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TITLE:
Vaccines for the future: learning from human immunology

ABSTRACT:
Conventional vaccines have been extremely successful in preventing infections by pathogens expressing relatively conserved antigens through antibody-mediated effector mechanisms. Thanks to vaccination some diseases have been eradicated and mortality due to infectious diseases has been significantly reduced. However, there are still many infections that are not preventable with vaccination, which represent a major cause of mortality worldwide. Some of these infections are caused by pathogens with a high degree of antigen variability that cannot be controlled only by antibodies, but require a mix of humoral and cellular immune responses. Novel technologies for antigen discovery, expression and formulation allow now for the development of vaccines that can better cope with pathogen diversity and trigger multifunctional immune responses. In addition, the application of new genomic assays and systems biology approaches in human immunology can help to better identify vaccine correlates of protection. The availability of novel vaccine technologies, together with the knowledge of the distinct human immune responses that are required to prevent different types of infection, should help to rationally design effective vaccines where conventional approaches have failed.

Introduction:
Vaccination has been one of the most effective measures to control infectious diseases in the 20th century. It is believed that vaccination saves 2–3 million lives per year globally. Smallpox has been eradicated worldwide and polio is almost eliminated. Most of the viral (Mumps, Rubella, Measles, Varicella and Rotavirus) and bacterial (Tetanus, Diphteria, Haemophilus influenzae, Streptococcus pneumoniae, Meningococcus group C) diseases traditionally affecting childhood all over the world are now preventable with vaccines (GIVS 2006–2015 at http://www.who.int/vaccines-documents). Besides paediatric and adult population, vaccination has been successfully extended to adolescents, elderly, immunocompromised subjects and pregnant women. Although therapeutic vaccines targeting ongoing chronic infectious diseases have not yet been developed, effective vaccines exist that prevent chronic infections by human papilloma virus (HPV) and hepatitis B virus (HBV), which in some cases cause cancer. In summary vaccination has been – and continues to represent – a success story (Plotkin et al., 2008; Levine et al., 2009). However, there is still a long list of viruses [e.g. hepatitis C virus (HCV), human immunodeficiency virus (HIV), Dengue, respiratory syncytial virus (RSV) and cytomegalovirus (CMV)]; parasites (e.g. Plasmodium, Leishmania, Schistosoma, Trypanosoma) and bacteria [e.g. Mycobacterium tuberculosis (TB), Group A streptococcus (GAS), Group B Streptococcus (GBS), Staphylococcus aureus, Meningococcus Group B (MenB), Shigella, pathogenic Escherichia coli] that are not preventable by vaccination, and infectious diseases are still a major cause of death and disability worldwide. Thirty-three million people are currently infected by HIV that causes 1.8 million deaths per year (WHO Global summary of AIDS epidemic: http://www.unaids.org/globalreport). Malaria and tuberculosis together kill almost 3 million people every year (WHO report 2010: http://www.who.int). The total number of deaths increases even more if we include those caused by vaccine preventable infections in areas of the world where vaccines are not available to the population. It is estimated that over 2.5 million of children under 5 years of age die every year as a result of vaccine preventable infections (GIVS 2006–2015). In the future, the increased availability of existing vaccines and the development of new vaccines can have a tremendous impact in reducing the mortality and disability caused by infectious diseases worldwide.

Success and failure of conventional vaccines:
Traditional vaccines based on inactivated or attenuated pathogens or on purified pathogen subunits, such as toxins, proteins and polysaccharides, have been very efficient in preventing infections of pathogens with low degree of antigen variability. These vaccines work mainly by eliciting functional antibodies that can (i) neutralize viral invasion, (ii) neutralize bacterial toxins and (iii) induce opsono-phagocytosis or complement-mediated killing of bacteria (Germain, 2010). It has also been possible to develop effective vaccines to prevent pathogens that have a moderate degree of antigen variability and exist in multiple strains. The problem has been solved by

developing multivalent vaccines, which combine multiple antigens, each directed against one strain or serotype, in the same formulation. Four different virus-like particles that elicit neutralizing antibodies have been assembled in one HPV vaccine (Pomfret et al., 2011). In the case of bacterial pathogens, polysaccharides derived from the capsule of various strains have been conjugated to protein carriers and mixed in the same vaccine. Multivalent glycoconjugate vaccines have been developed for Pneumococcus (7-valent; 10-valent and more recently a 13-valent vaccine) (Prymula and Schuerman, 2009; Duggan, 2010) and for Menigococcus (two 4-valent vaccines have been licensed for A, C, W and Y serotypes) (Pace, 2009). A glycoconjugate strategy could not be adopted for Meningococcus serotype B because its capsular polysaccharide is similar to a self antigen. In this case traditional techniques were unable to identify a universal vaccine against all MenB strains, and a novel approach based on genomic information called reverse vaccinology has been applied as described in detail in the next paragraph (Giuliani et al., 2006).

Traditional vaccines have been also able to prevent diseases caused by pathogens, such as Influenza (flu), that are not only present in different strains or clades, but change the antigenic target of neutralizing antibodies (haemagglutinin, HA) at every season. In this case the problem has been solved by producing a trivalent vaccine and by changing the composition of the vaccine every year. However a flu vaccine with increased breadth would greatly help the manufacturing process and increase vaccine efficacy. Some investigators have suggested various strategies to develop a universal flu vaccine, capable of preventing infection by all existing flu strains (Nabel and Fauci, 2010; Dormitzer et al., 2011). A universal flu vaccine would be ideal to prevent, or at least mitigate, the risk of pandemic outbreaks, which are originated by unpredictable flu strains of animal origin.

Until today vaccines failed to prevent infections by pathogens characterized by a high mutation rate that are able to modify the target antigens and evade the antibody response during the course of the same infection. In addition, vaccines have been very inefficient in preventing infections that are not controlled by antibodies but by cellular immunity. The challenge for the future is even higher if we consider that the infections caused by some of the most variable pathogens, such as HIV and HCV, are probably not preventable only by antibodies but require a combination of humoral and cellular responses (McElrath and Haynes, 2010).

The success of future vaccines against highly variable pathogens depends on the ability to induce a universal B cell response, characterized by the production of functional antibodies that can cross-react with multiple variants of the same antigen. However, in some cases a universal antibody response may not be enough for protection and must be complemented by the ability to elicit efficient CD4 and CD8 responses (Sallusto et al., 2010). In this case, similarly to antibody assays (neutralization, opsonophagocytosis, bactericidal killing) that have been used as correlate of protection for vaccines that work through B cells, new cellular assays to evaluate vaccine efficacy must be developed. It has been highlighted by several experts in the field that if we want to understand the cellular correlate of vaccine protection it is not sufficient to count the frequencies of antigen-specific T cells in response to vaccination but it is important to assess their quality: the cytokine that they make, their differentiation state (central memory, effector memory, effector T cells) and the receptors expressed on the surfaces that may predict their localization in case of infection (Germain, 2010; Sallusto et al., 2010).

The following paragraphs will focus on the strategies that have been recently employed to cope with bacterial and viral diversity and on the technologies that can be applied to the vaccines of the future to prevent highly variable pathogens. We will then describe new technologies including adjuvants, delivery systems and viral vectors that can be used to shape the cellular and humoral immune response to vaccination. Finally we will review new system biology approaches to better understand human correlates of vaccine efficacy.

Reverse vaccinology: coping with bacterial diversity:

A vaccine discovery process called reverse vaccinology has been efficiently adopted for bacterial pathogens that have a high degree of antigen variability and circulate in multiple strains (Sette and Rappuoli, 2010). In summary, genomic information of multiple strains has been used to select surface-exposed antigens that are more conserved. In silico selected antigens have then been expressed as recombinant proteins and used to immunize mice. The antigens that gave the best bactericidal antibody responses or – in the absence of a correlate of protection – the best survival rate in animal challenge studies have been selected for prototype vaccines. This approach has been adopted for the development of a vaccine that was able to prevent infection by most Men B strains in mice (Giuliani et al., 2006). The preclinical results were confirmed by clinical trials in

adults and infants and a licence application has been recently submitted in Europe (Findlow et al., 2010; Snape et al., 2010). The same approach has been adopted for the development of a vaccine able to protect all circulating strains of GBS (Maione et al., 2005). In most of the cases the antigens identified by reverse vaccinology were unknown and their characterization has often helped to understand the biology of the pathogen. For example, a protective antigen identified by reverse vaccinology in GAS was found to be the component of a previously unknown pilus-like structure mediating bacterial adhesion to host cells (Lauer et al., 2005). In the future, reverse vaccinology can be applied to generate universal protein-based vaccines against variable pathogens such as GAS, S. pneumoniae and pathogenic E. coli strains.

Analytical and structural vaccinology: coping with highly variable viruses:
Reverse vaccinology is not applicable to the development of vaccines against viruses with high mutation rate. In contrast, it has been shown that the interrogation of human B cell response to infection can lead to the identification of cross-reactive protective epitopes that may represent structural determinants of broadly protective vaccines. This process has been called analytical vaccinology and is based on the recent development of several methods of generating human monoclonal antibodies from human blood samples (reviewed in Sallusto et al., 2010). One approach that has been used to analyse the human B cell compartment was to select total memory B cells from infected or vaccinated subjects, immortalize them through a new efficient method and screen them for antibody functional assays (Traggiai et al., 2004). Alternatively it was possible to select plasma cells induced by vaccination (Wrammert et al., 2008) or antigen-specific memory B cells (Scheid et al., 2009) and to rescue all antibodies by single cells PCR. The antibodies were then expressed in heterologous cells and used for functional assays (Liao et al., 2009). Through these approaches it has been possible to analyse the human antibody repertoire generated by infection of variable pathogens such as flu and HIV. In both cases broadly neutralizing human monoclonal antibodies have been identified and the epitopes that they recognize have been mapped. Flu-cross-neutralizing antibodies recognize a conserved region in the stem of the HA protein (Ekiert et al., 2009; Corti et al., 2010). These antibodies do not prevent HA binding to receptors, but interfere with conformational changes in HA that are required for membrane fusion (Sui et al., 2009). In the case of HIV, extremely efficient monoclonal antibodies cross-neutralizing 91% of primary isolates tested in the form of pseudoviruses bind a conserved epitope in the CD4 binding site of the Env protein (Wu et al., 2010). The same approach has been used for the identification of human monoclonal antibodies that neutralize human CMV infection. In this case the analytical vaccinology has shown that protective neutralizing epitopes are generated on a pentameric protein complex which represents a new candidate for vaccine development (Macagno et al., 2010).
The identification of conserved epitopes in HIV and flu that are target of broadly neutralizing antibodies is only the first step for the development of universal vaccines. A lot of work needs to be done to rationally design and express the target antigens (e.g. HA or Env) in a form that stabilizes cross-reactive epitopes and makes them sufficiently immunogenic. The only way to achieve this goal is to have a deep structural knowledge of the candidate antigens (Fig. 1). Structural information can also be exploited to generate chimeric proteins which contain protective epitopes deriving from multiple variants of the same antigen. This approach has been recently used for a novel MenB candidate vaccine that was rationally designed by assembling epitopes from three different variants of MenB factor H-binding protein (Scarselli et al., 2011).

Novel adjuvants: shaping the immune response:
The identification of protective epitopes and the production of rationally designed antigens that induce cross-neutralizing antibody are not enough to produce an effective vaccine. The antigens need to be formulated with antigen delivery systems and immunomodulators or must be inserted in viral or nucleic acid expression vectors. It has been well known for a long time that adjuvants can increase the amount, the quality and the duration of the antibody responses to vaccination. Empirically derived adjuvants such as aluminium salts have been used in human vaccines for more than 70 years. However, in the last decade the knowledge of the human innate immune system has rapidly progressed leading to the identification of novel classes of molecules [Toll-like receptors (TLRs), Rig-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectins] that are validated targets for the development of new immunomodulators (reviewed in this issue by Carlos Gutzman et al., in press). In parallel, a significant amount of work has been done to advance antigen delivery systems including liposomes, virus like particles and nanoparticles, which can be used to formulate antigens and immunomodulators in the same vaccine allowing co-delivery

(Bachmann and Jennings, 2010). Despite the universally acknowledged potential of vaccine adjuvants, only two compounds are licensed for human use in the USA (alum and the TLR4 agonist MPL) and few others in Europe (virosomes and the oil in water emulsions MF59 and AS03) (Coffman et al., 2010; Mbow et al., 2010). The few licensed adjuvants have demonstrated a tremendous impact on vaccination. MPL adsorption to an alum-HPV vaccine promoted higher neutralizing antibody titres and increased memory B cell frequencies (Giannini et al., 2006). The addition of oil in water emulsions MF59 and AS03 to H5 pandemic flu vaccines was required for protection (seroconversion) and promoted cross-reactivity versus various heterologous H5 clades (Banzhoff et al., 2009; Schwarz et al., 2009). Besides antibodies, MF59 enhanced both H5-specific CD4 and memory B cell responses to vaccination (Galli et al., 2009a,b). Interestingly, the increased breadth of the response to H5 induced by MF59 was associated with the recognition of an increased number of epitopes on HA (Khurana et al., 2010). These data suggest that adjuvants can change not only the amount but also the quality of the humoral responses to vaccination, allowing for a better coverage of less immunodominant epitopes that may be very important for cross-protection. Many new adjuvants, in isolation or in combination, are in clinical trials with very promising results. One example is AS01, a combination of liposomes, MPL and QS21 that was used in a malaria phase II clinical trial that achieved for the first time 30–50% protection (Cohen et al., 2010). Another promising adjuvant in clinical development is the TLR9 agonist CpG oligonucleotide (Steinhagen et al., 2010). In addition, many other TLR agonists, such as PolyI:C, flagellin, lipoproteins, have already been tested in humans in combination with various antigen and delivery systems (Steinhagen et al., 2010). It is easy to predict that in the future more immunomodulators and antigen delivery systems will be available for use in human vaccines. The use of these compounds in various combinations will greatly help to rationally design vaccines with distinct properties, with the aim of generating protective adaptive immune responses.

New viral vectors and prime-boost strategies: eliciting multifunctional adaptive responses:
Adjuvants have been very efficient in boosting antibody and CD4 responses to subunit vaccines. However the induction of CD8 by subunit vaccines, which relies on cross-presentation mechanisms, is generally very poor. Some adjuvants, such as the saponin-based ISCOMs have shown the ability to increase cross-presentation in animals and human studies (Drane et al., 2007; Sun et al., 2009). However in humans ISCOMs produced only partial CD8 responses (Drane et al., 2009). Probably the best approach to induce MHC-I-mediated immune responses is to use nucleic acid or viral vectors, which mediate the expression of the selected vaccine antigens directly in the cytoplasm of target cells (Liu, 2010) (reviewed in this issue by Oliver Elbert, in press). Several DNAs and replicating or non-replicating viral vectors have been tested in clinical trials until now; however, none of them has been licensed for human use. One very well-known example of a non-replicating vector is the Adenovirus-derived Ad-5 vector that was used for the STEP HIV trial and induced strong CD8 responses in all subjects that did not have pre-existing Ad5 antibodies (McElrath et al., 2008). Unfortunately, the trial showed that a good CD8 response did not correlate with prevention of HIV infection, suggesting that probably a more balanced immune response including both CD4 and CD8 T cells and cross-neutralizing antibodies is required (Buchbinder et al., 2008; McElrath et al., 2008; McElrath and Haynes, 2010). One way to obtain multifunctional responses is to use prime boost regimens, in which the priming with an expression vector targeting CD4 and CD8 responses is followed by a boost with a subunit vaccine, which improves antibody production. Recently a prime-boost strategy was implemented in the HIV trial RV144 conducted in Thailand. Subjects have been primed with a canarypox vector encoding gag, env and protease and boosted with a gp120 subunit vaccine. The RV144 trial showed for the first time 31% efficacy against HIV-1 acquisition (Rerks-Ngarm et al., 2009). These results are very encouraging and can be considered as a proof of concept that an HIV vaccine strategy can be applied. However, more work needs to be done to optimize the vaccine by using more efficient expression vectors and a rationally designed Env antigen eliciting cross-neutralizing antibodies formulated in adequate immunomodulators and delivery systems.

Systems vaccinology: identifying new correlates of vaccine efficacy:
Although the RV144 trial has shown the benefit of a prime-boost strategy for a preventive vaccine against HIV, it is still not clear which are the effector mechanisms elicited by the vaccine that protected some of the subjects from HIV infection. For difficult intracellular pathogens such as HIV and HCV, protection probably arises from the integration of different effector mechanisms of the appropriate quality. Therefore, correlates of protection are not easily measured by simple antibody or T cell assays, but require more complex readouts. With the progress of genomics it is possible

to generate a lot of high-throughput data from human blood samples including: RNA expression profiles measured through DNA microarrays; protein expression profiles measured by mass spectrometry; and analysis of genomic polymorphism measured by deep sequencing. Systems biology is required to integrate genomic data with the data obtained from the same subjects through classical immunological assays for antibodies (neutralization, bactericidal activity, ELISA) or for T cell characterization (ELISPOT; FACS-intracellular staining; proliferation, cytotoxic activity) (Pulendran et al., 2010) (reviewed in this issue by David Klatzmann, in press). In addition, more innovative immunological assays are now available, such as antibody repertoire analysis or single cell phospho-specific flow cytometry (Krutzik et al., 2011) that allow to evaluate in detail the quality of humoral and cellular responses to vaccination. Systems biology approaches have been already successfully applied to humans vaccinated with the Yellow fever vaccine YF-17D (Gaucher et al., 2008; Querec et al., 2009). These studies have shown that it is possible to predict the efficacy of the vaccine by measuring the transcriptome of PBMCs few hours after vaccination. Interestingly, the predictive innate immune signatures were not obvious, involving genes, such as EIF2AKA that had not been associated with the generation of adaptive responses before. The new biological information discovered by systems biology approaches helped to better understand the mechanism of action of the vaccine and can now be exploited to rationally design improved vaccine adjuvants targeting the ‘protective’ genes or pathways (Pulendran et al., 2010).

Conclusions:
Preventive vaccines have been a major success in medicine in the last century. However there are still many infectious diseases that cause millions of deaths every year worldwide. Conventional approaches have failed to develop effective vaccines against these pathogens. However there is great hope for the future that is based not only on new technologies available for vaccine development, but also in an increased ability to interrogate the human immune responses and integrate complex readouts through computational methods.
We think that vaccinology of the future will be less empirical than it used to be and will learn more from human immunology. Antigen design will be directed by a deep knowledge of cross-protective epitopes in humans. Adjuvants and expression vectors will be selected based on the type of humoral and cellular immune responses which correlate with protection. A more rational vaccine design will hopefully allow the development of effective preventive vaccines for all remaining ‘difficult’ targets including HIV, malaria and tuberculosis.