Bridging the knowledge gaps in vaccine design

Rino Rappuoli

To design the vaccines of the future we need to fully exploit microbial genomes and understand the basic mechanisms of the immune system.

Existing vaccines have been developed mostly against pathogens that show no or limited antigenic variation and that can be controlled by neutralizing serum antibodies. In contrast, the conquest of pathogens that display more variable antigens and require T-cell immunity remains elusive, requiring several gaps in our knowledge to be bridged. The advent of genomics is allowing us to address the problem of antigen variability by increasing the number of characterized antigens by several orders of magnitude and making possible the selection of antigens that are conserved and that induce antibody-mediated protection. But filling the remaining knowledge gaps will require greater understanding of the molecular nature of epitopes and the mechanisms of T-cell and mucosal immunity. These problems can be best addressed by systematic studies of antigen three-dimensional structure and further progress in our ability to program and measure the immune system's ability to protect the host.

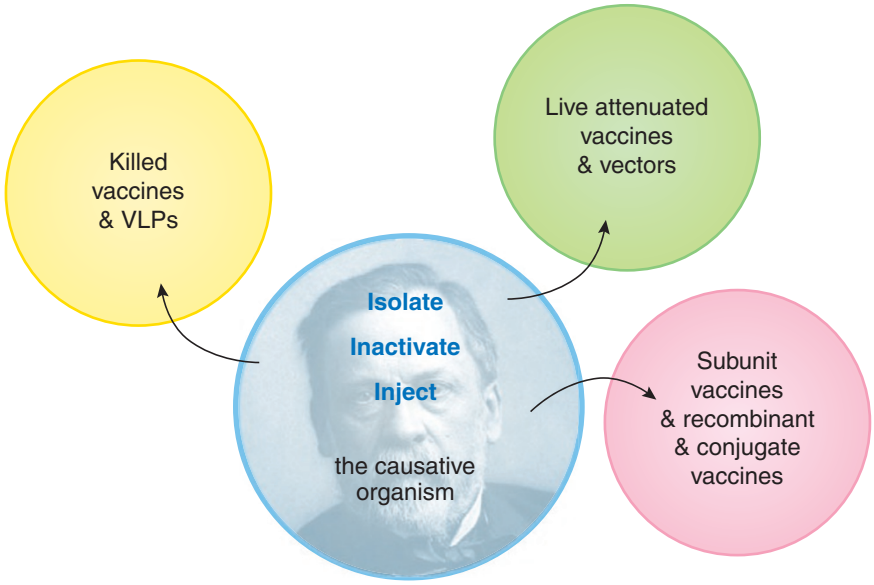

Figure 1 Vaccines licensed so far have all been developed by following Pasteur's principles: "isolate, inactivate and inject" the causative microorganism. This led to the development of killed, live attenuated and subunit vaccines. Recent technological breakthroughs have amplified the potential of the vaccines developed by Pasteur's principles by making it easier to manufacture structures mimicking killed viral vaccines (virus-like particles or VLPs) by engineering replicating and nonreplicating recombinant vectors that mimic live vaccines and by providing new forms of subunit vaccine such as conjugate and recombinant vaccines.

Technological breakthroughs

For more than a century, vaccine development has followed Pasteur's principles: "isolate, inactivate and inject" the causative microorganism[1]. Bacterial and viral vaccines have been produced that consist of whole killed pathogens, live attenuated pathogens or purified components from pathogens (**Fig. 1**). Together with the wider availability of potable water, the provision of vaccines has had the most profound positive effect on the quality of public health of any measure: during the past century, these products essentially eliminated most infectious diseases causing mortality in infants and children. Vaccines against diphtheria, tetanus, polio, measles, mumps, rubella, pneumococcus, hepatitis B and meningitis (*Haemophilus influenzae* and serogroup C meningococcus) have reduced the incidence and mortality of these diseases by > 97–99% (ref. 2).

Technological breakthroughs during the past three decades have extended considerably the power of the vaccines produced according to Pasteur's principles, especially subunit vaccines. The first breakthrough was the advent of gene-splicing technology and the production of a recombinant hepatitis B virus (HBV) vaccine[3]. In this case, although a vaccine made by inactivated viral surface antigen (HBsAg) was shown to be effective in the seventies, vaccine manufacture was limited since the antigen could only be isolated from the plasma of infected people, because HBV could not be cultivated outside patients. Recombinant DNA technology allowed the production of unlimited amounts of HBsAg of the vaccine in yeast, thus solving forever the vaccine-manufacturing problem.

Rino Rappuoli is at Novartis Vaccines, Via Fiorentina 1, 53100 Siena, Italy.
e-mail: rino.rappuoli@novartis.com

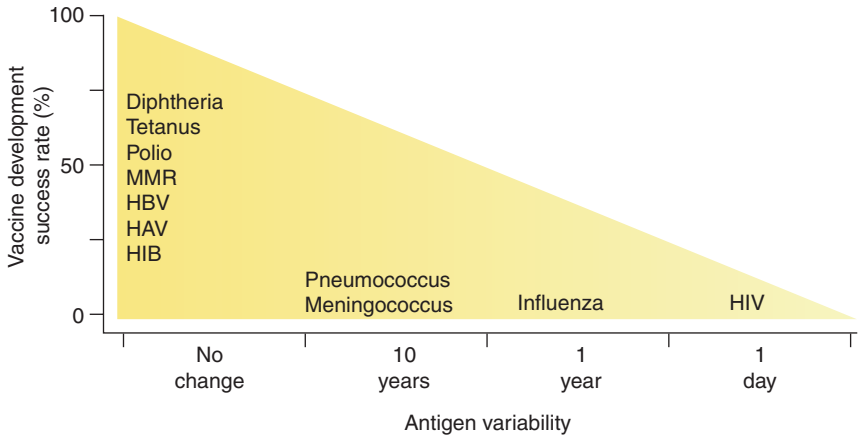

Figure 2 Schematic representation showing that the success rate of vaccine development decreases with the increasing variability of the surface antigens of pathogens. Vaccines mentioned in the figure are selected examples of each category and not a complete list. MMR, measles, mumps and rubella; HBV, hepatitis B virus; HAV, hepatitis A virus; HIB, *H. influenzae* type B.

The second technological breakthrough was conjugate vaccines. In this case, polysaccharide subunit vaccines were known to be able to induce protective immunity, but they were not widely used because they could not engage a T-cell response and failed to work in infants. But conjugating polysaccharide antigens to proteins created T-cell epitopes that made these vaccines very effective in infants, opening the way to the development of several safe and effective vaccines against *H. influenzae*, *Neisseria meningitidis* serogroup A, C, Y, W135 and *Streptococcus pneumoniae*[4].

A third breakthrough was the engineering of *Bordetella pertussis* to produce a mutant pertussis toxin that had lost all the toxic activities of pertussis toxin[5,6]. In this case, the recombinant DNA technologies produced a clean, nontoxic and highly immunogenic antigen. Previously, such antigens could only be derived from the pathogenic microorganism directly and detoxified by harsh chemical treatment, which often compromised the protective epitopes of the subunit vaccine.

Finally, a recent breakthrough has been the ability to produce nonreplicating, recombinant virus-like particles of many viruses[7]. This innovation has allowed the development of a vaccine against human papillomavirus and is likely to provide new and improved vaccines against many other viruses, including influenza and Dengue virus. Even live attenuated vaccines are being extended by a new family of replicating and nonreplicating viral vectors[8].

The first gap: antigen variability

Conventional vaccinology has been very successful in developing vaccines against pathogens that do not change the vaccine-targeted antigens over time (**Fig. 2**). Classical vaccines such as diphtheria and tetanus, for instance, target toxin antigens that have not had antigenic drift during the past century. Other antigens with little or no antigenic drift include the surface antigens of poliovirus (where the three known serotypes have been stable for the past 60 years), the envelope antigens of measles, mumps and rubella, the HBV and hepatitis A virus surface antigens, and the polysaccharide antigen of *H. influenzae* type B.

To a certain extent, conventional vaccinology has had success in targeting particular pathogens that have many variants of target-vaccine antigens and change them over time. A classical example is the polysaccharide antigens of pneumococci. Although this bacterium has >90 chemically different polysaccharide forms, most human disease is associated with only 23 variants. This made it possible to produce a very complex, but practical, 23-valent polysaccharide vaccine against the disease[9,10]. This approach reached its limits when conjugate vaccines were developed. In this case, it was practically impossible to manufacture a 23-valent conjugate vaccine, and thus lower valency conjugate vaccines were initially developed, primarily to cover the serotypes responsible for invasive disease in one region of the world. Thus, a vaccine might offer good coverage in, for example, the United States, but offer poorer coverage in other regions of the world.

In the long term, this solution also has its drawbacks. This is because even in countries where the seven-valent vaccine is originally protective, serotypes not covered by the vaccine slowly colonize the vaccinated population through selective pressure. In the long term, therefore, it is possible that existing serotypes in the seven-valent vaccine may have to be replaced with new serotypes occurring in the target population[11,12]. This is not necessarily an insurmountable problem because vaccines do effectively control the disease during the first few years after introduction. Moreover, we also have the tools to follow the epidemiology of pathogens and to design and license new vaccine formulations before they are actually needed to address the emergence of new pathogenic variants. Indeed, the development of 11-valent and 13-valent pneumococcal vaccines, which are in the late stage of development, moves in this direction. For pneumococcus, therefore, global control of the disease is possible simply by designing regionally targeted vaccines and updating their formulation every 10 years.

Vaccination against meningococcus, a bacterium with five chemically different capsular polysaccharides known as serogroup A, B, C, Y and W135, also follows a similar paradigm. When the first conjugate vaccines were developed in the early nineties, the disease in Europe and the United States was mainly caused by serogroup C and B. A decade later, however, serogroup Y became prevalent in the United States. Thus, today, although vaccines containing only serogroup C are still useful in Europe, in the United States these are insufficient and only C- and Y-containing vaccines are protective[13,14].

Conventional vaccinology has also been relatively successful in developing vaccines against those pathogens that change their surface antigens every year, such as influenza. In this case, to face the antigenic variability of the influenza viruses, the formulation of the vaccine is altered every year and the population is vaccinated every year with the new formulation[15,16].

It is all too clear, however, that pathogens changing their antigens faster than once a year are beyond the reach of conventional vaccinology. RNA viruses, such as HIV, are an extreme example of how in the absence of proof-editing of the genetic code a virus can generate antigenically different forms every day and bypass any attempt to make a conventional vaccine[17,18].

The second gap: T cell immunity

Most vaccines licensed so far induce antibodies, which are responsible for the protection induced by the vaccine (**Fig. 3**). We have been successful in developing vaccines against diphtheria, tetanus, polio, *H. influenzae* type B, hepatitis A, hepatitis B, rabies, measles, mumps, rubella, influenza, papillomavirus, pneumococcus and meningococcus because these pathogens can be entirely prevented by appropriate amounts of immunoglobulin G antibodies in the serum[19,20] (**Fig. 3**). In some

instances, we also know that the vaccine protection seems to go beyond the protection mediated by the antibodies alone; however, we have no way to quantify it. We know, for example, that the Sabin oral polio vaccine induces concentrations of serum antibodies that are lower than those induced by the killed Salk vaccine. We suspect that the Sabin vaccine, which multiplies in the gut, is likely to induce a protective immunity that goes beyond immune protection mediated solely by serum antibodies; however, we do not know how to measure the extra protection[21–23].

Other examples where T cells are likely to contribute to immune protection are live attenuated vaccines such as varicella zoster virus[24]. The recently developed live attenuated vaccine against influenza provides a similar paradigm. One dose of vaccine has been shown to induce a protection superior to that induced by two doses of inactivated influenza vaccine, despite lower recorded serum antibodies[25]. The literature suggests that the extra protection induced by the live vaccine must be due to the cytotoxic T cells induced by the replicating virus, and perhaps to mucosal immunity at the portal of pathogen entry. Even so, no one has yet been able to provide an assay that can correlate the observed protection with an *in vitro* measure of T-cell or mucosal immunity. As a consequence, although regulatory agencies normally approve vaccines based on serum antibody measurements, they are not in the position to use mucosal or T-cell immunity to license vaccines.

A large body of literature suggests that cytotoxic T cells are important in protection from infectious diseases and cancer, and many vaccines are being tested to see whether T-cell–based vaccines can actually protect on their own. Despite the scientifically sound theory behind T-cell based immunity, and many clinical trials trying to prevent infection and disease by eliciting T cells alone, today we have indications and trends, but not one single solid example of an effective T-cell based vaccine. Therefore, the requirement of T cell immunity for protection is still an open question in vaccine development (**Fig. 3**).

In this field, the evidence that $CD8^+$ T cells can control viral infections comes largely from HIV. Given the failure to protect using the antibody-based gp120 vaccines[26,27], T-cell–based vaccines have been widely tested in nonhuman primates. The consensus emerging from these studies is summarized in **Figure 4** (refs. 17,28). T-cell vaccination has never been able to prevent infection; however, it has been consistently able to reduce the peak viremia after infection and to reduce ~100-fold the viral load for a prolonged period. Eventually, in most

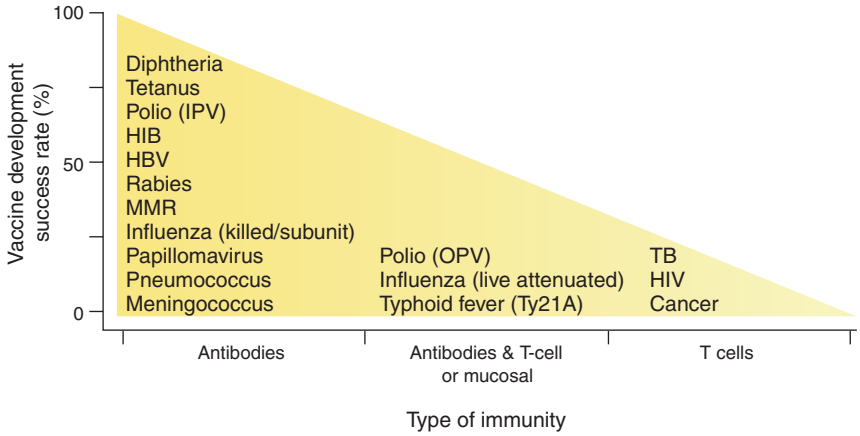

Figure 3 Schematic representation showing that the success rate of vaccine development decreases with the decreasing ability of antibodies to confer protective immunity. Vaccines mentioned in the figure are selected examples of each category and not a complete list. IPV, inactivated poliovirus; OPV, oral poliovirus; TB, tuberculosis; other abbreviations as in **Figure 2**.

cases, variants escaping T-cell control emerge, and viral load returns to that seen in unvaccinated controls. In contrast, passive immunization with antibodies or the administration of antibody-inducing vaccines is able to prevent infection against a challenge with the virus strain used to make the vaccine, but unfortunately not against other viral strains. Although there is a lot of debate as to whether the initial reduction in viremia induced by T-cell vaccines results in a clinical benefit, the pattern of protection (**Fig. 4**) seems to suggest the following: antibody-based vaccines are excellent in providing protection from infection; in contrast, T-cell–based vaccines are unable to prevent infection, but may be important in controlling an established infection. Comparable data from several other pathogens, including hepatitis C virus[29], back up this picture.

Indeed, a similar conclusion can be reached for vaccines against cancer and therapeutic vaccines in general, which are based on inducing cytotoxic T cells. To create T-cell–based vaccines, several systems in animal models and humans have been recently developed to specifically induce cytotoxic T cells, including DNA vaccines, replicating and nonreplicating viral vectors (for example, adenovirus and poxviruses), mixed regimes based on DNA priming and viral-vector boosts, bacterial vectors (for example, *Listeria*), and adjuvants[18,30]. Despite all of these technical developments, proof-of-principle that a pure T-cell vaccine is protective on its own is still lacking. And recently, the premature termination for lack of clinical efficacy of Merck's (Whitehouse Station, NJ, USA) V520 HIV prime/boost vaccine (a trivalent vaccine of *gag, pol* and *nef*) has not only cast more serious doubts on whether 'T-cell only' vaccines can be used in prevention but also challenged the validity of the results obtained in nonhuman primates shown in **Figure 4**. In fact, the vaccine based on adenovirus vector to deliver gag HIV T-cell epitopes not only failed to protect patients from disease, but also had little impact on viral load—a result that was somewhat expected from the studies in nonhuman primates (**Fig. 4**; ref. 31). It is possible that this result may represent a proof-of-concept that pure T-cell vaccines are not a solution for preventive vaccines.

In contrast, for those vaccines that are capable of inducing local mucosal immunity, we do have proof-of-concept that they can be effective and that mucosal immunity contributes to protection by neutralizing pathogens at their portal of entry. Even so, despite many successful vaccines developed in mice—and several adjuvants and delivery systems developed to specifically induce local immunity—a live oral vaccine against salmonella (T21A), a killed oral vaccine against cholera and a live attenuated intranasal vaccine against influenza represent the only mucosal vaccines currently on the market[22,23,25]. Although these mucosal vaccines have been shown to be efficacious in protecting from disease, we do not yet understand their mechanism of action and we do not have an assay that correlates with protection.

The first bridge: reverse vaccinology

Figure 5 illustrates vaccine development in the form of a graph where the two gaps for vaccine development (antigen variability and dependence on serum antibodies) are used as the horizontal and vertical axes, respectively. The vaccines available today, based on conserved antigens and serum antibodies, are mostly in the upper right red quadrant and extend to the left quadrant to capture some of the variable

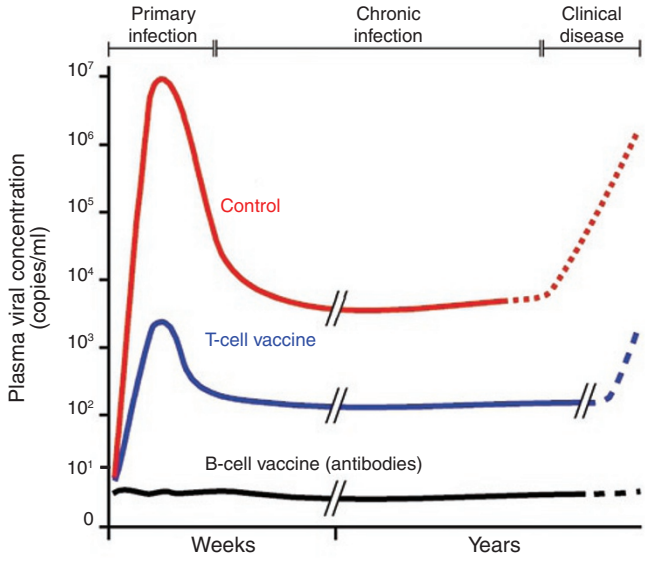

Figure 4 Schematic diagram of the HIV infection cycle and the predicted outcome of a T-cell and B-cell vaccination as deduced from vaccination of nonhuman primates. Although T-cell vaccination is not predicted to prevent infection, it is predicted to control the viral load until escape mutants arise. Vaccines inducing neutralizing antibodies are able to prevent infection but cannot cope with the antigenic variability.

antigens such as pneumococcus and influenza. Although existing vaccines include an impressive number of diseases and have improved the quality of life, they still cover only a small portion of the space that could be addressed by vaccination.

How can we tackle those diseases that have not been addressed by conventional vaccinology? The availability of genomic sequences has allowed us to use computers to search the entire genetic repertoire for protective antigens, thus increasing by several orders of magnitude the number of antigens available for vaccine development. With many more antigens available for each pathogen, it is now possible to select those antigens that respond to validated principles, such as limited or absent antigenic variability and antibody-mediated protection. Because this approach to vaccine discovery starts with the analysis of the information contained in a computer instead of with growing pathogens, it has been named 'reverse vaccinology'[32].

A classic example of genome-based antigen discovery is meningococcus B. In this case, the chemical composition of the capsular polysaccharide, which is a polysialic acid identical in structure to a self-antigen present in many human glycoproteins, prevented use of a conjugate vaccine, an approach that had been very successful against meningococcus serogroups A, C, Y and W135. Searching for alternative approaches, during the past several decades, conventional vaccinology had come up with vaccines based on detergent extracts of whole bacteria (outer membrane vesicles or OMVs). These are excellent vaccines, which can be very effective in eliminating epidemics caused by single strains (an example is the recent elimination of the New Zealand epidemic caused by meningococcus serosubtype P1. 4 (ref. 33 and http://www.moh.govt.nz/moh.nsf). But such vaccines are not effective against endemic disease, which is typically caused by strains that have huge antigenic variability in the PorA protein—the protective antigen in OMV vaccines. Until very recently, OMV vaccines were the only option and conventional approaches provided little chance of identifying protective antigens against all strains. Here, the availability of genomic sequence was truly revolutionary because it made available hundreds of new vaccine candidates and facilitated the identification within a short period of time of 29 new validated vaccine targets. The 29 antigens were then prioritized to identify those that were most effective and showed less antigenic variability, and a new vaccine was designed that is able to induce protection against the majority of disease-causing strains[34,35]. The vaccine is now being tested in phase 2 clinical studies.

Reverse vaccinology is now being applied to many pathogens, and thus far it has been successful in every case in providing new, valid antigens for vaccine design. A variation on the initial concept of reverse vaccinology, the pan-genome approach, is that today several genome sequences of the same pathogen may be used to capture the genomic variability of the species[36,37]. Other variations on the theme are the identification of new vaccine targets by screening of genome-wide libraries using antibodies from convalescent subjects[38], or the direct identification of antigens by mass spectroscopy, which is now possible thanks to the knowledge of the genomic sequence[39]. Among the pathogens that have been tackled by reverse vaccinology are group B streptococcus, group A streptococcus, pneumococcus, chlamydia and staphylococcus. Even so, the approach is applicable to many more pathogens, including parasites, which so far have been recalcitrant targets for vaccine development[40]. A systematic approach to bacteria, parasites and viruses with large genomes for which a vaccine is not yet available is thus likely to provide many new vaccines and considerably expand the space covered by vaccines (**Fig. 5**).

The second bridge: addressing antigenic variability

Despite great progress, we still face some very tough pathogens that so far have eluded vaccine design efforts. These pathogens usually have the common property of showing to the immune system antigens of high variability, which allows escape from immune recognition. A typical example is HIV, which changes its antigenic surface daily, making it impossible for us to design a conventional vaccine. An improved vaccine against influenza with broader protection that would not require repeated immunization every year would also be desirable. These pathogens usually have two types of surface epitopes: those that are highly visible by the immune system, conventionally named immunodominant epitopes (which also have the property of being highly variable), and those that do not mutate and are common to all strains. Unfortunately, the latter are usually completely invisible or poorly visible to the immune system, and immunity against them cannot be elicited by conventional vaccinology. For example, in the case of HIV, the immune response is almost exclusively directed against variable epitopes on the surface of the gp120 envelope protein. At the same time, we do know that monoclonal antibodies against epitopes common to all HIV strains have been identified and that these antibodies (for example, 2F5, 2G12, 4E10 and b12) neutralize most HIV viruses *in vitro*[17] and provide complete protection from infection in animal models[41–43] (**Fig. 4**). Therefore, why are we unable to elicit an immune response against these epitopes held in common?

It is obvious that if we were able to generate a broadly neutralizing antibody response, we would bring the HIV field into the top right quadrant of **Figure 5**, and we would have the potential to solve the HIV problem immediately. The problem is that we do not understand

the molecular nature of 'immunodominant' or 'immunosilent' epitopes. To our eyes, both types of epitopes are similar; nevertheless, they must look extremely different to the immune system. To solve this problem, we need to go back to basic science and try to understand the molecular nature of immunodominance. One way of addressing this problem is to determine the three-dimensional structure of many immunodominant and silent epitopes and try to understand the basic structural rules underlying them. Once we understand the rules, we should be in the position to engineer new types of immunogens capable of inducing an immune response against conserved epitopes and conquer those diseases that are still beyond our reach today. I thus believe we need to start a project for high-throughput determination of the three-dimensional structure of antigens and antigen-antibody complexes, and this project and its technology should become an intrinsic part of vaccinology. An example in this direction is the recent determination of the three-dimensional structure of the complex between gp120 and the broadly neutralizing antibody b12, which shows that the antibody recognizes seven noncontiguous conserved amino acid stretches in the CD4 receptor–binding region[44].

Hopefully, for structural vaccinology[45], we will see a trend similar to the one we have seen for reverse vaccinology. We started using genomes for vaccine development only 10 years ago; today, we do not start a vaccine project without knowing the genome. In 10 years' time, we should not be starting a vaccine project without knowing the structure of the relevant antigens.

The third bridge: harnessing T-cell and mucosal immunity

Vaccines that are licensed or are in late-stage clinical trials today make use of adjuvanted formulations based on aluminum salts or oil-in-water emulsions. We use them because they work and are safe; however, our understanding of their mechanism of action is basically the same as that of Glenny[46], who introduced alum in 1926, or Freund, who introduced oil emulsions more than 70 years ago[47]. In other words, the mechanism of action of adjuvants is still a black box. Today, for the first time, we have the opportunity to address the mechanisms by which adjuvants work *in vivo* and to use them to program the immune response[48]. The combined availability of a variety of animal models with single or multiple knockout mutations in key components of the immune response and of molecular adjuvants acting as agonists of toll-like receptors or natural killer T cells should help address some fundamental

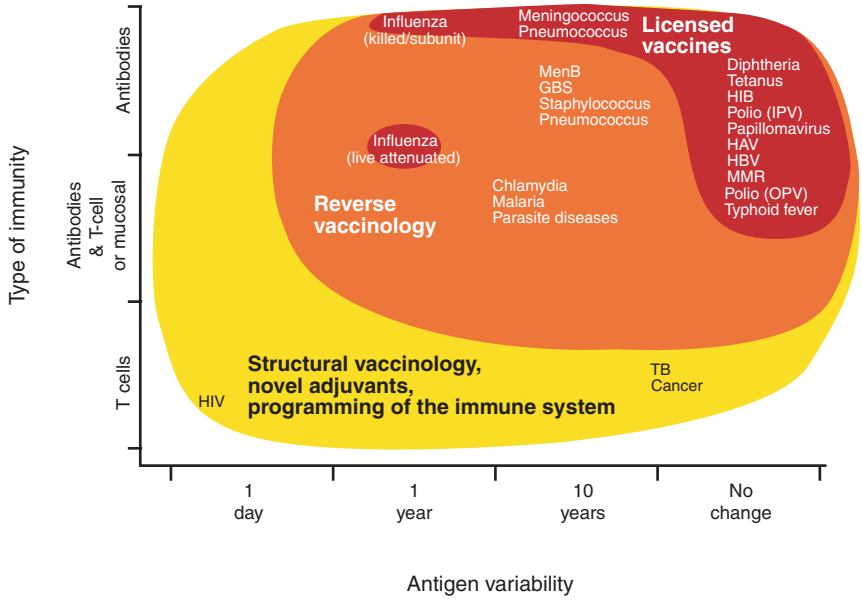

Figure 5 Known facts and knowledge gaps in vaccine development. The graph is built using antigen variability and type of immunity from **Figures 2** and **3** as horizontal and vertical axes, respectively. Existing vaccines are mostly in the upper right red quadrant. Moving down and left we exit the comfortable zone of the known, move into the unknown, and face increasingly difficult challenges. Reverse vaccinology, which has already shown to have the potential to address many diseases by mining the genomes to find relatively conserved antigens able to induce protective antibodies, may extend considerably the area covered by vaccines (orange). The most difficult challenges are in the lower and left part of the graph, where antigens are extremely variable and protection relies only on T cells. Addressing these diseases and expanding vaccine coverage to the area shown in yellow will require filling the gaps in knowledge by structural vaccinology, developing new adjuvants and learning how to rationally program the immune system. MenB, Meningococcus serogroup B; GBS, group B streptococcus; other abbreviations as in **Figures 2** and **3**.

questions that we need to answer to design the vaccines of the future. Typical questions are the following: first, is the function of $CD4^+$ T cells only to help and educate B and $CD8^+$ T cells or do such cells actually have a direct role in protection; second, are cytotoxic T lymphocytes needed at all in preventive vaccines; third, is there a role in protective immunity for B and T cells that home to mucosal tissues; and fourth, can vaccines do anything after infections have been established?

In a few instances, we have begun to get preliminary answers to these questions, but they are only hints of the avalanche of new information that we are likely to see in the near future. For example, it has been shown that in the case of *Helicobacter pylori,* protection can be achieved in the absence of antibody-producing B cells, suggesting a direct role of $CD4^+$ T cells in protection. Even so, we do not know what the effector mechanisms of $CD4^+$ T cells are and whether protection is mediated by an unknown subset of T cells[49]. The recent discovery of T-helper-17 (T_H17) T cells indicates that there are more than just T_H1 and T_H2 T-cell subsets, and that to fully exploit their potential we must understand the role of each subset and learn how we can stimulate or inhibit the development of each subset by vaccination[50,51]. Recent findings showing that an established chronic viral infection can be eradicated by the existing $CD8^+$ T cells simply by removing immunosuppression using an antibody to interleukin-10 that restores the natural antiviral function of $CD8^+$ T cells[52], or that herpes simplex virus reactivation is effectively controlled by $CD8^+$ T cells that are localized to the surface of sensory nerves[53], illustrate that we are starting to understand more of how T-cell–mediated immunity works and can be manipulated. Another example of how the immune system can be programmed to develop $CD8^+$ or $CD4^+$ T cells with defined specificity to control and eliminate established tumors is engineering lentiviral vectors to transduce hematopoietic stem cells with genes for the alpha and beta chains of T-cell receptor[54,55].

This example of 'instructive immunotherapy' shows that the rules of programming the immune system are within our reach and that it is likely that during the next decade we will see a paradigm shift in vaccinology, moving from the empirical use of what works by happenstance to the rational design of an appropriate immune response[56,57]. This may lead to the conquest of the most difficult quadrant,

shown in yellow in **Figure 5.** It will also not only extend vaccination to the prevention of the most challenging infections agents that we currently face—AIDS, tuberculosis, malaria—but also enable us to develop therapeutic vaccines to treat cancer, chronic infections and metabolic and neurodegenerative diseases.

COMPETING INTERESTS STATEMENT
The author declares competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

1. Robbins, J.B. *et al. J. Infect. Dis.* **191**, 81–88 (2005).
2. Rappuoli, R., Miller, H.I. & Falkow, S. *Science* **297**, 937–939 (2002).
3. Ellis, R.E. in *New Generation Vaccines* (eds. Woodrow, G.C. & Levine, M.M.) 439–447 (Marcel Dekker, New York and Basel, Switzerland, 1990).
4. Wenger, J., Campbell, H., Miller, E. & Salisbury, D. in *New Generation Vaccines* 3rd edn. (eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) 427–441 (Marcel Dekker, New York and Basel, Switzerland, 2004).
5. Pizza, M. *et al. Science* **246**, 497–500 (1989).
6. Greco, D. *et al. N. Engl. J. Med.* **334**, 341–348 (1996).
7. Noad, R. & Roy, P. *Trends Microbiol.* **11**, 438–444 (2003).
8. Liniger, M., Zuniga, A. & Naim, H.Y. *Expert Rev. Vaccines* **6**, 255–266 (2007).
9. Poolman, J.T. *Expert Rev. Vaccines* **3**, 597–604 (2004).
10. Levine, O.S., Klein, D.L. & Butler, J.C. in *New Generation Vaccines* 2nd edn. (eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) 443–469 (Marcel Dekker, New York and Basel, Switzerland, 1997).
11. Poehling, K.A. *et al. J. Am. Med. Assoc.* **295**, 1668–1674 (2006).
12. Singleton, R.J. *et al. J. Am. Med. Assoc.* **297**, 1784–1792 (2007).
13. Sharip, A. *et al. Pediatr. Infect. Dis. J.* **25**, 191–194 (2006).
14. Baltimore, R.S. *Curr. Opin. Pediatr.* **18**, 58–63 (2006).
15. Treanor, J.J., King, J.C. & Zangwell, K.M. in *New Generation Vaccines* 2nd edn. (eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) 537–558 (Marcel Dekker, New York and Basel, Switzerland, 1997).
16. Hilleman, M.R. *Vaccine* **20**, 3068–3087 (2002).
17. McMichael, A.J. *Annu. Rev. Immunol.* **24**, 227–255 (2006).
18. Spearman, P. *Curr. Pharm. Des.* **12**, 1147–1167 (2006).
19. Robbins, J.B., Schneerson, R. & Szu, S.C. *J. Infect. Dis.* **171**, 1387–1398 (1995).
20. Robbins, J.B., Schneerson, R. & Szu, S.C. *Adv. Exp. Med. Biol.* **397**, 169–182 (1996).
21. Dietrich, G., Griot-Wenk, M., Metcalfe, I.C., Lang, A.B. & Viret, J.F. *Vaccine* **21**, 678–683 (2003).
22. Freihorst, J. & Ogra, P.L. *Ann. Med.* **33**, 172–177 (2001).
23. Ogra, P.L. *Ann. NY Acad. Sci.* **754**, 97–107 (1995).
24. Kimberlin, D.W. & Whitley, R.J. *N. Engl. J. Med.* **356**, 1338–1343 (2007).
25. Belshe, R.B. *et al. N. Engl. J. Med.* **356**, 685–696 (2007).
26. Flynn, N.M. *et al. J. Infect. Dis.* **191**, 654–665 (2005).
27. Pitisuttithum, P. *et al. J. Infect. Dis.* **194**, 1661–1671 (2006).
28. Johnston, M.I. & Fauci, A.S. *N. Engl. J. Med.* **356**, 2073–2081 (2007).
29. Houghton, M. & Abrignani, S. *Nature* **436**, 961–966 (2005).
30. Kenney, R.T. & Edelman, R. in *New Generation Vaccines* 2nd edn. (eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) 213–223 (Marcel Dekker, New York and Basel, Switzerland, 1997).
31. Cohen, J. *Science* **318**, 28–29 (2007).
32. Rappuoli, R. *Curr. Opin. Microbiol.* **3**, 445–450 (2000).
33. Rappuoli, R., Pollard, A.J. & Moxon, E.R. in *New Generation Vaccines* 3rd edn. (eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) 421–426 (Marcel Dekker, New York and Basel, Switzerland, 2004).
34. Pizza, M. *et al. Science* **287**, 1816–1820 (2000).
35. Giuliani, M.M. *et al. Proc. Natl. Acad. Sci. USA* **103**, 10834–10839 (2006).
36. Tettelin, H. *et al. Proc. Natl. Acad. Sci. USA* **102**, 13950–13955 (2005).
37. Medini, D., Donati, C., Tettelin, H., Masignani, V. & Rappuoli, R. *Curr. Opin. Genet. Dev.* **15**, 589–594 (2005).
38. Etz, H. *et al. Proc. Natl. Acad. Sci. USA* **99**, 6573–6578 (2002).
39. Rodriguez-Ortega, M.J. *et al. Nat. Biotechnol.* **24**, 191–197 (2006).
40. Muzzi, A., Masignani, V. & Rappuoli, R. *Drug Discov. Today* **12**, 429–439 (2007).
41. Parren, P.W. *et al. J. Virol.* **75**, 8340–8347 (2001).
42. Mascola, J.R. *et al. Nat. Med.* **6**, 207–210 (2000).
43. Gauduin, M.C. *et al. Nat. Med.* **3**, 1389–1393 (1997).
44. Zhou, T. *et al. Nature* **445**, 732–737 (2007).
45. Serruto, D. & Rappuoli, R. *FEBS Lett.* **22**, 2985–2992 (2006).
46. Glenny, A.T., Popo, C.G., Waddington, H. & Wallance, V. *J. Pathol. Bacteriol.* **29**, 38 (1926).
47. Freund, J., Casals, J. & Hosmer, E.P. *Proc. Soc. Exp. Biol. Med.* **37**, 509–513 (1937).
48. Guy, B. *Nat. Rev. Microbiol.* **5**, 505–517 (2007).
49. Ermak, T.H. *et al. J. Exp. Med.* **188**, 2277–2288 (1998).
50. Harrington, L.E., Mangan, P.R. & Weaver, C.T. *Curr. Opin. Immunol.* **18**, 349–356 (2006).
51. Bi, Y., Liu, G. & Yang, R. *J. Cell. Physiol.* **211**, 273–278 (2007).
52. Brooks, D.G. *et al. Nat. Med.* **12**, 1301–1309 (2006).
53. Zhu, J. *et al. J. Exp. Med.* **204**, 595–603 (2007).
54. Yang, L. & Baltimore, D. *Proc. Natl. Acad. Sci. USA* **102**, 4518–4523 (2005).
55. Yang, L., Bailey, L., Baltimore, D. & Wang, P. *Proc. Natl. Acad. Sci. USA* **103**, 11479–11484 (2006).
56. Zanetti, M. & Franchini, G. *Trends Immunol.* **27**, 511–517 (2006).
57. Kaufmann, S.H.E. *Nat. Rev. Microbiol.* **5**, 491–504 (2007).