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Vaccinology at the beginning of the 21st century

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Today, the main challenges for vaccinologists include improving vaccines against as yet undefeated pathogens, rapid identification and response to emerging diseases and successful intervention in chronic diseases in which ongoing immune responses are insufficient. Reverse genetics and reverse vaccinology are now used to generate rapidly new vaccine strains and to mine whole genomes in the search for promising antigens. The rational design of adjuvants has become possible as a result of the discovery of the receptors that recognize microbial patterns and lead to dendritic cell activation. Antigen-loaded dendritic cells, DNA in naked, formulated or viral form, and other delivery systems are used to maximize immune responses. Although work on the 'easy' vaccines has already been completed, it is hoped that a combination of conceptual and technical innovation will enable the development of more complex and sophisticated vaccines in the future.

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Introduction

Although the eradication of smallpox in the 1970s and of poliomyelitis hopefully in the coming years mark two of the most important milestones in medical history, we now face an unprecedented succession of new pathogens which jump species barriers to infect humans, and the frustration deriving from the inability to control devastating diseases such as HIV, malaria and tuberculosis. This review will cover the recent progress in vaccinology (Figure 1), largely resulting from dramatic technical innovation that is now reaching the clinic, as well as the huge challenges posed by old and new pathogens that are facing us.

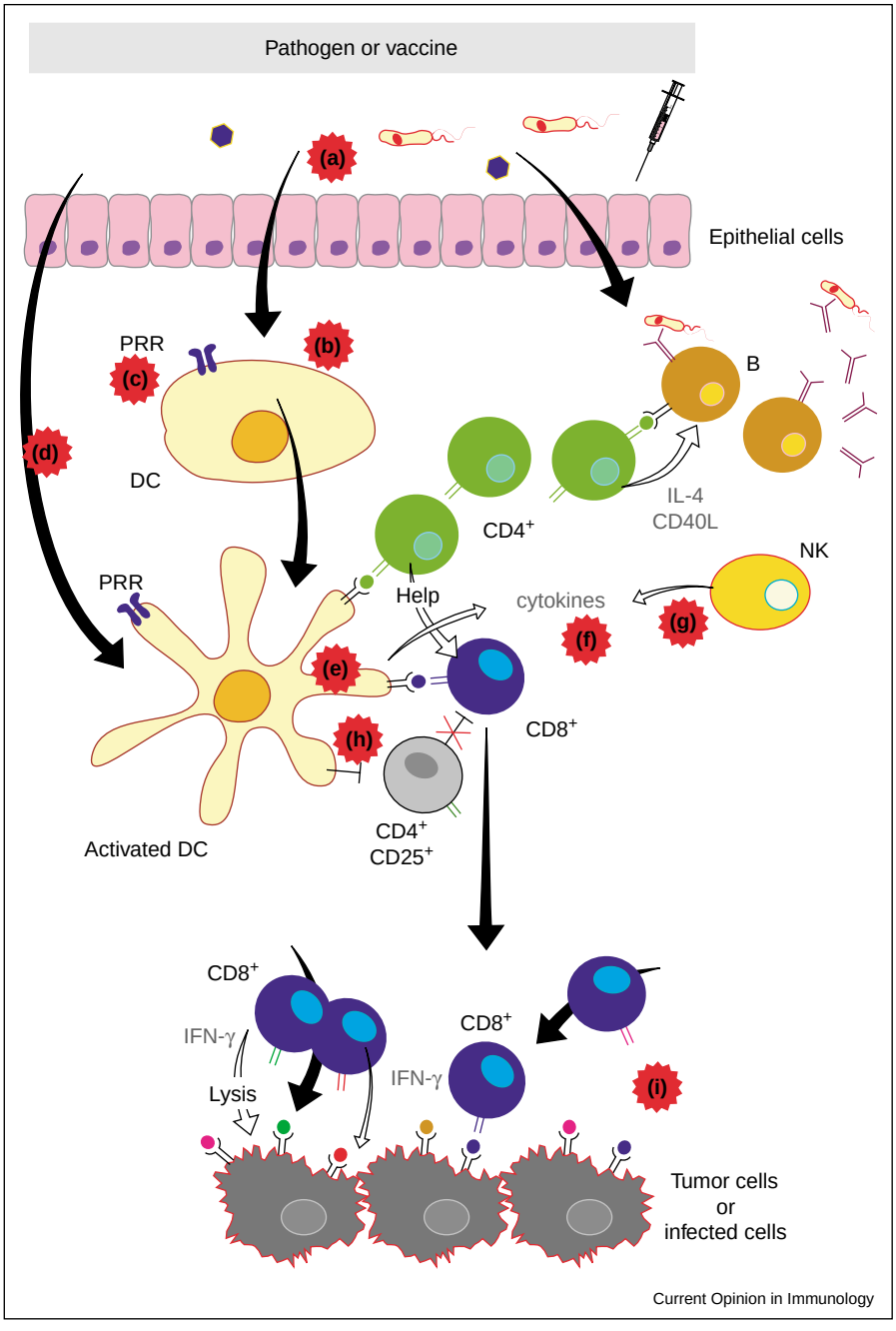
The diseases to fight

The three 'big killers', the pathogens that most heavily afflict global health, are HIV, mycobacterium and plasmodium. Whereas the latter two represent long-term companions of the human species, HIV is a virus that spread in the human population only about 25 years ago and has a nonhuman primate origin [1]. In fact, most, if not all, of the recently emerging diseases go back to animal reservoirs, from which they infect humans through close contact during hunting and farming, in live animal markets or through food processing, preparation or consumption. An example is Ebola virus, which, after three outbreaks between 1976 and 1979, has appeared in the human population nine times since 1994, with each outbreak resulting from the handling of dead gorillas, chimpanzees or duikers, which in turn are thought to have been infected by an unknown natural host [2•]. The variant Creutzfeldt–Jacob disease reached the population through the food chain, from 'rendered' sheep and cow carcasses fed to cows which were subsequently consumed by humans. The severe acute respiratory syndrome (SARS)–coronavirus sequences from the earliest identified cases are identical to those found in palm civets and raccoon dogs in animal markets and farms [3•], strongly suggesting an animal origin for this disease.

Rapidly changing ecosystems and human behavior, an ever-increasing density of human and (farmed) animal populations and their close vicinity, poverty, a high degree of mobility and many other factors contribute to the more frequent occurrence and often rapid dissemination of new diseases. Another example of this is the arrival and persistence of West Nile virus in the USA, which is probably a result of increased mobility, an exceptionally broad host range and climatic changes [4]. Among the re-emerging diseases of the past few years, diphtheria and cholera should be mentioned [5], as well as the frequent appearance of multidrug-resistant bacteria and the cases of anthrax infection as a result of deliberate release in 2001.

In the case of influenza, concern for the advent of a new pandemic has been fuelled by reports of 89 human infections by the avian H5N1 virus strain (where H stands for hemagglutinin and N for neuraminidase, the two major surface glycoproteins of the virus) in 2004, leading to 52 deaths (http://www.who.int/csr/disease/avian_influenza/en/). This was the third time in the space of a few years (after the previous outbreaks of infection in 1997 and 2003) that H5N1 avian flu viruses caused disease and death in humans. An H9N2 avian strain caused infection in humans in 1999 and 2003. Because the world's population has not been exposed to these strains, it is immunologically naïve, and these or any other avian flu strains with new surface glycoproteins would meet no

Figure 1



Possible intervention points for vaccine improvement. Schematic view of a pathogen- or vaccine-induced immune response. Vaccines or pathogens cross epithelial barriers and are taken up by antigen-presenting cells such as DCs. Interaction between pattern recognition receptors (PRRs) and their agonists activate DCs, resulting in increased antigen presentation, cytokine production and co-stimulation. $CD4^+$ and $CD8^+$ T cells recognize antigen presented by DCs and are activated. Cognate interaction between primed $CD4^+$ T cells and B cells activates the B cells, resulting in clonal expansion and antibody production. DC activation also leads to inhibition of the regulatory effect of $CD4^+CD25^+$ Treg cells. Fully activated $CD8^+$ cells can target tumor cells and pathogen-infected cells. The letters (a–i) indicate processes where improved vaccines can lead to more efficient immune responses: **(a)** The site of administration influences the type of immune response and enables the usage of lower vaccine doses [19,20]. **(b)** Particulate antigen is taken up more easily by macrophages and DCs than soluble antigen [47]. **(c)** TLR agonists and other immunostimulants binding to PRRs increase the activation of DCs [32–35,36•,37•,38,40]. **(d)** DCs matured and loaded with antigen *in vitro* are efficient vaccines [41••,42,43]. **(e)** DNA vaccination leads to efficient antigen presentation on MHC class I [48,49,50•,51••,52,53]. Crosspresentation of antigen on MHC class I of host DCs is facilitated after vaccination with antigen-loaded DCs undergoing delayed apoptosis [44•]. **(f)** Cytokines can be added in protein or DNA form as natural adjuvants [32,33]. **(g)** Recruited NK cells can be an early source of Th1-driving cytokines [39•]. **(h)** Vaccines can break tolerance when the suppressive effect of $CD4^+CD25^+$ Treg cells is overcome [54•]. **(i)** Pre-existing T cells specific for tumor antigens not contained in the vaccine expand after vaccination and predominate in the antitumor response [55,56•].

resistance provided by existing immunity and could rapidly expand. Once an avian strain has developed a more efficient means of human-to-human transmission, devastating pandemics, such as those arising in 1918, 1957 and 1968, could ensue. It should be noted, however, that older studies found seroprevalence rates for avian flu strains among Chinese rural populations to be between 2% and 7% for H5 viruses and between 15% and 38% for other avian strains [6,7]. Thus, it might in fact be the quality of surveillance, rather than the frequency of outbreaks, that has increased over the past few years [8]. However, the contact between humans and pathogens in animal reservoirs is likely to intensify, and, consequently, the emergence or re-emergence of diseases will occupy vaccinologists frequently in the future.

How to react to new pathogens

One of the most important future challenges will be to respond promptly to emerging diseases such as those mentioned above. A striking example for rapid reaction was in the case of the SARS outbreak, where the genome sequence was publicly available in less than a month after the virus was identified [9]. This enabled the speedy development of diagnostic tools, as well as the identification and recombinant expression of targets for vaccines and therapeutic agents [10–12].

For the influenza virus, in addition to the annual definition of the relevant strains to be included in the vaccine for the following season, the World Health Organization closely monitors cases of avian flu (for further information, see the World Health Organization website indicated above), and prototype vaccines for these strains are being developed. Apart from almost complete lack of protection in the population, an additional threat of the avian flu H5N1 isolate from 2003 is that it kills embryonated eggs, the traditional virus growth substrate used for influenza vaccine production. Such problems can now be solved, owing to the discovery some years ago that influenza virus can be generated entirely from transfected DNA (reverse genetics [13,14]). In this particular case, Webby *et al.* [15•] have used polymerase chain reaction-based mutagenesis to replace the hemagglutinin cleavage site (which was shown to be the cause of high pathogenicity) of the H5N1 strain with the sequence from a nonpathogenic strain. Vero cells were then transfected with plasmids encoding the neuraminidase and mutated hemagglutinin from the circulating strain, together with the plasmids encoding the remaining proteins from the laboratory-optimized PR8 strain. The resulting vaccine strain was successfully grown in eggs and shown to be nonpathogenic and stable. Thus, the use of reverse genetics enables rapid production of a reference vaccine virus in response to the emergence of a new influenza variant [15•,16]. In addition, reverse genetics can be used for more far-reaching vaccination strategies, such as the construction of 'consensus' strains expressing conserved amino acid sequences or more than one version of the surface glycoproteins, or additional immunoenhancing molecules, such as cytokines [8]. Much research effort is also being invested into the development of improved cell culture systems that can replace completely the use of embryonated eggs in vaccine production and would render the production process more flexible and controllable.

Several reports have addressed the question of how to stretch the available supply of vaccine doses in cases of shortage or in the face of a pandemic. Two studies indicated that intradermal, rather than intramuscular, application of 40% [17•] or 20% [18•] of the usual vaccine dose leads to equal or better immune responses. Both theoretical models [19] and trials [20] have shown that immunizing a high proportion of children, known to have a high rate of infection and an important role in transmission, also decreases the incidence of influenza in older age groups, a phenomenon known as herd immunity and described in previous trials in Michigan and Japan [21,22].

Reverse vaccinology

The genomic revolution has opened up a completely new approach to vaccine discovery. For pathogens that do not grow *in vitro*, the availability of the genome sequence has enabled the development of recombinant vaccines, as has been carried out for hepatitis B virus (HBV) and is underway for hepatitis C. In regard to bacteria, group B meningococcus posed insurmountable obstacles to conventional vaccinology approaches; these were eventually overcome by mining the information from the sequenced genome [23]. A total of 600 potential vaccine candidates were predicted by computer analysis, 350 of which were expressed and tested for immunogenicity [24]. Some of these candidates are now in clinical trials. This genome-based approach, called reverse vaccinology, is now used routinely in vaccine development, and is a major tool in the quest for vaccines against pneumococcus, group B streptococcus and chlamydia (see also Update).

Recently, genome sequencing of both *Plasmodium falciparum* [25] and its main vector, *Anopheles gambiae* [26], has sparked off new hopes for an efficient vaccine against malaria. For the rodent models of this disease, subtractive cDNA techniques were used to identify genes that are only expressed in pre-erythrocytic stages of the parasite [27,28]. Plasmodium mutants deficient in one of these genes, *uis3*, are blocked in their early liver-stage development and all subsequent stages, and therefore do not lead to disease. When *uis3*-deficient sporozoites are used as genetically attenuated vaccines in mice, they confer long-lasting, stage-specific protection [29••]. This is a promising example of how molecular approaches are employed for the rational design of new vaccines.

Another example of encouraging progress towards a malaria vaccine was reported by Alonso *et al.* [30•]. They

describe Phase IIb trials of a subunit vaccine consisting of a recombinant protein (expressed in yeast) composed of the carboxy-terminal of the *P. falciparum* circumsporozoite protein and the HBV surface antigen. This fusion protein, together with unfused HBV surface antigen proteins, forms particles. The final vaccine formulation includes the adjuvant AS02A, an oil in water emulsion containing the immunostimulants monophosphoryl lipid A (MPL) and *Quillaja saponaria* fraction 21. It had previously been shown that, indeed, a $CD4^+$ T cell response to an epitope contained in the vaccine correlates with protection from infection and disease [31•]. In this trial in children, the vaccine showed an efficacy of 30% and 58% in preventing clinical episodes and severe episodes, respectively [30•].

Adjuvants

The past ten years have changed our vision of the immune response to pathogens. It has become clear that the degree and type of antigen-specific, clonal B and T cell responses (acquired immunity) depend crucially on the prior action of a more ancient system of pathogen detection (innate immunity). This system relies on the activation of antigen-presenting cells such as dendritic cells (DCs) upon recognition of patterns common to viruses and bacteria and largely absent in mammals. With the discovery of the involved pattern recognition receptors, among which the Toll-like receptors (TLRs) represent an important subgroup, immune-enhancing molecules or adjuvants can no longer be considered as the alchemistic 'immunologist's dirty secret' but have become amenable to rational design, providing a huge potential for manipulating the immune response. As different TLR agonists elicit different types of immune responses (reviewed in [59]), future adjuvants might be able to tailor the immune response so that optimal protection to a given pathogen is induced. In fact, the number of clinical trials involving TLR agonists as new adjuvants is ever increasing [32,33] (Table 1).

The adjuvant function of nonmethylated cytidine-phosphate-guanosine (CpG) sequences, which are frequent in microbes but under-represented in humans and are agonists of TLR9, has been extensively demonstrated in animal models [34] and is currently being tested in several clinical trials. When CpGs are coadministered with licensed HBV or flu vaccines, the combination leads to increased antibody titers or increased (interferon-γ) IFN-γ production, as compared with the response to the vaccine alone [35,36•]. An additional effect of CpG oligonucleotides appears to be the promotion of affinity maturation and, as a result, a higher overall affinity of the vaccine-specific antibody pool [37•]. Similarly, the TLR4 agonist MPL has been shown in the past to enhance the immune response to HBV vaccination in humans [38]. In addition, both the malaria subunit vaccine mentioned above [30•] and a licensed melanoma vaccine contain MPL. Another experimental vaccine that includes a TLR2 agonist is described below, in the section on synthetic vaccines.

Activation of DCs increases their ability to process and present antigen and to attract and activate T cells through cytokine secretion; consequently, several cytokines are

Table 1

Human TLR agonists used as adjuvants in vaccine formulations in clinical trials or licensed vaccines[a].

Receptor	Known natural agonist	Form used in vaccines	Vaccine type
TLR1 (with TLR2)	Lipopeptides		
TLR2	Lipopeptides		
	Lipoteichoic acid		
	Porins		
	Zymosan		
TLR3	Double-stranded RNA		
TLR4	Lipopolysaccharide	Monophosphoryl lipid A	Melanoma
	Heat shock proteins	AS02 (MPL + saponin QS-21)	Malaria [30•], HBV, HPV, HIV-1,cancer, tuberculosis
	Fibrinogen		
	Fibronectin	AS04 (MPL + alum)	HBV [38]
		RC-529 (MPL derivative)	HBV
TLR5	Flagellin		
TLR6 (with TLR2)	Lipoproteins		
TLR7	Unknown		
TLR8	Single stranded RNA		
TLR9	Bacterial DNA	CpG oligonucleotides	HBV, flu [35,36•,37•]
			ISS (CpG linked to antigen DNA)
TLR10	Unknown		
TLR11	Components of uropathogenic bacteria		

[a] Data from [32,33] unless otherwise indicated.

currently being tested for their adjuvant function. As mentioned above, the way the innate immune system is activated influences the type of the ensuing acquired immune response. Martin-Fontecha *et al.* [39•] showed that the ability of adjuvants to elicit a Th1 type response depends crucially on the recruitment of, and IFN-γ production by, natural killer (NK) cells, which indicates a possible mechanism of the way in which adjuvants direct the type of adaptive immune response induced downstream. Another vaccine approach is the use of heat shock proteins, which bind specifically and activate dendritic cells and, because they are loaded with endogenous peptides, can be purified from tumor cells and function as a combined antigen delivery system and adjuvant [40].

Antigen-loaded DCs as vaccines

Because the main targets of adjuvants are DCs, it is a logical step to evaluate their direct use as a vaccine. Despite the labor-intensive necessity of individual cell culture for each patient, this approach can be attractive where other approaches have failed, for instance as a therapeutic vaccine in HIV or cancer patients. When HIV patients were treated with autologous DCs loaded with autologous, inactivated HIV, both virus-specific $CD4^+$ Th1 and $CD8^+$ responses were induced and the plasma viral loads were reduced [41••]. These results closely reflect previous findings from a similar vaccination of rhesus macaques [42], except for the lack of induction of neutralizing antibodies in the human study. DC vaccination is also being tested in a variety of cancer treatments [43].

In a study comparing the immunogenicity of DCs transfected with cytopathic or noncytopathic viral RNA, the former regimen was shown to be more efficient at inducing protective immune responses [44•]. This suggests that reprocessing of dying DCs by endogenous antigen-presenting cells enhances immunogenicity, either through additional danger signals triggered by the cell damage or by the increased level of crosspresentation by endogenous DCs. The same mechanism might be at work in mycobacterium infection of macrophages, where apoptosis was shown to enhance crosspresentation by bystander DCs [45]. Interestingly, following fractionation of the cytoplasm of dying cells, uric acid was identified as a highly efficient endogenous danger signal that enhances immunogenicity [46], and might explain the above results.

DNA vaccines

The high expectations associated with DNA vaccination, as a result of promising data obtained in mice, were somewhat tempered by disappointing early results when DNA was tested as a vaccine in humans. Therefore, the latest generation of DNA vaccines rely on improved delivery either through use of microparticles [47] or through viral vectors. A particularly promising approach is a heterologous prime-boost strategy, where administration of plasmid DNA is followed by recombinant virus (modified vaccinia virus Ankara [MVA] or adenovirus) expressing the same antigen. This regimen induced strong T cell responses against *P. falciparum* in naïve adults [48] and enhanced the response in Gambian men who are constantly exposed to *P. falciparum* [49]. Although partial protection against challenge with a different *P. falciparum* strain was observed, no significant differences in the infection rate was found in the Gambian trial [50•]. In spite of these setbacks, a very similar regimen has been shown also to be highly immunogenic against HBV, tuberculosis and HIV, and vaccination only with MVA expressing the mycobacterium A85 antigen elicited strong T cell responses [51••].

Because the induction of T cells is considered to be crucial in anti-tumor immune responses, a huge number of trials are presently being conducted to test DNA vaccination regimens for anticancer treatment [52,53]. When viral vehicles (vaccinia or adenovirus) were compared with loaded DCs in terms of their ability to overcome established tolerance and induce immune responses in a transgenic mouse model, the viral formulations were able to do so, whereas DCs required repeated administration of TLR agonists or irrelevant virus, or removal of suppressive $CD4^+CD25^+$ Treg cells [54•]. Such models of established tolerance might prove useful for testing the success of vaccines in the face of long-term antigen exposure, as in the case of cancer or chronic diseases.

Two studies analyzed in detail the T cell response after vaccination with a recombinant canarypox virus expressing melanoma-specific T cell epitopes [55•,56•]. Focusing on the blood and metastases from a patient with complete regression, these studies reconfirmed earlier observations that the frequency of anti-tumor T cells can be relatively high. Although vaccination leads to a slight increase in vaccine-specific T cells, these remain only a small fraction of total anti-tumor T cells. By contrast, T cells directed against epitopes not contained in the vaccine represent the vast majority of anti-tumor cells, and their frequency increases both in the blood and in metastases. Thus, it appears that with the appropriate vaccine regimen, the inefficiency of pre-existing specific T cells to combat the tumor was reversed in an indirect manner, presumably by activating another subset of T cells. It remains to be clarified, however, how much of this reactivation is due to the action of vaccine-induced T cells and how much is a general, antigen-independent immune-enhancing effect. In any case, for the development of therapeutic vaccines, it will be vital to understand how the balance can be tipped back from a state of tolerance to a successful immune response.

Synthetic vaccines

The development of vaccines aimed at the polysaccharide (PS) capsule of bacteria is one of the great

achievements in vaccinology. So far, the PS used in large-scale vaccine production has been purified from the pathogen itself, grown in large quantities – an approach that is costly and difficult to control. Through great simplification of the carbohydrate chemistry involved, Verez-Bencomo *et al.* [57•] have now demonstrated the first large-scale production of an anti-*Haemophilus influenzae* type B vaccine, consisting of synthetic PS conjugated to tetanus toxoid protein carrier. This vaccine has been shown to be as efficient as commercially available vaccines in inducing protective levels of antibody titers in infants.

An entirely synthetic vaccine with a branched structure containing a TLR2 ligand, a $CD4^+$ T cell epitope and either a $CD8^+$ T cell or a B cell epitope has been shown to elicit strong $CD8^+$ T cell and B cell responses, respectively [58••]. Here, the minimal requirements for an efficient vaccine are met in a single molecule: targeting to and activation of DCs, T cell help and activation of antigen-specific $CD8^+$ T cells or B cells.

Conclusions

The world of vaccines is undergoing dramatic changes. Never before have such sophisticated techniques and an in-depth knowledge of immunological processes been at hand to exploit fully the potential of protecting from, as well as curing, diseases through vaccination. A formidable task in the future will be the development of effective therapeutic vaccines, in situations where chronic antigen exposure by itself does not elicit a sufficiently strong immune response, as is the case in cancer and chronic infectious diseases. Compared with vaccines against self-limiting infections, where the aim is to be as good as the real pathogen (but less harmful), this requires the development of vaccines that are better than the natural antigens in inducing immunity. All of our knowledge will be necessary to succeed in this challenge.

Update

A recent article describes the use of multigenome analysis and screening against a large panel of strains to identify a universal group B streptococcus vaccine. Although none of the single antigens contained in the vaccine elicits protection against all strains, the combination of four proteins is able to cover a wide range of strains [60•].

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