correlates of protection are unknown either for natural infections or during early vaccine development. In addition, such measures may not necessarily account for all the immune mechanisms that ultimately contribute to infection control. For example, amnestic responses primed by vaccination can quickly induce specific antibodies to slow an initial infection through pathogen neutralization while subsequent additional (non vaccine-related) adaptive cell mediated immunity can develop to target virus infected cells to deliver clearance of the infection. Operationally, effectiveness of immunization against HBV surface antigen was first shown to correlate with continued protection against infection. However, on longer follow up, waning antibody levels did not correlate with increase susceptibility with an amnestic response still recoverable on vaccine boosting.²² The lesson here is that the antibody levels measure immunogenicity not necessarily efficacy. Fortunately, in some cases e.g. pneumococcal and influenza vaccines, there is a sufficiently strong relationship between vaccine induced protection and certain measures of immunogenicity to allow the magnitude of antibody response to be accepted for licensure, even if the serological response is not whole story accounting for protection.^{27, 28} Such preclinical studies provide the platform for refining the vaccine design in terms of host-pathogen interaction, the protective immune mechanisms and the appropriate antigen and

adjuvant combination to elicit the desired response before entering into clinical trials. In addition, issues of antigen production, vaccine formulation and delivery may need to be optimized as well as the development of assays to measure immunogenicity. The final vaccine design needs to be comprehensively tested for single and repeated dose toxicity, immunogenicity, pharmacodynamics (safety), pharmacokinetics and local tolerance. For the in vivo studies, a relevant age and physiological state of test animal for assessing the dose, route and treatment regimen of the vaccine including also stability measures of the candidate material are all necessary requirements prior to the initiation of clinical studies. Recent preclinical studies in the development of vaccines against Ebola²⁹ and Zika virus³⁰ illustrate some of these challenges.

Clinical trials

Further development requires the testing of vaccines in healthy volunteers to evaluate safety, immunogenicity and clinical efficacy in three distinct phases. When there is an effective treatment for a human disease, challenge trials in which volunteers agree to pathogen exposure after vaccination is a valuable means to test vaccines where no animal models are available. (e. g. HPV and malaria). Phase 1 studies are focused on safety, phase II trials concentrate on establishing an immunogenicity proof of concept and dose ranging (sometimes efficacy data) while larger phase III studies are designed to evaluate whether the dosing and vaccination schedule can deliver the desired impact on the clinical problem with an acceptable safety profile.³¹ At the same time, the vaccine's physicochemical and biological qualities must be established for consistency by the testing of different timed manufactured vaccine lots.^{32, 33} To enable success there is a need for specific and relevant immunological and clinical endpoints particularly when there is no known immune correlate of protection. Trial design requires accurate estimates of sample size based on disease incidence, with

sufficiency of subject numbers to deal with any drop out rates combined with rigorous data management. Safety is of paramount importance through all the clinical studies and continuous monitoring procedures so that any adverse events are flagged at the earliest opportunity are obligated.³⁴ Several categories of adverse events following immunization need to be recorded. These include a reaction or induced event to correctly delivered vaccination such as pain, redness, swelling or fever or caused by vaccine properties such as the presence of an adjuvant leading to local site inflammatory reactions or a mild fever and/or rash after immunization with attenuated viruses such as MMR or paralytic polio after live attenuated poliovirus vaccines. Such reactions are thus documented during the procedures leading to licensure and may limit or refine the vaccination procedure and/or target populations but overall need to be safe and tolerable thereby not outweighing the benefits of the protections of the vaccination. Other events include immunization errors that occur in the preparation, handling or maladministration of vaccines; coincidental events that can happen shortly after immunization but are not caused by the vaccine; immunization anxiety reactions such as syncope and panic attack; vaccine failures through for example inadequate storage; and finally unknown events without an attributable cause. Inability to make a sufficient response to a potent vaccine can also result from inherent differences in the recipients (e.g. genetics). Such variation is seen in some receiving standard HBV vaccination where alternative formulations, for example with ASO4, can counteract this problem.³⁵

When adverse events are recorded, it is vital to evaluate whether there is any causal relationship with the vaccine administration or if it is just coincidental. The monitoring systems need to be sensitive enough to activate further enquiries whenever necessary. There are sets of clinical definitions of adverse events and some standardization of processes that help to unify the consistency of reporting worldwide.^{36,37} Such investigations need to be

forensically analysed as the consequence can be far reaching without sufficient cause as is exemplified by the spurious associations claimed for MMR vaccination in 1998. As mumps, measles and rubella all have the potential to cause death, disability and death, the questioning of the safety of the trivalent MMR vaccine led to a drop in vaccination coverage in several countries which unfortunately has persisted in the face of almost universal use and accepted effectiveness. Recent work has noted that MMR vaccination is unlikely to be associated with autism, asthma, leukaemia, hay fever, type1 diabetes, gait disturbance, Crohn's disease, demyelinating diseases, bacterial or viral infections. However, there is still scope for improvements in the design and reporting of safety outcomes in MMR vaccine studies, both pre- and post-marketing.^{38, 39}

The path to licensure & beyond

Comprehensive information on the chemical and structural properties of the vaccine, all preclinical and clinical trial results, the data on manufacturing quality assurance are all required to produce a regulatory dossier which is used to apply for product registration. Issues relating to the filling of vials or syringes, any storage conditions (e.g a cold chain) must all be documented for quality testing control.^{40, 41} It is important not to underestimate the necessary details of packaging and distribution particularly as individual countries often have their own requirements for product information display. This can complicate the supply chains in times of shortage. Once a vaccine is licensed it may be recommended by public health authorities or the World Health Organization but the effectiveness and safety of the new vaccine must be continuously monitored in “the wild” conditions.⁴²⁻⁴⁴ Such monitoring is essential for risk/benefit assessments to be appropriately vigilant. Given that most vaccines are provided as a preventative measure in otherwise healthy individuals the threshold for any risk must be higher than for a medication used to treat a disease that might otherwise cause

harm. The provision of vaccines is generally widespread in populations compared to therapeutic interventions so detection of rare events associated with vaccination could possibly be clinically relevant. An important example of post-licensure studies impacting on the use of an oral rotavirus vaccine resulted from the observation of a small increase in the risk of intussusception.⁴⁵ The latter is most common in children 6-12 months in age so mitigation of risk was achieved by completing the course of vaccination before 6 months of age.⁴⁶

Optimizing protection for the many

Any impact on disease reduction ultimately depends on sustained immunization coverage as well as individual vaccine effectiveness. Thus with sufficient population coverage the indirect effects on unimmunized individuals (herd immunity) are also provided against the infectious agent.^{47,48} While immunizing those with the highest risk of disease or its consequences would be a very efficient way to deliver cost effective vaccination, the risk groups for many infections like measles, rubella, varicella and rotavirus are simply not easily identifiable. In practice, a combination of strategies which are based on targeting age and/or risk groups has often proven the best means for disease reduction. Table 2 summarizes some examples of different vaccination strategies.⁴⁹⁻⁵⁵ For any new vaccination it is necessary to understand the population demography, the age at which the disease most occurs and the biological and social factors which influence its transmission and replication. Knowledge of the disease burden, incidence and prevalence rates of infection can be used in mathematical modeling to estimate the necessary coverage for vaccination rates in order to impact disease spread and its reduction. Health economic analyses can then be used to persuade funding authorities of the cost effectiveness of such vaccination programs. Figures 3 & 4 illustrate aspects of the complexities and diversity of the major events in the development and

implementation of vaccination against HPV^{23,56} and Figure 5 summarizes the on going process of producing an effective malaria vaccination strategy against *Plasmodium falciparum*.^{57,58}

Particular populations such as pregnant women, preterm infants, adolescents with chronic medical conditions and older adults have recommended variations in vaccine dosing schedules to account response differences.⁵⁹ For example, maternal pertussis vaccination is a very effective way of protecting against pertussis infection in early infancy that is endemic in much of the world when infants are especially at risk when maternal antibody levels wane. Likewise specific vaccination scheduling or indeed contraindication may be recommended for individuals who are immune-compromised by a condition or its treatment. For example, the use of live vaccines is not used in such immunosuppressed individuals. With age, there is a decline in innate and adaptive immunity and this immunosenescence increases the susceptibility to vaccine preventable diseases. Thus there are recommendations for annual vaccination programs for those over 65 years for seasonal inactivated influenza vaccine, tetanus/diphtheria /pertussis, herpes zoster and pneumococcal vaccinations. While immunodeficient patients (genetic, transplant related or disease induced) present additional challenges for vaccination, alternative strategies can be developed to provide for useful protection.⁶⁰

The three vaccine development phases, preclinical, clinical and post licensure can take a considerable time (10-30 years) and some key elements are summarized in Figure 6. The process integrates the requirement to ensure safety, immunogenicity and efficacy in the final licensed product.

Concluding remarks

There is no doubt that the tools available to meet the needs of improving existing and developing new vaccines are very powerful. The challenge is coordinating this potential in the face of the diverse agendas of political, corporate and individual group lobbies. A key goal must be to provide the available protections as widely as possible (sufficient coverage in all countries at risk) but this can only be achieved by global cooperation (and funding) to help developing countries. However, vaccination is only one component of disease prevention, and also requires the medical infrastructure for successful implementation. In the future, pandemics will occur and although the infrastructure to successfully intervene in such circumstances may theoretically exist, at least in developed countries, it is yet to be significantly tested. The recent outbreaks of Ebola exemplify the processes whereby a vaccine can be developed and tested in the midst of an epidemic.^{29,61} This required a significant international response which utilized combinations of state of the art technology combined with some understanding of the disease epidemiology, pathogen biology and host immunity. Importantly, the rapid isolation of Ebola disease cases was a critical component limiting disease spread, thereby providing a platform for implementing a successful ring-vaccination strategy.

In most circumstances any individual who refuses vaccination against an infectious pathogen potentially threatens everyone in that society. In an era of increasing vaccine hesitancy it is a societal need for the general public to be appropriately educated to understand the need for immunization to counteract the continuing and changing risks from the pathogenic world.²

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- 155

Table 1. PAMPS and PRRs.

Human TLR1, 2, 4, 5, 6 are at cell surfaces; TLR3, 7, 8, 9 within endosomes. Hetero-dimerization extends the ligand spectrum recognized

PAMP	Pathogens	PRR	Innate Response
Microbial cell wall components	Streptococci, Staphylococci	Complement (C)	Opsonisation; C activation
Mannose-rich glycans	Salmonella species	Mannose binding protein	Opsonisation; C activation
Capsular polyanionic saccharides	Pneumococci, Haemophilus species	Scavenger receptor	Phagocytosis
Lipoproteins of gram positive bacteria; viral proteins	Streptococci, Staphylococci; CMV & HSV	TLR2	Macrophage activation, secretion of inflammatory cytokines
Double stranded RNA	Rotavirus, other RNA/DNA viruses	TLR3	Type 1 interferon
Lipopolysaccharide of gram negative bacteria	E. coli, Salmonella, Haemophilus, Neisseria species	TLR4	Macrophage activation, secretion of inflammatory cytokines
Flagellin (from bacterial flagella)	Pseudomonas species, E. Coli	TLR5	Macrophage activation, secretion of inflammatory cytokines
Uracil-rich single stranded RNA	RNA viruses including HIV Replicating RNA virus	TLR7 TLR8	Type 1 interferon

CpG motif-containing DNA	High frequency in viral & bacterial genomes	TLR9	Macrophage activation, secretion of inflammatory cytokines
Triacyl lipopeptides	Various bacteria, parasites	TLR1/2	Macrophage activation, secretion of inflammatory cytokines

Vaccination strategy	Goal	Pathogen examples
Routine vaccination (selective immunization) Single birth cohort	To eradicate, eliminate or contain disease	Diphtheria, Tetanus, Pertussis, Rotavirus
Double cohort (e.g. infants & adolescents)	To eradicate, eliminate or contain disease	Hepatitis B virus
Mass immunization (entire population in affected area or high risk priority groups); response to emerging epidemic	To rapidly limit morbidity and deaths due to verified detection of vaccine preventable disease	Yellow fever
Response to a predicted epidemic	To establish population immunity before risk occurs	Influenza
Response to a disease outbreak	To establish population immunity & reduce case numbers in monitored groups of people	Hepatitis A
Catch up vaccination	To protect unimmunized individuals	Measles, Mumps, Rubella
Specific campaigns for particular vaccinations	To eradicate, eliminate or contain disease when not delivered by routine vaccination	Oral poliovirus

Table 2: Strategies of vaccination for disease reduction goals. After, Hardt et al. Vaccine 34 (2016) 6691-6699.

The immune control of infection (*The Art of war by Sun Tzu*)

The immune Defense Forces

(Be prepared for any contingency)

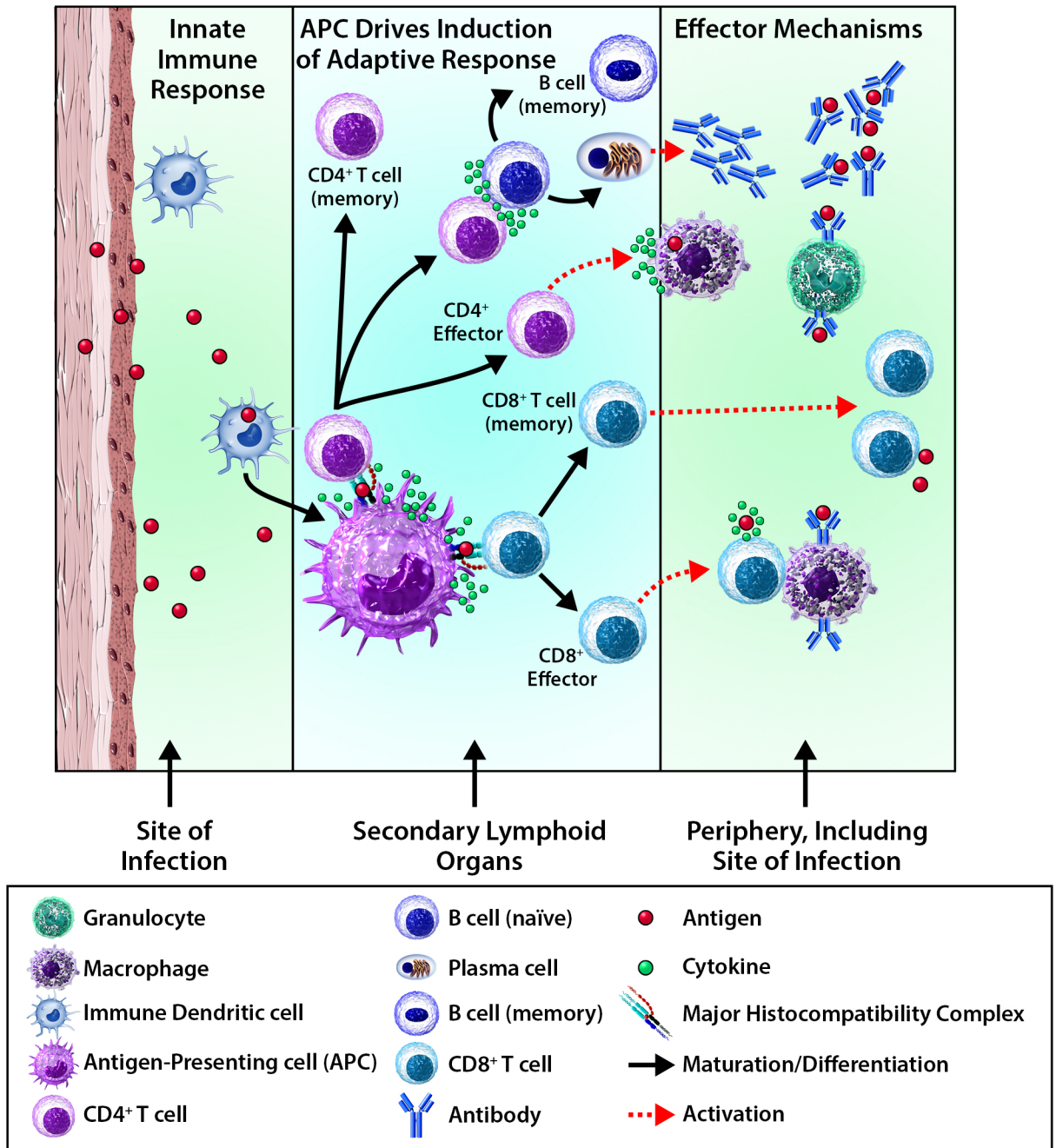
Vigilance/surveillance & detection of danger
(Force enemy to reveal themselves)

Holding the line, communicating the problem to the wider defense forces
(Of greatest importance in war is extraordinary speed)

Calling up appropriate numbers & specifically armed capability. Directing the integrated deployment & attacking the enemy

(Baulk the enemy's power)

Consolidating the gains
Those skilled in war conquer by strategy



Inadequate Control Cofactors

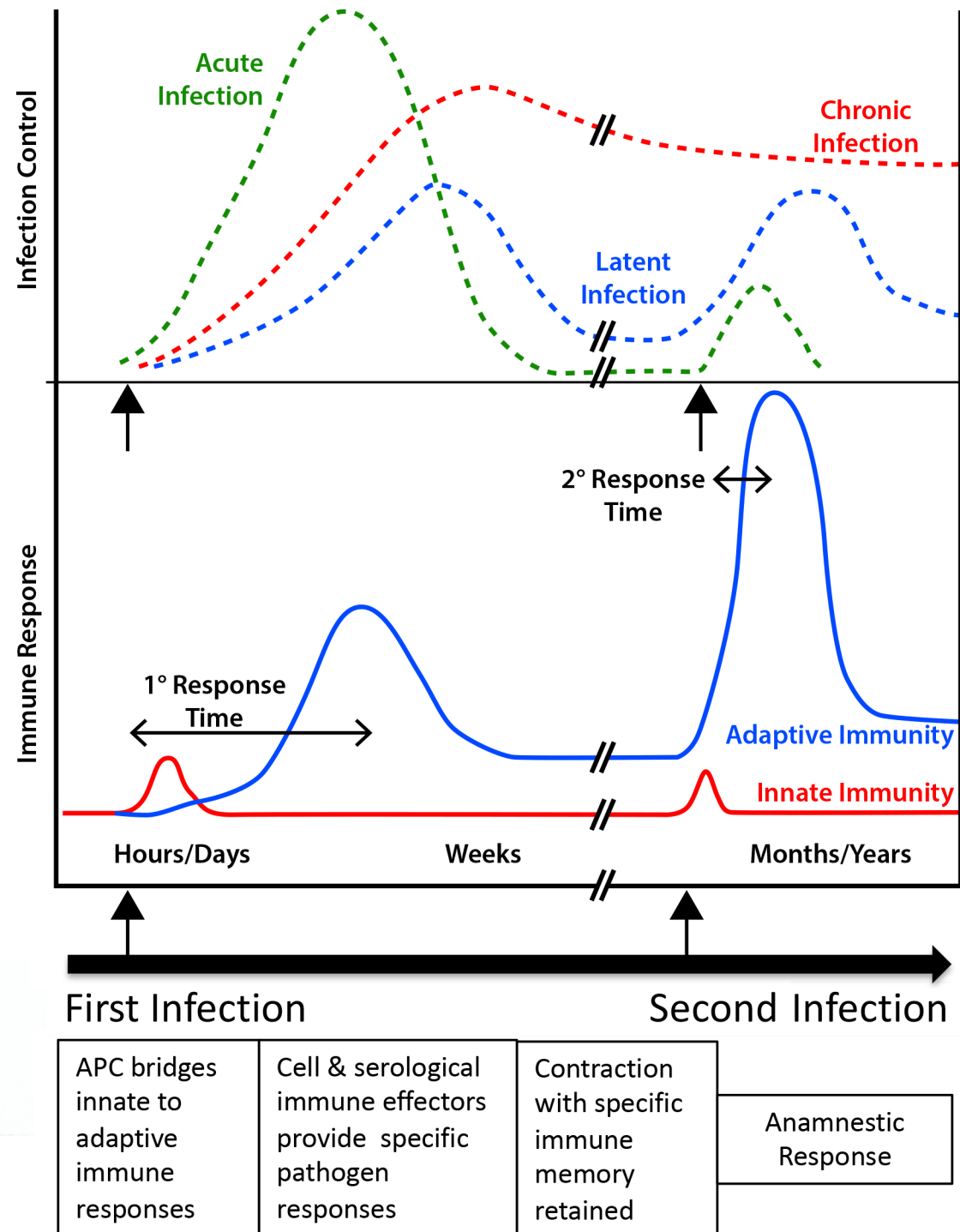
- Host Genetics
- Pathogen Variation
- Pathogen Immune Escape
- Host Immunosuppression

The race to control infection (*The Art of war by Sun Tzu*)

- *Knowing when to reduce the deployment*
- *Providing the defensive organization to prevent/limit future attack*
- *The war is not won in a day*
- *There are always more potential enemies*

Vaccination Control

- Immune priming allows for a rapid secondary immune response on productive infection by a pathogen
- Early infection control prevents threats from acute or chronic infection



Vaccine strategies to prevent HPV associated disease

Burden of Disease

- Cancer risk is through persistent infection with oncogenic HPV types 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59 (70% caused by HPV 16 & 18). The virus is DS-DNA with an 8kb genome & 55nm particle of 72 capsomeres made of L1 (major) & L2 (minor) capsid proteins. HPV 6/11 cause genital warts (GW).
- Anogenital & oral pharyngeal cancer: Cervical cancer most is prevalent with 230K deaths and >0.5 million cases per annum. Yearly cases in female and/or males (respectively) at oral (2900, 5200), oropharynx (1100, 5100), anus 14300, 13000, vulva/vagina (16000) and penis (10500) sites add to the disease burden. Cervical screening can detect pre-cancers (cervical intraepithelial neoplasia (CIN)) but this is mostly unavailable in the developing world where most deaths occur.
- Viral infection occurs at specific basal epithelia through minor trauma, with epithelial differentiation required for completion of the lifecycle. There is no cell death & no viraemia. After sexual debut, this stealthy life history provides the risk of an undetected high risk HPV persistent infection where viral E6 & E7 drive immortalization, genetic instability leading cancer.

Vaccine Design & strategy

- To prevent infection by induction of type specific neutralising antibodies (nAb) requires a suitable antigen & vaccine immunogenicity to improve on natural serological immunity which does not guarantee protection & optimally delivered before sexual debut. Vaccines to prevent cancer by HPV 16/18 are the highest priority & then the other most prevalent high risk types. Inclusion of VLPs to HPV6/11 can also prevent GW.
- Genetically engineered production of high risk HPV L1 capsids form virus like particles (VLP) which mimic the structure of native virions that cannot be produced easily in vitro. VLPs vaccines need to induce high levels of nAbs for protective levels at the infection sites & for transfer to mucosal secretions plus to maintain protection throughout a sexually active life as natural infection may not boost local responses. Adjuvants required to boost the magnitude & longevity of the induced immunity. Figure 4 shows production & characteristics of the current licensed vaccines (V) bivalent (bV), quadrivalent (qV) and nonavalent (nV)

Vaccine Immunogenicity & efficacy

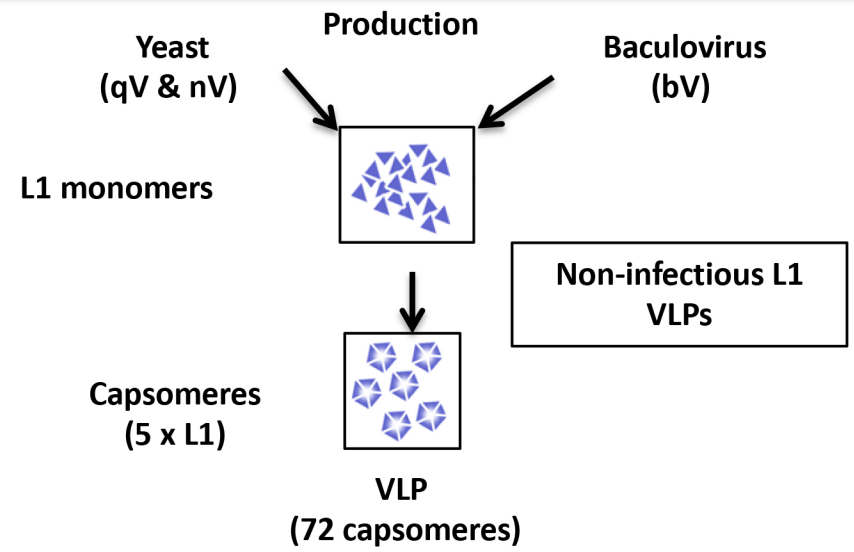
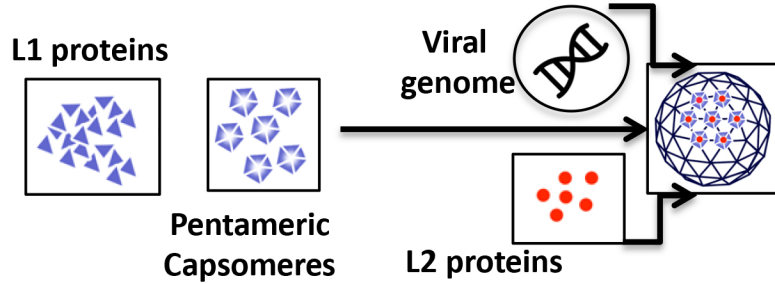
- Surrogate assays for neutralization developed as virus cannot be easily grown in vitro and is not cytopathic. There is no known immune correlate of protection. 2 or 3 immunizations in volunteers gave 100% sero-conversion at many fold higher than natural exposure. Two immunizations have non-inferior immunogenicity.
- Efficacy was tested in sexually active young women who were regularly screened for cervical neoplasia with an endpoint of CIN2/3 (requires treatment but which is very effective). qV and bV give virtually 100% protection against vaccine –included types of CIN2/3 as trialed in women naïve to the corresponding type at vaccination. Follow up & modeling studies suggest high levels of Abs may be sustained for decades. Justification for delivery to adolescents derives from bridging studies in the latter showing excellent safety & stronger immune responses. The bV had greater impact against CIN3 than expected from the vaccine type lesion prevalence indicating significant cross protection versus related non-vaccine types possibly through the action of ASO4 (overall 93% CIN3 protection). The nV protects against infection & disease associated with the HPV types covered by the vaccine including the excellent efficacy against the GWs associated HPV 6, 11 also seen with the qV.

Impact & future

- In Australia, the national vaccination with the qV of adolescent girls (from 2007) & boys (from 2013) plus some initial catch up operations has delivered 77% female and 66% male coverage in 2015. There has been a 92% reduction in GWs with significant herd protection. Measuring any direct impact on cancer will take several decades. There are measurable affects on HPV prevalence & CIN. For example, in Scotland national vaccination of 12-13 year old girls with the bV (2008-2012) plus an initial catch up (13-17 years) delivered 90% and 66% coverage respectively. HPV 16/18 prevalence was reduced from 30% to 14% and CIN1, -2, -3 reduced by 29%, 50% & 55% respectively. Unfortunately, there is poor coverage of vaccination in the developing world where most cancer deaths occur. Recent data suggest a single immunization may be sufficient. This might help together with cheaper vaccines & potentially delivery as a part of a multi-target paediatric vaccine.

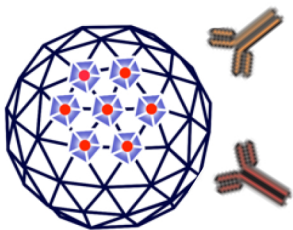
Native virus

Papillomavirus capsids, 55nm in diameter, are composed of 72 pentameric capsomeres arranged in a T=7 icosahedral. Each capsomere has five monomers of L1, the major capsid protein, which self-assemble with 12 copies of the L2 minor capsid protein, to form the particles that encapsulate the 8kb ds-DNA viral genome.



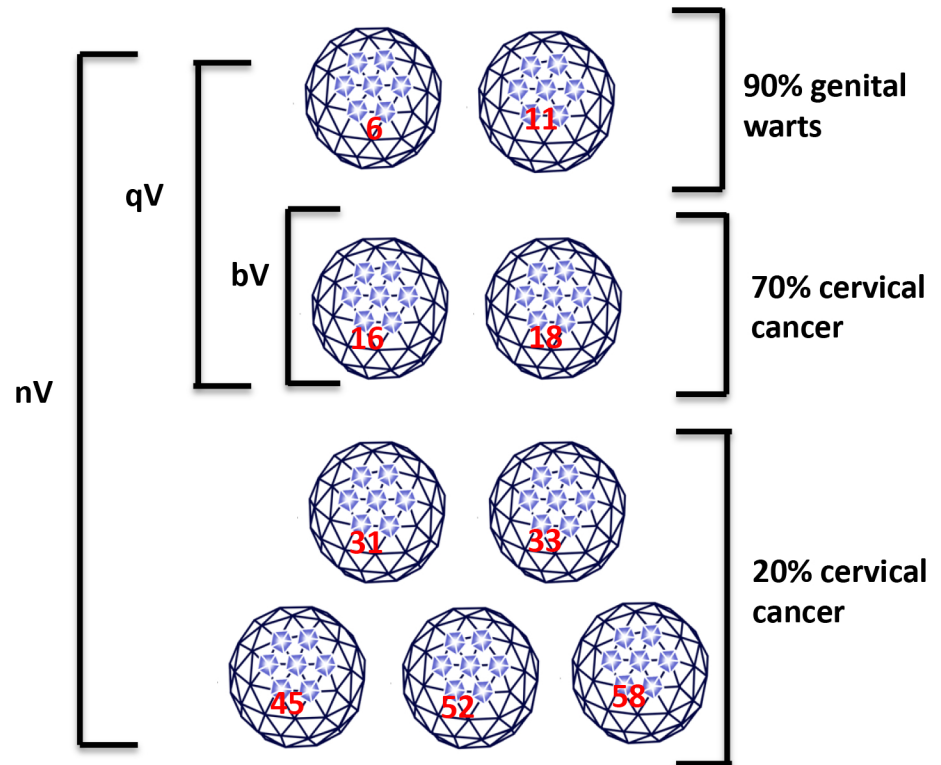
Licensed vaccines bivalent (bV), baculovirus produced HPV16 & 18 L1 VLPs with adjuvant ASO4; yeast produced VLPs & amorphous aluminium hydroxy-phosphate sulphate (AAHS) adjuvant with HPV 16, 18, 6 & 11 (quadrivalent, qV) or plus also HPV 31,33,45,52,58 (nonavalent, nV). All vaccines are safe with virtually 100% efficacy in protection against vaccine-targeted types of CIN2/3. Immunization with qV & nV also provides effective protection against GWs.

Likely mechanisms of protection



L1 VLP-induced nAb blocks binding to basement membrane, the initial step required for infection

L1 VLP induced nAb blocks infection post-binding to basement membrane



Vaccine Strategies to help prevent malaria

Burden of Disease

- Human malaria is caused by 5 species, the most virulent by *P. falciparum* (98% mortality, most drug resistant). The parasite haploid genome is 23mb & encodes 5500 proteins. Transmission is by female anophelene mosquito with a 3 phase lifecycle. 1) Pre-erythrocytic (invasive sporozoites give large schizonts in the liver); 2) Asexual blood merozoite invasion & replication in erythrocytes leading to significant parasitaemia & clinical symptoms; 3) Sexual stage with male & female gametocytes in blood ingested by mosquito.
- 198 million clinical illnesses & 584000 deaths mostly in sub-Saharan Africa. Natural immunity (antibody and/or cell mediated) depends on the stage & the intra- or extracellular status of the parasite. The parasite employs abundant genetic polymorphism which significantly compromises immune control.

Vaccine Design & Strategy

- Most advanced vaccine strategy targets the pre-erythrocyte stages of the parasite cycle seeking to exploit circum-sporozoite protein (CSP) immunity. CSP is on the sporozoite surface & expressed by early liver forms & exported to hepatocyte cytoplasm. CSP conformation is important in parasite binding, movement, cell biochemistry & penetration.
- Building on experience with Hepatitis B (HB) vaccines, a fusion construct of a repetitive immuno-dominant B cell epitope in the middle region (R) & a T cell epitope (T) from the C-terminal flanking region of CSP were cloned with HB surface (S) Ag. When expressed together with free HBs antigen in yeast the proteins assemble into VLPs similar to HBs Ag proteins.
- There is limited value in testing in animals. RTS,S was tested with different adjuvants & AS01 showed the best immunogenicity & efficacy against controlled human malarial infection. AS01 is a liposomal suspension of immune enhancers MPL & QS21, a natural saponin molecule. The hypothesized vaccine mode of action is to induce specific anti-CSP immune responses that can prevent initiation of blood-stage infection by killing parasites at the pre-erythrocyte stage. There is no immune correlate of protection known. Anti-CSP antibody & CD4 T cell activation have been correlated in humans.

Vaccine Immunogenicity & efficacy

- A licensed vaccine that is greater than 50% efficacious for a least one year against severe disease & death is the initial goal. The focus is on reducing the burden of *P.falciparum* in infants & children in sub-Saharan Africa. A phase II study confirmed that the vaccine was safe & gave partial protection in conditions of natural exposure. A Phase III performed in 11 centres within seven countries showing differing patterns of malarial transmission confirmed the protection against severe malaria in a proportion of infants.
- The vaccination protocol of 4 immunizations in children (5-17 months) & infants (6-12 weeks) had an efficacy against fever & parasitaemia at 20 months of respectively 50% and 30%. Lower antibody levels were found in the infants which might reflect immune immaturity. Children given 4 immunizations between 5-17months showed 39% and 29% protection against malaria or severe malaria respectively after 4 years with added value through a reduction in hospitalizations .

Impact & future

- The vaccine was recommended by WHO for a pilot introduction in selected areas of Ghana, Malawi, & Kenya. This started in 2019 with provision to young children through the routine immunization programs. It is being evaluated as a complementary malaria control tool.
- Necessary components to the successful progression of this activity are the collaboration agreement between the vaccine manufacturer (GSK), PATH and the WHO; that the participating countries have sufficient infrastructure & national commitment and that there is funding for this malarial vaccine implementation program(MVIP) through Gavi, the Vaccine Alliance, the Global Fund to Fight Aids, Tuberculosis & Malaria & Unitaidd until end of 2020.
- Clearly vaccines to prevent important infectious diseases need not be 100% effective but still have an important part to play in reducing the deaths & disease in combination with other strategies. A life threatening infection near you can justify the time, collaboration & finance required to produce a safe vaccine & override any vaccine hesitancy

Technical Approach

Protection mechanism information

Target antigen identification

Preclinical Development

Antigen characterization & purification

Formulation & adjuvant selection
Immune studies in animals

Investigation of immune mechanisms

Initial toxicology evaluation

Production of batches by GMP for Phase I/II clinical trials

Investigational New Drug (IND) submission

Clinical Development

Phase I: Safety, immunogenicity & dose ranging in small numbers

Phase II: Safety, immunogenicity in 100s-1000s volunteers with dose ranging & possibly efficacy evaluation

Phase III: Large multi-center efficacy, immunogenicity & safety trials in large numbers of subjects

Defining immune correlates of protection if possible

Scale up & validation for final vaccine GLP/GMP production including consistency lots

Medical Need

Epidemiology

Target population

Disease burden

Health economics

Approval & Licensure

Cost/effectiveness assessment for reimbursement

Immunization implementation

Post-licensure pharmacovigilance with sustained monitoring of AEs

Phase IV studies on new indications

Regulatory Submission Filing

Figure 1: The immune control of infection (*The Art of war by Sun Tzu*)

In the left panel, particular PAMPS/DAMPS of infection stimulate immature dendritic cells to mature differentiate and process pathogen derived antigen, while migrating to the lymph nodes. In the centre panel, these APCs induce the adaptive immune response including activation of T cells into effector cells and differentiation of T cells into memory cells. Naïve B cells differentiate into antibody-secreting plasma cells and memory B cells following activation by helper CD4⁺ T cells. Activated tissue-resident macrophages are also stimulated by the CD4⁺ T cells. In the right panel, various effector mechanisms are shown. Antibodies can enhance the effector functions of various innate cells as well as also neutralise pathogens directly. Cytotoxic T cells can directly kill infected tissue/cells via molecular and chemical signalling and also induce infected cells/phagocytes to kill intracellular pathogens, or inhibit pathogen replication. Not all T-cell subsets are represented in this illustration.⁵ To the left of the illustration is a summary of the key events in the immune control of an infection with parallels to *The Art of War* by SunTzu.

Figure 2: The kinetics of the immune response & how vaccination prevents an infection out running control & leading to overt disease

Figure 3: Vaccine strategies to prevent HPV associated disease

Figure 4: Virus-like particles in the formulation of HPV vaccines

Figure 5: A vaccine strategy to help prevent malaria. World Health Organization. Q&A on the malaria vaccine implementation programme (MVIP). www.who.int/malaria; 8 November 2019

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27 Figure 6: Vaccine Development Phases

28 The preclinical, clinical and post licensure phases can take a considerable time (10-30 years).

29 The process integrates the requirement to ensure safety, immunogenicity and efficacy in the
30 final licensed product.

31

32

Journal Pre-proof

Learning Objectives: At the conclusion of this activity, participants should be able to:

- Describe the key steps in the development of a vaccine to prevent an infectious disease
- Discuss the formulation of a vaccine to ensure immunogenicity, efficacy and safety in prevention of infectious diseases

Q1. Which of the following statements is true of vaccination against a particular pathogen:

- A. Protects against all subsequent infection with that pathogen
- B. Limits the consequence of the infection and thus the risk of disease
- C. Requires a precise knowledge of the natural immune control of the pathogen
- D. Is mediated by the innate immune response
- E. Prevents infection through sterilising immunity

Q1 ANS: B. Limits the consequence of the infection and thus the risk of disease

Rationale:

For most viral (pathogen) infections, there is an incubation period, a prodromal stage leading to invasion and the peak of infection which coincides with the intensity of symptoms. Natural immune control, starts with innate immune activation that communicates the specific qualities of the pathogen to the adaptive immune system. This generates specific antibodies that can neutralise the free virus and cellular immunity that can destroy the virus infected cells. This activation process typically takes a couple of weeks with the control and virus elimination delivered during a convalescent period. With some pathogens, through specific immune evasion strategies or particular circumstances, optimal activation of natural immunity does not occur. Failure to control an acute infection like influenza or Ebola is immediately life threatening while persistent infections or latency can provide for secondary consequences manifest in additional disease complications (e.g. cancer). Vaccination acts to prime the immune response with a memory component that can allow for rapid expansion and deployment of specific immunity to provide adequate pathogen control reducing the risks of disease.

References:

1. Leo O, Cunningham A, Stern PL. Vaccine Immunology. Chapter 2 pages 25-29. In: Understanding modern vaccines. Eds. Garçon N, Stern P, Cunningham T and Stanberry L. (2011) Elsevier <http://www.sciencedirect.com/science/journal/22107622>
2. Cunningham AL, Garçon N, Leo O, et al. Vaccine development: From concept to early clinical testing. Vaccine. 2016;34:6655-6664

Q2. Which of the following statements is true in relation to vaccine adjuvants:

- A. Are always added to vaccines to generally boost immune responses

- B. Are selected to induce the desired level & type of immune response against a specific antigen
- C. Are never intrinsically reactogenic.
- D. Are all aluminium based
- E. Work through specific damage associated pattern receptors

Q2 ANS: B. Are selected to induce the desired level & type of immune response against a specific antigen

Rationale:

Adjuvants enhance & modulate immune responses to antigens; this is particularly important when the antigens are purified & lack intrinsic innate immune triggers. Adjuvants differ in the types and magnitude of immune responses they engender, hence they must be selected in view of the immune response required to induce immunity to a given pathogen or antigen. Combinations of adjuvants can take advantage of the properties of each individual component. Adjuvants are a key tool to developing efficacious vaccines to meet many vaccine challenges.

References:

1. Garçon N, Di Pasquale A. From discovery to licensure, the Adjuvant System story. Hum Vaccin Immunother. 2017;13:19-33.
2. Di Pasquale A, Preiss S, Tavares Da Silva F, Garçon N. Vaccine Adjuvants: from 1920 to 2015 and Beyond. Vaccines (Basel). 2015;3:320-43.

Q3. Which of the following statements is true in relation to HPV VLP based vaccines:

- A. Are 100% protective against oncogenic HPVs
- B. Are prophylactic & therapeutic
- C. Primarily target adolescent girls
- D. Induce protective cell mediated immunity
- E. Are delivered without adjuvants

Q3 ANS: C. Primarily target adolescent girls

Rationale:

The available HPV VLP adjuvanted vaccines have shown virtually 100% protection against the cervical cancer surrogate endpoint of CIN3 associated with the types included in vaccine formulation. Through type inclusion and/or cross protection this can achieve an overall protection against over 90% of oncogenic HPV associated cancer. The vaccines are believed to work in preventing infection through the induction of neutralising antibodies at much higher levels than those found naturally. There is as yet no known immune correlate of protection but

the antibody levels induced are predicted to be long-lived. There is no evidence of any therapeutic activity. The principle risk group is women who can get an undetected high risk HPV infection through sexual transmission that can subsequently lead to anogenital cancer. Adolescent girls, pre-sexual debut and with higher immune responses than induced in adults, are an accessible and optimal group to immunise for maximising the impact on reducing the risk of anogenital neoplasia. With high population coverage there are added herd affects including on the male population.

References:

1. Roden RBS, Stern PL. Opportunities and challenges for human papillomavirus vaccination in cancer. *Nat Rev Cancer*. 2018;18:240-254.
2. Pouyanfar S, Müller M. Human papillomavirus first and second-generation vaccines-current status and future directions. *Biol Chem*. 2017;398:871-889.

Q4. Which of these adjuvants function through TLR4:

- A. 3-deacylated monophosphoryl lipid (MPL)
- B. Peptidoglycan
- C. Double stranded RNA
- D. cCpG motif containing DNA
- E. HIV

Q4 ANS: A3-deacylated monophosphoryl lipid (MPL)

Rationale:

An important group of PRRs are the TLRs that on binding of ligands on APCs, mainly dendritic cells, lead to APC maturation, induction of inflammatory cytokines and the priming of naive T cells to drive acquired immunity. Therefore, activation of TLRs promotes both innate inflammatory responses and the induction of adaptive immunity. Human TLRs recognize distinct PAMPs derived from various microorganisms broadly in three groups: those that recognize lipids and lipopeptides (TLR1, 2, 4 and 6), proteins (TLR5) and nucleic acids (TLR3, 7, 8 and 9). TLR1 forms heterodimers with TLR2 (TLR1/2) and recognizes triacyl lipopeptides. TLR2 together with TLR1 or TLR6 recognizes a wide variety of PAMPs, including peptidoglycan, lipopeptides and lipoproteins of gram-positive bacteria, mycoplasma lipopeptides and fungal zymosan. TLR4, together with its extracellular components such as MD-2 and CD14, recognizes LPS, a constituent of cell walls of gram negative bacteria. TLRs recognizing nucleic acids are localized in cytoplasmic compartments where they detect DNA and RNA derived from viruses and bacteria. TLR3 recognizes double-strand RNA and TLR7 and TLR8 are responsive to the single-strand RNA found during viral replication. TLR9 recognizes unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) motifs commonly present in bacterial and viral genomes. TLR5 recognizes bacterial flagellin. Heterodimerization of TLRs extends the spectrum of ligands recognized. MPL adjuvant is a chemically modified derivative of lipopolysaccharide that displays greatly reduced toxicity while maintaining most of the immunostimulatory activity of

lipopolysaccharide through TLR4. MPL acts as a potent stimulator of T cell and antibody responses and was the first TLR ligand in licensed human vaccines, in the form of AS04.

References:

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2. Baldrige JR, McGowan P, Evans JT, et al. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. *Expert Opin Biol Ther.* 2004;4:1129-38.

Q5. What is the goal of a vaccination strategy used to deal with a predicted influenza epidemic?

- A. To rapidly limit morbidity and deaths due to verified detection of vaccine preventable disease
- B. To eradicate, eliminate or contain disease
- C. To establish population immunity before risk occurs
- D. To protect unimmunized individuals
- E. To establish population immunity & reduce case numbers in monitored groups of people

Q5 ANS: C: To establish population immunity before risk occurs

Rationale:

An example of a pandemic alert due to an outbreak of swine-origin influenza A virus H1N1 was declared by the WHO in the summer of 2009. One year later more than 200 countries had reported over 18000 cases. Global surveillance systems were put in place to assess the safety profiles of the new pandemic vaccines which allowed their licensing under a fast track procedure incorporating comprehensive and stringent safety assessments plus immunogenicity and efficacy requirements ensuring a satisfactory benefit-risk profile. In the last 4 months of 2009, tens of millions of pandemic H1N1 2009 vaccine doses generated data that underwrote their safe use. In the pandemic situation, the goal is to provide widespread vaccination of populations to generate immunity prior to the arrival of the threat. Production of sufficient doses to deliver worldwide vaccination can be helped by the use of reduced antigen content while sustaining immunogenicity by the addition adjuvants.

References:

1. Rebmann T, Zelicoff A. Vaccination against influenza: role and limitations in pandemic intervention plans. *Expert Rev Vaccines.* 2012;11:1009-19.
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