Chapter 3

Vaccine Design in the 21st Century

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1 INTRODUCTION

Vaccines are well-established medical interventions capable of preventing infectious disease. There are many notable vaccine success stories, starting more than 200 years ago with the earliest work by Jenner that led to a cowpox-based immunization to prevent smallpox disease. Subsequent work by Pasteur during the 19th century refined and consolidated the basis of vaccinology through the principles of isolation, inactivation, and administration of key components from disease-causing pathogens. Relatively soon, this basis had enabled the development of several "first generation" vaccines that afforded protection against rabies, typhoid, cholera, and plague (within the 19th century), followed by tuberculosis, yellow fever, and pertussis by the first half of the 20th century. Breakthroughs in mammalian cell culture technology in the second half of the 20th century led to the development of "second generation" vaccines, protecting against polio, measles, rubella mumps, and varicella (as reviewed previously[1]). In the late 20th century the first polysaccharide and glycoconjugate vaccines were developed, some of which have been refined and are implemented on a global scale.

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Despite estimates that vaccines have saved several hundred million cases of disease and more than 100 million deaths, there are still numerous pathogens causing globally significant morbidity and mortality, for which effective vaccines are not yet available. Here, we describe the existing and emerging technologies and strategies that we believe will be crucial for design of next generation vaccines to address unmet medical needs relevant across the world in the 21st century.

2 STRATEGIES FOR MODERN VACCINE DESIGN

2.1 Glycoconjugate Vaccines

In the mid-20th century, plain polysaccharide vaccines were developed to protect against pneumococcal, meningococcal, and *Haemophilus influenzae* type B (Hib) infection and disease. Such vaccines were based on the use of capsular polysaccharide (CPS) preparations derived from the surface of these bacteria. The high abundance and surface-exposure of CPS make them readily accessible to antibodies and thus susceptible to opsonophagocytosis and complement-mediated bactericidal killing, the two main processes underlying polysaccharide vaccine-induced immunity. However, plain polysaccharide vaccines were effective in adults but not in infants and young children, and therefore improvements were required.

A major breakthrough in the 1980–90s was the development and implementation of glycoconjugate vaccines, using CPS components chemically conjugated to carrier proteins,[2] such as the chemically detoxified diphtheria or tetanus toxoids (DT or TT), or CRM197 a nontoxic mutant of diphtheria toxin.[3] Covalent coupling of CPS to a carrier protein enables recruitment of T-cell help, resulting in the generation of an affinity-matured and protective immune response in all age groups. The first glycoconjugate vaccine targeted Hib and dramatically reduced Hib meningitis and patient mortality following introduction in North America.[4]

While the great majority of Hib disease was caused by one serotype, more complex epidemiology exists for many other pathogens, for which several immunologically distinct serogroups (or serotypes) circulate and cause disease. For such pathogens, broadly protective glycoconjugate vaccines can be designed by including multiple CPS serogroups in a "multivalent" formulation. For example, a highly successful 7-valent glycoconjugate vaccine against *Streptococcus pneumoniae* conferred large reductions in pneumococcal meningitis and invasive pneumococcal disease in all age groups, between 1998 and 2007.[5] However, while such multivalent vaccines are broadly protective, there are now more than 90 distinct disease-causing pneumococcal serotypes, suggesting that an alternative pneumococcal vaccine based on one or a few highly conserved protein antigens, rather than a complex formulation of many different CPS components, would increase breadth of protection and ease of manufacturing.[6]

Glycoconjugate vaccines have also been developed and implemented to protect against *Neisseria meningitidis*. In 1999 a monovalent formulation was introduced in the United Kingdom to control the hyperendemic *N. meningitidis* serogroup C (MenC). Routine nationwide implementation directly reduced MenC disease, acquisition and carriage, and conferred a herd protection effect.[7] Subsequently, tetravalent glycoconjugate vaccines have been licensed to protect against *N. meningitidis* serogroups A, C, W, and Y.[8] Perhaps most remarkably of all has been the rapid development and broad deployment of the monovalent glycoconjugate vaccine (MenAfriVac™) to protect against MenA in sub-Saharan Africa, a region that experiences annual meningococcal outbreaks and devastating epidemics. The MenAfriVac vaccine was pioneered by the "Meningitis Vaccine Project" (MVP)[9] and within a decade it was administered on a large public health scale in several neighboring African countries with excellent results both in preventing MenA disease and in eliminating carriage, likely aided by strong herd protection.[10,11] Building on the success of the MVP, a similar pentavalent glycoconjugate vaccine to protect against MenACWYX is now under preclinical development. Promising preclinical studies have also shown that glycoconjugate vaccines of MenX CPS combined with CRM197 could be developed to protect against MenX, currently emerging in Africa.[12] However, a glycoconjugate vaccine against MenB is generally not considered viable because the MenB CPS resembles a neuraminic acid moiety present on human tissues, shows poor immunogenicity in humans, and generated debate regarding the risk of undesirable autoimmune responses.[13,14]

Glycoconjugate vaccines are also under clinical development to combat Group B streptococcus (GBS)[15] and *Salmonella* Typhi.[16] Further, while an early small-scale clinical trial using a glycoconjugate vaccine against *Staphylococcus aureus* was promising,[17] subsequent *S. aureus* trials have failed, as discussed recently.[18] Nevertheless, new trials are ongoing for a multivalent staphylococcal vaccine containing both protein and glycoconjugate antigens.[19]

Recent research has continued to build on the great achievements of the glycoconjugate vaccine field, especially by attempting to improve CPS production and conjugation methodologies. Standard glycoconjugates are prepared by CPS purification from the cultured pathogenic bacteria, followed by CPS fragmentation to generate poly- or oligosaccharides of specific composition and size. A recently developed alternative that avoids pathogen manipulation is the use of purified recombinant polymerases directing capsule biosynthesis to enable safer production of the CPS in vitro.[20] Alternatively, the impurities and batch variability associated with bacterial CPS generation could be eliminated by using chemically synthesized oligosaccharides.[21] Indeed, a synthetic oligosaccharide Hib vaccine showed clinical results comparable to those obtained using standard Hib vaccines.[22] In any case, following CPS/oligosaccharide preparation, conjugation to the carrier protein is typically a chemical reaction that covalently attaches the oligosaccharide to one sort of amino acid (usually lysine, aspartic/glutamic acid, or cysteine), available on the surface of the carrier protein.

The latter is therefore not a precisely defined site-specific conjugation, such that some variability in the glycoconjugate product is obtained. To reduce variability, a variety of chemistry-driven methods have been developed to enable controlled site-specific glycoconjugation, in principle offering to deliver glycoconjugate vaccines with better-defined labeling sites and stoichiometry.[21] Alternative genetic-based approaches are also conceivable, where rare codons could direct the incorporation of nonnatural amino acids into a recombinant carrier protein to enable its site-specific labeling with defined oligosaccharides.[21] Both these examples open the possibility to add saccharide units in selected well-exposed regions of a carrier protein without masking its beneficial protective epitopes (Fig. 3.1).

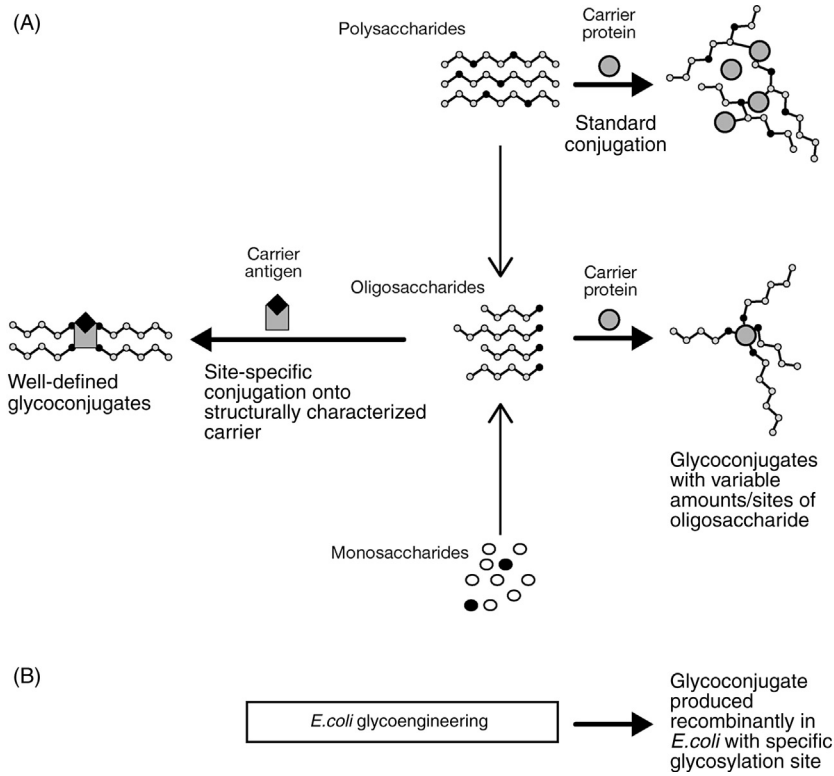

FIGURE 3.1 Glycoconjugate vaccines. (A) Progression from plain polysaccharide antigens to polysaccharide-carrier glycoconjugates (right) enabled the development of more efficacious vaccines against Hib, *S. pneumoniae*, and *N. meningitidis*. Ongoing refinements in oligosaccharide production processes, site-specific conjugation strategies, and the use of new carrier antigens that simultaneously present oligosaccharides and protective protein epitopes (♦) is expected to potentiate the development of novel well-defined glycoconjugates with enhanced safety and efficacy profiles (left). (B) An important new alternative approach to generate glycoconjugate vaccines is represented by the use of genetically modified *Escherichia coli* to directly produce recombinant glycoprotein antigens.

Novel site-specific chemical glycoconjugation approaches have shown initial promise yet appear to be hampered by relatively low yields. One study demonstrating improved conjugation yields used a copper-free reaction mechanism for tyrosine-specific labeling.[23] Further, advances in the ability to directly produce protein antigens with posttranslational addition of specific polysaccharides in *E. coli* has opened new possibilities to generate "bio-conjugate" vaccines.[24] In preclinical studies, the approach was successful in generating antigens protective against *S. aureus*.[25] Moreover, a promising bio-conjugate vaccine against *Shigella dysenteriae* was made using the polysaccharide component of the *Shigella* O1 lipopolysaccharide conjugated to exotoxin protein A of *Pseudomonas aeruginosa*, and both the CPS and carrier components were immunogenic,[26] suggesting that the bio-conjugate approach may be broadly applicable. Similarly, attempts to make hybrid antigens by combining CPS and protein carrier components that both target the same pathogen (rather than simply coupling the CPS to an unrelated carrier) have shown promise in preclinical studies targeting *Clostridium difficile*, by chemically conjugating the clostridial PSII polysaccharide and the TcdA and TcdB toxin proteins.[27] Indeed, evidence that a protein can act both as CPS carrier and as an immunogen emerged from clinical studies using pneumococcal polysaccharides conjugated to protein D from nontypable *H. influenzae*.[28] In such cases the design of conjugation sites should include structural considerations, such that CPS moieties do not perturb beneficial conformational epitopes on the carrier protein. Continued efforts in this arena will accelerate the journey toward "precision" bio/glycoconjugate vaccines safely produced in vitro with scalable production processes.

2.2 Protein Subunit Vaccines and Structure-Based Antigen Design

While glycoconjugate vaccines have been highly effective, they are not the only modern vaccine strategy available. Indeed, several early protein-based vaccines have been very successful. Notably, by purification from host pathogens followed by chemical inactivation, toxoid protein vaccines were developed in the early 20th century against diphtheria and tetanus; and, soon after, inactivated influenza virus vaccines were developed, using viral hemagglutinin purified from infected eggs as the main antigen. Later, in the 1980–90s, several bacterially produced protein subunit vaccines were licensed to protect against pertussis, and led to the proposal of a genetically detoxified form of the pertussis toxin (PT) that showed superior immunogenicity over chemically detoxified PT.[29] Finally, late in the 20th century, efforts to develop a vaccine against hepatitis B virus (HBV) led to the first widely implemented vaccines composed purely of a recombinant protein subunit (the HBV surface antigen, HBsAg).[30]

Subsequently, efforts to generate new recombinant protein-based vaccines were initiated for many other disease targets that had been difficult to address via previous technologies. One interesting example is the case of *N. meningitidis*

serogroup B (MenB), where a CPS-based vaccine was not feasible (mentioned earlier). Consequently, a protein-based subunit vaccine against MenB was sought. This challenge was greatly facilitated by the dawn of the genomic era at the turn of the 21st century, which accelerated the computational identification and selection of potential meningococcal protein antigens, an approach now termed "reverse vaccinology."[31] Extensive computational and experimental screening led to the identification of three main protective protein antigens[32] and later the development and licensure of the first genome-derived recombinant protein-based vaccine (Bexsero®) against MenB, approved by the European Medicines Agency in 2013, and subsequently in over 35 countries.[33] Reverse vaccinology has been applied to several other vaccine research programs, with promising results in the quest for protective antigens against GBS,[34] extraintestinal pathogenic *E. coli*,[35] and *S. aureus*[36] to name a few examples.

Reverse vaccinology indeed presents a speedy route to candidate identification, yet frequently reveals antigens for which prior biological information is unavailable. Given the high attention focused on vaccine safety, it is desirable that the antigenic composition of any formulation is extremely well-characterized and understood when proceeding with clinical trials, in order to ensure safety, antigen formulation stability, and reproducible vaccine efficacy. Detailed biochemical, biophysical, and structural biology investigations can combine effectively with functional studies to provide the high degree of antigen characterization required to support the vaccine development process.

In addition to providing exquisitely detailed antigen characterization, it has also been demonstrated over the last decade that structural biology, powered by X-ray crystallography, electron cryomicroscopy (cryoEM), nuclear magnetic resonance spectroscopy, and computational studies, can make a very significant contribution to the design and optimization of vaccine antigens.[37] A number of key studies demonstrating the combination of computational and structural biology in vaccine antigen design (an approach termed "structural vaccinology") have been reported, as reviewed recently.[38]

Structural vaccinology is a multidisciplinary strategy that combines the insights gained through high-resolution structural and computational biology studies with neighboring fields such as formulation science, immunology, animal studies, and serology, in order to design, evaluate, optimize, and deliver leading candidate vaccine antigens. There are at least three key ways in which structural biology can support vaccine research. First, structural biology can highlight potential weaknesses in an antigen, such that issues of poor biochemical behavior can be resolved; as exemplified by studies leading to the design of a novel form of the respiratory syncytial virus (RSV) glycoprotein F antigen in a highly stable nonaggregating postfusion conformation capable of raising high titers of neutralizing antibodies in preclinical studies.[39] Second, structural studies can reveal conformational heterogeneity in an antigen, which may suggest routes to engineer mutated forms of the antigen that adopt only the preferred conformation most likely to elicit the desired immune response. For example,

the crystal structure determination of the RSV F protein in complex with the Fab fragment of the human antibody D25 (specific for an antigenic site targeted by potently neutralizing antibodies) provided the first detailed insights into the atomic structure of the prefusion F conformation.[40] Moreover, that structure enabled the design of site-directed mutations that locked the F protein in the prefusion conformation, via the introduction of stabilizing intramolecular disulfide bonds and hydrophobic cavity-filling residues, yielding an immunogen capable of eliciting high-titers of RSV-specific neutralizing activity in mice and macaques.[41] Third, when combined with epitope mapping studies that identify the regions of an antigen that are crucial for raising protective or neutralizing antibody responses, structural information can be used to generate novel immunogenic protein surfaces with enhanced breadth of coverage due to the introduction of epitopes from multiple pathogenic variants onto a single vaccine antigen. This strategy of epitope grafting was demonstrated by engineering the meningococcal factor H binding protein variant 1 to display more than 20 surface-exposed residues from variants 2 and 3, thus generating a novel hybrid surface that conferred broader strain protection and overcame the issue of high sequence variability on meningococcal surface antigens.[42]

Structural vaccinology has been applied extensively in research toward a vaccine against human immunodeficiency virus (HIV). Efforts have focused on designing immunogens that raise protective antibody responses targeting the gp120 or gp41 components of the HIV envelope glycoprotein (Env) trimer, the only target for neutralizing antibodies. For example, the structure of CD4-bound gp120 was used for the rational design of a gp120 construct with mutations that lock it in the receptor-bound state, thus eliciting a greater proportion of antibodies focused on conserved CD4 and coreceptor binding sites.[43] Recently, cryoEM and crystal structures of HIV Env (in genetically engineered soluble and stabilized mutant forms) have been determined in complexes with broadly neutralizing Fab fragments.[44–46] These structures have provided the molecular basis for the design of novel immunogens capable of eliciting broadly neutralizing antibodies against HIV Env, and it is now a major ongoing challenge to develop such research into efficacious vaccines.

In an even more creative fashion, structural vaccinology has been combined with nanobiology, via the design of self-assembling protein nanoparticles presenting multiple copies of an antigen in an ordered array. For example, in seeking to design a broadly protective influenza vaccine, a single genetic construct was used to encode an influenza hemagglutinin (HA) antigen followed by a C-terminal bacterial ferritin protein, thus generating nanoparticles composed of 24 ferritin protomers that self-assembled to display 8 copies of the trimeric HA in a native-like conformation, with the HA head projecting outward. In preclinical studies, this antigen-nanoparticle was successful in raising anti-HA antibodies targeting both the stem and the receptor-binding site in the head, and provided broader and more potent immunity than standard influenza vaccines.[47] More recently, the same authors also performed iterative structure-based design

to obtain a stable HA stem-only fragment displayed on ferritin nanoparticles. This novel HA stem-only nanoparticle lacked the immunodominant sequence-variable head domain, focused the immune response onto the immunogenically subdominant highly conserved stem region of HA, and conferred heterosubtypic protection in preclinical studies.[48]

The benefits of combining structural vaccinology and nanobiology are manifold. The considerably larger antigen-nanoparticle is more immunogenic than the individual recombinant proteins, the multiple copies in ordered arrays enhance B-cell receptor avidity and mimic the surface of the natural pathogenic organism, and the ability to genetically encode antigen display on a nanoparticle means that a precisely controlled number and orientation of antigenic constructs can be achieved, potentially allowing focusing of the immune response against a carefully selected region of the antigen identified previously by epitope mapping. It emerges from these pioneering studies that structural vaccinology has the potential to drive the design of promising new vaccine candidates, and this ability is inextricably linked to obtaining high-quality structural information, which is somewhat unpredictable and a potential hurdle, but which is becoming easier to overcome due to continuous improvements in protein crystallography[38] and major breakthroughs in cryoEM.[49] These purely structural techniques can be effectively combined with the complementary ability to reliably perform mapping of conformational epitopes in solution via hydrogen–deuterium exchange mass spectrometry (HDX-MS).[50] Because structural vaccinology is also dependent on the ability to perform epitope mapping using antibody reagents, several recent technological advances in human B-cell cloning and antibody production have potentiated structure-based antigen design enormously, and these breakthroughs are discussed later.

2.3 B-Cell Repertoires, Antibody Discovery, and the Human Immune Response

For more than 30 years it has been known that antibody-mediated immune responses are crucial for preventing infection, while T-cell-mediated effector mechanisms are important in controlling the clearance of virus-infected cells. Antibodies are the primary elements of adaptive immunity, and the induction and maintenance of protective levels of antibodies underlie the basis of the immune response to vaccination. The B-cell response is initiated by the cognate interaction between activated antigen-specific T cells and B cells that have captured and processed the antigen through the B-cell receptor (BCR). The cognate T–B interaction leads to the expansion of antigen-specific B cells and to their differentiation into short-lived plasma cells, which represent the first line of defense through the production of unmutated antibodies, usually of the IgM isotype.

The extra-follicular aforementioned response is followed by formation of the germinal center (GC) in the lymphoid organs. The GC reaction is driven by

the presence of the antigen on the surface of the follicular dendritic cells (FDCs) in the form of immune complexes, and the antigen:antibody immune complexes continuously stimulate resident antigen-specific B cells.[51] The interaction of B cells with follicular helper T cells (T_{FH}) within the GC drives proliferation, isotype switching, somatic hypermutation, and affinity maturation of the BCR leading to the generation of memory B cells and long-lived plasma cells that produce high-affinity somatically mutated antibodies of switched isotypes (typically IgG).[52] Plasma cells with higher affinity for the antigen that emerge from GCs can migrate to the bone marrow, where they persist in specialized survival niches.[53] This pool of long-lived plasma cells continuously secretes antibodies, and is therefore responsible for sustained serum antibody levels even in the absence of antigen.[51] Memory B cells generated by a GC reaction recirculate in secondary lymphoid organs and peripheral blood, are highly capable of capturing the antigen due to their high affinity BCR, and can be triggered to proliferate and differentiate into antibody-secreting plasma cells once they reencounter the antigen. Typically, the newly generated plasma cells reach a peak level in the blood on day 7 after antigenic boost and antibody titers concomitantly increase in the serum.[54]

Not all antibody responses are equally effective. T-cell-independent antibody responses to free polysaccharides are known to be short-lived, whereas T-cell-dependent antigens can elicit immunity lasting for decades or a lifetime. The continued dissection of the basic mechanisms defining the dynamics of the immune response to vaccination and a deeper knowledge on the correlates of vaccine-induced protection or biological signatures of responsiveness are fundamental aspects in the development of novel vaccines in the 21st century.

Nearly all licensed vaccines confer protection against infectious diseases by stimulating the production of antibodies by B cells, but the nature of a successful antibody response has been difficult to capture. The isolation and characterization of the antibodies produced by the antigen-specific B-cell repertoire has therefore acquired importance in the last decades, to dissect the response to vaccine antigens. Antibodies consist of heavy (μ, α, γ, δ, ε) and light chains (κ, λ), are linked by disulfide bonds, and each chain contains variable and constant domains. Antigen binding occurs in the variable domain, which is generated by recombination of a finite set of tandemly arranged variable (V), diversity (D), and joining (J) germline gene segments. This process, called VDJ recombination, assures a high diversity of the antibody repertoire and allows antibodies to recognize an extraordinary variety of antigens. Diversity in the antibody repertoire is mainly concentrated at the variable site of the heavy chain (IgH VDJ gene segment), also known as the IgH complementarity-determining region 3 (CDR-H3), the most diverse component in terms of length and sequence and the principal determinant of antibody specificity.[55]

A milestone in the understanding of antibody responses has been the development of technologies for the production of human monoclonal antibodies (mAbs) by using Epstein–Barr (EB) virus transformation,[56] by phage display,[57]

in genetically modified mice,[58,59] by stimulation with TLR agonists,[54] or by producing human hybridomas[60] for immortalization of antibody-producing B cells. Since 2008, advances in sequencing technologies have enabled the amplification and cloning into expression vectors of both the heavy and light chain immunoglobulin (Ig) genes from single B cells,[61] allowing isolation and synthetic production of human mAbs by transfection of producer cells in vitro. To date, this technology has been mainly applied to identify high-affinity influenza-specific antibodies[62] and to isolate broadly neutralizing antibodies (bnAbs) against HIV.[63] These first examples of the isolation and characterization of bnAbs induced by infection have highlighted that understanding the mechanisms leading to the elicitation of neutralizing antibodies can aid the design of more effective vaccines. Such methods have been used to investigate mAbs generated against a variety of antigens, and have allowed characterization of "key" antibodies with a protective role in response to vaccines against influenza, tetanus, Hib, and some serotypes of *S. pneumoniae* as well as to natural infection (reviewed in[64]). Nonetheless, one key limitation is the low-throughput of single B-cell cloning technology used to isolate mAbs, such that we can only interrogate a miniscule slice of the full antibody repertoire.

Recent advances in next-generation sequencing (NGS) technology have enabled the sequencing of antibody genes from millions of cells simultaneously, giving a high-resolution characterization of the antibody sequence repertoire, and of the changes that occur following vaccination.[65] These approaches have yielded important insights into the B-cell response and have raised the possibility of using specific antibody sequences as measures of vaccine immunogenicity. The antibody repertoire has been examined using NGS after vaccination with influenza and tetanus.[66,67] These studies revealed minor changes in the VDJ segment usage, and the size and diversity of the different B-cell lineages after vaccination, but they have opened up the possibility, through the analysis of the B-cell repertoire of different individuals, to identify "antibody signatures" (common Ig VDJ sequences) providing insights into the adaptive immune responses elicited by vaccination. The majority of published studies are consistent with the notion that while the VH gene repertoire is highly private (unique to an individual) a small number of CDR-H3 appear to be shared among different individuals (ie, are stereotypical or public). Boyd and coworkers[68] observed convergent antibody signatures (stereotyped CDR-H3 sequences) in patients experiencing acute dengue infection, suggesting that Ig-sequencing aimed at detecting stereotypical responses could be used as a tool for identifying common sequences induced by vaccine antigens or pathogens in different individuals.

Further, analyses of the human antibody repertoire offer the novel possibility of tracing the evolutionary paths that lead to the generation of broadly neutralizing Abs (bnAbs) targeting conserved antigenic epitopes. The availability of new techniques to isolate human mAbs, combined with the ability to determine protein structures in atomic detail, allows to finely describe

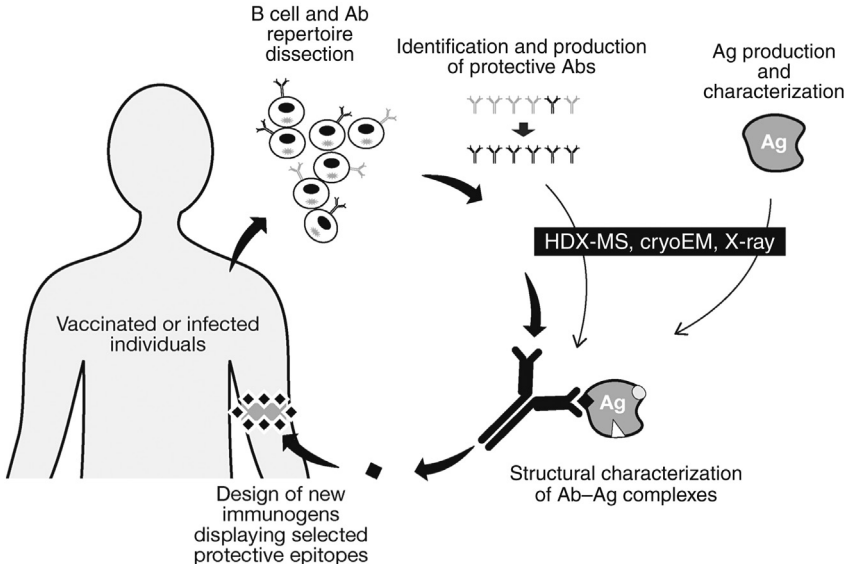

FIGURE 3.2 Starting center-left, a schematic flow-path representation of how human B-cell repertoire analyses, the selection of protective antibodies, antibody and antigen (Ag) production can be combined with the structural characterization of a protective epitope (♦), followed by its selection over nonprotective epitopes (○ and Δ within Ag, not targeted by protective Ab) in order to allow the rational design of novel immunogens.

antigen:antibody interactions. Such high-resolution epitope mapping enables the design of novel immunogens and vaccination schedules that will elicit an immune response driven by a B-cell clonal selection that leads to the production of the best bnAbs[69,70] (Fig. 3.2). Many advances in this field have been driven by the quest for a vaccine to prevent HIV infection, but should be applicable to combat other pathogens like pandemic influenza and RSV.

Finally, in recent studies, the analysis of the Ig gene repertoire has been combined with the mining of the antigen-specific mAb repertoire that comprises the human serum polyclonal response.[71] The new perspective offered by combining the analysis of the B cell and antibody repertoire induced by tetanus toxoid (TT) vaccination has highlighted that the anti-TT serum IgG repertoire is composed of a limited number of antibody clonotypes (80–100) while the B-cell BCR repertoire diversity in the memory and plasmablasts compartments is orders of magnitude greater than that of the serological repertoire.[72] This suggests that most peripheral B-cell-encoded antibodies are unlikely to be present in detectable amounts as soluble proteins in blood or secretions and thus are unlikely to contribute to humoral immunity, leaving unanswered questions regarding the nature and dynamics that regulate the serological memory. Collectively, these examples of our growing understanding of the immune response highlight a new era in which a detailed understanding of pathogenic antigen-specific

human immunology can drive vaccine development in the design of more efficacious vaccine antigens to prevent current and future pathogenic threats.

2.4 Nucleic Acid Vector Vaccine Delivery Systems

Most licensed vaccines used to date are based on immunizations that elicit a protective antibody response and indeed the correlates (or surrogates) of protection established are typically based on the functional antibody levels induced.[73] However, the immune system has evolved to be redundant, and nonantibody-based cellular immune mechanisms, which can act alone or in synergy with antibodies, can provide a major contribution to protection. With this in mind, significant efforts have been made to design novel vaccines focused on induction of cellular responses able to promote clearance of some of the most challenging pathogens, which have so far proved recalcitrant to traditional vaccine design strategies, such as malaria, tuberculosis, HIV, hepatitis C virus, and Ebola virus.

In particular, $CD8^+$ T-cell responses have been demonstrated to contribute to protection in both preclinical and clinical experiments.[74] One way to elicit such $CD8^+$ responses (which are poorly induced by conventional protein subunit antigens) is via the delivery of DNA vectors harboring genes encoding intracellular antigen expression. Several approaches have been explored to achieve this aim, including the use of naked DNA fragments or virally derived systems based on alphavirus, poxvirus, vaccinia virus, or lentivirus. Replication-defective human adenovirus 5 (Ad5) vectors have been used for gene delivery in a number of vaccine development studies and showed promising immunological performance in preclinical and clinical trials, most importantly including the ability to induce relatively potent antigen-specific $CD8^+$ T-cell responses in humans, for example, against HIV[75] and Ebola.[76] However, most humans have been previously exposed to Ad5 and thus present high titers of anti-Ad5-neutralizing antibodies, which limit the immunological potency of these vector delivery systems.

To circumvent the limitations of human adenoviral vaccine vectors, an alternative approach has been developed using related naturally occurring simian adenoviral vectors isolated from chimpanzees and against which most humans do not display neutralizing antibody titers. From thousands of adenoviral strains, a library containing numerous replication-defective chimpanzee adenovirus (ChAd) vectors able to grow in human cell lines was developed and several were demonstrated to potently induce $CD8^+$ T-cell responses in mice and macaques, and some were shown to be safe and immunogenic in humans.[77] The many noncross-reactive ChAd strains appear to be suitable candidates as vaccine delivery vectors, such that preexisting neutralizing antibodies should not be an issue for broad application of this strategy, which may enable a versatile "one vector—one disease" approach. Indeed, a number of studies have now demonstrated that ChAd vectors have the essential properties required for human vaccine development, including immunogenicity, safety and ease of large-scale

manufacturing.[78] Further recent developments in viral-based delivery of genetic vaccines include a heterologous prime-boost strategy based on the combination of a ChAd vector followed by a modified vaccinia Ankara (MVA) vector.[79] Promising results were obtained by generation of very high levels of both $CD8^+$ and $CD4^+$ T-cells specific for the hepatitis C virus antigens delivered genetically, suggesting that this approach may be suitable as a prophylactic HCV vaccine. The clinical efficacy of ChAd vectors is still to be fully demonstrated. However, in rapid response to the recent West African outbreak of Ebola virus that caused more than 8500 deaths, an expedited vaccine development program enabled a clinical trial to assess performance of a monovalent ChAd3 vaccine encoding the surface glycoprotein of *Zaire ebolavirus*. In Phase I trials, the vaccine was safe and immunogenic,[80] further supporting the optimism surrounding ChAd technology.

For over 2 decades it has been known that RNA molecules can be used to express proteins in vivo,[81,82] suggesting opportunities for RNA-based vaccines as an alternative strategy to elicit immune responses (reviewed elsewhere[83]). RNA vaccines display several advantages compared to DNA vaccines. RNA avoids the issue of possible integration of plasmid DNA into the genome of an immunized host, and it is translated directly in the cytoplasm. Finally, the kinetics of antigen expression following RNA injection appear to peak and decay rapidly, while DNA administration can induce antigen expression persisting for many weeks.[84] Overall, RNA-based vaccines better mimic antigen expression occurring during an acute infection, which could induce stronger antigen-specific immune responses. The effectiveness of RNA vaccines may also be related to the fact that RNA is known to be a potent stimulator of innate immunity. Hence, the functionality of RNA vaccines involves at least two components: (1) local expression of antigen to facilitate presentation by MHC molecules and (2) engagement of pattern recognition receptors to stimulate innate immunity leading to potentiation of antigen-specific immune responses.

Although studies in animal models seemed to be very promising, the feasibility of using RNA as a new nucleic acid vaccine was initially challenged, due to the instability of naked RNA in the presence of tissue fluids and the uncertainty of developing reasonable manufacturing processes yielding a stable formulation. Nevertheless, several efforts have been made to increase the efficiency and stability of RNA-vaccines, focusing the research on delivery systems, adjuvants, and engineering of the RNA molecule. Encapsulation in liposomes[81] and complexation with cationic polymers[85,86] can protect RNA from degradation and enhance cellular uptake. Moreover, self-amplifying replicons have the potential of capturing the advantages of both DNA vaccines and viral delivery while overcoming the drawbacks of each technology. Recently a self-amplifying RNA was encapsulated in lipid nanoparticles (LNPs) to implement the self-amplifying mRNA (SAM®) vaccine technology as a platform for multiple disease targets, showing promising results in animal models.[87] These favorable observations led RNA-vaccines to move into human clinical trials as

immunotherapeutics in the "cancer-vaccine" field, taking advantage of the expression of specific markers by cancer cells to direct the immune response and attack the tumor. RNA vaccines against proteins produced in excess in tumor cells were used to formulate a vaccine against lung cancer, designing a vaccine with different antigens which is consequently better at targeting the tumor cells.[88] Clinical studies in metastatic melanoma and renal cell carcinoma patients have shown the elicitation of antigen-specific immune responses (both antibodies and T cells).[89] RNA-vaccines against prostate cancer and melanoma are currently in clinical trials. The use of RNA-vaccines for the prevention of infectious diseases is also under evaluation. Clinical trials have been performed with RNA replicon vaccines packaged in viral particles encoding for cytomegalovirus (CMV) gB and pp65/IE1 proteins. The vaccine has shown to be well tolerated and immunogenic in healthy CMV seronegative volunteers, with the added value of inducing $CD8^+$ T-cell responses.[90] A vaccine against rabies is currently in a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02241135) while vaccines against influenza, HIV, or tuberculosis are still at the research stage.

The future of the RNA vaccines will rely on the formulation with new synthetic delivery systems to combine the effectiveness of live attenuated vaccines, an equal or better safety profile than plasmid DNA vaccines, and completely synthetic methods of manufacture.

2.5 Synthetic Viral Seeds for Rapid Generation of Influenza Vaccines

Because new influenza variants emerge and spread globally through human populations so rapidly, it is not always possible with current health organizations and manufacturing capabilities to provide new, well-matched influenza vaccines in a timely manner. In pandemic scenarios, little if any vaccine has been available during the initial waves of virus spread.[91] Recent efforts to improve vaccine responses to the emergence of new influenza variants have included research into universal influenza vaccines, increasing the number of strains in each vaccine, and increasing the speed of vaccine production. Indeed, synthetic biology now enables the rapid conversion of digitally transmitted sequences into genes that encode new influenza variants,[92] thus providing a unique tool to rapidly respond to the need of pandemic vaccine availability.

The synthetic approach to generate vaccine viruses from sequence data has proven to be feasible, starting from the available hemagglutinin (HA) and neuraminidase (NA) gene sequences, and applying cell-free gene assembly techniques for rapid and accurate gene synthesis. Viral RNA expression constructs encoding HA and NA and plasmid DNAs encoding viral backbone genes were then used to transfect Madin–Darby canine kidney (MDCK) cells, qualified for vaccine manufacture. Viruses for use in vaccines were rescued from MDCK cells with increased yield of the essential vaccine antigen, HA. The

implementation of synthetic vaccine seeds has demonstrated the capability of accelerating the response to influenza pandemics reducing the time required for vaccine manufacturing from months to weeks. In a recent emergency to respond to a potential influenza pandemic, the use of a synthetic seed virus, containing the HA and NA genes from a supplied A/H7N9 virus sequence, was investigated in conjunction with the MDCK cell culture technology. Together, these approaches resulted in impressively rapid vaccine production rates, much faster than currently possible with standard methods. Synthetic technology has been used to respond to the H7N9 influenza outbreak by producing a synthetic virus that was used to make a vaccine. In a Phase I trial the cell culture–derived H7N9 vaccine was safe and immunogenic, with significant and potentially protective immune responses after two doses in most subjects with no preexisting immunity to the H7N9 virus.[93] This particular vaccine was stockpiled by the US Government before the second wave of the outbreak, and overall these observations have provided a strong rationale for further clinical development of synthetic vaccine reagents.

3 CONCLUSIONS AND FUTURE OUTLOOK

The development of partially effective plain CPS vaccines led to the development of the first highly effective glycoconjugate vaccines around the end of the 20th century. Several glycoconjugate vaccines are now available to protect against many strains of pneumococcus, meningococcus, and *H. influenza* type B. In the first decades of the 21st century, further refinements in glycoconjugation technologies, and large clinical trials, are expected to deliver new glycoconjugate vaccines broadly protective against several additional globally important pathogens.

Nevertheless, glycoconjugate vaccines are not suitable to protect against many other important pathogens, where instead protein subunit vaccines containing protective immunogens may be effective. Recombinant protein vaccines against hepatitis B virus and serogroup B meningococcus are now widely available. The biochemical, biophysical, and three-dimensional structural characterization of protein antigens can play a major role in enabling the design and optimization of protein immunogens. The application of this strategy, termed structural vaccinology, coupled with immunological insights that can now be obtained via analyses of B-cell repertoires from infected or immunized humans, and antibody discovery and production technologies, is likely to be a key driver in vaccine development in the 21st century, and is already starting to deliver strong candidate vaccine antigens to protect against HIV, RSV, and influenza.

While most licensed vaccines are based on antibody-mediated protection, novel nucleic acid vaccine strategies capable of inducing potent cellular responses are under development to combat pathogens such as malaria, HCV, ebola, and HIV, which have so far resisted standard protein-based vaccine strategies. Notably, several replication-defective simian adenovirus nucleic

acid vectors have been shown to induce strong T-cell responses and are safe and immunogenic in humans, underlining the potential of this genetic vaccine approach. Similarly, RNA vaccines are emerging; they offer several benefits over DNA vaccines and, with improved synthetic delivery systems and manufacturability, appear to be applicable to protect against cancer or infectious disease.

In a distinct arena of vaccine technology, in order to be ready to meet the future demands of possible influenza pandemics, notable progress has been made in using cell culture technology to produce the virus, potentially from a rapidly generated synthetic nucleic acid seed, such that vaccine production can be expedited at large scale.

Collectively, all the advances outlined here demonstrate that the future is bright for the design and development of novel vaccines. Considering the additional possibilities presented by their formulation and delivery using next-generation technologies, including an increasing array of potent adjuvants (see chapter: Vaccine Adjuvants), these novel 21st-century vaccines have great promise to further reduce morbidity and mortality on a global scale.

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REFERENCES

1. Plotkin S. History of vaccination. *Proc Natl Acad Sci USA* 2014;**111**(34):12283–7.
2. Makela PH, Kayhty H. Evolution of conjugate vaccines. *Expert Rev Vaccines* 2002;**1**(3): 399–410.
3. Broker M, Costantino P, DeTora L, McIntosh ED, Rappuoli R. Biochemical and biological characteristics of cross-reacting material 197 (CRM197), a non-toxic mutant of diphtheria toxin: use as a conjugation protein in vaccines and other potential clinical applications. *Biologicals* 2011;**39**(4):195–204.
4. Wenger JD. Epidemiology of *Haemophilus influenzae* type b disease and impact of *Haemophilus influenzae* type b conjugate vaccines in the United States and Canada. *Pediatr Infect Dis J* 1998;**17**(9 Suppl.):S132–6.
5. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* 2010;**201**(1):32–41.
6. Ladhani SN, Ramsay ME. Editorial commentary: the story of sisyphus: why we need a universal pneumococcal vaccine to replace current conjugate vaccines. *Clin Infect Dis* 2015;**61**(5):776–8.
7. Borrow R, Abad R, Trotter C, van der Klis FR, Vazquez JA. Effectiveness of meningococcal serogroup C vaccineprogrammes.*Vaccine* 2013;**31**(41):4477–86.
8. Gasparini R, Panatto D. Meningococcal glycoconjugate vaccines. *Hum Vaccin* 2011; **7**(2):170–82.
9. LaForce FM, Konde K, Viviani S, Preziosi MP. The meningitis vaccine project. *Vaccine* 2007;**25**(Suppl. 1):A97–A100.

10. Kristiansen PA, Diomande F, Ba AK, Sanou I, Ouedraogo AS, Ouedraogo R, et al. Impact of the serogroup: a meningococcal conjugate vaccine, MenAfriVac, on carriage and herd immunity. *Clin Infect Dis* 2013;**56**(3):354–63.
11. Frasch CE, Preziosi MP, LaForce FM. Development of a group A meningococcal conjugate vaccine, MenAfriVac(TM). *Hum Vaccin Immunother* 2012;**8**(6):715–24.
12. Micoli F, Romano MR, Tontini M, Cappelletti E, Gavini M, Proietti D, et al. Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X. *Proc Natl Acad Sci USA* 2013;**110**(47):19077–82.
13. Robbins JB, Schneerson R, Xie G, Hanson LA, Miller MA. Capsular polysaccharide vaccine for Group B *Neisseria meningitidis*, *Escherichia coli* K1, and *Pasteurella haemolytica* A2. *Proc Natl Acad Sci USA* 2011;**108**(44):17871–5.
14. Finne J, Leinonen M, Makela PH. Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet* 1983;**2**(8346):355–7.
15. Heath PT. An update on vaccination against group B streptococcus. *Expert Rev Vaccines* 2011;**10**(5):685–94.
16. Bhutta ZA, Capeding MR, Bavdekar A, Marchetti E, Ariff S, Soofi SB, et al. Immunogenicity and safety of the Vi-CRM197 conjugate vaccine against typhoid fever in adults, children, and infants in south and southeast Asia: results from two randomised, observer-blind, age de-escalation, phase 2 trials. *Lancet Infect Dis* 2014;**14**(2):119–29.
17. Shinefield H, Black S, Fattom A, Horwith G, Rasgon S, Ordonez J, et al. Use of a *Staphylococcus aureus* conjugate vaccine in patients receiving hemodialysis. *N Engl J Med* 2002;**346**(7):491–6.
18. Bagnoli F, Bertholet S, Grandi G. Inferring reasons for the failure of *Staphylococcus aureus* vaccines in clinical trials. *Front Cell Infect Microbiol* 2012;**2**:16.
19. Scully IL, Liberator PA, Jansen KU, Anderson AS. Covering all the bases: preclinical development of an effective *Staphylococcus aureus* vaccine. *Front Immunol* 2014;**5**:109.
20. Fiebig T, Freiberger F, Pinto V, Romano MR, Black A, Litschko C, et al. Molecular cloning and functional characterization of components of the capsule biosynthesis complex of *Neisseria meningitidis* serogroup A: toward in vitro vaccine production. *J Biol Chem* 2014;**289**(28):19395–407.
21. Adamo R, Nilo A, Castagner B, Boutureira O, Berti F, Bernardes GJ. Synthetically defined glycoprotein vaccines: current status and future directions. *Chem Sci* 2013;**4**(8):2995–3008.
22. Verez-Bencomo V, Fernandez-Santana V, Hardy E, Toledo ME, Rodriguez MC, Heynngnezz L, et al. A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. *Science* 2004;**305**(5683):522–5.
23. Nilo A, Allan M, Brogioni B, Proietti D, Cattaneo V, Crotti S, et al. Tyrosine-directed conjugation of large glycans to proteins via copper-free click chemistry. *Bioconjug Chem* 2014;**25**(12):2105–11.
24. Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thony-Meyer L. Production of glycoprotein vaccines in *Escherichia coli*. *Microb Cell Fact* 2010;**9**:61.
25. Wacker M, Wang L, Kowarik M, Dowd M, Lipowsky G, Faridmoayer A, et al. Prevention of *Staphylococcus aureus* infections by glycoprotein vaccines synthesized in *Escherichia coli*. *J Infect Dis* 2014;**209**(10):1551–61.
26. Hatz CF, Bally B, Rohrer S, Steffen R, Kramme S, Siegrist CA, et al. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella dysenteriae* type 1 administered to healthy adults: a single blind, partially randomized Phase I study. *Vaccine* 2015;**33**:4594–601.

27. Romano MR, Leuzzi R, Cappelletti E, Tontini M, Nilo A, Proietti D, et al. Recombinant *Clostridium difficile* toxin fragments as carrier protein for PSII surface polysaccharide preserve their neutralizing activity. *Toxins* 2014;**6**(4):1385–96.
28. Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study. *Lancet* 2006;**367**(9512):740–8.
29. Seubert A, D'Oro U, Scarselli M, Pizza M. Genetically detoxified pertussis toxin (PT-9K/129G): implications for immunization and vaccines. *Expert Rev Vaccines* 2014;**13**(10):1191–204.
30. Keating GM, Noble S. Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B. *Drugs* 2003;**63**(10):1021–51.
31. Rappuoli R. Reverse vaccinology. *Curr Opin Microbiol* 2000;**3**(5):445–50.
32. Giuliani MM, Adu-Bobie J, Comanducci M, Arico B, Savino S, Santini L, et al. A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci USA* 2006;**103**(29):10834–9.
33. O'Ryan M, Stoddard J, Toneatto D, Wassil J, Dull PM. A multi-component meningococcal serogroup B vaccine (4CMenB): the clinical development program. *Drugs* 2014;**74**(1):15–30.
34. Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, et al. Identification of a universal Group B streptococcus vaccine by multiple genome screen. *Science* 2005;**309**(5731):148–50.
35. Moriel DG, Bertoldi I, Spagnuolo A, Marchi S, Rosini R, Nesta B, et al. Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*. *Proc Natl Acad Sci USA* 2010;**107**:9072–7.
36. Bagnoli F, Fontana MR, Soldaini E, Mishra RP, Fiaschi L, Cartocci E, et al. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 2015;**112**:3680–5.
37. Dormitzer PR, Grandi G, Rappuoli R. Structural vaccinology starts to deliver. *Nat Rev Microbiol* 2012;**10**(12):807–13.
38. Malito E, Carfi A, Bottomley MJ. Protein crystallography in vaccine research and development. *Int J Mol Sci* 2015;**16**(6):13106–40.
39. Swanson KA, Settembre EC, Shaw CA, Dey AK, Rappuoli R, Mandl CW, et al. Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. *Proc Natl Acad Sci USA* 2011;**108**(23):9619–24.
40. McLellan JS, Chen M, Leung S, Graepel KW, Du X, Yang Y, et al. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. *Science* 2013;**340**(6136):1113–7.
41. McLellan JS, Chen M, Joyce MG, Sastry M, Stewart-Jones GB, Yang Y, et al. Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. *Science* 2013;**342**(6158):592–8.
42. Scarselli M, Arico B, Brunelli B, Savino S, Di Marcello F, Palumbo E, et al. Rational design of a meningococcal antigen inducing broad protective immunity. *Sci Transl Med* 2011;**3**(91):91ra62.
43. Dey B, Svehla K, Xu L, Wycuff D, Zhou T, Voss G, et al. Structure-based stabilization of HIV-1 gp120 enhances humoral immune responses to the induced co-receptor binding site. *PLoS Pathog* 2009;**5**(5):e1000445.
44. Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, et al. Crystal structure of a soluble cleaved HIV-1 envelope trimer. *Science* 2013;**342**(6165):1477–83.
45. Pancera M, Zhou T, Druz A, Georgiev IS, Soto C, Gorman J, et al. Structure and immune recognition of trimeric pre-fusion HIV-1 Env. *Nature* 2014;**514**(7523):455–61.

46. Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, et al. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. *Science* 2013;**342**(6165):1484–90.
47. Kanekiyo M, Wei CJ, Yassine HM, McTamney PM, Boyington JC, Whittle JR, et al. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 2013;**499**:102–6.
48. Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M, Kong WP, et al. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* 2015;**21**:1065–70.
49. Bai XC, McMullan G, Scheres SH. How cryo-EM is revolutionizing structural biology. *Trends Biochem Sci* 2015;**40**(1):49–57.
50. Malito E, Faleri A, Lo Surdo P, Veggi D, Maruggi G, Grassi E, et al. Defining a protective epitope on factor H binding protein, a key meningococcal virulence factor and vaccine antigen. *Proc Natl Acad Sci USA* 2013;**110**:3304–9.
51. Amanna IJ, Slifka MK. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunol Rev* 2010;**236**:125–38.
52. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity* 2007;**27**(2):190–202.
53. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, et al. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat Rev Immunol* 2006;**6**(10):741–50.
54. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002;**298**(5601):2199–202.
55. Xu JL, Davis MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 2000;**13**(1):37–45.
56. Steinitz M, Klein G, Koskimies S, Makel O. EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* 1977;**269**(5627):420–2.
57. McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990;**348**(6301):552–4.
58. Green LL, Hardy MC, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, et al. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat Genet* 1994;**7**(1):13–21.
59. Lonberg N, Taylor LD, Harding FA, Trounstine M, Higgins KM, Schramm SR, et al. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 1994;**368**(6474):856–9.
60. Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature* 2008;**455**(7212):532–6.
61. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 2008;**329**(1–2):112–24.
62. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* 2008;**453**(7195):667–71.
63. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 2009;**326**(5950):285–9.
64. Wilson PC, Andrews SF. Tools to therapeutically harness the human antibody response. *Nat Rev Immunol* 2012;**12**(10):709–19.
65. Reddy ST, Georgiou G. Systems analysis of adaptive immunity by utilization of high-throughput technologies. *Curr Opin Biotechnol* 2011;**22**(4):584–9.

66. DeKosky BJ, Ippolito GC, Deschner RP, Lavinder JJ, Wine Y, Rawlings BM, et al. High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire. *Nat Biotechnol* 2013;**31**(2):166–9.
67. Laserson U, Vigneault F, Gadala-Maria D, Yaari G, Uduman M, Vander Heiden JA, et al. High-resolution antibody dynamics of vaccine-induced immune responses. *Proc Natl Acad Sci USA* 2014;**111**(13):4928–33.
68. Parameswaran P, Liu Y, Roskin KM, Jackson KK, Dixit VP, Lee JY, et al. Convergent antibody signatures in human dengue. *Cell Host Microbe* 2013;**13**(6):691–700.
69. Haynes BF, Kelsoe G, Harrison SC, Kepler TB. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. *Nat Biotechnol* 2012;**30**(5):423–33.
70. Kwong PD, Mascola JR. Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. *Immunity* 2012;**37**(3):412–25.
71. Wine Y, Horton AP, Ippolito GC, Georgiou G. Serology in the 21st century: the molecular-level analysis of the serum antibody repertoire. *Curr Opin Immunol* 2015;**35**:89–97.
72. Lavinder JJ, Wine Y, Giesecke C, Ippolito GC, Horton AP, Lungu OI, et al. Identification and characterization of the constituent human serum antibodies elicited by vaccination. *Proc Natl Acad Sci USA* 2014;**111**(6):2259–64.
73. Plotkin SA. Correlates of protection induced by vaccination. *Clin Vaccine Immunol* 2010;**17**(7):1055–65.
74. Kim PS, Ahmed R. Features of responding T cells in cancer and chronic infection. *Curr Opin Immunol* 2010;**22**(2):223–30.
75. Harro C, Sun X, Stek JE, Leavitt RY, Mehrotra DV, Wang F, et al. Safety and immunogenicity of the Merck adenovirus serotype 5 (MRKAd5) and MRKAd6 human immunodeficiency virus type 1 trigene vaccines alone and in combination in healthy adults. *Clin Vaccine Immunol* 2009;**16**(9):1285–92.
76. Ledgerwood JE, Costner P, Desai N, Holman L, Enama ME, Yamshchikov G, et al. A replication defective recombinant Ad5 vaccine expressing Ebola virus GP is safe and immunogenic in healthy adults. *Vaccine* 2010;**29**(2):304–13.
77. Colloca S, Barnes E, Folgori A, Ammendola V, Capone S, Cirillo A, et al. Vaccine vectors derived from a large collection of simian adenoviruses induce potent cellular immunity across multiple species. *Sci Transl Med* 2012;**4**(115):115ra2.
78. Capone S, D'Alise AM, Ammendola V, Colloca S, Cortese R, Nicosia A, et al. Development of chimpanzee adenoviruses as vaccine vectors: challenges and successes emerging from clinical trials. *Expert Rev Vaccines* 2013;**12**(4):379–93.
79. Swadling L, Capone S, Antrobus RD, Brown A, Richardson R, Newell EW, et al. A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci Transl Med* 2014;**6**(261):261ra153.
80. Rampling T, Ewer K, Bowyer G, Wright D, Imoukhuede EB, Payne R, et al. A monovalent chimpanzee adenovirus ebola vaccine—preliminary report. *N Engl J Med* 2015 Jan 28: 1–10 [Epub ahead of print].
81. Martinon F, Krishnan S, Lenzen G, Magne R, Gomard E, Guillet JG, et al. Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. *Eur J Immunol* 1993;**23**(7):1719–22.
82. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. *Science* 1990;**247**(4949 Pt. 1):1465–8.
83. Pascolo S. Messenger RNA-based vaccines. *Expert Opin Biol Ther* 2004;**4**(8):1285–94.
84. Probst J, Weide B, Scheel B, Pichler BJ, Hoerr I, Rammensee HG, et al. Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. *Gene Ther* 2007;**14**(15):1175–80.

85. Fotin-Mleczek M, Duchardt KM, Lorenz C, Pfeiffer R, Ojkic-Zrna S, Probst J, et al. Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. *J Immunother* 2011;**34**(1):1–15.
86. Scheel B, Teufel R, Probst J, Carralot JP, Geginat J, Radsak M, et al. Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. *Eur J Immunol* 2005;**35**(5):1557–66.
87. Geall AJ, Verma A, Otten GR, Shaw CA, Hekele A, Banerjee K, et al. Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci USA* 2012;**109**(36):14604–9.
88. Sebastian M, Papachristofilou A, Weiss C, Fruh M, Cathomas R, Hilbe W, et al. Phase Ib study evaluating a self-adjuvanted mRNA cancer vaccine (RNActive(R)) combined with local radiation as consolidation and maintenance treatment for patients with stage IV non-small cell lung cancer. *BMC Cancer* 2014;**14**:748.
89. Kreiter S, Diken M, Selmi A, Tureci O, Sahin U. Tumor vaccination using messenger RNA: prospects of a future therapy. *Curr Opin Immunol* 2011;**23**(3):399–406.
90. Bernstein DI, Reap EA, Katen K, Watson A, Smith K, Norberg P, et al. Randomized, double-blind, Phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers. *Vaccine* 2009;**28**(2):484–93.
91. Tizzoni M, Bajardi P, Poletto C, Ramasco JJ, Balcan D, Goncalves B, et al. Real-time numerical forecast of global epidemic spreading: case study of 2009 A/H1N1pdm. *BMC Med* 2012;**10**:165.
92. Dormitzer PR, Suphaphiphat P, Gibson DG, Wentworth DE, Stockwell TB, Algire MA, et al. Synthetic generation of influenza vaccine viruses for rapid response to pandemics. *Sci Transl Med* 2013;**5**(185):185ra68.
93. Bart SA, Hohenboken M, Della Cioppa G, Narasimhan V, Dormitzer PR, Kanesa-Thasan N. A cell culture-derived MF59-adjuvanted pandemic A/H7N9 vaccine is immunogenic in adults. *Sci Transl Med* 2014;**6**(234):234ra55.