Vaccines: Underlying Principles of Design and Testing

Sallay Kallon[1], Shahryar Samir[1] and Nilu Goonetilleke[1,2,*]

In this paper, we review the key elements that should be considered to take a novel vaccine from the laboratory through to licensure in the modern era. This paper is divided into four sections. First, we discuss the host immune responses that we engage with vaccines. Second, we discuss how *in vivo* and *in vitro* studies can inform vaccine design. Third, we discuss different vaccine modalities that have been licensed or are in testing in humans. Last, we overview the basic principles of vaccine approvals. Throughout we provide real-world examples of vaccine development against infectious diseases, including coronavirus disease 2019 (COVID-19).

OVERVIEW

In response to the first encounter with an infection (primary infection), our bodies induce a complex immune response to control and/or clear the infection. A component of this immune response is long-lived and highly specific for the infection. This "memory" immune response functions to prevent re-infection or limit serious disease following re-infection. Vaccines are treatments that induce memory immune responses in individuals. Vaccines are mostly given to "naïve" individuals, that is individuals who have never previously encountered that pathogen. A successful vaccine will prevent or greatly limit symptoms from an infection and significantly limit spread in the community (Box 1). In 1796, Jenner used cowpox pustules as an inoculum against smallpox. Subsequently, population-wide smallpox vaccination programs using less virulent vaccines, live attenuated vaccinia virus, and modified vaccinia Ankara strain were initiated. In 1980, this formerly deadly pathogen was declared eradicated globally.[1] In the United States, licensed vaccines are available for over 25 pathogens. Worldwide, vaccination programs are estimated to save between 2 and 3 million lives annually and limit morbidity in 10s of millions. Vaccination programs also provide enormous economic savings.[2]

There are diseases against which preventative vaccines have not been developed. These include HIV and malaria. Other diseases, such as tuberculosis and seasonal influenza, have suboptimal vaccines. Despite available treatments, global deaths from these four diseases alone accounted for 2.58 million deaths in 2019.[4–7] Other diseases do not cause death but life-altering morbidities. Chlamydia is a bacterial disease that primarily occurs in young people, and in women can result in pelvic inflammatory disease and infertility.[8] Decades-long (and, in the case of tuberculosis, century-long[9]) research continues against each of these infections, all of which pose unique challenges to our immune system and, in turn, vaccine design and testing.

By contrast, severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) is a novel coronavirus that emerged in humans in late 2019.[10] SARS-CoV-2 is a respiratory pathogen and the causative agent of COVID-19. The lack of pre-existing immunity to SARS-CoV-2 in humans has resulted in the global transmission of this virus. At the writing of this paper, the case-fatality rates of SARS-CoV-2 in the United States were 1.8% and globally were 2.2%.[11] To date, SARS-CoV-2 has killed > 2.5 million people globally, including > 500,000 in the United States.[11] There has been a remarkable and unprecedented global effort to design and test COVID-19 vaccines. Two COVID-19 vaccines have now gained emergency use authorization (EUA) in the United States,[12] with several more authorized in other countries (Box 2). The speed at which COVID-19 vaccines have been generated, tested, and approved in 2020 reflects advances both in our understanding of host responses to infection and vaccine-associated technologies.

In this paper, we review the key elements that must be considered to take a novel vaccine from the laboratory through to licensure in the modern era. This paper is divided into four sections. First, we discuss the host immune response that we engage with vaccines. Second, we discuss the *in vivo* and *in invitro* studies that inform vaccine design. Third, we discuss different vaccine modalities that have been licensed or are in testing in humans. Last, we overview the basic principles of vaccine approvals.

Vaccines, in part, model our host immune response to infection

Our immune response describes the complex interaction between different cell populations, collectively called immune cells, that function to recognize, clear, and control foreign pathogens. The word "foreign" is important here. Our immune system has evolved to very effectively distinguish self (our cells and human microbiome) from non-self (viruses, bacteria, parasites, and tumors). The immune response can be broadly divided into innate and adaptive immune responses; both are critical to the clearance or control of primary infections.

[1]Department of Microbiology & Immunology, UNC-Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA; [2]UNC HIV Cure Center, UNC-Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA. *Correspondence: Nilu Goonetilleke (nilu_goonetilleke@med.unc.edu)

Received January 7, 2021; accepted February 9, 2021. doi:10.1002/cpt.2207

Box 1 Herd immunity

Herd immunity describes a level of immunity within a community, whether produced through natural infection or vaccination, that is sufficient to reduce pathogen spread below a "critical point." Roy and May described the critical point being achieved in a community where, on average, an infected person infects less than one other person.[3] Not everyone in a community needs to be vaccinated to achieve herd immunity. As has been made clear by the coronavirus disease 2019 (COVID-19) pandemic, many factors impact herd immunity. These include the transmission and replication kinetics of the evolving pathogen as well as the demographics, infrastructure, and behavior of the community.

Box 2 Public Health Emergencies

Vaccine licensing authorities, including the US Food and Drug Administration (FDA) and the UK Medicines and Healthcare Products Regulatory Agency (MHRA), can expedite the development, testing, and availability of vaccines in public health emergencies. In the United States, manufacturers may submit a request for EUA to the FDA to facilitate the availability and use of their vaccine during this time.

Operation Warp Speed (OWS)

As part of a wider strategy to accelerate the development, manufacturing, and distribution of COVID-19 vaccines, OWS formed a public-private partnership between different US Federal departments and the biomedical industry. It aimed to produce and deliver 300 million doses of safe and effective vaccines with the initial doses available by January 2021.

Governmental bodies did not skip the traditional vaccine development and distribution steps; rather, steps were accelerated and initiated simultaneously. With initial funding of about $10 billion from the Coronavirus Aid, Relief, and Economic Security (CARES) Act, the most promising vaccine candidates were funded by the US Government to offset the financial risk of upscaling infrastructure for large-scale manufacturing in parallel with running large clinical trials.

The FDA was also continuously provided with efficacy and safety data during trials to accelerate the review process for EUA approval or licensure.

Innate immunity. Innate immunity is elicited earlier than adaptive immunity in response to primary infection. The innate response serves two complementary functions. First, direct antimicrobial activity against the pathogen. Innate immune cells recognize "foreign" components of pathogens collectively described as pathogen-associated molecular patterns (PAMPs).[13] PAMPs can derive from all parts of the pathogen, including lipopolysaccharide and peptidoglycans in the bacterial cell wall, viral RNA, and DNA. PAMPs bind to ligands in host cells, called pattern recognition receptors (PRRs), to rapidly induce a range of innate responses, including the production of antiviral molecules, such as interferons, synthesis of chemokines, like MIP-1β and phagocytosis, to engulf, degrade, and clear the pathogen (reviewed in ref. 14). PRRs include toll-like receptors (TLR), mannose receptors, NOD-like receptors, and RIG-I-like receptors. PRRs are expressed by innate immune cells, which include dendritic cells (DCs), macrophages, neutrophils, and epithelial cells. Following infection, pathogens reside and replicate in different parts of a cell. Accordingly, PRRs are located on the cell surface, cytosol, and in endosomal compartments of cells. PRRs can also be grouped by the type of PAMP they recognize, such as lipids, DNA, and RNA. Expression of some PRRs is limited to a specialized group of cells, called antigen-presenting cells (APCs) that include DCs, B cells, and macrophages.

The second role of the innate immune system is that activation of innate immunity is required for induction of the adaptive immune response. Here, APCs, particularly DCs, are key. Myeloid-derived DCs (mDCs) are the most effective APCs and are located throughout tissues, including mucosal surfaces.[15] The mDCs in the tissues are highly phagocytic and pinocytic. Following infection, mDCs take up degraded pathogens at the site of infection. This process also triggers mDC-PRRs, which together "activate" the DCs. Activated mDCs then migrate via afferent lymphatic vessels to local lymph nodes. During their travels (1–2 days[16]), mDCs undergo many changes described as maturation. Mature mDCs efficiently present antigens taken up in the tissues to the main mediators of our adaptive immune responses, B cells, CD4+, and CD8+ T cells.[15] The first time mDCs in the lymph nodes (LNs) present a novel antigen to naïve B and T cells is referred to as "priming." Once primed, B and T cells, in turn, become activated and differentiate into memory cells that can more efficiently respond to secondary antigenic challenge.

Vaccines emulate the process of antigenic priming, transforming naïve B and T cells to memory cells.

Humoral response. Most licensed vaccines against infections mediate protection by the induction of humoral immunity. In the humoral response, small proteins called antibodies are produced by B cells or plasma cells. Plasma cells are specialist B cells that produce large quantities of antibodies. The basic antibody unit has a "Y" shape. The variable tips of the Y (Fab) are specific for the pathogen[17] whereas the base (Fc) is nonvariable. There are several classes of Fc, each recognized by different cell receptors that initiate different cellular responses.

The antibodies we are typically most interested in for vaccination are antibodies with IgG Fc. The Fab region of these antibodies coats the surface of the pathogen preventing it from infecting cells, whereas the Fc IgG binds to phagocytes, which degrade the antigen-Ab complex. This process is called neutralization. Effective neutralizing antibodies (NAbs) can completely prevent systemic or disseminated infection by a pathogen. Non-neutralizing antibodies also exist. These antibodies still bind to the pathogens but do not interfere with their infectivity. Non-neutralizing antibodies can contribute to protective immunity by augmenting clearance of infected cells or inducing complement.

B cells become activated through B cell receptor (BCR) signaling following initial antigen encounter with activated mDCs. The BCR-antigen is internalized and the antigens are processed and presented via class II to CD4+ T cells.[18] This results in the B cells receiving additional signaling from CD4+ T cells or locally produced cytokines.[19] Activated B cells migrate through follicular dendritic cell networks where they initiate proliferation and affinity maturation creating germinal centers in LNs.[20] Affinity maturation is the process of Ig gene mutation and selection resulting in high-affinity BCRs. Some B cells also undergo class switch recombination and can differentiate further to become high-Ab producing plasma cells, living days to months.

Although affinity maturation occurs independently in each one of us, most people can generate effective antibodies in response to an infection. A successful humoral vaccine is therefore one that can induce good high affinity, ideally NAbs in the majority of vaccine recipients. IgG antibodies are the major class antibodies circulating in the blood and can persist for months or years. Vaccination with live, attenuated vaccinia virus induced anti-smallpox immunity that was sustained for > 25 years in over 90% of vaccinees.[21] In some individuals, vaccine-induced immunity against smallpox lasted 75 years.[21] This is why childhood vaccination is sufficient to induce immunity against many pathogens. Later in this review, we discuss why some pathogens, such as influenza, require annual vaccination.

T cell response. A single line of defense is never a good strategy. Pathogens that can bypass NAbs and infect cells are detected and cleared by T cells. There a two major classes of T cells, CD8+ and CD4+ T cells. CD8+ T cells specialize in killing infected cells, whereas CD4+ T cells subsets modulate their nearby cells by secreting different cytokine milieus. As mentioned above, CD4+ T cells also have important roles as helper cells for the induction of humoral immunity, as well as maintaining gut immunity.

Following infection, almost all cells (muscle, skin, nerve cells, etc.) are capable of diverting some amount of pathogen to the proteasome pathway. Proteasomes are similar to garbage disposals. Proteasomes are found in the cell cytoplasm and function to chew up proteins into smaller peptides. Proteasomes have an essential function in cells because they help recycle older proteins that can be "upcycled" for other uses in the cell. For pathogens that replicate in the cytosol (e.g., viruses), pathogen-derived peptides generated by proteasomes are presented on the surface of the infected cells with host proteins called major histocompatibility (MHC) molecules. This peptide-MHC complex flags the cell as infected to circulating T cells. The T cells have special receptors, called T cell receptors, that can bind specific peptide-MHC complexes. Proteins are also degraded by lysosomal proteolysis and loaded onto MHC-II molecules that have been transported to the endosome from the endoplasmic reticulum.[22] Peptide-MHC-II complexes are then shuttled to the cell surface for presentation to a different class of T cells, called CD4+ T cells.[22]

Once bound, naïve T cells initiate maturation and proliferation, differentiating into memory T cells. Peptide-MHC presentation to memory T cells results in rapid functional responses, including the release of lytic molecules that kill the infected cell in minutes and/or cytokines that modulate the activity of surrounding cells. Cytokines, such as IFN-γ, can activate infected cells promoting microbicidal activity. Other cytokines promote cell maturation, survival, and trafficking.

It should be noted, that most pathogens induce both CD8+ and CD4+ T cells but the ratio of CD4:CD8 T cells differ. Viruses that replicate in the cytosol typically induce a T cell response dominated by CD8+ T cells (e.g., HIV ref. 23), whereas obligate intracellular pathogens that replicate in endosomes like *Mycobacterium tuberculosis* and *Chlamydia trachomatis* induce T cell responses dominated by CD4+ T cells.[24–26] All proteins, surface and internal, of the pathogen can be degraded either by proteasomes or through lysosomal proteolysis. T cells therefore can recognize any part of the pathogen. This is an important trait, as many pathogens (e.g., HIV and influenza) avoid antibody neutralization by mutating or recombining their surface proteins.

There is an ongoing debate on how long T cell memory lasts in the absence of persistent antigen (e.g., a nonreplicating vaccine).[27] How long T cell memory lasts in humans may well reflect the original infection or the vaccine modality used. T cell responses lasting decades have been reported following inoculation with the live, attenuated vaccines bacillus Calmette-Guérin (BCG) and vaccinia (smallpox vaccine).[28,29]

Summary. To elicit a *de novo* protective memory immune response, vaccines must first be recognized by the innate system to initiate uptake of the vaccine antigen by mDCs and second to promote mDCs maturation and migration to LNs. This means the vaccine must activate PRRs on cells and be recognized as foreign. Once in the LNs, mDCs will present vaccine antigen to naïve B cells, CD4+, and CD8+ T cells inducing their differentiation into memory cells. The relative bias toward a humoral or the T cell immune response is dictated by the vaccine modality.

Preclinical vaccine research

The successful design of a vaccine requires a detailed understanding of the natural history of the disease in people. *In vitro* studies and *in vivo* animal models are also used extensively to inform vaccine design and iteratively test different vaccine modalities (**Figure 1**).

Natural history studies. Natural history studies are noninterventional, either cross-sectional or longitudinal studies of disease cohorts. These studies combine virology/microbiology, immunology, and epidemiology research to provide a detailed workup of the course of a disease. Such studies are critical to the appropriate design of preventative vaccines. They inform route of transmission and transmission kinetics, disease course, including mortality and morbidity, susceptible and resistant/controlling populations, and mechanisms of immune evasion, as well as health care and broader economic costs.

A priority of natural history studies is to determine the goal of vaccination. Ideally, all vaccines would prevent productive or disseminated infection and limit community transmission. This high bar is required for highly transmissible respiratory pathogens, as

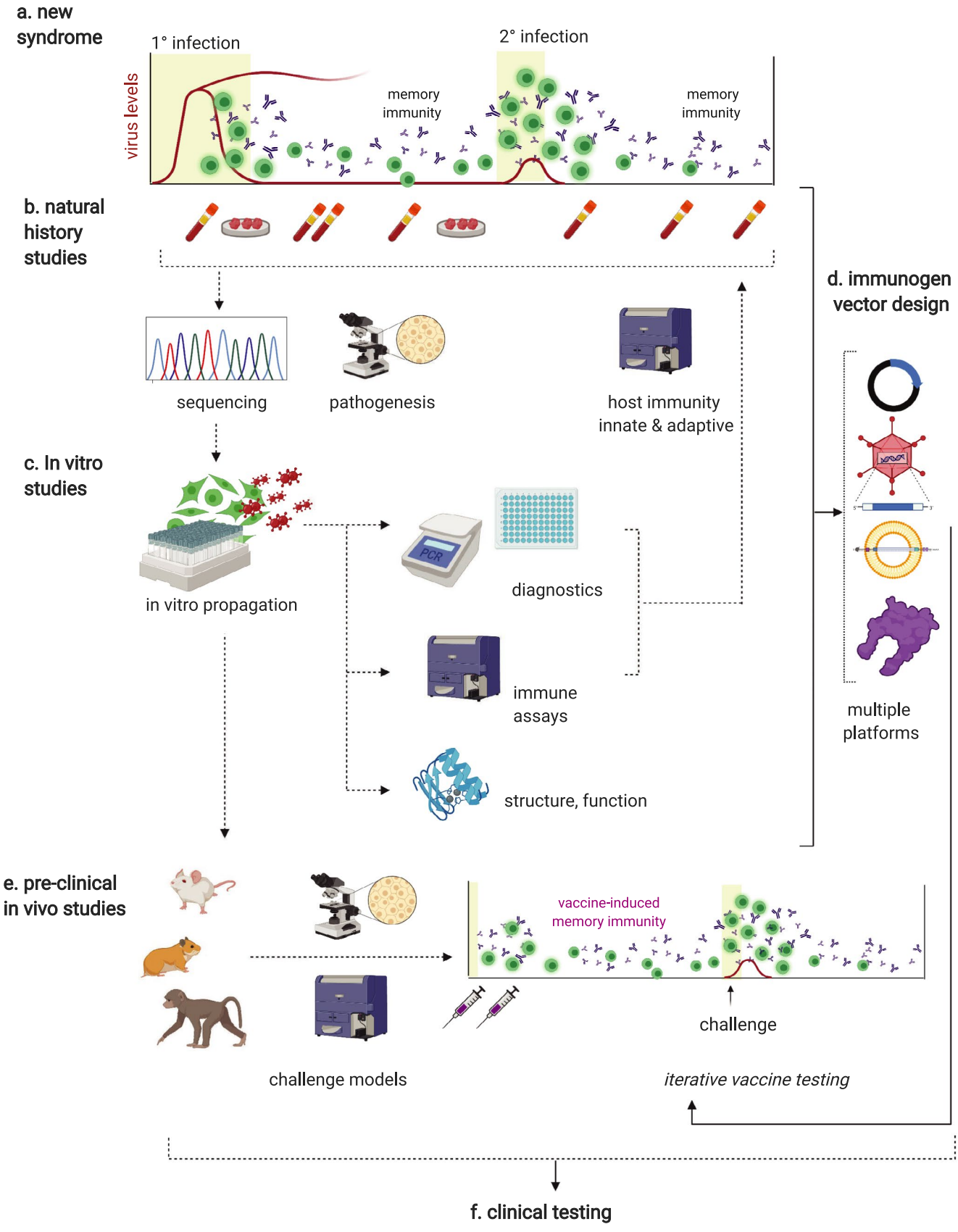
a. new syndrome
1° infection
2° infection
virus levels
memory immunity
memory immunity
b. natural history studies
sequencing
pathogenesis
host immunity innate & adaptive
d. immunogen vector design
c. In vitro studies
in vitro propagation
PCR
diagnostics
immune assays
structure, function
multiple platforms
e. pre-clinical in vivo studies
vaccine-induced memory immunity
challenge
challenge models
iterative vaccine testing
f. clinical testing

Figure 1 Overview of vaccine design and testing. (**a**) The emergence of a novel syndrome may indicate the entrance of a new pathogen in the human population. (**b**) Natural history studies initially focus on the collection of clinical samples and sequencing performed to identify and classify the pathogen. If novel, (**c**) *in vitro* studies begin, first to identify susceptible cell lines for pathogen propagation and generation of stocks. Both sequence information and pathogen stocks are used for the generation of diagnostic tests and assays to measure adaptive immunity. These are in turn used in cross-sectional and longitudinal natural history studies to document the disease pathogenesis and host immunity (innate: yellow shading; humoral: purple; and T cell immunity: green) associated with both disease susceptibility and control. (**d**) Vaccine vectors that model protective host immunity are prioritized for testing. Similarly, regions of the pathogen most commonly targeted in protective immunity are prioritized as vaccine targets or immunogens. Parallel *in vitro* structure-function studies are performed to investigate the pathogen's virulence and immune evasion mechanisms, that in some cases result in further modification of the vaccine immunogen. (**e**) Challenge studies using either the human pathogen or a closely related species are performed to identify animal models that best reflect both the disease course and host immune response observed in natural infections studies. Note, all animal models have limitations. Putative vaccines are tested iteratively in animal models; the most successful progressing to non-human primates. (**f**) Progression to clinical testing requires reproducible vaccine immunogenicity, a strong safety profile, and typically protective efficacy in one or more animal models. For severe acute respiratory syndrome-coronavirus 2, this entire process, **a**–**f**, occurred in < 12 months.

evidenced by the global spread of SARS-CoV-2 in 2020.[11] For other pathogens, such as *M. tuberculosis*, where primary infection is controlled in 90% of people, vaccine efforts focus on preventing reactivation-disease, which occurs in ~ 10 million people each year.[30] Note, the licensed tuberculosis vaccine, BCG, has higher efficacy in children and poor efficacy in adults in equatorial regions.[31] Some pathogens are not fatal but cause lifelong morbidity. *C. trachomatis* is a nonfatal and often asymptomatic infection. However, bacterial ascension in the female genital tract can result in pelvic inflammatory disease that, over time, can result in chronic pain, ectopic pregnancy, and infertility.[8] A vaccine that limits *C. trachomatis* ascension preventing pelvic inflammatory disease would be a remarkable achievement for women's health.

Natural history studies help identify which arm of the adaptive immune response should be targeted with vaccination. With COVID-19, most individuals can control infection and many are asymptomatic, although likely still shed virus.[32] Severe disease from SARS-CoV-2 associates with age, immune deficiency, obesity, and an exacerbated immune response in the lungs.[33] Another way to look at this is that the immune response to control SARS-CoV-2 can control infection in the majority of otherwise healthy people. Studies in these individuals, both young and old, found both NAbs and T cell responses targeting the SARS-CoV-2, Spike protein.[34] Altogether, natural infection studies of COVID-19 suggested that vaccines targeting Spike, particularly those inducing strong humoral immunity, were likely to be protective against SARS-CoV-2 by either preventing or limiting severe disease.

By contrast, natural infection studies of HIV have supported a greater focus on the development of T cell vaccines. The high mutation, recombination, and replication rates of HIV enable the generation of escape mutations against adaptive immune responses in natural infection.[35] HIV can tolerate extensive mutation in its surface Envelope protein, limiting the success of humoral vaccines to date. Natural infection studies have shown associations between the CD8+ T cell response against HIV and virus control.[36,37] This is consistent with an overall slower rate of HIV escape to T cells that target structural HIV proteins that are less tolerant of mutations.[38]

Natural history studies also identify those most susceptible to infection, which, in turn, identifies priorities for vaccination. It is therefore critical that natural history cohorts reflect all population demographics, most critically age. The strength of the adaptive immune response is very closely linked with age. Relative to otherwise healthy young adults, children (< 2 years) and adults > 65 years exhibit weakened immune responses following infection and more commonly experience severe disease sequelae. The much higher rates of severe COVID-19 in those aged > 65 years rapidly identified this age group and long-term care homes as priorities for vaccination.[39] Older people also produce weaker antibody responses to seasonal influenza vaccines, which prompted the development of high-dose vaccines targeted to older populations.[40] Similarly, varicella-zoster virus, which causes chickenpox mostly in children, can reactivate in nerve tissue in olderpopulations causing shingles. Natural infection studies associated shingles with diminished T cell immunity. Vaccines targeting shingles use higher doses and more potent adjuvants than childhood vaccines, to elicit a stronger T cell response (reviewed in ref. 41).

***In vitro* studies.** A broad number of *in vitro* studies are necessary to support vaccine design and testing. These range from (i) *ex vivo* propagation of the pathogen in cell lines for identification and basic characterization as well as testing in *in vivo* studies, (ii) diagnostic assays to detect infection, and (iii) immune assays to quantify the immune response following vaccination.

Here, the COVID-19 pandemic again serves as an example of how *in vitro* assays are integral to all aspects of vaccine development and testing. SARS-CoV-2 was isolated from patient samples, including bronchoalveolar lavage fluid, and sequenced using a combination of pan-coronavirus primers, next-generation sequencing, and metagenomics analysis.[10] Viral sequences were then aligned with publicly available databases to show the new pathogen was closely related to SARS-CoV, which caused an epidemic in 2002–2004 that infected lungs via the ACE-2 receptor.[42] Subsequent studies found that SARS-CoV-2, like SARS-CoV, could be propagated *ex vivo* in cell lines transduced with human ACE-2,[43,44] and *in vitro* infection could be prevented by antibodies targeting the Spike glycoprotein.[45] Virus stocks were then made available to the community for further *in vitro* testing and as challenge stocks for the development of *in vivo* animal models.

Recombinant techniques were used to express and derive the structure of SARS-CoV-2 surface proteins, which were used to define targets susceptible to antibody neutralization.[46,47] Within weeks of the identification of the SARS-CoV-2 sequence, a polymerase chain reaction-based diagnostic test was available.[48] This and other tests not only greatly improved clinical care but enabled the enrollment of participants in study cohorts for natural history

studies. Antibody microneutralization[49] and binding assays[43] were also rapidly developed to examine the humoral response in natural infection and as diagnostic tests to confirm virus infection or to identify those previously infected. These assays are also being used to examine the humoral response to vaccination, both in humans and animal models, and whether vaccine-induced antibodies can recognize and neutralize newly emerged SARS-CoV-2 variants.[50,51] T cell-based assays have also been developed to quantify and characterize T cell immunity following both natural infection and vaccination.[52] These assays typically examine the functional response of T cells, such as cytokine production and killing capacity.

While global vaccination is now underway for SARS-CoV-2, *in vitro* studies are ongoing. These include studies that aim to identify the drivers of severe disease and also examine the longevity of the adaptive immune response to both the natural infection and vaccination.

***In vivo* animal models.** Advancing a vaccine generally requires some evidence of protective efficacy in one, ideally > 1 animal model of disease. Animal models are used to identify dose ranges, optimal routes of delivery, and to directly compare vaccine responses in blood (typically the only available sample in clinical vaccine testing) to tissues, including the site of infection.

The animals most commonly used in research are inbred mice, which have a large selection of reagents available for study. Moreover, mice are small, facilitating rapid and iterative testing. Other rodent models include rabbits, guinea pigs, and hamsters, although these have fewer reagents available. Many varieties of "humanized" mice have been generated that contain human T and B cells and/or express human cytokines or receptors to better model the human immune response to infection or vaccination.[53] These chimeras, although very informative, over time suffer from graft-vs.-host disease limiting their application in long-term immunology experiments. Non-human primates (NHPs), typically rhesus macaques, cynomolgus macaques, or African green monkeys, are also used in vaccine testing. NHPs generally better recapitulate the human immune response and disease pathology to infection. Importantly, NHPs are outbred and, similar to humans, exhibit variability both in terms of disease progression and vaccine immunogenicity. Unsurprisingly, studies in NHP are limited due to availability, cost, and bioethics. Therefore proof-of-concept is typically required in a lower order animal before initiation of NHP studies.

Animal models, however, rarely recapitulate the full disease pathology or course of the human infection. This is particularly problematic for species-tropic pathogens that only infect humans. For example, mice are not naturally susceptible to SARS-CoV-2. Although, a mouse-adapted SARS-CoV-2 strain has been recently reported that recapitulates many elements of COVID-19 disease pathology.[54] Alternatively, whereas *M. tuberculosis* readily infects a range of animal models, including inbred mice, the disease pathology in the mouse lungs is distinct from humans limiting the use of pathology as a readout of vaccine efficacy.[55] The dissemination of some pathogens is also different between humans and animal models. *Chlamydia muridarium* (*C. muridarium*) is used to model *C. trachomatis* in inbred mice. *C. muridarium* infection induces pathology in the genital tract of female mice,[56] however, in contrast to women, *C. muridarium* undergoes significant dissemination to other tissues,[57] inducing much stronger circulating adaptive immunity than reported to date in humans.[58]

Unsurprisingly, these differences can impact the outcome of challenge experiments to test vaccine efficacy. Further, the design of the challenge experiment itself will impact results. For example, a vaccine is more likely to be successful against a lower dose and/or less virulent virus challenge. Conversely, very high or repeated vaccine doses may be protective in the animal challenge but may not be possible in humans due to manufacturing limitations or for reasons of safety and tolerability. Last, the readout of protection may differ in models. Some models may prioritize vaccine-induced sterilizing immunity following challenge whereas others examine pathogenic burden or focus more on disease-associated pathology. It is therefore very important that *in vivo* animal studies clearly identify caveats, produce challenge experiments that are reproducible between laboratories, and, if possible, test > 1 one animal model.

Animal models, however, provide many advantages. First, experimental parameters can be controlled to reduce group size to rapidly address key questions, for example, allowing the direct comparison of vaccine modalities. Second, informative procedures, not possible in humans, can be performed. For example, CD8-Ab depletion following simian immunodeficiency virus infection led to increased viremia in rhesus macaques, supporting human studies that CD8+ T cells were important for control of HIV, in turn, supporting T cell vaccine strategies.[59] Transgenic technology, particularly gene knockouts and adoptive cell or plasma transfer experiments in mice, have been essential to advancing our mechanistic understanding of immune response to infection and vaccination.

A less exciting but critical aspect of animal studies is their use for toxicity testing of vaccines. Good manufacturing practice-compliant, nonclinical evaluation of vaccines in animal models is generally required before first-in-human clinical testing. Detailed guidance is provided by national regulatory agencies, such as the FDA,[60] as well as the World Health Organization.[61] The goals of this type of testing are not to establish immunogenicity or protection but to characterize toxic effects and inform a safe starting dose and dose range for human testing. Parameters to be considered in the design of toxicity testing include the appropriateness of the animal species/strain, the dosing schedule, and route of vaccine administration, as well as the timing of evaluation (clinical chemistry, auto-antibody screening, and necropsy). The dose tested should maximize the animal's exposure to the candidate vaccine and the immune response induced (e.g., peak antibody response). In general, a lethal dose does not need to be determined. Reporting parameters include local inflammatory reactions and systemic effects of the vaccine, effects on reproduction and the developing fetus, and toxicity specific to the route of administration. In-life parameters should be monitored daily, including body weight and food consumption. Similarly, hematology and serum chemistry analysis should be conducted at regular intervals over the observation period. Last, a detailed necropsy, including the collection of organs for histological evaluation, is needed.

Summary. Successful vaccine development requires natural history, *in vitro*, and *in vivo* studies to be highly integrated and to plan for iterative testing of different vaccine platforms.

Vaccine design and modalities

The first human vaccines were either inactivated or attenuated whole pathogens. These vaccines work both as the immunogen, the pathogen-derived component of the vaccine, as well as an adjuvant that promotes DC maturation and antigen presentation to B and T cells. To improve both vaccine safety and production, as well as focus the vaccine-induced immune response on the most critical regions of the pathogen, newer generation vaccines commonly contain only components or subunits of the pathogen. These targeted immunogens are delivered with a range of adjuvants or expressed by recombinant vectors that initiate the immune response.

Immunogen. The first generation of vaccines against infectious diseases included smallpox, BCG, yellow fever, and oral polio. For all, vaccination induced an immune response against the entire pathogen. Recombinant technology has enabled the synthesis of vaccine immunogens containing single proteins or subunits of the pathogen. The shift to subunit immunogens has benefits in both focusing the immune response against key targets of the pathogen and in vaccine manufacture and safety. It should be noted that codon optimization is routinely used to improve the expression of recombinant immunogens.

The design of a subunit immunogen is influenced by many factors, informed by natural history studies. The most important is whether the correlate of protection is predicted to mostly rely on humoral or T cell-mediated immunity. If humoral immunity, then immunogens will be comprised of surface proteins. If T cell immunity, then immunogens can include any protein/s, surface or nonsurface. For humoral immunity, the vaccine immunogen could further focus on the region of the pathogen that binds the cell receptor that enables infection, thereby maximizing bNAb activity. By way of example, the Moderna COVID-19 mRNA vaccine encodes the full-length, prefusion stabilized Spike glycoprotein of the SARS-CoV-2 protein,[62] whereas Pfizer initially also tested an mRNA vaccine encoding the receptor-binding domain of the Spike protein, that mediates virus entry.[63] Targeting a focussed immunogen was initially supported by a large cohort study in which 90% NAb activity targeted the receptor-binding domain.[64] However, final efficacy studies by both companies used a full-length Spike and both reported > 90% protective efficacy in clinical testing.[65]

Another factor considered in immunogen design is immunogenicity. Antigens induce different levels of immune response dependent expression level (higher expression typically induces a stronger immune response), access to antigen processing pathways, and/or the number of available B or T cell epitopes. The genetic diversity between individuals also impacts the immune response to a pathogen. This is particularly relevant for T cell immunity where almost 13,000 MHC alleles have been identified in humans.[66] Natural history studies have been critical to identifying regions of pathogens that are most commonly targeted by T cells. These proteins or regions are described as immunoprevalent. An example here is T cell immunogen design for new generation vaccines against tuberculosis. Whereas *M. tuberculosis* contains around 4,000 genes,[67] both natural history and animal model studies have shown that three secreted proteins collectively called the antigen 85 complex are strongly and consistently targeted by IFN-γ secreting CD4+ T cells, the arm of the immune response considered to confer protection from reactivation.[68] Accordingly, the 85 complex or its component antigens are commonly used as immunogens in the development of new generation tuberculosis vaccines.[68,69]

Pathogen diversity is also a critical factor in immunogen design; high-throughput sequencing of clinical samples to track pathogen diversity and evolution is now a standard element of vaccine development. A vaccine immunogen should ideally elicit immune responses that will recognize a broad range of pathogen serotypes or clades. Otherwise, serotype or clade-specific immunogens may be required, significantly increasing costs of vaccine manufacture and distribution. Licensed conjugate vaccines against *Streptococcus pneumoniae*, which is the leading cause of pneumonia in children, induce humoral immunity against numerous polysaccharide antigens representing up to 23 of over 90 different serotypes.[70] Influenza vaccination is approached differently. Antigenic shift and drift of surface hemagglutinin and neuraminidase proteins results in seasonal changes in influenza serotypes.[71] In turn, populations are vaccinated yearly with humoral vaccines containing immunogens predicted to match circulating strains. A "bad flu" season typically reflects a year when the vaccine immunogen was a poor match for the dominating serotype. Recent studies are now trialing "universal" influenza vaccines targeting the more conserved hemagglutinin stalk.[72]

HIV immunogen design encompasses all the factors mentioned above. As noted, natural history studies of HIV have shown that HIV readily escapes from humoral immunity. Moreover, relative to other viral infections, the induction of bNAbs is rare.[73] When detected, HIV bNAbs typically exhibit extensive somatic hypermutation, which is very difficult to recapitulate with a single vaccine and vaccine immunogen. Although HIV also escapes from CD8+ T cell immunity, CD8+ T cells targeting regions of HIV that exhibit lower diversity (conserved) experience less escape most likely because virus mutation in these regions exerts a fitness cost on the virus.[74] Parallel strategies are therefore being pursued to develop preventative HIV vaccines that target both humoral and CD8+ T cell immunity. As expected, humoral vaccine strategies target the surface Envelope protein of HIV. However, different strategies are being taken to approach HIV diversity and generate bNAbs (reviewed in ref. 75). These include sequential vaccination with different immunogens to precisely direct B cells to generate bNAbs, using previously defined bNAbs as templates for trimer immunogens, and using glycans to mask irrelevant epitopes that prevent the induction of non-neutralizing antibodies. HIV T cell immunogens are largely comprised of conserved and immunogenic regions of HIV, predominately from Gag. These designs mostly exclude the highly variable regions in Envelope and Nef proteins.[76,77] The challenge to T cell immunogen design is to elicit high frequencies of T cells targeting multiple regions of HIV (described as breadth) to increase the chance that vaccine-induced T cells will (i) recognize the infecting virus and (ii) be able to detect and clear virus-infected cells prior to escape. To date, T cell vaccines have induced

Table 1 Overview of vaccine modalities

	Live attenuated vaccines	Inactivated vaccine	Toxoid vaccine	Subunit vaccine	RNA vaccine	DNA vaccine
Description	Contains live pathogens (bacterial or viral) that have been modified to be less virulent or harmless[a]	Can contain bacteria, virus particles, or other pathogens that have been killed or inactivated to remove any disease-producing capability[a]	Contains a toxoid (an inactivated toxin) that has been chemically or thermally treated to suppress toxicity but retain immunogenicity[b]	Contains a fragment of the pathogen that can be any molecule, most commonly a protein or polysaccharide[a]	Contains mRNA that will be used to produce antigenic proteins of the pathogenic virus[a]	Contains a DNA plasmid that contains genes which code for antigenic proteins of the pathogenic virus[a]
Mechanism	Closest thing to natural infection. Induces a CD8 T cell and T-dependent antibody response to confer long-term immunity.[a]	The inactivated pathogen can still be recognized by the immune system but is unable to reproduce. This requires periodic booster shots to reinforce immune response.[a]	The toxoid induces an immune response that can give protection from the original toxin as the molecular markers of the toxin and toxoid are preserved.[b]	Pathogenic proteins/other molecules are either isolated directly or built from the antigen's gene using a vector (bacterial, yeast, or viral). These are then administered into the body to induce an immune response.[a]	Synthetic mRNA is delivered to the bodies using a lipid nanoparticle, viral vector, or just buffer. The mRNA then transfects into immune cells where pathogenic proteins are built which induce both cellular and humoral immunity.[c]	Uptake of DNA and subsequent expression by host cells in target site produce antigenic proteins. This can induce both a cellular and humoral immune response.[c]
Immunogenicity	Provides a long-lasting and effective immune response as live microorganisms give enough antigenic stimulation for memory cell production[b]	Weaker immune response than a live attenuated vaccine. Usually requires multiple injections and adjuvants to elicit an effective immune response.[b]	Not highly immunogenic. Requires the addition of an adjuvant (aluminum or calcium salts) and multiple doses.[b]	Weaker immune response usually requiring the addition of adjuvants and, or multiple doses[b]	Induces both cellular and humoral immunity[c]	Induces both humoral and cellular immunity[c]
Safety	Not suitable for immunocompromised individuals. May pose a risk for pregnant women.[b] In rare instances, attenuated pathogen can revert to original form and cause disease.[b]	Safer than live attenuated vaccines as there is no chance of inducing disease.[b]	Nondisease causing as toxoids cannot become virulent.[b] Long-lasting and able to withstand changes in temperature, humidity, or light.[b] Rarely causes local or systemic reactions.[b]	Unable to revert to a virulent form thus cannot cause disease.[b] Safe for those that are immunocompromised.[b] Able to withstand changes in temperature, light, and humidity.[b]	Non-infectious.[a] Possibility of adverse reaction in those with autoimmune disease.[c] Can elicit an unintended immune response but this has been minimized.[a]	Noninfectious.[a] Good safety profiles.[a]
	Smallpox, measles, mumps, rubella, varicella vaccine[a]	Polio, hepatitis A, cholera vaccine[a]	Tetanus toxoid, diphtheria toxoid[b]	HPV, hepatitis B vaccine[a]	COVID-19[a]	In development (Inovio COVID-19)[a]

COVID-19, coronavirus diseae 2019; mRNA, messenger RNA.
[a]https://sites.bu.edu/covid-corps/projects/science-communication/types-of-vaccines-infographics/. [b]https://vaccine-safety-training.org/toxoid-vaccines.html. [c]https://www.gavi.org/vaccineswork/what-are-nucleic-acid-vaccines-and-how-could-they-be-used-against-covid-19.

insufficient breadth to overcome HIV diversity.[78] The development of an efficacious HIV vaccine remains one of the greatest challenges in modern medicine.

Vector/adjuvant. An effective vector/adjuvant platform should reproduce protective immunity elicited following natural infection. Adjuvants can take many forms, from the original PAMPs in live attenuated viruses to proprietary formulations that are combined with subunit immunogens.

The vector/adjuvant system also determines the strength of the immune response elicited (**Table 1**). Live attenuated vaccines (e.g., tuberculosis, smallpox, and yellow fever), attenuated by heat treatment or *in vitro* passage, are strongly and consistently immunogenic in people. However, vaccination has produced severe adverse events in immunocompromised individuals and pregnant women.[79] Fully inactivated pathogens are better tolerated but are less immunogenic and multiple vaccinations or higher doses may be needed.[80] Subunit protein or toxoid vaccines also have excellent safety profiles but also elicit weaker immunity. These vaccines are almost always delivered with specific adjuvants to stimulate PRRs and or induce localized inflammation.[81] DNA vaccines have been in development for almost 30 years (reviewed in ref. 82). In addition to having a direct adjuvant effect (bacterial-derived DNA binds the PRR, TLR-9), DNA vectors can also express host cytokines to further augment the immune response. To date, the strong immunogenicity observed in preclinical models has not consistently translated to clinical testing, however, therapeutic vaccination with DNA expressing HPV16 and HPV18 E6 and E7 proteins showed efficacy against cervical intra-epithelial neoplasia.[83] Another class of vaccine vector is replication-deficient recombinant viruses, which include modified vaccinia Ankara, VSV-G, and adenoviral vaccines. These viruses can infect cells, express the recombinant antigen, but not replicate further. Replication-deficient viruses have excellent safety records inducing both humoral and T cell immunity. In clinical testing to date, recombinant chimpadenovirus expressing the SARS-CoV-2 Spike glycoprotein afforded 70% protective efficacy and 100% protection from severe disease.[84]

Many recombinant DNA and viral vectors have been tested in combination, in "prime-boost" strategies.[85] The rationale, which has been confirmed in clinical testing, is that vaccination with different vectors expressing a common immunogen elicits a stronger immune response than homologous vaccination. The limitation of these prime-boost strategies is of course that two or more vaccines must be manufactured.

The COVID-19 pandemic has identified mRNA vaccines as a powerful vaccine vector platform capable of inducing potent bNAbs and T cell immunity.[62] Microbial mRNA has potent adjuvant activity stimulating TLR-3 and -7 expressed by APCs. Although long-term safety testing is ongoing, in theory, mRNA vaccines will be fully degraded by host RNAases and therefore are safe. mRNA vaccines will likely be used increasingly to target other diseases.

Production and delivery. An advantage of live, attenuated pathogen-vaccines, or recombinant viral vaccines is they have mechanisms to infect the cell. By contrast, considerable research has been conducted to maximize antigen loading of subunit vaccines, including the development of slow-release formulations.[86] Cell uptake of vaccines and subsequent antigen presentation can also be impacted by particulate size. As a result, investigators are examining the use of nanoparticles and microparticles for vaccine delivery (reviewed in ref. 87). Although nucleic acid (DNA and RNA) vaccines have more rapid development and manufacturing timelines than other modalities, efficient cellular delivery has been a greater challenge, limiting *in vivo* immunogenicity. Direct structural modifications and lipid nanoparticles are being used to increase stability of mRNA vaccines in the extracellular space and maximize delivery to the cytosol, whereas electroporation and optimization of signal/leader sequences have improved delivery of DNA vaccines to the nucleus (reviewed in refs 88, 89).

From bench to people

Licensure of a vaccine is typically a highly standardized and generally laborious process overseen by governmental bodies, such as the FDA in the United States, the MHRA in the United Kingdom, and the European Medicines Agency (EMA) in the European Union. An overview of this process is given in **Table 2**. In the United States, permission to proceed to a first-in-human phase I study requires submission of an investigational new drug (IND) application. The critical elements of this application are (i) detailed records of all aspects of chemistry, manufacture, and controls of the vaccine product, (ii) a clinical trial protocol that details how the vaccine will be tested including the target population, and (iii) supporting safety data, including animal toxicology and previous human experience with the vaccine or related drugs (e.g., the same vaccine vector albeit a different immunogen).

If permission to proceed is received, the FDA or regulatory body maintains oversight of the clinical study requiring the annual submission of safety data. In the United States, details of FDA-approved clinical studies (all trials, not just vaccine studies) must be made publicly available on the website, clinicaltrials.gov. Results of the study must be posted within 12 months of the final study visit of the trial. Many non-US, non-FDA trials also are posted on this website making it the best global source of information of ongoing and completed clinical trials.

Clinical testing of a vaccine is an iterative process typically involving multiple phase I studies assessing the safety and immunogenicity of many parameters including (i) vaccine dose—typically a dose-escalation is performed, (ii) route of vaccination (e.g., i.m. vs. i.d. vs. i.n.), (iii) different intervals between booster doses, (iv) different study populations—typically, beginning with lowest risk groups, and (v) also the impact of different adjuvants. This process typically takes more than 10 years. Critically, the lessons learned from these studies can greatly accelerate vaccine development against a new pathogen. When SARS-CoV-2 was identified, researchers at the University of Oxford had been testing attenuated viral vectors in people for 20 years.[90–103] This work had identified chimpadenovirus vectors as safe and strongly immunogenic, inducing both humoral and T cell immunity in different study populations. Parameters, such as dose, route of vaccination, and dosing intervals, had been previously optimized in the testing of different vaccine immunogens targeting malaria, HIV, influenza, MERS, and

Table 2 United States approvals process for clinical testing of vaccines[a]

Preclinical	• Toxicology, pharmacokinetic data to support safety and efficacy in humans • An Investigational New Drug application is submitted to the FDA containing preclinical data, full manufacturing details, previous clinical experience, and proposed clinical trial protocol. If no safety concerns are found after FDA review, clinical trials may proceed.
Phase I	• The objective is to assess the safety and dosage of vaccine in humans. If possible, data are also collected on the capacity of the vaccine to produce an immune response. • The study population involves a small group (n = 20–100) of healthy, immunocompetent adult volunteers. Study design is usually nonrandomized and open-label but can be performed as an RCT. • If no safety concerns and serious side effects reported, vaccine progresses to phase II.
Phase II	• The objective is to provide a clinically significant outcome on the safety, efficacy, and immunogenicity of the vaccine. Preparation, optimal dosage, and schedule of vaccine are identified. • The study population involves hundreds of recruits from the target population of vaccine. Study design is a single-blind or double-blind RCT where the vaccine can be tested against a placebo or another vaccine. • Multiple phase II trials may be conducted to address impact of each variable (dosage, schedule, and demographics). These follow stringent go/no-go criteria to avoid unnecessary injection of an ineffective vaccine in larger and more costly phase III trials.
Phase III	• The objective is to provide definitive data on the safety and efficacy of vaccine with a much larger and more defined sample size. The most common end point for this phase is a decrease in the occurrence of disease but other immunological correlates of protection may apply. • The study population involves recruiting thousands of participants from the target population. The study site of such a large-scale trial must have extensive epidemiological data; this trial is usually conducted at multiple centers to test the vaccine in different conditions and populations. The study design follows the single-blind or double-blind RCT format. • This phase also allows the FDA to assess the manufacturing process of the vaccine such that it is produced reliably and consistently.
BLA Submission	• The BLA is a comprehensive submission of the preclinical and clinical data on the vaccine as well as details of its manufacturing process. • Once the BLA is submitted by the manufacturer, the FDA evaluates the data to determine if the potential benefits of the vaccine outweigh the risks. Upon approval, the manufacturer is granted a license to market the vaccine to the approved population. • The FDA may also solicit the opinion of the VRBPAC during this process. This is a group of outside, independent experts from various scientific and public health disciplines.
Phase IV	• These are postapproval studies that aim to gather additional data on safety and efficacy of the vaccine. By assessing the vaccine in real-life scenarios, the studies can assess the long-term side effects as well as discover potential rare side effects that may have been missed in earlier trials. • The study design for this trial is usually either a case-control study or an observational cohort study.

BLA, Biologics License Application; RCT, randomized controlled trial; VRBPAC, Vaccine and Related Biological Products Advisory Committee.
[a]Adapted from Guidance provided by the FDA Center for Biologics and Vaccine Research.

tuberculosis. Moreover, good manufacturing practice manufacture of chimpadenovirus vectors was standardized. These decades of work, combined with an incredible human effort, led to the first volunteer receiving the ChAdOx1 nCoV-19 within months of publication of the full-length SARS-CoV-2 sequence and EUA in the United Kingdom < 6 months later (Box 2). Critically, safety and efficacy testing coupled with ongoing independent review of this and other COVID-19 vaccines continues.

CONCLUDING COMMENTS

In this review, we provide a broad overview of key principles that underly vaccine design and testing. Additional detail can be found in the primary papers and excellent reviews cited throughout this text. The development, testing and approval of COVID-19 vaccines in < 12 months reflects global scientific, industry, and governmental efforts not only in 2020 but also over several decades. COVID-19 has highlighted other challenges for vaccine development, particularly problematic for pandemics. These challenges include equitable access, vaccine preparedness, and infrastructure, and critically, public understanding and trust. Although beyond the scope of this review, these are important considerations for vaccine researchers.

FUNDING

This study was funded by the National Institutes of Health (NIH): U01AI131310 (Goonetilleke) and U19AI144181.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

1. Jezek, Z., Khodakevich, L.N. & Wickett, J.F. Smallpox and its post-eradication surveillance. *Bull. World Health Organ.* **65**, 425–434 (1987).
2. Ozawa, S. *et al*. Estimated economic impact of vaccinations in 73 low- and middle-income countries, 2001–2020. *Bull. World Health Organ.* **95**, 629–638 (2017).
3. Anderson, R.M. & May, R.M. Vaccination and herd immunity to infectious diseases. *Nature* **318**, 323–329 (1985).

4. Organization (WHO). Tuberculosis factsheet <https://www.who.int/news-room/fact-sheets/detail/tuberculosis> (2020). Accessed February 8, 2021.
5. Organization (WHO). Malaria factsheet. <https://www.who.int/news-room/fact-sheets/detail/malaria> (2020). Accessed February 8, 2021.
6. Organization (WHO). HIV/AIDS factsheet <https://www.who.int/news-room/fact-sheets/detail/hiv-aids> (2020). Accessed February 8, 2021.
7. Organization (WHO). Influenza (seasonal) factsheet <https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)> (2020). Accessed February 8, 2021.
8. Haggerty, C.L., Gottlieb, S.L., Taylor, B.D., Low, N., Xu, F. & Ness, R.B. Risk of sequelae after Chlamydia trachomatis genital infection in women. *J. Infect. Dis.* **201**(Suppl 2), S134–S155 (2010).
9. Calmette, A. & Guerin, C. Vaccination des bovides contre la tuberculose et methode nouvelle de prophylaxie de la tuberculose bovine. *Ann. Inst. Pasteur* **38**, 371–398 (1924).
10. Zhou, P. *et al*. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).
11. Dong, E., Du, H. & Gardner, L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect. Dis.* **20**, 533–534 (2020).
12. Agency FD. COVID-19 vaccines <https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/covid-19-vaccines#eua-vaccines> (2021). Accessed February 8, 2021.
13. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* **22**, 240–273 (2009).
14. Chow, K.T., Gale, M. Jr & Loo, Y.M. RIG-I and other RNA sensors in antiviral immunity. *Annu. Rev. Immunol.* **36**, 667–694 (2018).
15. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
16. Lappin, M.B., Weiss, J.M., Delattre, V., Mai, B., Dittmar, H. & Maier, C. Analysis of mouse dendritic cell migration in vivo upon subcutaneous and intravenous injection. *Immunology* **98**, 181–188 (1999).
17. Inbar, D., Hochman, J. & Givol, D. Localization of antibody-combining sites within the variable portions of heavy and light chains. *Proc. Natl. Acad. Sci. USA* **69**, 2659–2662 (1972).
18. Batista, F.D., Iber, D. & Neuberger, M.S. B cells acquire antigen from target cells after synapse formation. *Nature* **411**, 489–494 (2001).
19. Woodland, R. & Cantor, H. Idiotype-specific T helper cells are required to induce idiotype-positive B memory cells to secrete antibody. *Eur. J. Immunol.* **8**, 600–606 (1978).
20. Herzenberg, L.A., Black, S.J., Tokuhisa, T. & Herzenberg, L.A. Memory B cells at successive stages of differentiation. Affinity maturation and the role of IgD receptors. *J. Exp. Med.* **151**, 1071–1087 (1980).
21. Hammarlund, E. *et al*. Duration of antiviral immunity after smallpox vaccination. *Nat. Med.* **9**, 1131–1137 (2003).
22. Kondylis, V. *et al*. Endosome-mediated autophagy: an unconventional MIIC-driven autophagic pathway operational in dendritic cells. *Autophagy* **9**, 861–880 (2013).
23. Riou, C., Ganusov, V.V., Campion, S., Mlotshwa, M., Liu, M.K. & Whale, V.E. Distinct kinetics of Gag-specific CD4+ and CD8+ T cell responses during acute HIV-1 infection. *J. Immunol.* **188**, 2198–2206 (2012).
24. Kagina, B.M. *et al*. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am. J. Respir. Crit. Care Med.* **182**, 1073–1079 (2010).
25. Jordan, S.J. *et al*. The predominant CD4(+) Th1 cytokine elicited to chlamydia trachomatis infection in women is tumor necrosis factor Alpha and not interferon gamma. *Clin. Vaccine Immunol.* **24(4), e00010-17**, 10–17 (2017).
26. Jasenosky, L.D., Scriba, T.J., Hanekom, W.A. & Goldfeld, A.E. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. *Immunol. Rev.* **264**, 74–87 (2015).
27. Zinkernagel, R.M. What if protective immunity is antigen-driven and not due to so-called "memory" B and T cells? *Immunol. Rev.* **283**, 238–246 (2018).
28. Hammarlund, E., Lewis, M.W., Hanifin, J.M., Mori, M., Koudelka, C.W. & Slifka, M.K. Antiviral immunity following smallpox virus infection: a case-control study. *J. Virol.* **84**, 12754–12760 (2010).
29. Mangtani, P. *et al*. The duration of protection of school-aged BCG vaccination in England: a population-based case-control study. *Int. J. Epidemiol.* **47**, 193–201 (2018).
30. Anonymous. World Health Organization <https://www.who.int/news-room/fact-sheets/detail/tuberculosis> (2020).
31. Mangtani, P. *et al*. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin. Infect. Dis.* **58**, 470–480 (2014).
32. Lee, S. *et al*. Clinical course and molecular viral shedding among asymptomatic and symptomatic patients with SARS-CoV-2 infection in a community treatment center in the Republic of Korea. *JAMA. Intern. Med. 180(11), 1–6* (2020).
33. Zhou, F. *et al*. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* **395**, 1054–1062 (2020).
34. Ni, L. *et al*. Detection of SARS-CoV-2-specific humoral and cellular immunity in COVID-19 convalescent individuals. *Immunity* **52**, 971–977.e3 (2020).
35. Mansky, L.M. & Temin, H.M. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J. Virol.* **69**, 5087–5094 (1995).
36. Kiepiela, P. *et al*. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13**, 46–53 (2007).
37. Mothe, B. *et al*. Definition of the viral targets of protective HIV-1-specific T cell responses. *J. Transl. Med.* **9**, 208 (2011).
38. Ganusov, V.V. *et al*. Fitness costs and diversity of the cytotoxic T lymphocyte (CTL) response determine the rate of CTL escape during acute and chronic phases of HIV infection. *J. Virol.* **85**, 10518–10528 (2011).
39. Dooling, K. *et al*. The advisory committee on immunization practices' interim recommendation for allocating initial supplies of COVID-19 vaccine - United States, 2020. *MMWR Morb. Mortal. Wkly. Rep.* **69**, 1857–1859 (2020).
40. Robertson, C.A., DiazGranados, C.A., Decker, M.D., Chit, A., Mercer, M. & Greenberg, D.P. Fluzone(R) high-dose influenza vaccine. *Expert. Rev. Vaccines* **15**, 1495–1505 (2016).
41. Warren-Gash, C., Forbes, H. & Breuer, J. Varicella and herpes zoster vaccine development: lessons learned. *Expert. Rev. Vaccines* **16**, 1191–1201 (2017).
42. Wan, Y., Shang, J., Graham, R., Baric, R.S. & Li, F. Receptor recognition by the novel coronavirus from wuhan: an analysis based on decade-long structural studies of SARS Coronavirus. *J. Virol.* **94(7)**, e00127-20 (2020).
43. Premkumar, L. *et al*. The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci. Immunol.* **5(48)**, eabc8413 (2020).
44. Hoffmann, M. *et al*. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* **181**, 271-280.e8 (2020).
45. Walls, A.C., Park, Y.J., Tortorici, M.A., Wall, A., McGuire, A.T. & Veesler, D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* **183**, 1735 (2020).
46. Wrapp, D. *et al*. Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. *Cell* **181**, 1004–1015.e15 (2020).
47. Quinlan, B.D. *et al*. An engineered receptor-binding domain improves the immunogenicity of multivalent SARS-CoV-2 vaccines. *bioRxiv*. https://doi.org/10.1101/2020.11.18.388934.
48. Corman, V.M. *et al*. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance* **25**. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045.

49. Amanat, F. *et al*. An in vitro microneutralization assay for SARS-CoV-2 serology and drug screening. *Curr. Protoc. Microbiol.* **58**, e108 (2020).
50. McMahan, K. *et al*. Correlates of protection against SARS-CoV-2 in rhesus macaques. *Nature*. https://doi.org/10.1038/s41586-020-03041-6.
51. van Doremalen, N. *et al*. ChAdOx1 nCoV-19 vaccine prevents SARS-CoV-2 pneumonia in rhesus macaques. *Nature* **586**, 578–582 (2020).
52. Sekine, T. *et al*. Robust T cell immunity in convalescent individuals with asymptomatic or mild COVID-19. *Cell* **183**, 158–168.e14 (2020).
53. Wahl, A. *et al*. Precision mouse models with expanded tropism for human pathogens. *Nat. Biotechnol.* **37**, 1163–1173 (2019).
54. Leist, S.R. *et al*. A mouse-adapted SARS-CoV-2 induces acute lung injury and mortality in standard laboratory mice. *Cell* **183**, 1070–1085.e12 (2020).
55. Dharmadhikari, A.S. & Nardell, E.A. What animal models teach humans about tuberculosis. *Am. J. Respir. Cell Mol. Biol.* **39**, 503–508 (2008).
56. Liu, Y. *et al*. Plasmid-encoded Pgp3 is a major virulence factor for Chlamydia muridarum to induce hydrosalpinx in mice. *Infect. Immun.* **82**, 5327–5335 (2014).
57. Howe, S.E., Shillova, N. & Konjufca, V. Dissemination of Chlamydia from the reproductive tract to the gastro-intestinal tract occurs in stages and relies on Chlamydia transport by host cells. *PLoS Pathog.* **15**, e1008207 (2019).
58. Farris, C.M. & Morrison, R.P. Vaccination against Chlamydia genital infection utilizing the murine C. muridarum model. *Infect. Immun.* **79**, 986–996 (2011).
59. Schmitz, J.E. *et al*. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**, 857–860 (1999).
60. Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for industry M3(R2) nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals <https://www.fda.gov/media/71542/download> (2010).
61. (WHO). WHO Guidelines on Nonclinical Evaluation of Vaccines Annex I World Health Organization <https://www.who.int/biologicals/publications/nonclinical_evaluation_vaccines_nov_2003.pdf> (2003).
62. Widge, A.T. *et al*. Durability of responses after SARS-CoV-2 mRNA-1273 vaccination. *N. Engl. J. Med*. https://doi.org/10.1056/NEJMc2032195.
63. Mulligan, M.J. *et al*. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. *Nature* **586**, 589–593 (2020).
64. Piccoli, L. *et al*. Mapping neutralizing and immunodominant sites on the SARS-CoV-2 spike receptor-binding domain by structure-guided high-resolution serology. *Cell* **183**, 1024–1042.e21 (2020).
65. Ledford, H. Moderna COVID vaccine becomes second to get US authorization. *Nature*. https://doi.org/10.1038/d41586-020-03593-7.
66. Gonzalez-Galarza, F.F. *et al*. Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. *Nucleic. Acids Res.* **48**, D783–D788 (2020).
67. Cole, S.T. *et al*. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **393**, 537–544 (1998).
68. Goonetilleke, N.P., McShane, H., Hannan, C.M., Anderson, R.J., Brookes, R.H. & Hill, A.V. Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J. Immunol.* **171**, 1602–1609 (2003).
69. McShane, H., Pathan, A.A., Sander, C.R., Goonetilleke, N.P., Fletcher, H.A. & Hill, A.V. Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials. *Tuberculosis* **85**, 47–52 (2005).
70. Centers for Disease Control and Prevention (CDC). Use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal polysaccharide vaccine for adults with immunocompromising conditions: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb. Mortal. Wkly. Rep.* **61**, 816–819 (2012).
71. Bouvier, N.M. & Palese, P. The biology of influenza viruses. *Vaccine* **26**(Suppl 4), D49–D53 (2008).
72. Nachbagauer, R. *et al*. A chimeric hemagglutinin-based universal influenza virus vaccine approach induces broad and long-lasting immunity in a randomized, placebo-controlled phase I trial. *Nat. Med*. https://doi.org/10.1038/s41591-020-1118-7.
73. McMichael, A.J., Borrow, P., Tomaras, G.D., Goonetilleke, N. & Haynes, B.F. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat. Rev. Immunol.* **10**, 11–23 (2010).
74. Liu, M.K. *et al*. Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. *J. Clin. Invest.* **123**, 380–393 (2013).
75. Burton, D.R. Advancing an HIV vaccine; advancing vaccinology. *Nat. Rev. Immunol.* **19**, 77–78 (2019).
76. Ondondo, B. *et al*. Novel conserved-region T-cell mosaic vaccine with high global HIV-1 coverage is recognized by protective responses in untreated infection. *Mol. Ther.* **24**, 832–842 (2016).
77. Mothe, B. *et al*. Definition of the viral targets of protective HIV-1-specific T cell responses. *J. Transl. Med.* **9**, 208 (2011).
78. Buchbinder, S. P. *et al*. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* **372**, 1881–1893 (2008).
79. Carlin, E.P., Giller, N. & Katz, R. Estimating the size of the U.S. population at risk of severe adverse events from replicating smallpox vaccine. *Public Health Nurs.* **34**, 200–209 (2017).
80. Bandyopadhyay, A.S. *et al*. Safety and immunogenicity of inactivated poliovirus vaccine schedules for the post-eradication era: a randomised open-label, multicentre, phase 3, non-inferiority trial. *Lancet. Infect. Dis*. https://doi.org/10.1016/S1473-3099(20)30555-7.
81. O'Hagan, D.T., Lodaya, R.N. & Lofano, G. The continued advance of vaccine adjuvants - 'we can work it out'. *Semin. Immunol.* **50**, 101426 (2020).
82. Ingolotti, M., Kawalekar, O., Shedlock, D.J., Muthumani, K. & Weiner, D.B. DNA vaccines for targeting bacterial infections. *Expert. Rev. Vaccines* **9**, 747–763 (2010).
83. Trimble, C.L. *et al*. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet* **386**, 2078–2088 (2015).
84. Voysey, M. *et al*. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet*. https://doi.org/10.1016/S0140-6736(20)32661-1.
85. Schneider, J. *et al*. A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the Plasmodium falciparum TRAP antigen in chimpanzees. *Vaccine* **19**, 4595–4602 (2001).
86. Boopathy, A.V. *et al*. Enhancing humoral immunity via sustained-release implantable microneedle patch vaccination. *Proc. Natl. Acad. Sci. USA* **116**, 16473–16478 (2019).
87. Oyewumi, M.O., Kumar, A. & Cui, Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. *Expert Rev. Vaccines* **9**, 1095–1107 (2010).
88. Reichmuth, A.M., Oberli, M.A., Jaklenec, A., Langer, R. & Blankschtein, D. mRNA vaccine delivery using lipid nanoparticles. *Ther. Deliv.* **7**, 319–334 (2016).

89. Gary, E.N. & Weiner, D.B. DNA vaccines: prime time is now. *Curr. Opin. Immunol.* **65**, 21–27 (2020).
90. McConkey, S.J. *et al*. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat. Med.* **9**, 729–735 (2003).
91. Sheehy, S.H. *et al*. Phase Ia clinical evaluation of the Plasmodium falciparum blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. *Mol. Ther.* **19**, 2269–2276 (2011).
92. Sheehy, S.H. *et al*. Phase Ia clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. *PLoS One* **7**, e31208 (2012).
93. Ogwang, C. *et al*. Safety and immunogenicity of heterologous prime-boost immunisation with Plasmodium falciparum malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan adults. *PLoS One* **8**, e57726 (2013).
94. Antrobus, R.D. *et al*. Clinical assessment of a novel recombinant simian adenovirus ChAdOx1 as a vectored vaccine expressing conserved influenza A antigens. *Mol. Ther.* **22**, 668–674 (2014).
95. de Barra, E. *et al*. A phase Ia study to assess the safety and immunogenicity of new malaria vaccine candidates ChAd63 CS administered alone and with MVA CS. *PLoS One* **9**, e115161 (2014).
96. Kimani, D. *et al*. Translating the immunogenicity of prime-boost immunization with ChAd63 and MVA ME-TRAP from malaria naive to malaria-endemic populations. *Mol. Ther.* **22**, 1992–2003 (2014).
97. Afolabi, M.O. *et al*. Safety and immunogenicity of ChAd63 and MVA ME-TRAP in West African Children and infants. *Mol. Ther. 24(8), 1470–1477* (2016).
98. Ewer, K. *et al*. A monovalent chimpanzee adenovirus ebola vaccine boosted with MVA. *N. Engl. J. Med.* **374**, 1635–1646 (2016).
99. Warimwe, G.M. *et al*. Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley Fever. *Sci. Rep.* **6**, 20617 (2016).
100. Mothe, B. *et al*. Therapeutic vaccination refocuses T-cell responses towards conserved regions of HIV-1 in early treated individuals (BCN 01 study). *EClinicalMedicine* **11**, 65–80 (2019).
101. Cappuccini, F. *et al*. Safety and immunogenicity of novel 5T4 viral vectored vaccination regimens in early stage prostate cancer: a phase I clinical trial. *J. Immunother. Cancer* **8(1)**, e000928 (2020).
102. Folegatti, P.M. *et al*. Safety and immunogenicity of a candidate Middle East respiratory syndrome coronavirus viral-vectored vaccine: a dose-escalation, open-label, non-randomised, uncontrolled, phase 1 trial. *Lancet Infect. Dis.* **20**, 816–826 (2020).
103. Wilkie, M. *et al*. A phase I trial evaluating the safety and immunogenicity of a candidate tuberculosis vaccination regimen, ChAdOx1 85A prime - MVA85A boost in healthy UK adults. *Vaccine* **38**, 779–789 (2020).