A GLOBAL PERSPECTIVE ON VACCINES: PRIORITIES, CHALLENGES AND ONLINE INFORMATION

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A GLOBAL PERSPECTIVE ON VACCINES: PRIORITIES, CHALLENGES AND ONLINE INFORMATION

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Table of Contents

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EDITORIAL
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Editorial: A Global Perspective on Vaccines: Priorities, Challenges and Online Information

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Keywords: vaccines, antimicrobial resistance, vaccine hesitancy, vaccine education, technological platforms

Editorial on the Research Topic

A Global Perspective on Vaccines: Priorities, Challenges and Online Information

Vaccines are one of the most successful stories in global health. For 99.99% of mankind history, life expectancy has been <30 years, but in the last 300 years human life has increased by 55 years, of which 35 were gained in the last century. Vaccines are accountable for a significant part of this extraordinary result. Since the first successful vaccine for smallpox in 1796, vaccines for many other diseases have been generated, taking advantage of the developments of science. Smallpox has been totally eradicated, and polio could be soon eliminated. Many other effective vaccines have significantly reduced the incidence of diseases that have killed millions of people in the past. Paradoxically, the decreased impact of infectious diseases at the global level is making people think that vaccines are no longer necessary. One of the reasons behind the decline in vaccine confidence is that many people have become complacent, and we face now the phenomenon of vaccine hesitancy. Thus, we can say that vaccines are victims of their own success.

The topic "A global perspective on vaccines: priorities, challenges and online information" focuses on the most crucial issues in the vaccine field, with a view on the years to come.

Since infections travel the whole world with no borders, the war against microbes is a global one. In this context, the action of military forces in vaccine development (Ratto-Kim et al.) has been an important initiator, and the worldwide effort includes organizations that fight long-known diseases and, more challenging, emerging infections such as SARS, MERS, Ebola, and Zika (Marinho de Andrade Zanotto and Leite). The globalized modern way of life, with the increased number of travelers throughout continents, is aggravating the situation. It is extremely important to ensure preparedness and efficacy of vaccines leveraging on innovation.

Research & Development remain the core of the evolving field. The -omics revolution, combined with the power of the extremely fast-growing technologies that will rapidly involve artificial intelligence, is providing a large variety of new vaccine candidates. There is a great need for harmonization and simplification for the newly generated vaccines. To this end, new platforms are being developed that will allow for new generation vaccines with built-in adjuvanticity for preventing multiple infections in a single shot.

Safety and affordability are characteristics that must be ensured in modern vaccines. Affordability will allow for fast access to effective vaccines worldwide, in particular to populations in developing countries. In this view, promoting local manufacturing is a key element in vaccine affordability (Rey-Jurado et al.). Side effects must be minimized, including the mild ones, to ensure the full safety of preventive treatments. In parallel, the new vaccines should have the highest effectiveness in all populations, from people living in the clean industrialized world to the populations that are constantly facing large microbial burden in infection-endemic areas. In this

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view, information on the co-evolution of microbes with the human host, coming from genome-wide association studies, is opening new avenues to the understanding of the mechanisms of resistance to infections. Studying the resistant subjects will pose the basis to design unprecedented preventive and therapeutic approaches. Malaria is an important example for this approach and is offering the possibility to rethinking vaccine design (1, 2).

Clinical trials are already being designed to prove the feasibility of new technological approaches in naïve and previously primed subjects. The understanding of the mechanisms and timing of prime-boosting immunizations is important, also in light of the problems observed with a candidate Dengue vaccine (3). Some examples are provided in this topic (Rauch et al., Launay et al., Yao et al.).

In the last decades, a complex global health system against known and unknown infectious disease threats has arisen, encompassing various formal and informal networks of organizations that serve different stakeholders, have varying goals, modalities, resources, and accountability; operate at different regional levels (i.e., local, national, regional, or global); and cut across the public, private-for-profit, and private-not-for-profit sectors. Organizations such as the Bill and Melinda Gates Foundation, the Global Alliance for Vaccine Immunization, and the Coalition for Epidemic Preparedness Innovations are potent drivers that are transforming the vaccine world (Bloom and Cadarette).

Among the new global health challenges that we are facing, the most alarming is the growing inefficacy of antibiotics. The excessive and incorrect use of antibiotics has accelerated the generation of resistant pathogens, which in many cases show resistance to multiple drugs. The increasing inefficacy of current antibiotics is expected to cause 10 million deaths per year in the world by 2050 (Tagliabue and Rappuoli). Vaccines could be the solution to **Antimicrobial resistance** (AMR) and its impending death toll. Thus, AMR is revolutionizing vaccine R&D to the point that priorities are being re-evaluated, and vaccines are being developed for diseases that we have for long considered harmless because curable with antibiotics. Furthermore, scientists are exploring the potential protective role of vaccines beyond the classical induction of pathogen-specific adaptive humoral and cellular immunity. An increasing body of experimental data is now supporting the notion that vaccines can have non-specific protective effects (Uthayakumar et al.). The concept of innate immune memory is starting to be exploited for the design of personalized effective adjuvants in novel vaccination strategies.

The impressive scientific and technological advancements and the huge global health efforts need to be paralleled by worldwide initiatives in vaccine **Education**. Thus, the high-level training for present and future vaccinologists plays a fundamental role in broadening conceptual and applied knowledge in the vaccine field (Lambert and Podda). But vaccine education should not be limited to raising expert vaccinologists. It should also target the general public. As already mentioned, vaccine hesitancy is spreading and could become a major threat for vaccine effectiveness. Public perception and public acceptance will make the difference between successful control of infections and failure with consequent scourge propagation. The use of modern social media could be extremely important to spread and popularize the correct information, avoiding ideological and political exploitation (Arif et al.), thereby increasing acceptance and compliance. False information, such as the idea that vaccines cause autism, is difficult to eliminate, despite the solid epidemiological data against it (4). The impact of social media on our society is huge and rapidly transforming. But eliminating vaccine hesitancy is a moral imperative.

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REVIEW
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Assessing the Importance of Domestic Vaccine Manufacturing Centers: An Overview of Immunization Programs, Vaccine Manufacture, and Distribution

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Vaccines have significantly reduced the detrimental effects of numerous human infectious diseases worldwide, helped to reduce drastically child mortality rates and even achieved eradication of major pathogens, such as smallpox. These achievements have been possible due to a dedicated effort for vaccine research and development, as well as an effective transfer of these vaccines to public health care systems globally. Either public or private institutions have committed to developing and manufacturing vaccines for local or international population supply. However, current vaccine manufacturers worldwide might not be able to guarantee sufficient vaccine supplies for all nations when epidemics or pandemics events could take place. Currently, different countries produce their own vaccine supplies under Good Manufacturing Practices, which include the USA, Canada, China, India, some nations in Europe and South America, such as Germany, the Netherlands, Italy, France, Argentina, and Brazil, respectively. Here, we discuss some of the vaccine programs and manufacturing capacities, comparing the current models of vaccine management between industrialized and developing countries. Because local vaccine production undoubtedly provides significant benefits for the respective population, the manufacture capacity of these prophylactic products should be included in every country as a matter of national safety.

Keywords: vaccine manufacturing, immunization programs, vaccine distribution, vaccine shortages, good manufacturing practices

INTRODUCTION

The incidence of numerous infectious diseases that are life threatening to humans has drastically declined since the development of safe and effective vaccines and the implementation of global vaccination programs worldwide. In fact, the variola virus, which caused smallpox disease that killed millions of individuals throughout history, was successfully eradicated from Earth during the

1980s (1), due to a worldwide immunization campaign against this major pathogen. Moreover, poliovirus, which severely affects the health of children with lifelong disabling consequences, has almost been eradicated from the world. Since 1999 very few cases of polio disease have been reported, probably due to two of the three poliovirus types. Indeed, the goal of the World Health Organization (WHO) is to achieve the eradication of polio during 2018. Therefore, millions of human lives have been saved by means of the implementation of national immunization programs in all countries, and the demand for new prophylactics to protect against infectious diseases is constantly growing. Although vaccine manufacturing is usually associated with biopharmaceutical companies, some public and academic institutions also produce these prophylactic formulations (2). Despite the existence of those manufacturers aiming at increasing vaccine availability, shortage of these products has taken place several times causing that not enough doses were available in some countries.

In this article, we attempt to comprehensively discuss the WHO current recommendations for routine immunization and some of the national immunization programs. Further, we associate such vaccination programs to the global vaccine manufacture and distribution capabilities, focusing in some industrialized and developing countries. The comparison between these two types of nations was done to point out key management differences among them, when aiming at guaranteeing prophylaxis against serious infectious diseases in their populations. In addition, we also examined the dependency on foreign vaccine supply of some countries, classifying them according to their capacity to supply the local demand with domestic facilities or *via* importation from other states.

VACCINES CURRENTLY RECOMMENDED BY THE WHO

According to the WHO, children should be immunized with bacille Calmette–Guerin (BCG), diphtheria-tetanus-acellular pertussis (DTaP), MMR (combines Mumps, Measles, and Rubella), and vaccines to prevent Hepatitis B, poliovirus, *Haemophilus influenza*e type B (Hib), several serotypes of *Streptococcus pneumoniae*, rotavirus, and papillomavirus (3). In addition to these vaccines for children, the influenza vaccine is also recommended to be administered in certain susceptible groups, such as pregnant women, healthcare workers, children aged 6–59 months and the elderly (>65 years old) (3). Furthermore, the coverage of routine Expanded Program on Immunization (EPI), which includes vaccines against tuberculosis (TB), diphtheria, tetanus, and pertussis, polio and measles, varies from country to country (**Figure 1**).

Vaccination for Poliomyelitis: An Example of a Nearly Eradicated Disease

Although poliomyelitis cases decreased greatly in 1988, 74 cases of this disease were reported in 2015. The majority of them occurred in Pakistan and in Afghanistan. Therefore, the goal proposed by the WHO is to eradicate poliomyelitis by 2018. Poliomyelitis is an infection caused by poliovirus that affects the human nervous system (4). The trivalent attenuated oral polio vaccine (tOPV), which includes the types 1, 2, and 3, has been used since the beginning of the 1960s. However, due to the polio type 2 vaccine components were pointed out as the infectious source leading to a large number of cases of vaccine-derived polioviruses, global initiatives have suggested to switch from the trivalent to a bivalent polio vaccine. Such vaccine only includes type 1 and 3 viruses (5). Interestingly, the wild type poliovirus type 2 has not been reported since 1999 and was declared eradicated in September 2015. Besides, the poliovirus type 3 has not been detected since 2012 and the poliovirus type 1 is likely the only strain remaining in circulation.

As an additional effort to keep population protected against all types of poliovirus during the eradication program, the WHO instructed to include at least one dose of the inactivated polio vaccine (IPV) in the sequential shift from the tOPV toward the dOPV (6). The IPV is composed by the three types of poliovirus, which is intramuscularly administered. Clinical trials in children have shown that this vaccine is an excellent booster and capable of enhancing the mucosal immune response in primed subjects (4, 7).The future goal is to shift from dOPV to IPV at the time when type 1 and 3 polioviruses were eradicated.

Vaccination for Respiratory Diseases: TB, Pneumonia, and Influenza

A different vaccine type, the BCG vaccine, has been used in over a billion people since 1921 to prevent TB (8). Although not able to induce a strong protective immunity in adults, the BCG vaccine has been shown to protect against meningitis TB disease in children (9). However, the BCG vaccine currently is not utilized in children from countries with low rates of TB incidence, such as the USA, Spain, Australia, Norway, Canada, and England (10). In those countries, the BCG vaccine is only recommended for those children showing a negative tuberculin skin test and that are continually exposed to adults with untreated or ineffectively treated for TB disease. Further, BCG vaccination is also recommended for health care workers in settings of frequent exposure to TB patients (11). Furthermore, because the BCG vaccine derives from attenuated bacteria passaged in the 1960s, the large number of passages affecting the banks available today has led to multiple genetic changes in the bacilli. Several studies supported the notion that this genetic divergence could be responsible for the variant protective capacity against TB shown by the various BCG vaccine strains (8, 12). Thus, an efficient BCG vaccine that provides full protection is still required. The major BCG manufacturers prequalified by the WHO are the Staten Serum Institute (Denmark), Serum Institute of India Ltd., Japan BCG Laboratory, and Intervax Ltd. (Canada). In addition, some Asian and Eastern-European countries possess their own locally-produced BCG vaccine, such as China (China National Biotec Group), Serbia (Torlak Institute), and Vietnam (IVAC) (8).

Bacteria-caused pneumonia, due to infection with various serotypes of *S. pneumoniae* (Pneumococcal disease) and Hib display a high rate of morbidity and mortality worldwide, although nowadays, the majority of the deaths take place in sub-Saharan Africa and Asia (13). Both pneumococcal and Hib vaccines are recommended by the WHO (3). However, not all

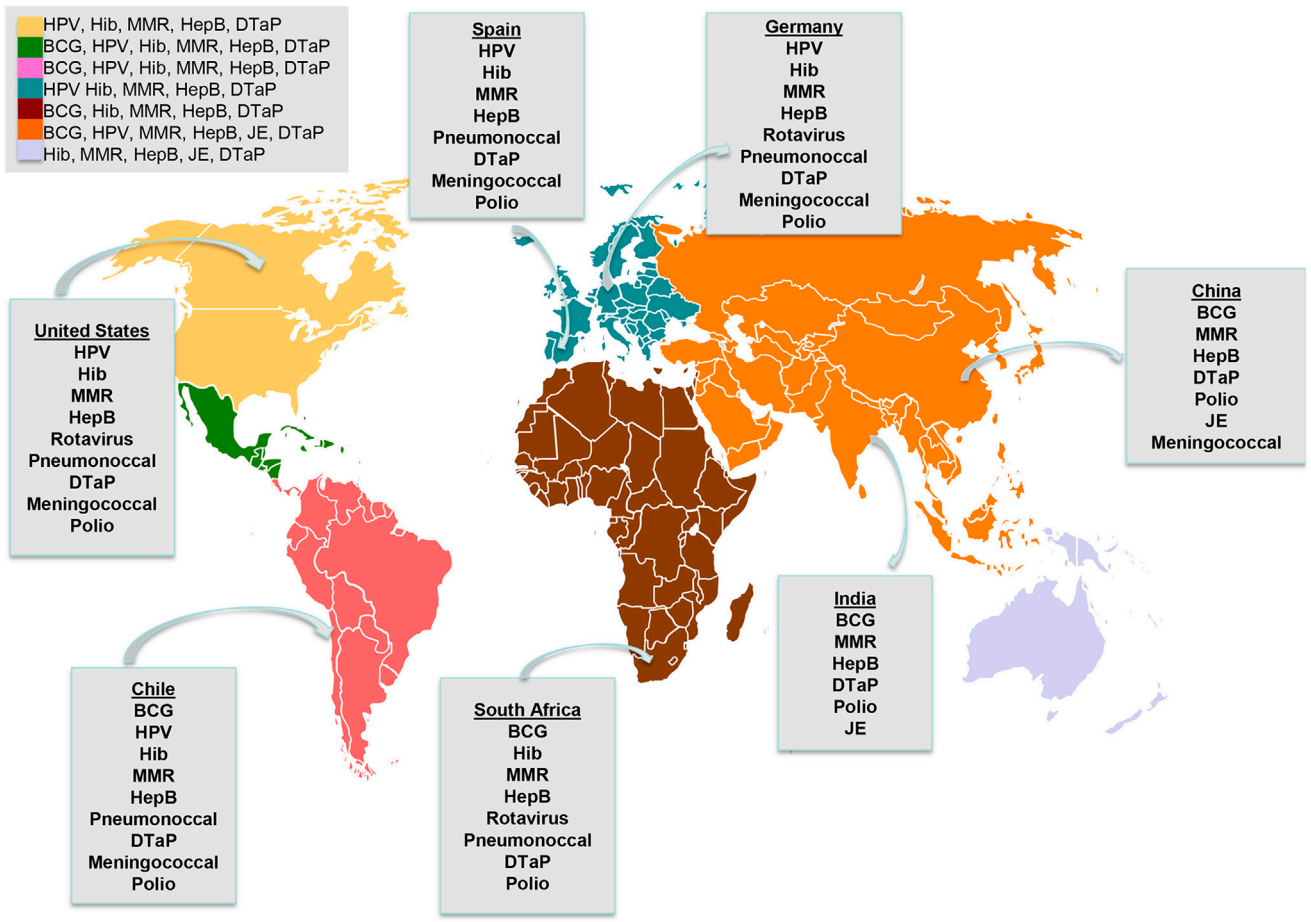

FIGURE 1 | Immunization programs around the world. Vaccines funded by national governments and included in national immunization programs by continent and regional examples. BCG, bacille Calmette–Guerin; HepB, hepatitis B virus; DTaP combines protection against diphtheria, tetanus, and pertussis; MMR, combines mumps, measles and rubella; Hib, *Haemophilus influenzae* type B; HPV, human papillomavirus; JE, Japanese encephalitis live vaccine. Exemptions: BCG is given in some countries of Europe. HPV is given in some countries of Africa.

countries include these vaccines in their national immunization programs and, for instance, the public health systems of some South Asian countries do not use them at all (**Figure 1**). Thus, whereas these vaccines were introduced in the 1990s in most industrialized countries, still these prophylactics are not funded by public health systems in some developing countries, such as South Asian nations. Consequently, still 18/100,000 and 26/10,000 cases of Hib were reported in children younger than 5 years old in Vietnam and in China, respectively. To handle these high incidence rates, organizations including the Global Alliance for Vaccine and Immunization (GAVI) and the United Nations International Children's Emergency Fund have financed pneumococcal and Hib vaccines to provide coverage for developing countries (14). Several studies conclusively have supported the notion that public health systems should add these vaccines to their national immunization programs with their own funding, in every developing country. Thus, adopting these measures, the incidence of these major infectious diseases could be reduced (15, 16). Further, some GAVI-supported countries experienced a transition from GAVI-derived support to a fully self-financed Hib vaccination program. Thereby, strategic immunization plans are required to provide vaccines to their population (17).

Viral respiratory diseases generated by the influenza virus causes low rates of mortality but high rates of morbidity worldwide every year (18–20). This seasonal disease is mainly caused by two types of influenza viruses: A and B (21). The influenza A virus displays a high rate of variation causing frequent antigenic changes, in a process known as antigen drift. For this reason, the influenza vaccine confers only limited-time protection (up to 2 years) and it is necessary to reformulate and manufacture new influenza vaccines every year. Influenza vaccination is recommended by the WHO for high-risk individuals, including children, pregnant women, healthcare workers, the elderly and individuals suffering from chronic conditions, such as asthma, diabetes and heart disease (3). Further, organizations such as the American Academy of Pediatrics recommend the seasonal influenza vaccination for children of 6 months and older (22). However, the coverage of this vaccine still remains low despite the influenza vaccination strategies, including government involvement and national programs (23). Importantly, pandemic influenza

H1N1 emerged in 2009, affecting mainly children and the elderly. The global number of deaths during the first 12 months of virus circulation was reported from 151,700 to 575,400 people (24). Moreover, the older age groups presented higher severity in post-pandemic influenza outbreaks (25).

Vaccination to Prevent Diphtheria, Tetanus, and Whopping Cough

Another vaccine of global relevance is the DTaP (14). This vaccine protects against three severe infectious diseases: diphtheria, tetanus, and pertussis. First, diphtheria is caused by *Corynebacterium diphtheria*, which produces pharyngeal infection, myocarditis, polyneuropathy, and systemic toxicity (26). Second, tetanus is caused by *Clostridium tetani* and the typical symptoms include muscle spam and contraction (26). Finally, pertussis, also known as whopping cough, is caused by *Bortedella pertussis*, which can produce loss of weight, subconjunctival hemorrhages, and syncope (26). Currently these three diseases circulate in the population worldwide and the highest rates are observed in children from countries with low vaccination coverage, especially in developing countries (27–29). However, and despite high vaccination coverage, several outbreaks have recently been reported in industrialized countries (30). For instance, an outbreak in the USA was reported in 2012, resulting in 48,277 cases of pertussis (31). According to the United States Center for Disease Control and Prevention (CDC), DTaP protects from whopping cough in 7 out of 10 vaccinated subjects, while it efficiently protects against the severe illness. In fact, the introduction of DTaP vaccine in the USA reduced from 100,000 to 32,000 cases of pertussis per year. Despite these good results, DTaP could fail to provide long-lasting protection in humans (31). It is important to indicate that the WHO estimates that there still are about 16 million cases of pertussis and 30,000 of diphtheria per year worldwide, being the highest rates in India (32). Therefore, these infections are still an important public health burden that requires close monitoring.

Vaccination to Prevent Cervical Cancer

The nine-valent human papillomavirus vaccine (HPV) is recommended for routine vaccination of girls at age of 9 or 10 years old to confer protection against cervical cancer caused by the HPV (33). This new vaccine is significantly more expensive as compared to the other vaccines. Thereby, although it is highly recommended vaccine, not all children are being immunized to prevent this cancer (33). Despite the fact that the first HPV vaccine was available in 2006, today only two biopharmaceutical companies manufacture this vaccine (33). A study performed in France showed 95.93% effectiveness for the HPV vaccine in sexually active young women (34). Despite such effectiveness, a strong parent refusal remains in several countries to vaccinate children against HPV due to safety and effectiveness concerns, as reported in a survey in the USA (35).

Vaccination to Prevent Diarrheal Diseases

Another recent vaccine included in the immunization programs of several industrialized and developing countries is the one to prevent rotavirus-infections (3). This virus is one the most common causes of severe gastroenteritis with diarrhea-related hospitalizations in children worldwide, which shows in particular high mortality rates in developing countries (36). The WHO has recommended that this vaccine should be included in all national immunization programs, being strongly recommended for countries showing a high mortality rate in children under 5 years old due to severe dehydrating diarrhea (37). Nowadays, an increasing number of countries, such as the USA and Germany have incorporated the rotavirus vaccine in their national immunization programs. A meta-analysis conducted on individuals of Europe, North America and Latin America showed that this vaccine has an efficacy of 53% against rotavirus infections, 73% against rotavirus-related hospitalizations, and 74% against severe diarrhea episodes (38).

Vaccination to Prevent Typhoid Fever

Typhoid fever is a life-threatening systemic disease caused by human adapted *Salmonella enterica* serovars, such as Typhi, Paratyphi A, Paratyphi B, and Paratyphi C (39, 40). These are Gram negative enterobacteria that infect humans by contamination of food and water supplies, causing disseminated infections that compromise internal organs, such as spleen, liver, bone marrow, and blood (39, 41). The incidence of these diseases is low in industrialized countries (less than 10 cases per 100,000) and high in developing countries, specifically in Asia and in Africa (more than 100 per 100,000) (42–44). Importantly, a significant increase in *S.* paratyphi A has been reported in the last years in Asian countries, reporting up to 44-fold increase in the period 2007–2013 in Cambodia (45). Currently, there are three licensed vaccines to prevent typhoid fever: The Vivotif®, Typbar®, and the Typhim V® vaccines. The Vivotif® is a live attenuated vaccine approved by the FDA for use in humans, based on the Ty21a strain, which was generated in the 1970 by chemical mutagenesis (46). This vaccine was previously produced and distributed by Crucell Switzerland It Ltd., but recently the American company PaxVax has acquired the license for this product. This vaccine is provided as a lyophilized formulation (in capsules) and used orally to promote mucosal immunity against these bacteria. A large clinical study performed in Chile showed that the rate of protection after three immunizations was 69% (47). In contrast, the Typhim Vi® and Typbar® are inactivated vaccines consisting of the Vi capsular polysaccharide, which are produced by Sanofi Pasteur and Bharat Biotech, respectively (48). The Typhim Vi® vaccine is administered intramuscularly and confers an antibody-based protection (49). The rate of protection for this vaccine is close to 75% (50). Further, those vaccines do not confer cross-immune protection against *S.* Paratyphy A, for which does not exist a licensed vaccine available to prevent disease caused by this bacterium (51). Because of the immune memory conferred by both vaccines are very limited, their inclusion in immunization programs has not been recommended. However, the use of this vaccine has been encouraged by the WHO, especially when sanitation measures are threatened, for instance during natural disasters that impair the accessibility to clean water. Nevertheless, due

to the growing emergence of antibiotic-resistant strains of *S.* Typhi in developing countries like India, the permanent use of these vaccines, as well as the generation of improved ones, would be highly appropriate to apply in their populations (52).

Vaccination to Prevent Meningitis

Meningitis is an inflammation of the membranes covering the brain and spinal cord known as meninges, which can be caused by viral, bacterial or fungal infection, but also by due to non-infectious causes, as it has been reported (53). The main bacterial agents responsible for this disease are *S. pneumoniae,* Hib, and *N. meningitidis*, which could be prevented by available vaccines (54). Meningococcal disease has been reported worldwide, but largest epidemics have affected mainly sub-Saharan African countries, known as the "meningitis belt" having 430 million of high-risk population (53, 55).

According to the recent report in May 2017 by the CDC (56), there are two types of meningococcal vaccines available in the USA. The first vaccine is based on bacterial conjugates: Menactra® and Menveo®, both conferring protection against A, C, W, and Y meningococcal serogroups. The second is a serogroup B recombinant meningococcal vaccine: Bexsero® and Trumenba®. An additional vaccine, named MenAfriVac® (produced by the Serum Institute of India Private Ltda.), confers protection against *N. meningitidis* serogroup A (Nm A), which is the most prevalent in the African "meningitis belt" (55). The MenAfriVac® vaccine was a result of collaborative efforts between the WHO and the PATH in the Meningitis Vaccine Project, with the purpose of developing a vaccine against the specific agent affecting importantly the health of the African population, presenting a low-cost manufacturing and being independent of the cold chain distribution (57, 58). Since the national routine immunization strategic plan started in 2010, the incidence of Nm A meningitis fell from 0.27 per 100,000 in 2004–2010 to 0.02 per 100,000 in 2011–2013 (59). According to the recent WHO weekly record, 19 of the 26 countries belonged to the African "meningitis belt" have shown a sustained decreased incidence for Nm A cases, which means a reduction by at least 57% of the meningitis burden in that area (55). Also, clinical trials demonstrated that MenAfriVac® decreases carriage rates in immunized populations and provides herd immunity probably because of the high antibody titers observed during the development and safety testing of the vaccine (60, 61). Due to the national immunization program for this vaccine was a success, Ghana and Sudan currently include the MenAfriVac® in their routine immunization schedule (55). Despite the significant decrease in the prevalence on Nm A, it is important to highlight the necessity to continue with immunization programs to guarantee protection against different serogroups (62). Further, experts alert of the possible serogroup replacement, following application of massive immunization programs (63). In fact, in 2015 an epidemic with a novel strain of *N. meningitidis* serogroup C was reported in Niger and Nigeria. In addition, in 2016 the principal *N. meningitidis* serogroup W was found in Ghana and Togo, although with a low number of cases (55). For that reason, the continuous research in this area is a central challenge toward elimination of meningococcal meningitis epidemics in Africa.

VACCINE TYPES, MANUFACTURING PROCEDURES, AND CURRENT RESEARCH ON MANUFACTURING STATUS

Types of Vaccines and Manufacturing Procedures

Vaccines can be classified as live-attenuated, inactivated, sub units, recombinant, conjugated, toxoids, or DNA, according to the final preparation of the microorganism or antigen (64). Live-attenuated and inactivated microorganisms cover the major fraction of licensed vaccines for use in humans. Smallpox, BCG, yellow fever, polio, chickenpox, rotavirus, typhoid fever (Ty21a vaccine), and influenza are examples of licensed vaccines produced with live attenuated microorganisms (8, 65–67). On the other hand, examples of inactivated vaccines include those preventing plague, whooping cough, influenza, polio, typhoid fever (Vi capsular polysaccharide vaccine), and hepatitis A diseases (5, 49, 68–72). Only few vaccines are produced using recombinant technologies (hepatitis B virus, influenza, HPV) or *via* purification of partial components of a microorganism [*S.* Typhi Vi capsular polysaccharide, diphtheria, tetanus, pneumococcus, meningococcus, Hib, pertussis toxoid, and anthrax protective antigen (PA)] (73, 74). However, there has been an increased interest in the usage of these technologies in the past years (75).

There are different methods of vaccines production, which include isolation of microorganisms from either infected tissues (e.g., smallpox), bacteria growth in fermenters (e.g., vaccines for TB, typhoid fever, plague, whooping cough, diphtheria, tetanus, pneumococcus, meningococcus, pertussis, anthrax), isolation from virus grown in cell cultures (e.g., polio, chickenpox, rotavirus, hepatitis A virus (HAV), influenza) or isolation from virus grown in eggs (e.g., influenza, yellow fever). For the case of bacteria grown in fermenters, is not the microorganism itself that is used for the vaccine elaboration, rather some of its components from cell-free filtrates (e.g., vaccines for tetanus, pertussis, anthrax). For example, the anthrax vaccine adsorbed consists in the PA purified from filtrates by precipitation with alum, which also serves as an adjuvant (76).

An interesting change in the way of manufacturing has been recently carried out for influenza vaccines, which has been produced for more than 50 years in embryonated chicken eggs (77). However, GlaxoSmithKline (GSK) and Seqirus are currently producing influenza virus using cell culture technology in bioreactors (approved by the FDA in 2012) to generate new licensed influenza vaccines (78). Likewise, the Kaketsuken vaccine company is working on the development of a cell culture-based process, using the EB66 cell line, to elaborate a vaccine for pandemic flu, which is currently under clinical studies (79, 80). More recently, the Protein Sciences Corporation received approval for commercialization of a licensed novel influenza vaccine consisting of purified recombinant hemagglutinin antigens expressed in insect cell cultures (81). Similar efforts are in progress toward the development of cell culture-based yellow fever vaccines using Vero cell cultures in microcarriers (82). For anthrax, a plant-derived recombinant protective antigen has been

developed as a vaccine, which is currently under evaluation in clinical trials (76, 83).

Thus, cell culture technologies, together with the enhancement of upstream and downstream processes, will bring production efficiencies to a next level as compared to the egg-based technology, and will increase manufacturing speed and capacities, thereby avoiding the shortage of these vaccines in the future (84, 85).

Vaccine Research in the Industry versus the Academia

Vaccine portfolios in many pharmaceutical companies have decreased in the last decades due to the cost and time involved for vaccine development, which are much more costly and time consuming to develop than other drugs (86). However, pharmaceutical companies as well as academic institutions are continuously investing in vaccine research. For example, the number of vaccines in development has increased about twofold, according to a study comprising the 1995–2008 period in the USA (87). This fact can be explained, in part, by the advancement of alternative technologies, such as baculovirus-based recombinant vaccines, virus-like particles, viral vectors and RNA or DNA vaccines (74, 88–93). Moreover, with a world population projected to be of 10 billion by 2050, a 90% of it is estimated to live in developing countries (United Nations projection) (94). Thus, the subsequent increase in the vaccine market from USD 25 billion by today to USD 100 billion by 2025, will continue to encourage vaccine research and development (95).

Many research groups in academic institutions have made considerable efforts on vaccine discovery and research, but only few of them have been able to move forward into the development vaccine process. A reduced technology transfer efficiency may be due to difficulties on establishing private-academy license agreements (LA) (96). Indeed, Public-Private-Partnerships (PPP) has shown to be relevant for some vaccine developments, such as for the prototype of HIV vaccine (97). Thus, these LA and PPP enable the implementation of new and improved vaccines in high-tech centers before a product is transferred into the market. Another factor is the requirement of facilities with Good Manufacturing Practicing (GMP) certification and high-quality personnel to develop vaccine production processes. The staff capacities and facilities to investigate, develop and manufacture vaccines are key to respond rapidly to the global emergencies, such as the recent Ebola outbreak (98).

The increase of vaccine manufacturers has impacted on the global market, allowing to lower the prices of vaccines and to improve the global demand. Further, partnerships, such as GAVI Alliance, UNICEF, and the WHO have also been key for enhancing that kind of vaccine production in developing countries (99). As example, the new vaccine manufacturing countries such Brazil, the Russian Federation, India, China, and South Africa (known as BRICS) play a substantial and increasing role in the global vaccine market. These countries not only produce traditional vaccines at competitive low costs and under the WHO-prequalified standards, but they also generate innovative products due to current strategic alliances with multinational corporations (99, 100). The most successful case of this strategic alliance, is the Bio-Manguinho plant in Brazil, that will be producing an affordable measles and rubella vaccine with the support of the Bill & Melinda Gates Foundation together with the Brazilian Ministry of Health (100). An arising number of pharmaceuticals along with the NIH are interested in enlarging the number of vaccines manufactured in those institutes, which in turn involved discussion of the agreement of the Trade Related Aspects of Intellectual Property Rights.

DIVERSITY OF IMMUNIZATION PROGRAMS WORLDWIDE: REGIONAL EXAMPLES AND THE GAP BETWEEN INDUSTRIALIZED AND DEVELOPING COUNTRIES

Worldwide, the diversity in national immunization programs is extensive, therefore the list of vaccines included and distributed in each country shows significant differences (**Figure 1**). Furthermore, the vaccination plan for the USA might even be different depending on the state, while in Europe the immunization plans have significant differences among the countries belonging to the European Union (**Figure 1**). On the other hand, there are variations in the financing mechanisms for vaccine production within Europe. For instance, the National Health System funds the rotavirus vaccine in Germany, but not in Spain. Other vaccines, such as the live attenuated Japanese encephalitis, cholera, and yellow fever vaccines are recommended only in some Asian countries, such as in India and in Thailand. Furthermore, meningococcal C conjugate vaccines are included in the National Health System of Australia, Chile, and Spain, but not in those of Asian countries like in India. Another example of diversity on immunization schedule is the BCG vaccine against TB. This vaccine is being administered only in some countries in Europe, Asia, Africa, and South America, but it is not administered in industrialized countries such as in the USA (14). **Table 1** summarizes the differences of the immunization programs between seven countries, including industrialized and developing countries (101, 102).

Germany is an example where vaccination is mostly voluntary with a reduced role of the state in the implementation of vaccination programs. Around 90% of the vaccines are given by private physicians and only the remaining small fraction of the vaccines is given by public institutions, schools or daycare centers (103). Massive school immunization programs are not mandatory, but the immunization status is checked at schools. This information is collected and documented by the Robert Koch Institute. The Berlin measles outbreak of 2015 and the death of a non-vaccinated infant raised the discussion as to whether vaccination in Germany must be mandatory (104). This discussion has been intensified considering that the Europe is confronting the largest immigration since the World War II. The collapse of national immunization programs in the countries undergoing political turmoil has led to children-disease outbreaks, which could have been prevented by vaccination. Moreover, refugees are susceptible to diseases due to overcrowding, physical and

TABLE 1 | National immunization programs of seven countries.

	BCG	HepB	Polio	DTaP	MMR	HPV	Hib	Pneumococcal	Rotavirus	JE
USA		2, 4, and 6 months old	2, 4, 6 months and 11 years old	2, 4, and 6 months old	12 months old	>11 years old	2, 4, and 6 and >12 months old	2, 4, and 6 and >12 months old	2, 4, and 6 months old	
Chile[a]	Newborn	2, 4, and 6 months old	2, 4, 6 months and 12–13 years old	2, 4, and 6 months old	12 months old	10 years old	2, 4, and 6 months old	12 months old		
Germany		2, 3, 4, 11–14 months old	2, 3, 4, 11–14 months and 5–6 and 9–11 years old	2, 3, 4, and from 11 to 14 months years old	11–14 and 15–23 months years old	9–14 years old	2, 4, 4, and 12–14 months old	2, 4, and 11–14 months old	6 weeks, 2 and 4 months old	
Spain		2, 4, 6 months old	2, 4, 6, and 18 months old	2, 4, 6, and 18 months old, and 6 years old	12 months and 3–4 years old	12–14 years old	2, 4, 6, and 18 months old	2, 4, and 11 months old[b]		
China[a]	Newborn	Newborn, 1 and 6 months old	2, 3, 4 months, and 4 years old	3, 4, 5, and from 18 to 24 months years old	18–24 months old					8 months and 6 years old
India[a]	Newborn	Newborn	6, 10, 14 weeks, and 16–24 months old	6, 10, 14 weeks, and 16–24 months old	9, 16–24 months[c]					9, 16–24 months old
South Africa[a]	Newborn	6, 10, 14 weeks and, 18 months old	Newborn, 6 weeks	6, 10, 14 weeks, and 18 months old	9 and 18 months[c]		6, 10, 14 weeks, and 18 months old	6, 14 weeks, and 9 months old	6 and 14 weeks old	

Developed and developing countries were selected according to their geographical area and income. Orange: not funded by the public health system. Blue: funded by the public health system.

[a]*Developing countries.*

[b]*Depends on the region, this vaccine is included in the public health system.*

[c]*Only vaccine against measles.*

BCG, bacille Calmette–Guerin; HepB, hepatitis B virus; DTaP, diphtheria, tetanus, and pertussis; MMR, mumps, measles, and rubella; Hib, Haemophilus influenzae type B; HPV, human papillomavirus; JE, Japanese encephalitis live vaccine.

psychological stress, malnutrition and low availability of sanitary systems. These health aspects and conditions constitute a serious threat to immigrants, as well as to international programs aimed at eradicating vaccine-preventable diseases. Recent studies of measles, mumps, rubella, and varicella seroprevalence in refugees in Germany have shown satisfactory immunity in adults but low seroprevalence in children, suggesting thorough and prompt vaccination of children entering Europe (105). The opposite has been found for hepatitis A immunity in refugees in Germany, where the high rate of HAV protection supports the thesis that the probability of large HAV outbreaks in current German refugee centers is low (71). Nevertheless, vaccination of refugees against HAV is highly recommended.

The immunization programs in the USA follow the CDC guidelines (106). In this country, as mentioned earlier, vaccine coverage differs widely among states, varying for instance with ≥2 doses of HAV from 41.2% in Mississippi to 72.8% in Nebraska (107). Recent nonmedical exemptions in immunization laws have prompted serious concerns about potential vaccine coverage weakening. However, after the recent outbreaks of vaccine-preventable diseases mandatory immunizations at entry-schools and primary care facilities have emerged. Indeed, those states that allow exemptions, including religious and philosophical reasons, have shown a significantly higher incidence of vaccine-preventable diseases, as compared to those states allowing less exceptions for vaccination (108). Interestingly, the coverage of vaccines in the USA will depend on the insurance plan of each individual. Accordingly to the CDC, the coverage of children aged 19–35 months was lower in those children uninsured or covered by public insurance programs, such as Medicaid, as compared to private insurance-covered kids (107). However, some the USA vaccine manufacturers and the National Vaccine Programs offer help to those people who cannot afford some vaccines, such as the one for HPV. Importantly, up to 32.9% of children of 19–35 months of age in the USA live below poverty level and can fail to receive all the required vaccines (107). To overcome this problem, the Vaccines for Children Program in the US offers free vaccines to children living in poverty (107).

In South America, the Pan-American Health Organization (OPS) provides a caring cooperation system, named the "Fondo Rotatorio," designed to obtain the vaccines recommended by the WHO at low prices (109). As for the case of Chile, the Public Health Institute and the Ministry of Health direct the Chilean National Immunization Program (CNIP) following international recommendations. Vaccines included in the CNIP are funded by the government and given to hospitals, family health centers and some schools in Chile. The introduction of the latest vaccines in the CNIP has significantly reduced the incidence of certain diseases, such as bacteria-caused pneumonia and cervical cancer. One example is the 10-valent pneumococcal vaccine, which was introduced in January 2011 and thereafter, the number of hospitalizations due to pneumonia were successfully reduced (110). Such effectiveness of the 10-valent pneumococcal vaccine has also been demonstrated in other South-American countries (111). In 2015, the Chilean government supported the introduction of the HPV vaccine in the CNIP and thereby, most of 9–10 years old girls have been vaccinated since then as a program to prevent cervical cancer.

Thus, each country has its own national immunization program (112), which in most cases includes vaccines that are sponsored by their public health systems reaching different levels of coverage (**Figure 2**). Nevertheless, many developing countries have difficulties to finance all the vaccines recommended by the WHO. As a result, different organizations have arisen to provide economical support to the developing countries requiring vaccines. For instance, the Global Vaccine Activation Plan (GVAP) has established itself the goal of reducing some vaccine-preventable diseases by 2020 (113). Moreover, most traditional vaccines are sold at lower prices to organizations, such as UNICEF and the Pan-American Health Organization to reach developing countries (14). Although global coverage has improved, in countries such as India, Nigeria, Pakistan and Indonesia, a low immunization coverage still exist (113). It is noteworthy that 35 of the 45 classified as lower-middle income countries by the World Bank Classification are not being supported by GVAP Alliance, thereby these countries are struggling to reach underused and new vaccines to immunize their children (14). Also, one of the GVAP goals was to eliminate the maternal and neonatal tetanus, measles, and rubella in 2014, but unfortunately this goal was not achieved (113). One of the main reasons for this failure has been the unstable political situation in some countries and the inefficient introduction of these vaccines in national immunization programs (113). Therefore, economical gaps still remain between industrialized and developing countries to accomplish efficient immunization programs for their children. With globalization, leading to increased and fast movements of goods and people traveling to all remote areas in the world, these differences in health protection can be a risk for outbreaks, epidemics or even worse, pandemics. Importantly, several organizations including the GAVI Alliance, UNICEF, the Bill & Melinda Gates Foundation, the United States National Institute of Allergies and Infectious Diseases, the WHO, together with governments and other institutions support the goals of the GVAP to reduce some vaccine-preventable diseases by 2020 (113).

VACCINE MANUFACTURE AND DISTRIBUTION: STATUS OF ACADEMIC, PUBLIC, AND PRIVATE MANUFACTURING COMPANIES

Vaccine Production and Distribution

Although mainly private pharmaceutical companies have engaged in vaccine manufacturing and distribution, there are also successful efforts made by academic or public institutions to achieve this goal (**Table 2**). Vaccine manufacturing requires specific and expensive facilities with high scale production, and quality standards to ensure consistency and controlled elaboration of these products. This is typically achieved following the guidelines of the current (c) GMP in compliance with the local regulatory authorities. Therefore, most of the countries have contract agreements with specific cGMP-certified manufacturers to purchase the vaccines required for their populations. For example, the private sector is in charge of the 5–10% of the vaccines market in Asia (114).

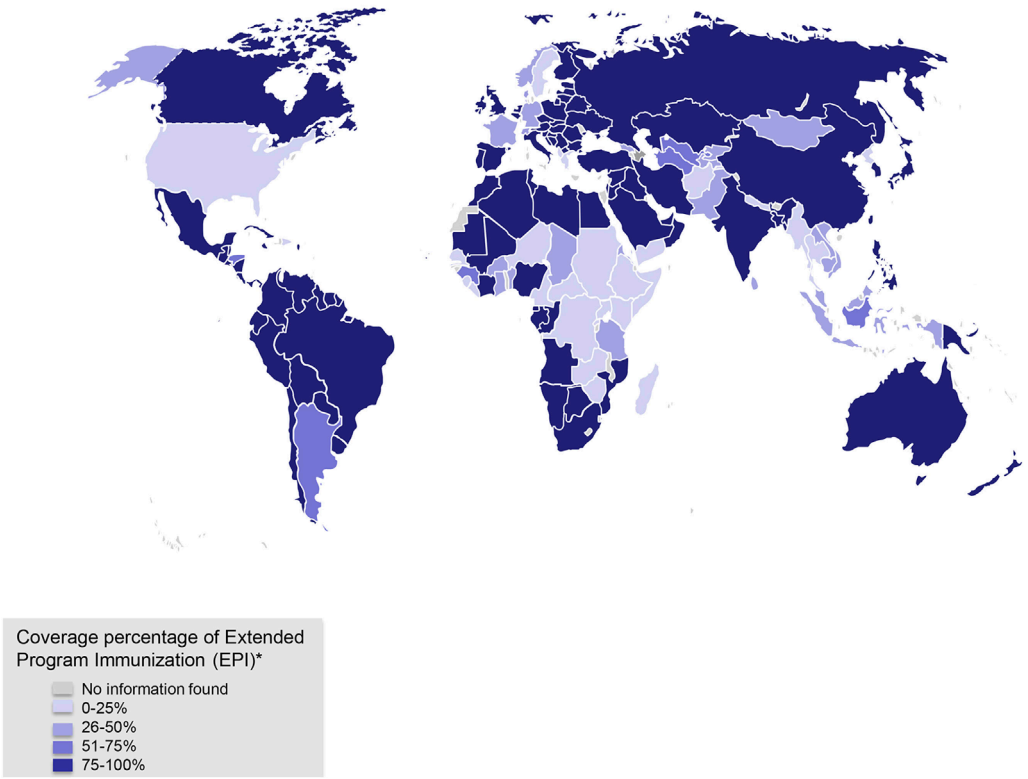

FIGURE 2 | Coverage for Extended Program of Immunization (EPI). *EPIs include those against tuberculosis, diphtheria, tetanus, and pertussis (DTP), polio, and measles, as well as those protecting newborn children and their mothers against tetanus by vaccination of pregnant women. Data obtained from WHO/UNICEF reports 2007 and 2014 reports.

The USA is one example of a country, in which both private and public sectors provide vaccines for their population (115). This is an advantage, because the public health system can choose from different sources and prices. The main pharmaceutical companies that produce and distribute vaccines around the world include GSK, the United Kingdom; Pfizer, the USA; Sanofi Pasteur, France; Merck & Co., the USA; Roche, France; Seqirus, Australia; Valneva SE, France (**Table 2**). In addition, emerging pharmaceutical companies, such as Astellas Pharma, Japan; Takeda, Japan, and AstraZeneca, United Kingdom currently invest in vaccine R&D. Other international companies, including the Serum Institute of India and the Bharat Biotech International supply vaccines to countries without local vaccine manufacture facility, such as Chile. Particularly, the Serum Institute of India is a state-owned vaccine manufacturing center that produces most of the vaccines recommended by the WHO including BCG, polio, Hib, DTaP, and MMR. Similarly, national public enterprises, including the Immunobiological Technology Guinhos (Bio-Manguinhos/Fiocruz) and the Butantan Institute supply most of the vaccines in Brazil (**Table 2**). Importantly, the two institutions previously mentioned supply about up to 83% of the Brazilian National Immunization Program demand, thereby reaching up to 179,855,000 national doses (116). A different situation can be found in Germany, where most of the vaccines are purchased from the private sector (90%) and 90% of them are financed by statutory insurance policies (117). The government provides the rest of the vaccines as part of special immunization programs. Recent studies have shown that no more than 0.47 and 0.25% of the German and Spanish healthcare budget, respectively, are addressed to vaccine production (117).

Due to the problems stated above, in the year 2000 an organization aimed to create alliances of vaccine manufacturers in developing countries was established. This organization, known as the Developing Countries Vaccine Manufactures Network (DCVMN), includes near 50 vaccine manufacturers in 17 developing countries in Latin America, Africa, the Middle East, and Asia (118–120). The companies that are members of this organization produce more than 40 different vaccines, including the ones recommended by the WHO including BCG, polio, Hib, DTaP, and MMR (**Table 2**) (118, 120). Although the DCVMN main goal is to provide a high quality (cGMP compliant) and sustainable supply of vaccines for developing countries, there are still not enough to provide the increasing demand of vaccines.

Vaccine Shortages

The coverage of the national immunization programs relies on the available supply of vaccines. Several countries have experienced vaccine shortages at some point, which have included BCG, Hib, DTaP, pneumococcal conjugate, MMR, meningococcal, yellow fever, and influenza vaccines (121, 122). As an example, Sanofi Pasteur, one of the major producers of BCG, the current

TABLE 2 | List of vaccine manufacturing centers companies of the countries reviewed in this work.

Name of company institute	Country	Vaccines manufactured
Statens Serum Institute	Denmark	BCG
GlaxoSmithKline	UK, Italy	Meningococcal, tetanus toxoid, acelullar pertussis, reduced diphtheria toxoid, HPV, HepB, influenza, HepA, Hib, meningococcal, rabies, rotavirus
Seqirus	UK	Difteria and tetanus, cholera, HPV, HepB, JE, meningococcal, MMR, influenza, pneumococcal, rabies, rotavirus, HepA
Sanofi	France	Cholera, diphtheria, pertussis and tetanus, Hib, meningococcal, BCG, typhoid fever, dengue, HepA, HepB, influenza, JE, polio, rabies, yellow fever
Immunobiological Technology Guinhos (Bio-Manguinhos/Fiocruz)	Brazil	Yellow fever, polio, meningitis A, MMR, rotavirus, Hib, pneumococo
Butantan Institute	Brazil	Diphtheria toxoid and tetanus toxoid, DTP-whole cell, influenza, hemorrhagic fever/dengue, HepB, rabies
Sinergium Biotech	Argentina	Influenza, pneumococcal, HPV
ANLIS	Argentina	BCG, rabies, tetanus toxoid, yellow fever
Fundaçao Ataulpho de Paiva	Brazil	BCG
Birmex	Mexico	Diphtheria toxoid and tetanus toxoid, polio
Pfizer	US	Meningococcal, pneumococcal
Merck	US	BCG, HPV, Hib, MMR, pneumococcal, HepB, rotavirus, HepA, varicella
Serum Institute of India	India	DTP, MMR, Hib, meningococcal, influenza, BCG, HepB, Polio
Bharat Biotech International	India	Rotavirus, Hib, polio, DTP, influenza, rabies, typhoid
Kaketsukken	Japan	DTP, influenza, JE, HepB, rabies
China National Biotec Group Company Limited	China	DTP, BCG, influenza, Hib, hemorrhagic fever, JE, meningococcal, MMR, polio, rabies, rotavirus, varicella, yellow fever
BioNet	Thailand	Acelullar pertussis
Biofarma	Indonesia	BCG, diphtheria, tetanus, DTP-HepB-Hib, HepB, measles, polio
GreenSignal Bio Pharma Limited	India	BCG
IVAC	Vietnam	BCG, DTP
Pasteur Institute of Iran	Iran	BCG, HepB
Queen Saovabha Memorial Institute	Thailand	BCG, rabies
Vabiotec	Vietnam	Cholera
Vacsera	Egypt	Cholera, diphtheria, tetanus
Eubiologics	South Korea	Cholera, diphtheria, tetanus
Biological E. Limited	India	Diphtheria, tetanus, DTP, HepB, Hib, HepB, JE, tetanus toxoid
Instituto Finlay de Vacunas	Cuba	Tetanus toxoid, DTP
Indian Immunological Ltd.	India	Diphtheria toxoid and Tenatus toxoid, DTP, rabies
SK Chemicals	Korea	HepB, influenza, tetanus-diphtheria
Razi	Irán	DTP, MMR, polio
Haffkine	India	Polio
TiantianBio	China	Rubeolla
Torlak Institute	Serbia	BCG, diphtheria, tetanus
Biovac	South Africa	BCG

BCG, bacille Calmette–Guerin; HepB, hepatitis B virus; DTP, diphtheria, tetanus, and pertussis; MMR, mumps, measles, and rubella; Hib, Haemophilus influenzae type B; HPV, human papillomavirus; JE, Japanese encephalitis live vaccine.

vaccine for TB, experienced significant manufacturing problems during 2012 and 2014. As a result, distribution of this vaccine was seriously compromised in several countries (123). Indeed, approximately 16.5 million doses shortfall of BCG occurred at the end of 2015 was estimated, using mathematical models, to be associated with 7,433 excess of TB deaths worldwide (124). In 2015, short supplies for the meningococcal vaccine worldwide threatened the health of the population in Nigeria, a place where an important epidemic of meningitis took place (125). An additional example is the Hib boost vaccine, for which doses were not available in the USA from December 2007 to September 2009 (122). Moreover, several physicians have reported shortages of influenza vaccines, especially for high-risk populations in the USA during the years 2004–2005 (126). Further, Africa and the USA have also experienced shortages for the yellow fever vaccine during the last 2 years (127–129). Similarly, significant shortages of the pneumococcal conjugate vaccine occurred during the period 2003–2004, causing an important decrease of 10.6% of the coverage of >4 doses of the seven-valent pneumococcal conjugate vaccine in 16-month-old children (130). Likewise, such shortage issues have prompted the concern of elaborating protocols for ensuring availability of those vaccines for at least the high-risk populations (131). Because the pandemic of influenza is highly extensive, the demand for this vaccine worldwide is very high, causing sometimes problems of vaccine shortage (132, 133). This situation is particularly dramatic when pandemics on influenza arise, such as the H1N1 in 2009 (134).

Different reasons can explain disruptions of the vaccine supplies, such as vaccines that leave the market, problems in the production, loss of the GMP in manufacturing centers/companies, and changes in the formulation of vaccines (135). An important correlation is that fewer vaccine manufacture suppliers exist for one vaccine the larger the impact of supply shortage can have on the population (135). To solve the vaccine shortage in case of epidemics, global vaccine stockpiles have been established for vaccines, including smallpox, meningococcal, yellow fever, oral cholera, and pandemic influenza vaccines (136). Moreover, the challenge for institutions, such as the Brazilian government, is

to make investments for local vaccine development and manufacturing to avoid international dependency and the threat of shortage (116).

GLOBAL EMERGING DISEASES AND ANTIBIOTIC RESISTANCE

The Ebola, Zika, and influenza virus pandemics are examples of worldwide emergencies that have recently affected various regions of the planet. In 2009, the H1N1 influenza pandemic resulted in the highest number of cases in Mexico (134). In April of 2009, the first cases with severe respiratory disease started to be concentrated in the Federal District of Mexico's most populated area. The Mexico's National Institute of Respiratory Disease struggled with such situation to contain the propagation of the influenza virus (137). Months later, the H1N1 virus was spread to over 213 countries causing 16,226 deaths and the WHO declared it to be the first flu outbreak in the last 41 years (138). The H1N1 2009 pandemic was identified as a new influenza A subtype of swine origin, and consequently, at that moment no vaccines were available. After that outbreak, a vaccine was rapidly developed, include the 2009 H1N1 influenza virus antigen in order to protect against that virus (139). However, if new mutations arise resulting in a new pandemic subtype, then the available vaccine will be useless and again no vaccine will be accessible to protect against a potential new virulent strain with a high rate of mortality, such as seen with the previous H1N1 influenza A virus pandemics.

In 2014, West Africa experienced a devastating outbreak of Ebola and multiple countries were affected. In response to that situation, several countries and institutions such as the WHO and the CDC activated emergency operations to control the situation (98). Although the end of transmission of Ebola was reported in Liberia and in Guinea, still the WHO in Guinea, Liberia and Sierra Leone has still reported a total of 28,616 Ebola cases, with 11,310 deaths (140). Ebola virus is associated with hemorrhagic fever and is transmitted by corporal fluids. No vaccine or treatment is available for this virus; thereby efforts in that situation were to limit transmission of the disease.

On the other hand, according to the CDC, most of the Zika virus cases have been reported in many countries of South America, Africa, Asia, and the USA (141). This virus is transmitted by a mosquito-borne (*Aedes aegypti*) and symptoms include mild fever, headache, arthralgia, myalgia, non-purulent conjunctivitis, and a pruritic maculopapular rash (142). However, the most concerning effect that has been associated with Zika virus is the prenatal microcephaly (143).

According to the WHO-vaccine pipeline tracker, vaccines against AIDS, malaria, enteric pathogens, including human norovirus, the respiratory syncytial virus, Zika virus, Dengue virus, and pulmonary TB are in different stages of development. Some of these diseases, such as AIDS or pulmonary TB have been a concerning problem, since for several years have not been obtained a definitive cure or an efficient vaccine to prevent them. In addition, other diseases, such as the ones caused by the Zika virus, have had emergency problems that have required a rapid response. One prompt response strategy for the past Ebola outbreaks has been the use of anti-Ebola antibodies from the blood of disease survivors. Therefore, strategies with monoclonal antibodies to treat Ebola are currently being studied (144). Moreover, research on nanoparticles, adenovirus-based, modified Vaccinia Ankara-based, and recombinant-rabies vaccines against Ebola are ongoing, even in phase I, II and III of clinical trials (98, 145). From Ebola vaccines in clinical trials so far, the most advanced one is a recombinant vesicular stomatitis virus–Zaire Ebola virus (rVSV-ZEBOV) vaccine that has been licensed to Merck and recently, showed to be effective in susceptible individuals (146). On the other hand, strategies such as adenovirus-based recombinant vaccines and cell culture-derived inactivated vaccines using BHK and Vero cells are under research for Zika virus vaccine development (147). Despite the research ongoing about Zika and Ebola viruses, or other common and fastidious viruses such as respiratory syncytial virus and human norovirus, no vaccines or efficient treatment are still available. Thus, high technology centers are urgently needed to provide a solution to these problems and offer a rapid response to global health emergency states.

As emerging diseases, microorganisms with multiple resistances to antimicrobial agents have been reported in the past years. Bacteria resistance to the available antimicrobial agents, such as *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium tuberculosis* have alarmed health care worldwide for their resistance to antimicrobial agents (148–151). Furthermore, availability of an effective therapy for patients infected with those microorganisms is limited and more research and development is needed (152). Despite policies concerning the use of antimicrobials and the development of new drugs, it is urgent to increase the vaccine manufacturing capacity to prevent the spreading of these infections with multiple antibiotic resistance (153).

CONCLUDING REMARKS

There is no doubt that many diseases have been prevented due to the implementation of extensive vaccination programs. Domestic health public systems worldwide are committed to increase vaccination coverage for the population through national immunization programs. Thus, the WHO recommends to immunize children with BCG, DTaP, MMR, and vaccines to prevent hepatitis B, poliovirus, Hib, several serotypes of *S. pneumoniae*, rotavirus, and HPV. However, not all these vaccines are included in the national immunization programs of most countries. Not only the problem is the inclusion of some vaccines in local programs of immunizations but also the cost associated with its production, implementation, and delivery are part of the barriers. In this line, it is important to highlight the effort of some organizations such the WHO, the PATH, the GAVI Alliance, the UNICEF, the Bill & Melinda Gates Foundation, among others, to include as much population as possible in these immunization global strategies. Furthermore, shortages around the world have taken places during the past years, which have underscored the necessity to improving the capacities and infrastructure to produce and distribute vaccines. It is important to underscore the role played by new countries manufacturing vaccines, which include Brazil, the Russian Federation, India, China and South Africa (a group known as BRICS). Such local production has

contributed to ensuring access to traditional vaccines and to maintaining the stability of immunization programs in developing countries. Also, an important gap between industrialized and developing countries prevails in this field. Further, Ebola, Zika, influenza virus pandemics, and antimicrobial resistance have raised alarms, questioning whether we are prepared to control rapidly and efficiently viral pandemics worldwide.

AUTHOR CONTRIBUTIONS

ERJ, FT, NMD, and AK wrote the manuscript; ML, LC, SB, CR, and YG reviewed the manuscript; and AK reviewed and approved the version to be published. All authors listed have made substantial and intellectual contribution to the work.

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REVIEW
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Education in Vaccinology: An Important Tool for Strengthening Global Health

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Over the past 20 years, education of scientists and public health professionals in Vaccinology has increased dramatically. There are now many international, regional, and national courses that provide education in vaccinology. The proliferation of these courses and the high number of applications submitted demonstrate the increasing and continuous need for improved education in this field since, generally, comprehensive vaccinology training is not offered to medical and/or biological sciences students as part of their Universities courses and consequently there is insufficient knowledge of vaccine topics among health-care providers. Multidisciplinary vaccinology courses have not only educational purposes but they may also contribute to strengthening the development, testing, and use of vaccines, which remain the most efficient tool for infectious disease prevention. The courses available have a varied focus and prioritize topics based on the trainees' different levels of professional exposure and requirements. Overall, they might be classified in two key categories: (i) courses targeting students who, after their university studies in Medicine, Biology, etc., develop a strong interest in vaccines, would like to learn more about the various aspects of vaccinology, and potentially develop a career in this field (postgraduate courses); (ii) courses targeting postdoctoral professionals, who already have a sufficiently broad knowledge of vaccinology, but would like to develop stronger skills to be able to play a leading role in decision-making for vaccine development (advanced professional courses). Both postgraduate and professional courses are available and are based on comprehensive curricula. In the future, particular attention should be paid to include in the training curricula topics that might help vaccine development, efficient and sustainable vaccine introduction through epidemiologically sound vaccination programs, and best practices to address associated challenges, including vaccine hesitancy which could become a threat to successful implementation of vaccination programs, particularly in developed countries. In addition, it appears that the next phase of vaccinology training could benefit from a global and more structured platform that could facilitate exchanges and collaboration and amplify the current capacity for disseminating vaccine education for future vaccinology leaders around the world. This would be favored by synergizing the efforts currently devoted to vaccinology education. To initiate this process of analysis and systematization, a multinational effort is needed.

Keywords: training, vaccinology, education, global health, vaccines

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INTRODUCTION

Education in vaccinology is an important priority to strengthen development, testing and use of vaccines, which remain the most efficient tool for the prevention of infectious diseases both in developed and developing countries. The several courses available worldwide today have a different focus and curricula are tailored to the trainees' different levels of professional exposure and requirements (1, 2). Overall, they might be classified in two key categories: (i) courses for more junior scientists who, after completion of their biological studies at University, would like to know more about vaccines and vaccinology and might potentially develop a career in this field (postgraduate courses); (ii) courses for experienced scientists who already have a quite good knowledge of vaccinology and are ready to develop a deeper competence to lead vaccine development projects at various levels of responsibility and to actively participate in strategic groups deciding on vaccination policies at national, regional, or international levels (advanced professional courses).

POSTGRADUATE COURSES IN VACCINOLOGY

Some of the disciplines representing the fundamental scientific background for efficiently working in a vaccine development environment, such as clinical aspects of infectious diseases, microbiology, immunology, epidemiology, biostatistics, and others, are regularly taught in University courses; however, most often these courses do not have a focus on the whole vaccine development process or on the public health context for the introduction of new vaccines and rarely are these disciplines presented with a multidisciplinary and holistic approach (3). In addition, theoretical teaching is not enough, and there is also a need for practice-based training and exposure to vaccine development-orientated activities. This is particularly true for disciplines that are not usually taught in university courses, such as Pharmacovigilance, Regulations, and Ethics in vaccine R&D studies and, particularly, aspects related to animal and human research. In this regard, internships within an experienced project team are an opportunity not only to allow young scientists to learn day-by-day vaccine development work but also to introduce them into the dynamics of a scientific community working together toward a common goal.

Multidisciplinary vaccinology courses are an important priority particularly for scientists from developing countries where vaccines have significantly contributed to the dramatic decrease in the number of deaths, due to infectious diseases, particularly in children below 5 years (4). However, in these countries, almost five million children still die every year and many of these deaths are due to vaccine preventable diseases. Therefore, there is a huge need not only for new vaccines against diseases mostly affecting developing countries, for which a vaccine is not yet available, but also for significant efforts and resources to introduce in Africa, Asia, and in general in low- and middle-income countries (LMIC) vaccines that are already available to children of developed nations. Development and introduction of new vaccines in these countries is obviously dependent on availability of locally generated data, particularly the high-quality clinical data needed by regulatory authorities for vaccine registration and by WHO for vaccine pre-qualification. An essential requirement to make this happen is to have a cohort of well-trained scientists from developing countries who have a clear understanding of the whole process behind vaccine development and subsequent vaccine distribution. With these capabilities, local scientists may become active players and efficiently implement the various activities needed for registration of new vaccines and then support post-licensure vaccine introduction in the context of country tailored immunization campaigns. Therefore, vaccinology courses for scientists from developing countries should include classes on epidemiology and clinical development, but also education on public health systems operations, cold chain logistics, and vaccine distribution. Given the challenges associated with such extensive vaccinology training, identification of suitable candidates for the training activities is really key. Participants may have different educational backgrounds and different R&D experience; therefore, well thought selection criteria based on a grading system should be established upfront to make sure that selected candidates can get the most from the training activities.

An example of this approach is given by the Master in Vaccinology and Pharmaceutical Clinical Development of the University of Siena (5), which one of us, AP, contributed to set up and implement. This course, a collaborative effort between academia and vaccine industry, particularly tailored for young physicians from developing countries, is an 18-month program, combining theoretical and practical training. The theoretical teaching component includes 10 modules in the key vaccinology disciplines (Public Health and Vaccine Development Process; Immunology and Preclinical Research; Manufacturing and Quality Control Processes; Infectious Diseases and Vaccine Prevention; Clinical Development Methodology, Biostatistics and Clinical Data Management; Pharmacovigilance; Epidemiology, Health Systems and Economics; Good Clinical Practices, Clinical Quality Assurance and Clinical Trial Operations; Regulatory Affairs; Policies and Recommendations for Vaccines in the World) and, in addition, parallel educational seminars for personal and professional development. This extensive theoretical training is supplemented by a 7-month training, at the University of Siena and within different departments of the sponsors and collaborative institutions, followed by investigational site training. Finally, the value of this course to the students is maximized by a faculty including worldwide experts from well-known international universities, supranational organizations, and vaccine industry (5).

ADVANCED COURSES IN VACCINOLOGY

In the past 20 years, there was an explosive development and introduction of new vaccines that have or may have a considerable impact on public health strategies. As a result, there is now an increasing need for experts with a broad understanding of major issues in vaccinology. This need exists as well in industry, including major players and subject matter experts, as in academia and public health. In fact, it is of critical importance for decision

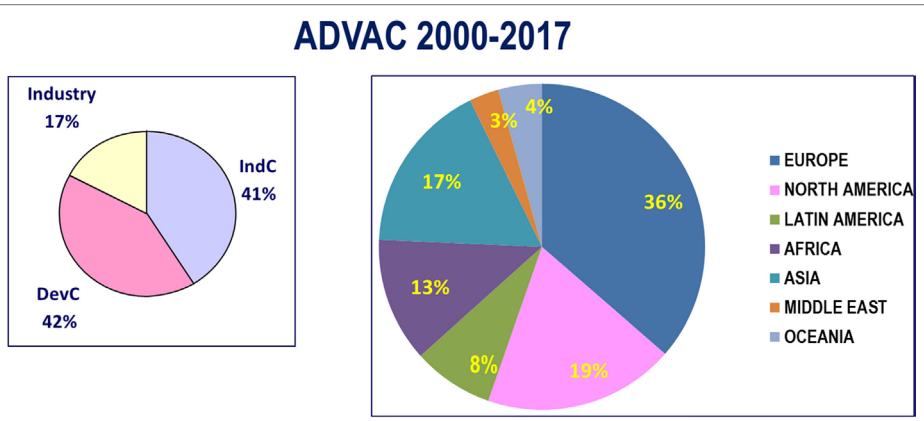

FIGURE 1 | Geographic distribution of ADVAC participants from 2000 to 2017.

makers in industry to understand the needs and the determinants that will influence the use of a given new vaccine in various country settings. Similarly, experts involved in public health strategy and in decisions related to the introduction of a new vaccination program at national, regional, or international levels must know key issues in the development process, essential safety considerations, limitations of the manufacturing process, and vaccine-related economic issues, e.g., cost-effectiveness. Managing real or alleged post-licensure safety issues is of critical importance. These aspects are also of great concern for academic professionals involved in training scientists with a potential role in vaccine development or monitoring.

A good example of this type of training is the Advanced Course of Vaccinology, ADVAC, which one of us, PHL, contributed to set up and implement. This course, organized on an annual basis since 2000 by University of Geneva and Fondation Mérieux, at Veyrier-du-Lac (France), in partnership with WHO, Johns Hopkins SPH & CDC and support from the European Commission and the Bill and Melinda Gates Foundation (6). At inception, it was aiming at filling major gaps in global vaccination strategies: (i) a lack of scientists with a broad vision of issues related to vaccines and immunization, (ii) a lack of qualified decision makers to identify priority targets in vaccinology, and (iii) a lack of qualified policy makers for deciding on the introduction of new vaccines in vaccination programs. Since 2000, 18 courses have been organized, gathering in total 1,070 participants from over 100 countries (**Figure 1**). To ensure a maximal impact, it appeared of particular importance to select highly motivated candidates, likely to have soon increasing responsibilities. It was also critical for appropriate networking to maintain a course format allowing the mixing of people with diverse professional backgrounds and diverse geographic origin: 41% came from high-income countries, 42% from LMIC, and 17% from industry (**Figure 1**). The ADVAC curriculum is providing a broad view of the various aspects of vaccinology: (1) priority targets for vaccine R&D, (2) understanding vaccine-induced immune responses, (3) new vaccine approaches, (4) clinical assessment of vaccine efficacy, (5) vaccine safety and regulatory aspects, (6) decision-making process for introduction of new vaccines, (7) defining optimal vaccination strategies, and (8) dealing with real or alleged adverse effects. The success of these courses is certainly dependent on the quality of the lecturers who are all top level vaccinologists on the international scene. However, a key factor is the interactive nature of all sessions, particularly in small groups or during group exercises including role play sessions and informal debates. The concurrent evaluation of training sessions is particularly helpful to adjust the level of training to the needs of the students. A follow-up program for ADVAC alumni has proven effective to maintain and increase the network of vaccinologists that is resulting from the initial training effort.

DISCUSSION

Several postgraduate and advanced professional courses are available for training of junior and senior scientists, interested to deepen their respective knowledge in vaccinology. As shown by the examples mentioned in this review, some of these courses have already good multidisciplinary curricula; however, looking at the challenges and gaps that still limit the expansion and the sustainability of vaccination programs, there are a number of topics that should be more deeply addressed in future trainings.

An important gap toward expansion of vaccination in developing countries, particularly in Africa, is the lack of a sufficient manufacturing capacity that could enable local development and production of new vaccines, thus making vaccination programs sustainable in most of LMIC once GAVI support is over. Among other factors, development of local manufacturing capacity is affected by lack of a well-trained and competent pool of local scientists and technicians who could reliably support technical operations ranging from technology transfer activities to development, formulation, manufacturing, quality control, and release of vaccines.

Recently, some training courses on these aspects have been organized by WHO, also in collaboration with both public and private institutions (7, 8). In addition, as part of the ADITEC project funded by the European Commission (9), the WHO and the University of Lausanne organized various theoretical and practical 1-week courses in "Adjuvants and vaccine formulations" with the objective of training students on production, purification, characterization, and control of recombinant antigens, and on methods of preparation of adjuvants, including oil-in-water emulsions and

aluminum gels, their formulation with antigens, and quality control of the resulting vaccines. Outcome of these technical trainings was excellent, based on the feedback received, and similar initiatives should be more frequently organized and offered to fruition in the future.

Anti-vaccination sentiments are heterogeneous beliefs, commonly defined as vaccine hesitancy, may represent an important cause of reduced vaccination coverage, both in developing and in developed countries, and sometimes may lead to recrudescence of infectious diseases for which vaccines have been available for a long time (10). Despite their unquestionable contribution to the reduction of morbidity and mortality from infectious diseases and, more in general, to an increased level of public health worldwide, for several reasons, mostly unfounded, vaccines have been associated to negative perceptions about their safety and, consequently, a growing sense of mistrust is associated with their use and should be properly addressed. Adequate education of health-care professionals is of paramount importance to address and reduce parental anxiety, concerns, and fears and therefore vaccinology trainings should more and more include well-documented sessions on vaccine safety. Similarly important is that vaccinologists are appropriately educated also on the potential side effects of vaccination, including identification and quantification of risks, so that, providing balanced and respectful information, they may contribute to re-establishment of trust (11).

An alarmingly high number of emerging bacterial infections are caused by the increasing anti-microbial resistance (AMR) worldwide and they may play an even worse effect on global morbidity and mortality in the near future (12). This is largely due to excessive and often inappropriate use of new antibiotics in medical practice and to the poorly controlled antibiotic use in animal food industry. Education of vaccinology scientists on the achieved reduction of antibiotics use and AMR by vaccination, with tangible benefits going beyond the non-vaccinated populations, through herd immunity, might push toward development of new future vaccines having also AMR reduction in their target product profile. This might also lead, on the one side, to better quantify the magnitude of antimicrobial use and of AMR for a given vaccine preventable disease and, on the other side, to select more appropriate vaccine candidates, including vaccines against highly resistant serotypes of the pathogen and/or virulence factors relevant for resistance acquisition.

Some other aspects deserve more and more attention in future vaccinology trainings; they include (i) preclinical and clinical vaccine assessment in LMIC, (ii) financing of vaccination programs, (iii) vaccine delivery, (iv) vaccine introduction strategies, and (v) vaccine regulations.

CONCLUSION

Our vision of the future of vaccinology, and associated medical and social impacts, is that more and more scientists will be required for the implementation of all aspects of the vaccinology lifecycle process, from vaccine research to optimal vaccine use in the field. Therefore, the importance of appropriately developing the technical skills of next generation vaccinologists is paramount, best initiatives currently devoted to vaccinology education should join forces and, with a multinational effort, a global and structured platform for future training of vaccine scientists around the world should be developed. To achieve this goal, a global commitment to provide continuous education and training is needed from all stakeholders, including Academia, Industry, and Public Health Institutions, with the ultimate objective of ensuring sustainability of life saving vaccination programs at the global level.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to this manuscript.

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Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top

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Antimicrobial resistance (AMR) is currently the most alarming issue for human health. AMR already causes 700,000 deaths/year. It is estimated that 10 million deaths due to AMR will occur every year after 2050. This equals the number of people dying of cancer every year in present times. International institutions such as G20, World Bank, World Health Organization (WHO), UN General Assembly, European Union, and the UK and USA governments are calling for new antibiotics. To underline this emergency, a list of antibiotic-resistant "priority pathogens" has been published by WHO. It contains 12 families of bacteria that represent the greatest danger for human health. Resistance to multiple antibiotics is particularly relevant for the Gram-negative bacteria present in the list. The ability of these bacteria to develop mechanisms to resist treatment could be transmitted with genetic material, allowing other bacteria to become drug resistant. Although the search for new antimicrobial drugs remains a top priority, the pipeline for new antibiotics is not promising, and alternative solutions are needed. A possible answer to AMR is vaccination. In fact, while antibiotic resistance emerges rapidly, vaccines can lead to a much longer lasting control of infections. New technologies, such as the high-throughput cloning of human B cells from convalescent or vaccinated people, allow for finding new protective antigens (Ags) that could not be identified with conventional technologies. Antibodies produced by convalescent B cell clones can be screened for their ability to bind, block, and kill bacteria, using novel high-throughput microscopy platforms that rapidly capture digital images, or by conventional technologies such as bactericidal, opsono-phagocytosis and FACS assays. Selected antibodies expressed by recombinant DNA techniques can be used for passive immunization in animal models and tested for protection. Antibodies providing the best protection can be employed to identify new Ags and then used for generating highly specific recombinant Fab fragments. Co-crystallization of Ags bound to Fab fragments will allow us to determine the structure and characteristics of new Ags. This structure-based Ag design will bring to a new generation of vaccines able to target previously elusive infections, thereby offering an effective solution to the problem of AMR.

Keywords: antibiotic resistance, vaccination, reverse vaccinology, human immunology, public health

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"MICROBES MAKETH MAN"

Modifying the old saying "Manners maketh man" (reported by William Horman in *The Vulgaria* written in 1519), a few years ago the magazine The Economist published in its Leaders section a comment regarding the new vision of the interaction between microbes and man (1). The new saying clearly indicates that microbes have determined in many ways the evolution of the human

species. At a first level, bacteria, viruses, fungi and archaea have literally become a part of us, forming the so called microbiota. A recent study has defined more precisely the number of bacteria present in our body, which is in the order of 39 trillion cells (2). Since the estimated number of human cells in the body (about 84% of which are red blood cells) is in the order of 30 trillion, the ratio between bacterial and human cells is about 1.3. The numbers may vary significantly from person to person and could change significantly with each defecation, ranging from 30 to 50 trillion in each individual. Women may also have a higher ratio of bacterial vs. human cells, because they have fewer red blood cells. This evaluation does not take into account fungi, viruses, and archaea, which all make up the human microbiota and would increase the ratio of microbes to human cells. Thus, we can consider ourselves like superorganisms, in which microbes do many jobs in exchange for the raw materials and the shelter their host provides. This alone shows how closely host and microbiota have co-evolved.

But microbes are also part of a living universe outside us, and often they act as parasites able to regulate the human life span. Over the entireness of the three million years of our species' evolution, life expectancy has always been between 25 and 35 years until very recently, and infections have been the main regulators of our life span. By learning from observation of nature, human beings progressively improved their living conditions to the point that about 250 years ago life expectancy started to increase. In 1900, mankind had already reached a life expectancy of approximately 50 years (3). Nowadays, a child born in a high-income country can expect to live 85 years. The additional 35 years of life that we gained during the last century are substantially due to the conquest of infectious diseases, which used to kill 50% of people before the age of 20. These were viral diseases such as smallpox, rabies, measles, rubella, mumps, and bacterial infections such as diphtheria, tetanus, typhoid fever, and cholera (4). This result has been achieved primarily by improved hygiene, but also by treatment of infectious diseases with antibiotics and by their prevention throughout vaccination. As negative control of this important result, we have to consider the poor areas of our planet, where hygiene, vaccines, and antibiotics are not properly used even today. As a consequence, in these areas infections still represent a major cause of mortality, maintaining life expectancy below 50 years.

DISCOVERING A GREAT TOOL AGAINST MICROBES

Antibiotics are an important example of how man can learn from nature to improve his own living conditions. The first observation that microbes are able to produces substances capable of killing pathogens came from the Italian scientist Vincenzio Tiberio in 1895, who showed the antibacterial activity of a natural substance produced by molds (5). Then Ernest Duchesne in France published in his doctorate thesis presented in 1897 (6) that the mold *Penicillium glaucum* possesses antibacterial properties. It was Alexander Flaming in 1928 that succeeded in definitely identifying the world's first antibiotic. It was a substance isolated from the mold *Penicillium notatum*, defined as benzylpenicillin (penicillin G) (7). The first industrial production came however after Howard Florey and Ernst Boris Chain continued the work of Flaming in Oxford. Thus, after the Pearl Harbor attack in 1941 a mass production could be initiated. By 1944, enough penicillin was produced to treat the wounded soldiers in the Allied forces. The 1945 Nobel Prize in Physiology and Medicine was assigned to Flaming, Florey and Chain. The era of antibiotics had begun, and several other molecules produced by microbes followed penicillin. With antibiotics, mankind could claim an historical success in the eternal war against pathogens. However, it was soon clear that antibiotics were not the definitive weapon.

In a book published in 1975, Stanley Falkow wrote that "*we owe to chemotherapy (antibiotics) the debt of reducing the high mortality rate of many bacterial infections*" and to hygiene and vaccines the debt of preventing them, however "*in helping to solve some of the problems of infectious diseases, chemotherapy has created some problems of its own*" (8). The problem created by antibiotics was the generation of bacterial strains resistant to multiple antibiotics, an event reported for the first time in 1956, with the isolation in Japan of a strain of *Shigella flexneri* resistant to streptomycin, tetracycline, chloramphenicol, and sulfonamides.

Today, antimicrobial resistance (AMR) has grown out of proportion, and many pathogenic bacteria are resistant to multiple antibiotics, including *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella*, *Escherichia coli*, *Acinetobacter*, *Proteus*, *Klebsiella*, *Serratia*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Helicobacter pylori*, and others. In a few cases, bacteria became resistant to most of the available antibiotics and are on the verge of becoming untreatable. As a consequence, AMR is perhaps the most alarming emerging problem of infectious diseases. Globally, AMR already causes 700,000 deaths/year, and the forecast is that in 2050 it will cause 10 million deaths/year, higher than the 8.2 million deaths caused by cancer today. As an example we can look at *S. pneumoniae*, also known as pneumococcus, a human pathogen that is the major cause of community-acquired pneumonia, bacterial meningitis, bacteremia, and otitis media (9, 10). In the past, most strains of *S. pneumoniae* were sensitive to penicillin, whereas today penicillin resistance goes from 5 up to 60% in various parts of the world. Thus, with time old antibiotics become less effective or lose efficacy, making the search for new molecules with different mechanisms of action a priority (11).

Alarming documents, calling for action and asking for new antibiotics, have been issued by governments such as those of the UK and USA, by the European Union (EU), and by international organizations such as the World Health Organization (WHO), the United Nations General Assembly, and the World Bank and the G20. The interest in fighting the increase in AMR has intensified, and new incentives for research and development of new drugs have been deployed. In 2016, about 500 million US$ have been allocated to new and existing initiatives aiming to accelerate the development of new antibiotics[1]. For example, the Innovative Medicines Initiative (the biggest public–private program in biomedical science of the EU Commission) funded several projects defined as *New Drugs*

[1] Boston Consulting Group, Federal Ministry of Health. *Breaking Through the Wall: a Call for Concerted Action on Antibiotics Research and Development* (2017). Available from: http://www.bcg.de/documents/file219507.pdf (Accessed: May 21, 2018).

for Bad Bugs[2]. Other initiatives include CARB-X, a collaboration between US and UK partners[3], and the Global Antibiotic Research and Development Partnership, a collaboration between the Drugs for Neglected Diseases *initiative* and the WHO[4]. It is also interesting a German proposal for a Global Union for Antibiotics Research and Development (GUARD), aimed at funding and coordinating a facility for antibiotics research and development.

On February 27th 2017, the WHO published a document[5], which we partially report hereafter: "*This is the first ever list of antibiotic-resistant "priority pathogens", a catalog of 12 families of bacteria that pose the greatest threat to human health. The list highlights in particular the threat of Gram-negative bacteria that are resistant to multiple antibiotics. These bacteria have built-in abilities to find new ways to resist treatment and can pass along genetic material that allows other bacteria to become multi-drug-resistant. The WHO list is divided into three categories according to the urgency of intervention, of critical, high and medium priority. The most critical group includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes and among patients whose care requires devices (such as ventilators and blood catheters). The group encompass Acinetobacter, Pseudomonas and various Enterobacteriaceae (including Klebsiella, E. coli, Serratia, and Proteus). These bacteria can cause severe and often deadly infections such as bloodstream infections and pneumonia, and have become resistant to a large number of antibiotics, including carbapenems and third generation cephalosporins (the best available antibiotics for treating multi-drug resistant bacteria). The second and third tiers in the list—the high and medium priority categories— include other increasingly drug-resistant bacteria that cause more common diseases, such as Neisseria gonorrhoeae (the agent of gonorrhea) and Salmonella (causing food poisoning). This WHO action intends to promote initiatives of basic science and advanced R&D from both publicly funded agencies and the private sector, aiming to discover new antibiotics.*"

The WHO text continues as follows:

> *"Tuberculosis (TB) was not included in the list, although its resistance to traditional treatment has been growing in recent years, because TB is targeted by other dedicated programs. However, we must remember that TB now kills more people than any other pathogen (1.8 million in 2015), and it is therefore a most urgent priority. Other bacteria that are not included in WHO list, such as Group A and group B Streptococcus and Chlamydia, have low levels of resistance to existing treatments and do not currently pose a significant public health threat, but there is a risk that with time also these pathogens may become resistant. The list was developed in collaboration with the Division of Infectious Diseases at the University of Tübingen, Germany, using a multi-criteria decision analysis technique vetted by a group of international experts.*
>
> *The criteria for selecting pathogens in the list were: a) how deadly the infections they cause are; b) whether their treatment requires long hospital stays; c) how frequently they are resistant to existing antibiotics when people in communities catch them; d) how easily they are transmitted between animals, from animals to humans, and from person to person; e) whether they can be prevented (e.g., through good hygiene and vaccination); f) how many treatment options remain; and g) whether new antibiotics to treat them are already in the R&D pipeline."*

The WHO document strongly underlines the need for new treatments. Thus, the search for new antimicrobial drugs is and must remain a great priority. However, it is important to realize that the pipeline for new antibiotics is not very promising, thereby making unlikely that the problem will be solved along this line (12). On the other hand, another tool that, together with antibiotics, contributed to conquer and eliminate many infectious diseases, i.e., vaccines, have a very promising pipeline thanks to the new technologies (3). Thus, vaccines have the possibility to make a big contribution to the control of AMR.

RESISTANCE TO ANTIBIOTICS AND VACCINES

The analysis of how vaccines and antibiotics contributed to conquering infectious diseases during the last century was originally published by the group of one of the authors of this paper (13), and more recently re-analyzed in depth by Kennedy and Read [(14), **Figure 1**]. This analysis shows that resistance to antibiotics inevitably emerges every time that a new antibiotic is introduced, starting a process of selection in the target bacteria that will eventually make that antibiotic useless. The consequence is that there is a continuous need of a fresh supply of novel antibiotics, to maintaining effectiveness of the therapeutic treatment. This strategy worked very well up to 1970s, when the identification of new antibiotics was abundant. However, since then the pipeline for new antibiotics has been drying out, and we have not been able to discover new classes of antibiotics (11). In marked contrast, Kennedy and Read show that we can use vaccines for a long time, generating no or very little resistance (14). Thus, vaccines can control infections over a long period of time without becoming obsolete. This occurs because vaccines work prophylactically and prevent the start of infections, while drugs work therapeutically on an ongoing infection in which bacteria proliferate and mutate, allowing the drug to select the resistant variants.

Furthermore, drugs are targeting few metabolic pathways on the pathogens, whereas vaccines induce a protective immune response against multiple antigenic targets. It can be concluded that selection has fewer opportunities to act upon vaccination than with antibiotic treatment. Still, both vaccines and antibiotics are very important in the control of infections. **Table 1** reports the major differences in the mode of action of vaccines and antibiotics and provides information that can guide us to take advantage of their strengths and to minimize their weaknesses. From this comparison, it is evident that antibiotics are the only life-saving tool

[2]http://www.imi.europa.eu/content/nd4bb (Accessed: May 21, 2018).
[3]http://www.carb-x.org/about (Accessed: May 21, 2018).
[4]http://www.dndi.org/wp-content/uploads/2016/03/GARDP_Briefer_Document.pdf (Accessed: May 21, 2018).
[5]http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/ (Accessed: May 21, 2018).

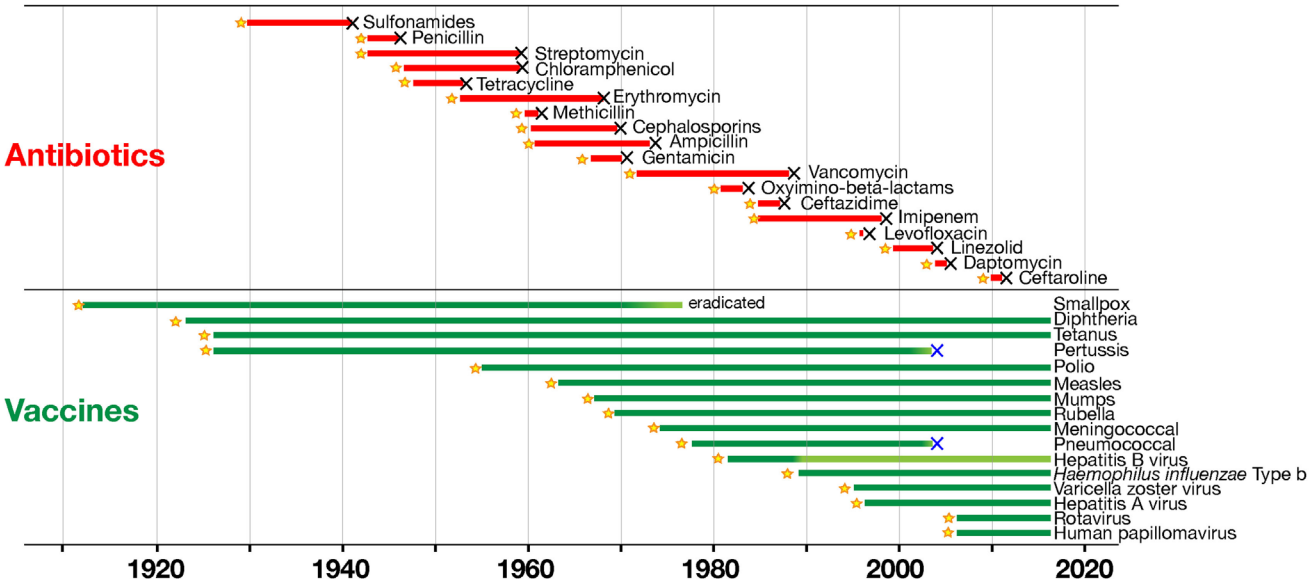

FIGURE 1 | Time to detection of resistance of human pathogens to antimicrobials (in red) and to vaccines (in green). Black X symbols indicate insurgence of resistance, with lines starting at product introduction (yellow stars; except for smallpox vaccination that began much earlier; with modifications from Ref. (14) with the permission of the publisher).

TABLE 1 | Comparison of the characteristics of vaccines and antibiotics in their capacity to fight pathogens.

Target/use	Vaccines	Antibiotics	Comments
Emergency use	No	+++	Antibiotics are immediately effective and are life saving during acute infections Vaccines require from 1 week to several months before they are fully protective
Memory (protection from diseases in the long term)	+++	No	Antibiotics are only effective while present in the body Vaccines induce a memory that last for many years
Eradication (of the infectious agent)	++	No	Vaccines allowed eradication of smallpox, and the elimination of polio, diphtheria, *Haemophilus influenzae*, meningococcus A and C, several strains of pneumococcus
Resistance (selection of resistant microbes)	+/–	+++	In nature, there are bacteria resistant to every antibiotic. Use, misuse, and abuse of antibiotics selects resistant bacteria and may generate superbugs that are resistant to most antibiotics There are very rare cases of resistance to vaccines.
Generation of new pathogens	–	++	Use, misuse, and abuse of antibiotics can select new pathogens as in the case of group B *Streptococcus*
Population use	++	–	Vaccines are most effective when used to vaccinate the entire population and generate herd immunity Antibiotics are most useful for the acute treatment of individual infections
Scientific progress (in the last 30 years)	+++	+/–	New powerful technologies such as glycoconjugation, genomics, structure-based antigen design, and adjuvants propelled the discovery and development of many novel vaccines Antibiotics did not benefit from the new technologies and during the last 30 years there was no discovery of new classes of antibiotics

that we can use during acute bacterial infections, although their often improper or excessive use is causing bacterial resistance in a continuously increasing fashion. The availability of vaccines to control infections may allow us to decrease the use of antibiotics and to generate less AMR. This will permit a more efficient use of existing and new antibiotics during acute infections. As shown in **Figure 1**, there are, however, some cases in which resistance evolved after vaccination. This can be due to several causes, such as the fact that vaccines can protect from disease but may not have the capability to completely prevent pathogen colonization and transmission, as in the case of the acellular pertussis vaccine, or it can be caused by serotype replacement after vaccination with vaccines not including all serotypes, as in the case of the vaccines against *S. pneumoniae*. Thus, even for vaccines the search for better protective antigens (Ag) is very important, particularly for antibiotic-resistant infections.

In May 2016, a group of experts coordinated by the economist Jim O'Neill published a comprehensive report entitled "*Tackling drug-resistant infections globally*" (11). Among many indications to prevent the increasing global problem of AMR, an entire section was devoted to vaccines. Hereafter we report part of the section "*We must reduce the demand for antimicrobial so the*

current stock of drugs last longer," and in particular in the point Intervention 6 entitled "*Promote development and use of vaccines and alternatives*":

> "*Vaccines can prevent infections and therefore decrease the demand for therapeutic treatments, reducing the use of antimicrobials thereby slowing the rise of drug resistance. Thus, vaccines should be eligible for the same incentives applied for antibiotic development. In particular, it is recommended 1) to use existing vaccines in humans and animals; 2) to renew impetus for early-stage research; 3) to sustain a viable market for vaccines.*"

Similarly, the WHO document on priority pathogens stresses that the role of vaccines in the global AMR crisis remains of great importance.

PROMOTING THE DISCUSSION ABOUT VACCINES AS A REMEDY TO AMR

Since 2004, the year of 100th anniversary of the foundation of the Serology and Vaccinology Institute Achille Sclavo, a forum for the discussion of the most important issues of the vaccine world takes place every year in Siena, Italy. Each annual meeting aimed to analyze the state-of-the-art of important themes in the field of vaccines and expand the vision for the years to come. The participation of excellent speakers and expert discussants ensured the high quality of the meetings, whose conclusions were published in international journals (15–24). The meetings were organized close to a popular event, the horse race named "Palio di Siena," for which the town is worldwide famous. Thus, those meetings were called the Palio Meetings. More recently, with the acquisition of the vaccine company in Siena by GSK, the venue of the meeting was moved to other locations in USA and Europe, but the traditional name was maintained. The topic of the meetings can vary but the mission is always based on one or more of the following pillars: (1) must define the state-of-the-art of cutting edge topics related to infectious diseases; (2) must advocate science policies to promote progress and improvement in human health; and (3) must be a strategic forum aimed to build new initiatives. **Table 2** reports the topics of the Palio Meetings during the years.

It was therefore almost mandatory that the subject of the 2017 Palio Meeting should be on the growing emergency caused by antibiotics failure, with the title "*Prioritizing vaccines to fight antimicrobial resistance*." This event shortly followed a meeting organized by David Salisbury on 2017 March 29–30 at the Chatham House, in London (25). The London meeting provided a clear consensus that vaccines complement the actions of antibiotics and can contribute to control, reduce, and sometimes eliminate diseases caused by AMR pathogens, more than any other intervention. Thus, the main scope of the 2017 Palio Meeting was to build on the conclusions of the London meeting, and posed the question of how can we make vaccines achieve their full potential and become one of the top tools to tackle AMR. Indeed, the discussion led to conclude that there is a need to make stronger, more evidence-based cases supporting the importance of vaccines in AMR prevention (24).

One example is represented by vaccines against the main strains of *S. pneumoniae*, vaccines that have reduced pneumonia cases in the first decade of this century and in parallel have decreased the number of infections resistant to front-line antibiotics (26). The introduction in 2009 in South Africa of a pneumococcal vaccine achieved an analogous result. Furthermore, it is interesting to note that the high use of antibiotics, prescribed to treat opportunistic bacterial infections in people weakened by flu, is prevented when flu vaccines are employed. There is a

TABLE 2 | The Palio Meetings during the years.

Date	Meeting title	Location	Reference
2004 July 3	First International Congress on Emerging and Re-emerging Infections: Impact on Society, Economy and Medicine	Siena, Italy	
2005 August 17	Toward Global Health: Cooperation among Non-profit Organizations to Address Orphan Social Needs in Health: How to Build a Global Social Enterprise	Siena, Italy	
2006 August 17	Protagonists in Building Resources for Global Health and Delivering Health Tools to People who Most Need	Siena, Italy	
2007 July 3	Global Partnerships for Vaccination	Siena, Italy	(15)
2008 July 3	Meningococcus Scientific Exchange Meeting	Siena, Italy	(16)
2009 July 3	Rethinking Influenza: Can Planning Avoid the Panic?	Siena, Italy	(17)
2010 July 2	How Trust in Immunization Can be Built and Maintained	Siena, Italy	(18)
2011 July 2–3	Towards a Meningitis-Free World	Siena, Italy	(19)
2012 July 3	Prevention of Perinatal Group B *Streptococcus* Disease Through Maternal Immunization	Siena, Italy	(20)
2014 July 12	Enhancing Vaccine Immunity and Value	Siena, Italy	(21)
2015 July 18	Global Health 2015 – Mission Grand Convergence	Siena, Italy	(22)
2016 July 7	Emerging Infectious Diseases	Rockville, MD, USA	(23)
2017 July 6	Prioritizing Vaccines to Fight Microbial Infections	Wavre, Belgium	(24)

need to make public the data generated by vaccine companies on vaccine effectiveness against AMR, and to continuously monitor the circulation of resistant bacterial strains. The discussion is continuing, and ideas and actions are becoming better defined (27). A global strategic effort to develop a portfolio of vaccines that target AMR is becoming mandatory.

EVOLUTION IN VACCINE RESEARCH

Why vaccines are becoming an advantageous weapon to curb AMR? This is because their effectiveness in preventing infections has hugely improved, as a consequence of the enormous technological developments of the last two decades. Since the introduction of Jenner's vaccine against smallpox in 1798, the field of vaccines has steadily progressed, but in the last years vaccine development has enormously benefited from the -omics approaches. Thus, new potential vaccine candidates can be discovered in much shorter time than in the past, when vaccines have been developed more empirically (3).

The new techniques of genome sequencing introduced in the late 1990 completely changed the process for discovering novel vaccine Ags. The "*reverse vaccinology*" approach showed that, starting from sequence information, it is possible to discover the protective Ags without handling the microbes (28). A recently licensed vaccine against meningococcus B is the first vaccine produced with reverse vaccinology (29). During the last decade, vaccine design was further potentiated by new technologies, leading to an approach that has been named "*reverse vaccinology 2.0*" (30). As summarized in **Figure 2**, this approach takes advantage of human immunology for designing optimal vaccine Ags.

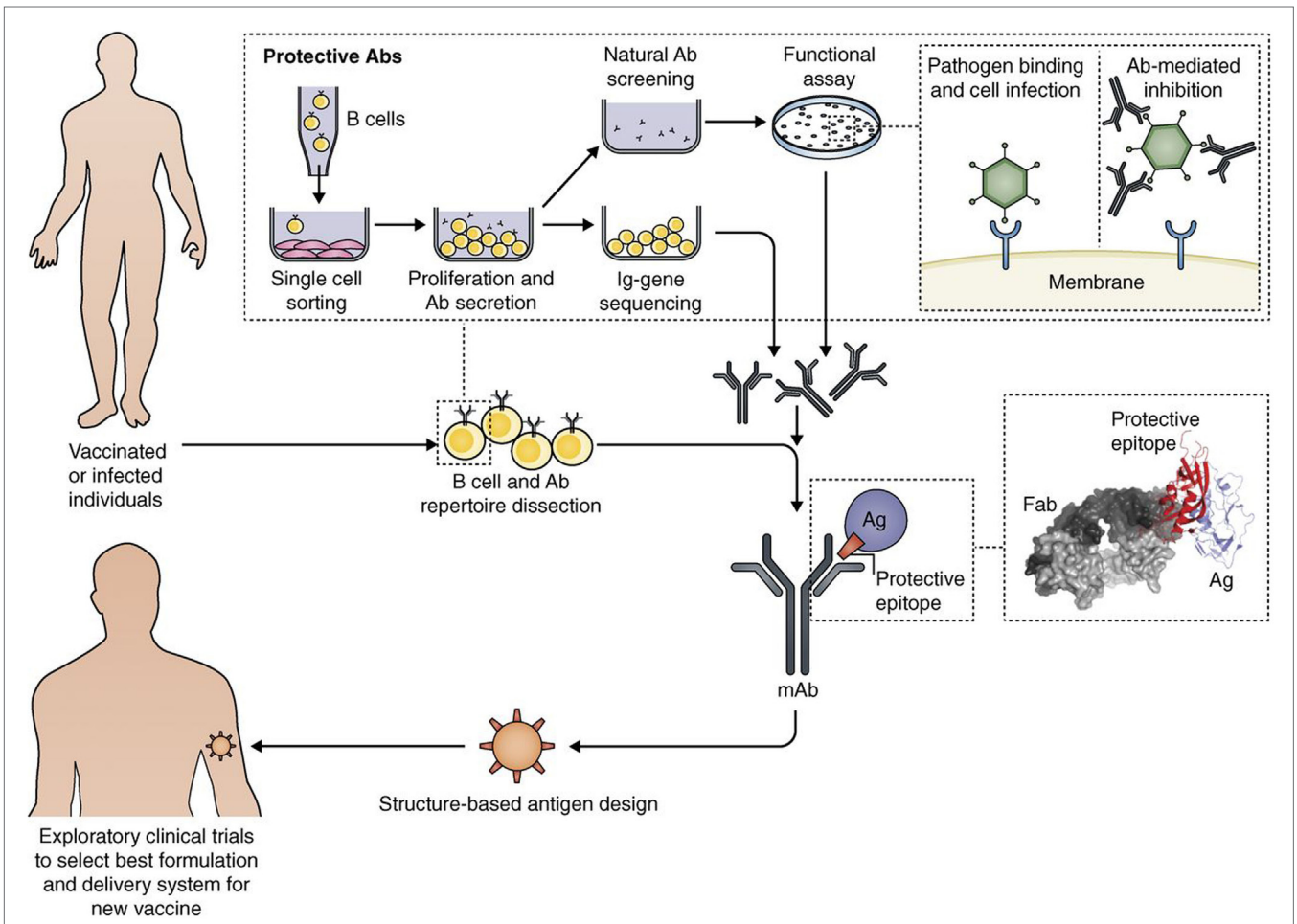

FIGURE 2 | Interplay of B cell technology and structural biology in vaccine design, as shown with the Reverse Vaccinology 2.0 approach (from 35). Flow path representation of how the analysis of the human B cell repertoire leads to the identification of protective Abs from vaccinated or infected subjects. From upper left: Single B cell sorting and culturing enables a direct screening and selection of naturally produced Abs with desired functionality, and the recovery of the corresponding Ig gene sequences. This approach allows us to interrogate single-sorted B cells through direct screening of Ab functionality. From the recovered Ig sequences, we can produce the Abs of interest as recombinant proteins, and fine-tune their properties. The structural characterization of recombinant monoclonal Abs bound to their target antigen (Ag) leads to a detailed definition of the protective epitope. The right inset shows the co-crystal structure of an Ag–Ab (Fab) complex, identifying a protective epitope (red). Engineering of the protective epitope can lead to the design of a novel optimized immunogen. For example, we can mount the epitope in an oriented multi-copy array on a nanoparticle that will act as carrier and increase an epitope-focused immune response ("structure-based Ag design"). The new Ag can be developed with the best formulation or delivery system to then be tested in humans (from Ref. (30) with permission of the publisher).

Thanks to better knowledge in handling human B cells and by selecting the most favorable donors, it is now possible to produce highly specific recombinant monoclonal antibodies (mAbs) and also their Ag-binding fragments (Fabs) (31). Further analysis by structural biology approaches brings to 3D studies of the target Ags complexed with the Fabs. It is also possible to discover the protective epitopes capable of inducing broadly neutralizing Abs (32–34). Furthermore, new computational approaches have allowed to obtain completely novel immunogens capable of inducing protection (35).

In viral infections, new structure-based powerful vaccine molecules have been already designed by screening human mAbs from convalescent people and obtaining the molecular structure of Ags and Ag–Ab complexes, as described earlier. Examples are the identification of the pentamer as key Ag for cytomegalovirus (CMV), and of the pre-fusion Ag of respiratory syncytial virus (RSV).

Until recently, the most promising CMV vaccine candidate was a recombinant form of the fusion protein gB. However, the human trial of gB combined with a potent adjuvant showed only moderate efficacy, and therefore the vaccine development was put on hold. Later, isolation of human mAbs from people previously exposed to CMV demonstrated that the most potent CMV neutralizing antibodies were not recognizing gB, but a complex Ag made by five proteins (pentamer). A recombinant form of the pentamer induced neutralizing antibodies that are orders of magnitude more potent than those induced by gB. The new Ag is a very promising candidate for a CMV vaccine and will soon undergo human trials (35).

In the case of RSV, it was possible to obtain a humanized mAb, palivizumab, that binds to an epitope present in the F protein in both the pre-fusion (pre-F) and post-fusion (post-F) conformation. Initial studies to develop an RSV vaccine were mainly focused on the use of the post-F protein that, unlike pre-F, is highly stable both as soluble Ag and when displayed onto virus-like particles. However, experimental vaccines based on whole virus, live attenuated virus, or post-F protein have failed to yield appropriate levels safety or efficacy. The scenario changed when the isolation and characterization of human neutralizing mAbs elicited by natural infection showed that the majority of antibodies are specific for the pre-F form of the protein and failed to cross-react with the post-F conformation. A structure-based design of a stabilized RSV pre-F protein was eventually obtained by complementing the crystal structure of the pre-F protein complexed with a highly neutralizing antibody with the neutralizing studies. The designed pre-F protein (DS-Cav1) could induce neutralizing antibodies 10–15 times more potent than those elicited by previous vaccines and is presently being tested in human trials (36, 37).

We can conclude that interrogation of human antibody responses can allow us to identify pathogen epitopes that are more likely to be protective and that are difficult to discover by conventional technologies. So far, isolation of human mAbs has been successfully used to identify viral Ags that could not be discovered by conventional technologies. On the other hand, there are no data yet regarding the identification of new Ags for antibacterial vaccines. After the proof-of-concept obtained with viral Ags, it would be very important to apply the same approach to the identification of novel bacterial Ags. This will allow us to design innovative vaccines against antibiotic-resistant pathogens, thereby effectively tackling the most pressing global health emergency.

CONCLUSION

It is time to consider how to find an effective solution to fight antibiotic resistance, and win this battle in the never-ending war against pathogenic microorganisms. As discussed, the human species has experienced an impressive prolongation of its life expectancy and improvement in life conditions, due to hygiene, antibiotics, and vaccines. Now, one of these pillars has weakened to the point that it will affect some important medical methodologies, first of all important surgeries, but also immunosuppressive chemotherapy and consequently organ transplantation, a great success of the medicine of our era. Again, infectious diseases could severely reduce our life span, as we will not be able to survive important medical treatments or even accidental wounds. A possible solution in sight is that of developing a combined preventive-therapeutic approach, in which vaccines will be one of the two arms and chemotherapy the other one. There are reasons to believe that the combination of the two approaches will result in an overall success.

The main reason is the difference in the mode of action between vaccines and antibiotics. First of all, vaccines on their own are rarely capable to generate resistance. Another critical difference between antibiotics and vaccines is the rate of discovery of new effective molecules. In the past, new antibiotics were identified and regularly reached the clinic, particularly in the three decades after 1950. Since then, however, very few new molecules have been introduced in the clinical use. An opposite situation occurred in the case of vaccines, which have been developed at an increasing speed. As for today, 22 new vaccines became available since 1980. This is a consequence of the introduction of new technologies, such as recombinant DNA, that led to the generation of new synthetic sequences. Therefore, we have obtained a great reduction of the incidence of bacterial meningitis (caused by *Haemophilus influenzae*, *S. pneumoniae*, and *Neisseria meningitidis*) thanks to a new generation of very effective conjugated vaccines, generated by chemical technologies for covalently linking bacterial polysaccharides to proteins. More recently, genomic sequencing opened the access to a higher level in vaccine design, since it made possible to predict the thousands of proteins encoded by bacterial genes, and to identify those likely exposed on the cell surface, in search of new vaccine candidates. This approach, defined as "reverse vaccinology" has resulted in a first important protein vaccine against meningococcus B that is now in use worldwide (29). Finally, a better understanding of the mechanisms regulating the induction of a protective immune response has opened the possibility to introduce, in vaccine formulations, new moieties that can make them more effective. These substances are defined with the general name of adjuvants.

The difference in the mechanisms of action of vaccines and antibiotics is enormous. Antibiotics are families of molecules produced by microorganisms to kill microorganisms. What

makes them effective is their capability to reach and poison targets across the strong barrier of the bacterial cell wall and avoid being ejected by potent efflux pumps. Any biochemical modification of the target microorganism can make the antibiotic inefficient. Among billions of bacteria present during an infection, such modifications can stochastically arise frequently. Vaccines are molecules with the capacity to evoke in the host a protective activity against infections. They do so by interacting with the immune system of the host, a system that during evolution has developed sophisticated mechanisms to recognize and destroy any kind of "danger" agents, essentially by distinguishing molecules that are different from self. The human immune system can potentially recognize any Ag in the universe, even those never encountered before, thanks to its complex gene rearrangement mechanisms. Thus, it is quite obvious that the potential of vaccines to protect us is extraordinary. And the more we learn about our immune system, the better we can design strategies and develop tools to protect our health from infections. For instance, in some cases it is now possible to cure established infections by administering the patient with specific antibodies produced in the lab with new technologies. In a way, these anti-infective antibodies could be considered as a new kind of antibiotic family, even though much more expensive.

For the time being, the strategy of combing antibiotics and vaccines remains the most sustainable option, which can allow us to avoid in an affordable way the AMR threat. We wish to stress again how serious this threat is, as we expect AMR will cause 10 million deaths/year from 2050.

Before embarking in complex combination studies, it is important to further investigate the role that existing vaccines could have against resistant infections. Indeed, there are some indications that an unconventional use of existing vaccines could provide important advantages. For instance, in New Zealand a new vaccine against meningitis B was introduced few years after the meningitis B outbreak of the end of the 1990. The vaccine was still produced with traditional techniques and was composed by bacterial outer membrane vesicles. Recent studies in the population vaccinated with this vaccine revealed protection against gonorrhea, a sexually transmitted infection induced by *N. gonorrhoeae* that is becoming resistant to antibiotics (38). The reason of this protection is likely due to the fact that bacteria causing meningitis and gonorrhea are genetically related. Furthermore, the current evidence shows that existing pneumococcal vaccines reduce AMR, due to the fact they prevent infection thereby reducing the carriage and transmission of antibiotic-resistant bacteria. Another example is the influenza vaccine that indirectly reduces the incidence of fever and sickness, thereby minimizing the use and, more often, the misuse of antibiotics.

The increasing AMR is one of the several alarming signals of the profound effects that human activities can have on our world and life on it. How can we try to solve the problem of infections that are resistant to antibiotics? The most critical point is the difficulty in obtaining new antibiotics. As already discussed, the classical approach does not work anymore, thus we need to devise a completely novel approach.

Passive immunization, i.e., the administration of immune antibodies, could perhaps be a solution, if we can solve the issue of sustainability. Nowadays, immune antibodies can be produced only in low amounts and with high costs. At the beginning of the last century, immune antibodies able to neutralize bacterial toxins were produced in big animals and largely employed, and contributed to building an industrial sector ("serum" institutes) that evolved in today's vaccine industry. We hope that the new technologies will allow us to revive the serology concept and make it a new tool against infection.

A revolutionary approach would be to make the bacteria living within or on us, our microbiota, to become our allies in fighting the infections. As already mentioned, a large component of our body is bacteria (over 50%). An increasing number of studies indicate that gut microbiota is influencing our health and pathological conditions (39). Intestinal microbes can influence host energy metabolism (40), intestinal epithelial proliferation (41), and immune responses (42). It has been shown that the microbiota composition could influence vaginosis (43), obesity (44), inflammatory bowel disease (45), functional bowel disorders (46), allergies (47), and other diseases. An increasing number of studies suggest we can educate our microbiota to combat metabolic and chronic diseases. Could it be also the case in fighting infections?

Several candidate vaccines are in development pipelines since the last few years. For sure the emergency that we are facing will change the health priorities, and vaccines against antibiotic-resistant bacterial strains will move to the top.

AUTHOR CONTRIBUTIONS

AT wrote the manuscript. RR critically revised it.

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Fake News or Weak Science? Visibility and Characterization of Antivaccine Webpages Returned by Google in Different Languages and Countries

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The 1998 *Lancet* paper by Wakefield et al., despite subsequent retraction and evidence indicating no causal link between vaccinations and autism, triggered significant parental concern. The aim of this study was to analyze the online information available on this topic. Using localized versions of Google, we searched "autism vaccine" in English, French, Italian, Portuguese, Mandarin, and Arabic and analyzed 200 websites for each search engine result page (SERP). A common feature was the newsworthiness of the topic, with news outlets representing 25–50% of the SERP, followed by unaffiliated websites (blogs, social media) that represented 27–41% and included most of the vaccine-negative websites. Between 12 and 24% of websites had a negative stance on vaccines, while most websites were pro-vaccine (43–70%). However, their ranking by Google varied. While in Google.com, the first vaccine-negative website was the 43rd in the SERP, there was one vaccine-negative webpage in the top 10 websites in both the British and Australian localized versions and in French and two in Italian, Portuguese, and Mandarin, suggesting that the information quality algorithm used by Google may work better in English. Many webpages mentioned celebrities in the context of the link between vaccines and autism, with Donald Trump most frequently. Few websites (1–5%) promoted complementary and alternative medicine (CAM) but 50–100% of these were also vaccine-negative suggesting that CAM users are more exposed to vaccine-negative information. This analysis highlights the need for monitoring the web for information impacting on vaccine uptake.

Keywords: information quality, google, Internet, news, news media, vaccines, autism, public understanding of science

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INTRODUCTION

Acceptance and uptake of vaccination is important for reaching public health targets. The information available, either from books, television news, newspaper articles, or online sources, has a major impact on how the public perceives vaccines. In this respect, the most impactful information was the publication by Andrew Wakefield in the medical journal The Lancet in 1998, supporting a link

between the mumps, measles, and rubella (MMR) vaccine and autism (1). The journal eventually retracted the paper in 2010 (2), because its findings were discredited (3), but its message has become commonplace and remains a significant concern among parents (4).

It has often been pointed out that antivaccine information available on the Internet has a high prevalence and could impact negatively vaccination decisions (5–8). Observational studies have shown an association between exposure to antivaccine information on Twitter (9), and on the Internet in general (10), and a negative perception of vaccine risks. A Canadian study on 250 mothers also reported that reliance on governmental websites, which promote vaccination, is associated with higher vaccination rates (11). It is difficult, however, to draw a causal link from these associations and quantify the impact of online information on vaccine uptake.

Furthermore, the information on the prevalence of antivaccine websites is not consistent. A study in the USA analyzing 89 websites on human papilloma virus (HPV) returned by Google, Yahoo, and Bing reported less than 10% of websites with negative tone about vaccines (12) while one on MMR, also in the USA, reported that searching Google in 2014 returned a proportion of 41% of antivaccine websites (13).

The purpose of this study is to analyze the information available to the public, 20 years on from the publication of the above mentioned Lancet paper, on the link between vaccines and autism. The study does not analyze the impact of online information of vaccination rates or on public health views on vaccines but provides an approach to monitor vaccine-related information on the web. Using a methodology used previously for similar studies, we obtained a sample of the existing information using Google as the search engine (14–17). This captures most information as news outlets, television, books, professional or government organizations, scientific journals, and personal websites or blogs are all online. We sampled the first 200 results returned by Google searching for "autism vaccines," and analyzed them for the vaccines mentioned, their stance on vaccination, and the source of the website. We also used a standard indicator of health information quality, the JAMA score, to assess their basic trustworthiness index. The JAMA score considers whether a website declares author, date of writing, financial ownership, and whether its information is backed up by references (18).

The analysis was performed in different countries on localized versions of the search engine in different languages (google.com, google.co.uk, and google.com.au in English; google.be in French; google.it in Italian; google.com.br in Portuguese; google.com.sg in Mandarin; google.com.sa in Arabic). This research was done by a pre-existing international research collaboration, and that dictated the choice of the languages or localized versions of Google.

We also investigated the visibility, in terms of ranking, given by the search engine to webpages with a negative tone on vaccines. This has been overlooked by most studies, and it is known that users typically spend a short time on each website (19) and seldom go beyond the first ones in the search engine result page (SERP) (20).

The results indicate differences in the composition of the antivaccine websites across the world and the footprint left by Wakefield's Lancet paper. They also show differences in the ranking of antivaccine websites in the different localized versions of Google.

MATERIALS AND METHODS

We searched the two keywords "vaccines" and "autism" in Google between June and September 2017. It was decided to use only those keywords because we wanted to obtain a sample of the websites returned independently of the expression used. For this reason, we decided not to use questions such as "do vaccines cause autism?" because the results would be different depending on how the question was formulated and we needed to be consistent across the different languages. Although "vaccines" could be synonymous to "immunization," particularly in the scientific literature, we decided to use the search term "vaccines" as this best represents what the lay public would search on the Internet.

Before performing the search, the investigators deleted cookies and browsing history from their browsers to avoid the results of the search being influenced by previous searches done on the same computer (21–23), although it must be noted that the search engine will still identify the locations where the searches was made from the IP address, and this may customize results. Locations where the searches were performed were as follows: google.com (English), google.co.uk (English), google.it (Italian), and google.com.sa (Arabic), Brighton, UK; google.com.au (English), Sydney, NSW, Australia; google.be (French), Brussels, Belgium; google.com.sg (Mandarin), Singapore; google.com.br (Portuguese), Porto Alegre, Brazil.

The first 200 websites returned in each SERP were transferred to a spreadsheet and then the websites visited individually. When searching google.be, the French terms (vaccins, autisme) were used and any webpage in Flemish would be excluded from the analysis. Webpages that were deemed not relevant, for instance, not mentioning vaccines or aggregators, like those no longer accessible, behind a paywall or requiring registration were excluded from the analysis.

The total number of webpages considered for the analysis were as follows: English (Google.com), 175; English, UK, 188; English, Australia,194; French, 154; Portuguese, 132; Italian, 191; Mandarin, 179; Arabic, 146.

For each website, we recorded the typology of the website using the classification previously described (16, 17). The typologies considered were: Commercial (C), Government (G), Health portal (HP), News (N), No-profit (NP), Professional (P), scientific journals (SJ), as shown in **Table 1**. Those not fitting any of these categories or difficult to classify are listed as "others" (O). These included blogs, personal websites, or websites not affiliated with any of the other typologies.

To assess the JAMA score, we searched the webpage for the presence of the following information: author, date, references, owner of website (18).

We also annotated webpages according to the following features:

(1) The name of the vaccine mentioned; (2) the overall stance on vaccines (positive, negative, or neutral); (3) the chemicals or adjuvants mentioned; (4) whether the page mentioned complementary and alternative medicine (CAM) and its stance toward it (positive, neutral, or negative); (5) whether religion was mentioned; (6) whether the page contained a testimonial (e.g., a personal story); (7) whether a celebrity was mentioned. For websites associated

TABLE 1 | Definitions and examples of typology of websites.

Typology	Description	Examples
Government (G)	Website of a governmental body	nhs.uk, cdc.gov, who.int
Health Portal (HP)	Website that contains information on a variety of health topics	Kidshealth.com, webmd.com
News (N)	A website from newspapers, magazines, or TV	Pbs.org, newsweek.com, arstechnica.com
Non-Profit (NP)	Website from a no-profit organization[a]	Autismcenter.org, avoiceforchoice.org
Professional (P)	Websites created by a health professional organization (medical school, clinic/hospitals, medical board)	Ama.com.au, livewellpediatrics.com
Commercial (C)	Selling of producing drugs, supplements, or other	mercola.com, bodyecology.com
Scientific journal	Academic journals	Sciencedirect.com, nature.com

[a]*In the UK, they indicate a "registered charity" number, in the USA "tax-deductible 501(c)(3) organization."*

with the typology "News," we recorded the most mentioned stories in each SERP.

Statistical Analysis

When indicated, statistical analysis was performed using GraphPad Prism version 7 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

A two-tailed Fishers Exact test was used when comparing frequencies; when comparing multiple groups, the Bonferroni correction for multiplicity was applied.

When comparing JAMA scores across more than two groups, ANOVA was performed followed by Kruskal–Wallis test corrected for multiplicity by controlling the false discovery rate using the method of Benjamini and Hochberg.

A Pearson correlation coefficient test was used to assess the correlation between two variables, following D'Agostino and Pearson normality test (when the number of samples was too small, a Kolmogorov–Smirnov test was used to determine normality, a pre-requisite for the Pearson's test). For non-normally distributed samples, correlation was assessed using a Spearman Rank test. An alpha value of 0.05 was used for all statistical tests unless otherwise specified.

The statistical test used is described in the text or in the legends to figures and tables.

Word count to detect the number of occurrences of the names of celebrities was performed using natural language processing. Briefly, text corpora were extracted using WebBootCaT, an online tool for bootstrapping text corpora from Internet. Then word counts were obtained using the corpus analysis software Sketch Engine by Lexical Computing, Brno-Královo Pole, Czechia (24).

The raw data containing the list of websites analyzed and how they were annotated in provided in Data Sheet S1 in Supplementary Material.

RESULTS

Focus on MMR

Because we only used the word "vaccine" without specifying further, we first analyzed the vaccines mentioned in the webpages returned. As shown in **Table 2**, MMR was the most discussed vaccine, as expected, followed by influenza, viral hepatitis, diphtheria–tetanus–pertussis (DTP), poliomyelitis, Haemophilus influenza b and meningococci, HPV. However, there were differences between the various languages. The largest spread of vaccines mentioned was observed in Mandarin, while webpages in Arabic only mentioned MMR and influenza. Mandarin webpages also mentioned BCG while those in Portuguese mentioned Yellow fever and measles.

TABLE 2 | Vaccines discussed by webpages in the different search engine result page (SERPs).

	Com	UK	AUS	FR	IT	Man	Port	ARA	Total
Mumps, measles, and rubella	123	133	112	96	93	116	88	71	832
Influenza	23	20	3	11	6	21	9	4	100
Hep	16	10	10	13	2	34	13	0	98
Diphtheria–tetanus–pertussis	10	9	4	4	0	37	8	0	72
Polio	5	10	5	6	18	25	0	0	69
Hib/Men	8	4	3	2	4	29	0	0	50
Human papilloma virus	6	6	0	1	2	8	0	0	23
Chickenpox	4	3	2	5	2	9	0	0	25
Pertussis	10	5	2	7	0	0	0	0	24
Rotavirus	3	2	0	1	2	10	0	0	18
Pneumococcal	3	3	0	5	0	10	0	0	21
Smallpox	4	2	0	20	4	4	0	0	34
BCG	0	0	0	0	0	10	0	0	10
Yellow fever	0	0	0	0	0	0	3	0	3
Measles	0	0	0	3	0	0	3	0	6

Values indicate the number of webpages in each SERP mentioning a specific vaccine. Color intensity indicate the frequency vaccines are mentioned in each SERP.

Typologies of Websites

Table 3 shows the composition of the SERP in terms of website typologies. In all SERPs, most websites (60–80%) were "news" or "other" (including non-affiliated websites, blogs etc.). Websites from governmental (e.g., national and international public health services, health ministries, CDC, FDA, etc.) or inter-governmental organizations (e.g., WHO) were not highly represented, their frequency ranging from 1.3% (French) to 6.7% (English/Australia).

Non-profit organizations, health portals and professional websites followed in various proportion. Commercial websites had a presence (except in Italian) between 2 and 6%. SJs online were present in a significant percentage (3–7%) only in the three SERPs in English, which is not surprising if we consider that scientific literature is mostly in English.

TABLE 3 | Composition of the search engine result page (SERP) by typology of webpages.

Typology	Google.com	UK	AUS	FR	IT	Man	Port	ARA
Comm	4.0	5.3	3.1	6.3	0.0	2.2	4.5	0.0
Gov	1.7	6.4	6.7	1.3	3.1	5.0	3.0	3.4
HP	3.4	3.7	6.2	1.9	4.2	10.1	10.6	11.6
News	41.7	30.3	26.3	31.6	49.7	36.9	31.1	34.9
NP	11.4	13.8	10.8	7.0	6.3	7.3	2.3	2.1
Other	26.9	26.6	29.9	41.1	32.5	31.8	29.5	39.0
Prof	7.4	6.9	10.3	8.9	4.2	5.6	17.4	8.2
ScJ	3.4	6.9	6.7	1.9	0.0	1.1	1.5	0.7
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Data are expressed as percentage of the total for each SERP. Color intensity indicate the frequency of the different typologies in each SERP.

On the other hand, the pattern in the top 10 websites is completely different (**Table 4**). Commercial websites are not present in the top 10 websites returned by Google. In many SERPs the frequency of government websites was 10–30%, higher than that in the whole search. News websites, representing 30–40% of the SERPs in English, were also less frequent (0–10%) in the top 10. The exception was the SERP in French where news websites represented 60% of the top 10 websites compared to 31% in the whole SERP, and a similar trend was observed in Portuguese (40% in the top 10, 30% in the whole search).

Testimonials, Celebrities, and CAM

We investigated whether websites contained a testimonial (personal story), mentioned a celebrity, or mentioned CAM.

As shown in **Figure 1A**, testimonials were present in around 30% of websites returned by the Australian and French Google searches, but were much less frequent in Italian, Mandarin, Portuguese, and Arabic websites.

Celebrities (**Figure 1B**) were present with high frequency in English, French, and Mandarin websites. The celebrities most frequently mentioned, and present in most languages, were Donald Trump (present in all SERPs, ranging from 27 webpages in Australia, 19 in UK, 18 in Mandarin, with a minimum of 1 in Arabic), Jenny McCarthy (present in SERPs in English, Portuguese, and Mandarin, 19 times in Australia, 12 in google.com, and 9 in UK), Robert F. Kennedy Jr. (23 webpages in Australia, 17 in google.com, 16 in UK), and Robert De Niro (present in all searches except Mandarin and Portuguese). Other celebrities mentioned were Dan Burton, Jim Carrey, Chuck Norris, and Luc Montagnier. Other names were language- or country-specific. In French, Martine Ferguson-André was mentioned in 23 websites while Agnès Buzyn was mentioned by 7 webpages. In Italian, Beatrice Lorenzin, was mentioned in 19 webpages. A short description of the main celebrities mentioned is given in **Table 5**. Interestingly, most of them were named by vaccine-positive or -neutral websites when describing the antivaccine movement.

Few websites mentioned CAM, and their frequency was higher in Mandarin and Portuguese websites (4–5%), while in other SERPs, they accounted for no more than 2% of the websites (**Figure 1C**).

TABLE 4 | Composition of the top 10 webpages by typology.

Typology	Google.com	UK	AUS	FR	IT	Man	Port	ARA
Comm	0	0	0	0	0	0	0	0
Gov	1	2	3	0	0	1	0	1
HP	1	0	1	0	1	2	1	1
News	1	1	0	6	3	2	4	2
NP	3	4	1	1	1	0	1	1
Other	1	1	1	3	3	4	2	5
Prof	2	0	3	0	2	0	2	0
ScJ	1	2	1	0	0	1	0	0

Data indicate the number of websites (total = 10). Color intensity indicate the frequency of the different typologies in each SERP.

Stance on Vaccines

The most important aspect of the content analysis was to assess the stance of websites toward vaccines, whether pro-vaccine, vaccine-negative, or neutral. A pro-vaccine stance would be that of websites promoting vaccination or denying the causal link with autism. A vaccine-negative stance would be that of supporting a link with autism or discouraging vaccinations, like the so-called "anti-vaxxers." An example of neutral stance would be that of a news website reporting the existence of this controversy or a scientific paper reporting findings from an epidemiological study.

Figure 2 reports the presence of total websites that are pro-vaccine, neutral, or vaccine-negative in the whole SERP (panel A) and in the top 10 websites returned by Google (panel B). The frequency of vaccine-negative webpages in the top 10 results was lower than that observed in the rest of the SERP in most languages except for Italian (11% in the whole SERP, 20% in the top 10) and Arabic (7.5% in the whole SERP, 30% in the top 10, $P = 0.0485$ by Fisher's test). The frequency of pro-vaccine websites in the top 10 was significantly higher than in the rest of the SERP in google.com but lower in google.be; Fisher's test, $P = 0.0472$ and 0.0220, respectively.

Figure 3 provides a visual representation of the ranking of the vaccine-negative websites (in yellow) in the first 100 websites across the different SERPs. There is a clear trend for searches in English websites which give a lower visibility to vaccine-negative webpages.

The observed frequency of vaccine-negative webpages across the different typologies of websites is reported in **Table 6**. For each language SERP, we color-coded values based on how the observed frequency of vaccine-negative URLs in that typology compared with the expected frequency (the overall percentage of vaccine-negative websites in the whole SERP). In almost all SERPs, a higher proportion than expected of commercial websites were vaccine-negative in stance (up to 71.4% were observed in google.com compared with 16.6% expected). It should be noted, however, that commercial websites account for only 2–6% of the total websites returned, and they never appear in the top 10, as shown in **Tables 3** and **4**. A higher frequency of websites classified as "other" were observed to be vaccine-negative in their stance (up to 40% of websites in the UK). This is particularly relevant as this website typology accounts for about one-third of the total SERPs. As expected, there were no vaccine-negative websites among the government typology, and very few in the professional typology

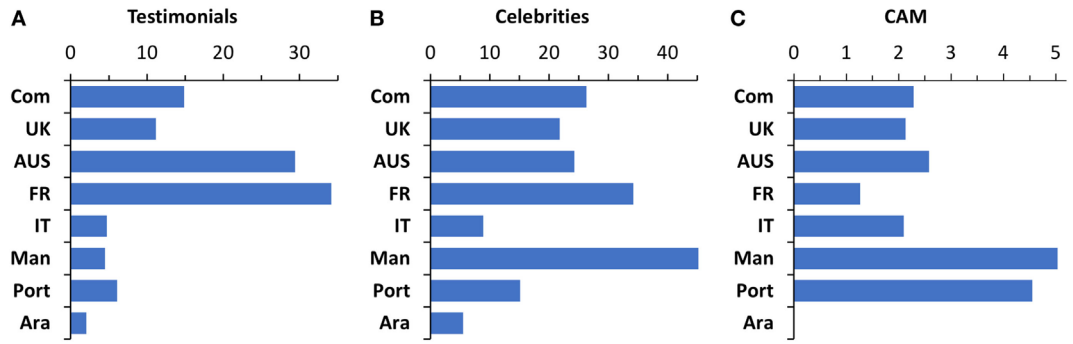


FIGURE 1 | Percentage of webpages containing testimonials **(A)** or mentioning celebrities **(B)** or complementary and alternative medicine **(C)**.

TABLE 5 | Celebrities most mentioned in the search engine result pages.

Name	Context
Donald Trump	US president, suggest vaccine cause autism on Twitter
Robert F. Kennedy Jr.	US Environmental attorney, claim links between vaccines and autism, rumored to be appointed by Donald Trump to lead a committee on vaccine safety
Jenny McCarthy	US actress and Playboy model, blames vaccination for his son's autism
Robert De Niro	US actor, founder of Tribeca festival. He has a son with autism and was linked to belief of the link between vaccines and autism and critical of the Center for Disease Control. He reversed his initial decision to include the film "Vaxxed" from the festival
Jim Carrey	US actor with autistic son (from Jenny McCarthy), led a "green our vaccines" march in Washington, DC and is critical of the Center for Disease Control
Chuck Norris	US actor, accused government to hide data on links between vaccines and autism
Dan Burton	US representative, grandfather of a child with autism, believer that thimerosal causes autism. Previously expressed support of laetrile, a complementary therapy for cancer
Luc Montagnier	French scientist, Nobel prize for the discovery of HIV. Attended vaccine skeptical conferences and highlighted an association between vaccine and autism (however, he warned that this may not mean causation). Previously linked to condescendence toward homeopathy
Martine Ferguson-André	French politician. Suspects vaccines caused his son's autism
Agnès Buzyn	French health minister, introduced 11 vaccines compulsory
Beatrice Lorenzin	Italian health minister, passed a law making 10 vaccines compulsory

(average of all SERPs, 6.9%). Vaccine-negative views were also infrequent in news websites (averaging 5.2% all SERPs).

We also analyzed whether the mention of testimonials, celebrities, CAM, or religion was associated with a particular stance on vaccines. **Figure 4** represents the stance on vaccines in all webpages from all SERPs mentioning testimonials, celebrities, CAM, or religion. The frequency of vaccine-negative websites was significantly higher in webpages reporting testimonials ($P = 0.0002$ by Fisher's test), CAM ($P = 0.0001$), or religion ($P = 0.02$) when compared to the total. On the other hand, websites mentioning celebrities had a similar pattern as the total search, indicating that even celebrities such as Trump were not mentioned in a vaccine-negative context.

Adjuvants

There is a diffuse concern that the chemicals, including adjuvants and preservatives, added to vaccines to act as adjuvants may be a cause of autism. We, therefore, took note of when a webpage mentioned the presence of it in the text. The chemical name occurring with the highest frequency was thimerosal (441 webpages, 60% of total), followed by the partially synonym mercury (184 webpages, 25% of total), aluminum (101, 14%), and formaldehyde (15, 2%). These adjuvants and preservatives were mentioned in a large proportion of the websites: 56% in Google.com, 50% in UK, 93% in Australia, 58% in French, 28% in Italian, 45% in Mandarin, 71% in Portuguese, and 32% in Arabic.

A sub-analysis of the adjuvant mentioned by websites and the stance of the website on vaccines showed that vaccine-negative websites mentioned aluminum with a frequency that was nine-times higher than pro-vaccine, and four-times higher than neutral, websites (**Table 7**). Although this trend was also observed for "mercury," it was not observed for "thimerosal."

News

Because of the high frequency of news websites, accounting for about one-third of all SERPs, we have summarized in **Table 8** the main topics covered by these websites.

As mentioned above, vaccine-negative news webpages were less frequent than expected in the whole SERPs. Vaccine-negative news articles were highest in Mandarin, Portuguese, UK, and google.com (12.1, 7.3, 7, and 6.8%, respectively) and lowest in French, Australian, Italian, and Arabic (0, 2, 2.1, 3.9%, respectively) webpages.

JAMA Score

The median JAMA score for all SERPs is shown in **Figure 5**. The Arabic SERP had a significantly lower JAMA score than any other SERP. Google.com and Google.co.uk had a significantly higher

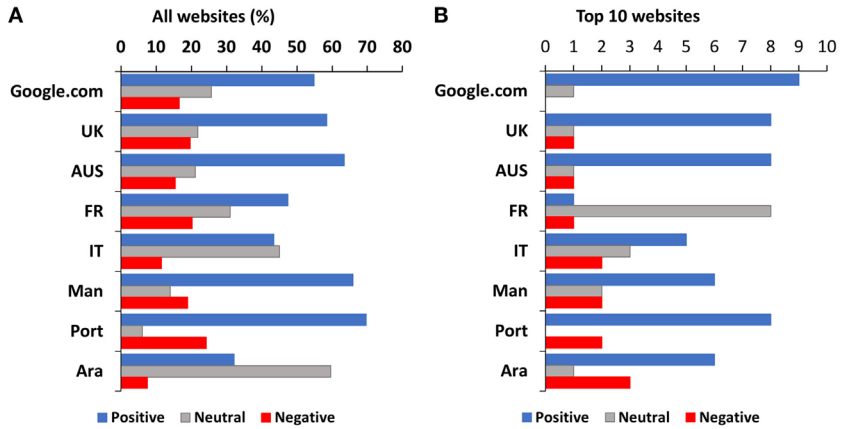


FIGURE 2 | Webpages with different stance on vaccines in the entire search engine result page **(A)** and in the top 10 webpages **(B)** returned by Google. Data are expressed as percentage of websites for the entire search or number of websites in the top 10.

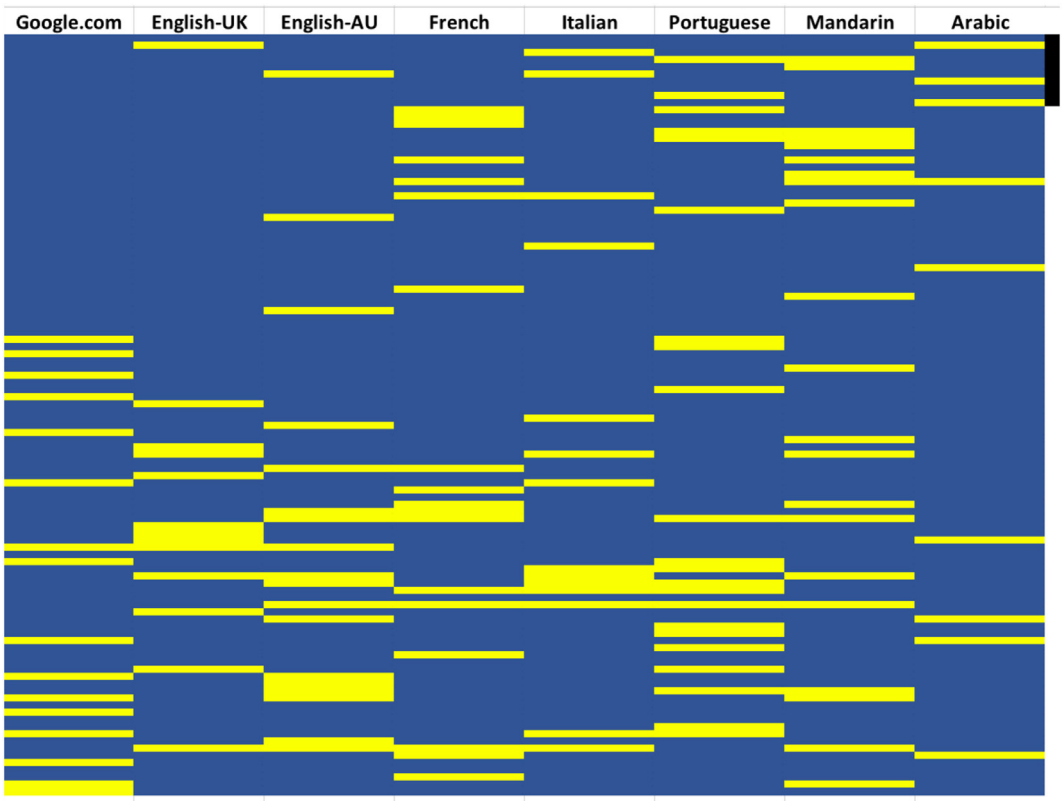


FIGURE 3 | Visualization of the ranking of webpages with a negative stance on vaccines in the first 100 websites in each search engine result page (SERP). Webpages are listed in the same order they are ranked in the SERP. Yellow, vaccine-negative websites; blue vaccine-positive or -neutral. The black bar on the right indicate the top 10 webpages.

JAMA score than the SERPs in English-Australia, French, and Italian.

We also analyzed, for each SERP, the JAMA score of vaccine-positive, -neutral, or -negative and could not find any difference in the JAMA score of websites with different stance on vaccines (data not shown). Furthermore, for any SERP, we could not find any significant difference in the JAMA score of the top 10 websites compared to the rest of the SERP.

DISCUSSION

The varied composition of the SERP returned by Google, with only 30% being non-affiliated websites or blogs, and the rest representing a wide range of news outlets, professional or government organizations, and scientific journals, represents a good sample of the information on the topic of vaccines and autism that the public is exposed to.

Because we analyzed the first 200 websites returned by Google, the list is not just a sample of all that is available in what has been called the infosphere (25), but it also reflects the visibility, or ranking, given by Google. For this reason, we did not just look at the composition of the SERP but also how webpages are ranked, particularly, the first 10 results that are more likely to be read (26).

TABLE 6 | Frequency of vaccine-negative webpages in each typology.

	google.com	UK	AUS	FR	IT	Man	Port	Ara
Comm	71.4	60.0	33.3	77.8	0.0	25.0	16.7	0.0
Gov	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HP	0.0	0.0	16.7	0.0	12.5	27.8	7.1	0.0
News	6.8	7.0	2.0	0.0	2.1	12.1	7.3	3.9
NP	10.0	11.5	4.8	30.0	0.0	15.4	33.3	0.0
Other	34.0	40.0	39.7	30.8	30.6	29.8	56.4	15.8
Prof	7.7	7.7	5.0	7.1	0.0	10.0	17.4	0.0
ScJ	0.0	23.1	7.7	0.0	0.0	0.0	0.0	0.0
Average vaccine-negative in the total search engine result page (SERP)								
	16.6	19.7	16.0	19.6	11.5	19.0	24.2	7.5

Data indicate the percentage of vaccine-negative webpages in each typology. Cells are color coded to show difference from "expected" based on the frequency in the total SERP shown in the bottom row (red, above the expected frequency; green, below the expected frequency).

Despite retraction of his paper in 2010, Dr. Wakefield is still highly mentioned (a word count found his name recurring 462 times in the Google.com search, 551 in UK, 706 in Australia, 378 in French, 361 in Italian, 21 in Arabic, 195 in Portuguese, and 11 in Mandarin). Although his original paper did not appear in any SERP, a letter he published in the Lancet in 1999 was present in both the UK and the Australian SERP (but not Google.com). In French, two websites (one Belgian and one French) displayed a video of Andrew Wakefield's interview with subtitles in French (http://initiativecitoyenne.be/2017/02/vaccins-autisme-le-dr-andrew-wakefield-repond-aux-accusations-et-aux-calomnies.html, accessed 19/03/2018 and archived at https://web.archive.org/web/20180319102307/http://initiativecitoyenne.be/2017/02/vaccins-autisme-le-dr-andrew-wakefield-repond-aux-accusations-et-aux-calomnies.html; http://www.agoravox.tv/tribune-liber/article/vaccination-et-autisme-dr-andrew-72269, accessed 19/03/2018 and archived at https://web.archive.org/web/20180319102342/http://www.agoravox.tv/tribune-liber/article/vaccination-et-autisme-dr-andrew-72269.).

TABLE 7 | Main chemicals mentioned in webpages with different stance.

	Positive	Neutral	Negative
Thimerosal	274 (37%)	86 (22%)[a]	81 (36%)
Mercury	92 (12%)	32 (8%)	60 (27%)[a]
Aluminum	21 (3%)[a]	23 (6%)	57 (25%)[a]
Formaldehyde	7 (1%)	2 (1%)	6 (3%)
Total	744 (100%)	384 (100%)	225 (100%)

Number of webpages mentioning a chemical of all the webpages with that stance on vaccines. Percentage is given in parenthesis.

[a]*Significantly different frequency compared to that of the other two groups combined ($P < 0.05$ by Fisher's test with Bonferroni correction for multiplicity for 12 comparisons).*

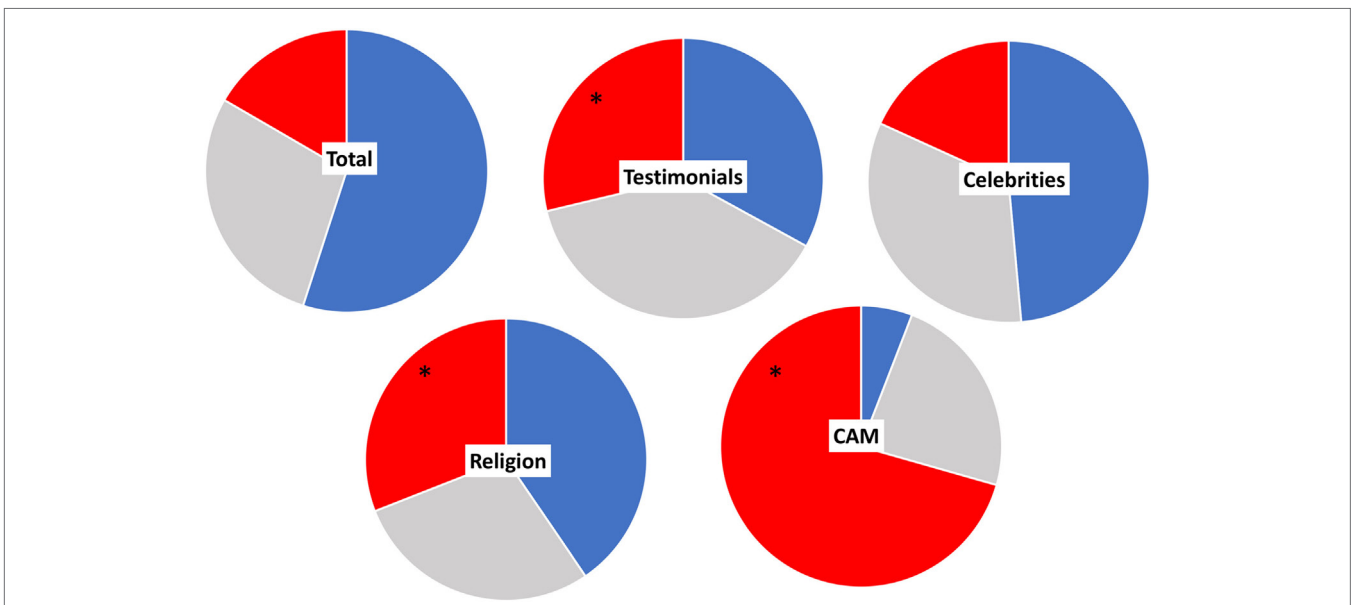


FIGURE 4 | Vaccine stance in webpages from all search engine result page (SERPs) mentioning testimonials, celebrities, religion, or complementary and alternative medicine. Blue, vaccine-positive, gray, neutral, red, negative. * Denotes a higher frequency of vaccine-negative webpages compared to the total SERP ($P < 0.05$ by Fisher's test).

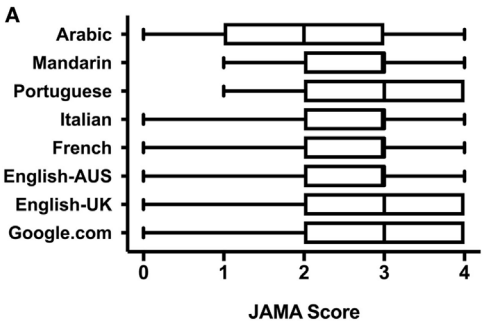
TABLE 8 | Main topics in news webpages.

Search engine result page	Topic	Examples
Google.com	Tribeca film festival and the anti-vaccine film "Vaxxed"	https://www.nytimes.com/2016/03/26/health/vaccines-autism-robert-de-niro-tribeca-film-festival-andrew-wakefield-vaxxed.html https://www.statnews.com/2016/03/31/vaxxed-vaccine-autism-movie/(Archived at: http://www.webcitation.org/6ww1JScrv)
	Donald Trump and political debate on vaccinations	https://www.usnews.com/news/articles/2017-01-24/donald-trumps-health-care-pick-rejects-claims-that-vaccines-cause-autism (Archived at: https://web.archive.org/web/20180202164318/https://www.usnews.com/news/articles/2017-01-24/donald-trumps-health-care-pick-rejects-claims-that-vaccines-cause-autism) http://uk.businessinsider.com/trump-vaccines-autism-wrong-2017-1?r=US&IR=T (Archived at: http://www.webcitation.org/6ww0yJnpi)
	Theory that the Center for Disease Prevention and Control (CDC) have withheld evidence that that African-American boys are at an increased risk of developing autism	https://www.colorlines.com/articles/new-documentary-alleges-cdc-withheld-proof-link-between-vaccines-and-autism-black-boys (Archived at http://www.webcitation.org/6ww0fb2AC)
Portuguese	Report the story of the origin of the myth of the link autism-mumps, measles, and rubella (MMR) Wakefield paper	https://web.archive.org/web/20180202161923/https://g1.globo.com/bemestar/noticia/a-historia-que-deu-origem-ao-mito-da-ligacao-entre-vacinas-e-autismo.ghtml https://web.archive.org/web/20180202162138/http://www.bbc.com/portuguese/geral-40663622
French	Report on a new law to make 11 vaccines compulsory in France, and of the opposition by Martine Ferguson-André, member of Europe Ecologie-les Verts	http://rmc.bfmtv.com/emission/vaccins-obligatoires-le-lien-entre-le-vaccin-contre-la-rougeole-et-l-autisme-ne-tient-pas-scientifiquement-1247033.html [Archived at: http://www.webcitation.org/6tSAGAQaw] http://www.la-croix.com/Sciences-et-ethique/Sante/Vaccination-pourquoi-parents-denfants-autistes-souhaitent-poursuivre-laboratoires-2017-07-24-1200865117 [Archived at: http://www.webcitation.org/6tUT5QS7t]
Italian	Reports of courts cases and final sentences of the Supreme Court in June 2016 and July 2017, which denied the causal link between vaccines and autism. Most news take the stance that connection between vaccines and autism is a hoax ("bufala") except one vaccine-negative article in "Corriere Quotidiano"	https://www.agi.it/salute/vaccini_bambini_e_autismo_storia_di_una_bufala-1987339/news/2017-07-26/(archived at http://www.webcitation.org/6tofW4vYP) http://www.repubblica.it/salute/2017/07/25/news/cassazione_non_c_e_correlazione_tra_vaccini_e_autismo_no_al_risarcimento_-171599464/(archived at http://www.webcitation.org/6wAj2KM3O) http://www.corrierequotidiano.it/1.67940/salute-e-medicina/toscana-siena/3715/vaccini-e-autismo-cassazione-nega-corte-europea-avvalla (archived at http://www.webcitation.org/6wApTvo0b)
	Report on the law, approved by the Italian Parliament in July 2017, making ten vaccinations compulsory for all children aged 10–16	http://www.metronews.it/17/09/07/dietrofront-del-veneto-stop-alla-moratoria-vaccini.html (archived at: http://www.webcitation.org/6xTn4tELD)
Mandarin	China Shandong Illegal Vaccine Scandal on vaccines purchased from illegal sources and not stored properly	http://www.zaobao.com.sg/wencui/politic/story20160324-596554 (archived at: http://www.webcitation.org/6xTSPfm4k)
	Donald Trump's stance on vaccines	http://hssszn.com/archives/17810 (Archived at http://www.webcitation.org/6tUAlNx7P) http://3g.forbeschina.com/review/201204/0016345.shtml (Archived at: http://www.webcitation.org/6tU9cS9qm)
	Andrew Wakefield. Talks about the revocation of his medical license and his fraudulent research paper published in The Lancet linking MMR vaccines to autism, which has since been withdrawn	http://www.webcitation.org/6tUNCaBbf http://www.webcitation.org/6tUMuQHdk https://read01.com/LNedyP.html (Archived at: http://www.webcitation.org/6tUPLRZEM) http://m.6park.com/index.php?act=wapnewsContent&nid=239399 (Archived at: http://www.webcitation.org/6tUNliETc)

It is important to be aware that the autism-MMR scare was not borne out of an obscure sect but from scientific papers published in respectable and authoritative journals, leading to a widespread concern even among health professionals.

This seems to be true today when articles published in academic journals of varied respectability can have a significant impact as they may be perceived as providing a scientific basis for antivaccine, or just vaccine-skeptical, positions. A study has shown that, in the US, a drop in the MMR vaccination rate was observed soon after the publication of original scientific reports, even before this was the subject of media coverage (27). These may also be ranked higher by search engines because scientific articles may be considered authoritative and, therefore, proxies for high quality information.

It may be surprising that in the UK and Australian websites, but not in Google.com, a proportion of SJs were vaccine-negative. As mentioned above, very few websites of SJs were present in non-English SERPs, not surprisingly as scientific articles are



B

Mean ranks		Google.com	English-UK	English-AUS	French	Italian	Portuguese	Mandarin	Arabic
787	Google.com			0.0043	0.0002	0.001			<0.0001
774	English-UK			0.0101	0.0006	0.0025			<0.0001
675	English-AUS								<0.0001
634	French								<0.0001
657	Italian								<0.0001
733	Portuguese								<0.0001
722	Mandarin								<0.0001
436	Arabic								

FIGURE 5 | JAMA score of webpages in the different search engine result pages (SERPs). **(A)** Box-and-whiskers graph indicate median, 25 and 75% percentiles, minimum and maximum. **(B)** Multiple comparison of different SERPs. *P*-values are reported only for statistically significant differences. Multiple comparison of JAMA scores among the different SERPs was performed using ANOVA followed by Kruskal–Wallis test corrected for multiplicity by controlling the false discovery rate using the method of Benjamini and Hochberg for 28 comparisons.

usually in English. Of the six scientific articles in Google.com, none were vaccine-negative, whereas UK and Australian websites (13 scientific articles each) had some vaccine-negative scientific articles (three and one, respectively). In the UK SERP, three vaccine-negative scientific papers were found. One was a 2002 paper in *LabMedicine*, published by the Oxford University Press and the American Society for Clinical Pathology and, to our knowledge, never retracted (28); a second a letter by Wakefield published in the Lancet in 1999 in response to criticism over his previous paper (29); a third is a 2017 editorial published in the "*Madridge Journal of Vaccines,*" a journal published in the US but, unlike *The Lancet* and *LabMedicine*, not listed by PubMed and the National Library of Medicine (30).

In particular, the 2002 paper published by Oxford University Press was ranked second in the UK SERP. Repeating the "autism vaccines" search on Google.co.uk 6 months later still returned this article second in the ranking (data not shown). This online article was not found in Google.com or in the Australian SERP.

In the Australian search, two websites were collections of scientific papers supporting a causal link between vaccines and autism, a third the Wakefield letter mentioned above, and a fourth a paper by the organization "Informed Consent Action Network" that, even if not published in a journal, and it might be questionable whether it could be legitimately defined a scientific paper as it is unclear whether it was peer reviewed, has all the features of a scientific review. Classifying these papers as vaccine-negative was a shared but subjective decision of the authors who reviewed those websites, and we provide the references in Data Sheet S1 in Supplementary Material in case the reader wishes to reassess our coding from a different perspective.

As noted in a Nature editorial by Leask (31), "just four months after the publication that triggered the MMR scare, 13% of general practitioners and 27% of practice nurses in north Wales thought it very likely or possible that the vaccine was associated with autism (32)". Leask noted that, to improve uptake of vaccinations, we should engage "fence-sitting parents" (31). This means that pro-immunization information needs to address those issues and concerns that anti-vaccine websites raise, such as the mention of aluminum or mercury as a component of thimerosal, as highlighted by our study. Furthermore, the present study also advocates the dissemination of pro-vaccine information on the same websites typologies that perpetuate the "fake science" that vaccines cause autism.

Despite the science behind it being discredited, there are several reasons as to why the association between the MMR vaccine and autism is still present amongst the lay public. Flaherty pointed out that this is partly due to autism being a complex condition without a single, established causal mechanism (33). It should be noted that a search of websites mentioning "vaccines and autism" returns websites mentioning other vaccines, not just the MMR, as this could suggest a potential extrapolation of the link with autism to other types of vaccines.

The strong association between vaccine-negative stance and CAM, as well as commercial websites often selling "natural products," confirms that cultural factors may reinforce an antivaccine stance by the association of vaccines with capitalism, big pharma, and profit.

Another finding of the present study is that government organizations accounted for only 1.3–6.7% of websites (**Table 3**). This is markedly less than what we found previously in a study on influenza vaccine where governmental websites represented 17% of the SERP in English and 42% of that in Italian (16). The reason for this is probably that, in the present study, we specifically introduced the search term "autism," which may not be mentioned in most of the government websites unless for educational purpose, which is to explain that there is no link to autism. The other possibility is that, in the case of influenza, there is a strong vaccination campaign because it is done on a voluntary basis, while the MMR is either part of the routine immunization schedule of babies (e.g., UK) or compulsory (e.g., Italy since 2017 or France for babies born after 01/01/2018).

The fact that Trump is the most frequently mentioned celebrity reminds us of the difference between countries, where in some countries antivaccine sentiment is prevalent among alternative, left-wing groups, and right-wing, individualist, groups in others. We could not find a significant association between mention of religious issues and sentiment about vaccines. In fact, religious beliefs may be important in the confidence in vaccines (34), although this may be a confounder as there are few religious groups who officially reject vaccinations (35).

The fact that news outlets represent 30–50% of the websites indicates that the link between vaccine and autism is a topical and newsworthy topic. From this point of view, it is reassuring that news websites returned by Google have a low frequency of vaccine-negative articles. This is not to say that there are no antivaccine news articles (many vaccine-negative articles have been published by top tabloid newspapers in the UK) but rather that these are not given visibility by the algorithm used by Google.

However, the information quality criteria used by Google do not always penalize vaccine-negative websites. This study shows that, while in Google.com the first vaccine-negative webpage came up only as 43rd, in the local UK and Australian SERPs some were found in the first 10 websites, and this was even more marked in non-English SERPs.

Interestingly, this is similar to what we observed in a previous research where we analyzed the information returned by Google on influenza vaccine or influenza prevention in English and Italian. While in google.com in English there were no vaccine-negative websites or websites promoting non-evidence-based medicine approaches to influenza prevention, this was not true for a search in Italian (16).

Of course, here we only use Google as a mesh to collect a sample of the web and the websites returned in the SERP might just reflect "what is out there." However, it is important to note that the overall frequency of vaccine-negative webpages was not so different in the different SERPs, and we have no explanation for this observation. One wonders whether the vaccine-negative study published in a SJ was ranked high in the UK SERP because the publisher is Oxford University Press, or whether the one in the top 10 in the Australian SERP was ranked high because the .org domain was taken as a proxy of authority and quality. It is also possible that the higher ranking of vaccine-negative webpages in some SERPs is due to the fact that they receive a high number of clicks in that country or language.

Another interesting finding of this study is the difference in the JAMA score of different SERPs. Websites in Arabic showed the lowest JAMA score than all other languages. Websites from Google.com and Google.co.uk ranked higher than those from the localized versions in English-Australia, French, and Italian. The fact that the mean JAMA score of websites returned in the Australian SERP is also significantly lower than that of those returned by Google.com or Google.UK seem to exclude that the language alone explains the difference.

One obvious question is how much the antivaccine information impacts on the uptake of vaccines. Data from the Organization for Economic Co-operation and Development (OECD) show that, in 2015, Italy had the lowest vaccination rate for measles (85%), People's Republic of China the highest (99%), Australia and France 91%, the USA 92%, the UK 95%, Belgium and Brazil 96%, Portugal and Saudi Arabia, 98% (36, 37). The low immunization rate is the reason why the Italian government made the MMR vaccine compulsory in July 2017, France followed in 2018 and Australia is also going along that route.

We assessed whether there was a correlation between the percentage of vaccine-negative webpages from **Figure 2A** and either the safety-related skepticism in the countries analyzed (34) or with the uptake of measles vaccination in 2016 (data from https://data.worldbank.org/indicator/SH.IMM.MEAS). There was no statistically significant correlation using the Spearman-Rank test or the Pearson correlation coefficient (data and results of the statistical analysis are provided in Data Sheet S2 in Supplementary Material).

It should also be noted that the search in Mandarin was performed using the localized version of Google in Singapore; because the Google search engine is not available in the People's Republic of China, our results cannot be extrapolated to the information available in that country. We should also bear in mind that most of the languages investigated are not specific to a single country. Hence, making correlation between webpages in one language and vaccination rate or sentiment in one country, is not immediate.

This lack of correlation might support the view that the impact of online information on vaccination acceptance may be exaggerated. For instance, a study among French mothers reported that the main source of information on vaccination is the family physician or pediatrician (84–90%) and the Internet accounts for only 10–12% (8), while a study on 1737 Canadian parents showed that, to obtain trustworthy and reliable information on vaccines, 68% of them would ask a physician, and just 27% the Internet (38). If we also consider the fact that only a small percentage of parents refuse to vaccinate their children, one could conclude that we should not overestimate the impact of webpages with a vaccine-negative stance. Other issues may be at the basis of vaccine skepticism such as the perceived role of big pharma and governments or the underestimation of potential risks, as in the case of the dengue vaccine (39).

A major limitation of this study is that we only looked at webpages and did not investigate social networks. Studies have previously explored this area of the Internet and have analyzed their features in English and French (6, 8). Another limitation of the present study is that we analyzed the sample of the online information on the topic but not all websites will have the same impact. Even within the first ten results, readers may just briefly glance through them using clues to decide what to read. To assess which top-ranking websites attract attention of the user and are actually read, research should be undertaken by asking volunteers to rank websites or, alternatively, their attention could be monitored using eye-tracking software (40). A further limitation of our study is that we used the same, neutral, search string ("vaccine autism") without taking into account potential differences in the most searched terms used, which could well be different in different languages. It is likely that users could find more biased information by using more negative search terms, although a recent study using eye-tracking software to investigate the search behavior of 56 volunteers found that users are more likely to use neutral search terms (19).

In summary, the main findings of this study are the marked differences in the visibility of websites with a negative stance on vaccines given by the ranking by Google across not only different languages but also in different localized searches in English. Public health authorities, particularly those acting internationally, will need to take these differences into account when designing websites aiming at promoting vaccinations. They will also need to consider the relevance that issues like the adjuvants included in vaccine preparation have in the information available and clarify these issues to correct misinformation. Counteracting disinformation about vaccines by health authorities is part of the solution, but the loss of confidence in vaccines goes far beyond

misinformation. Communities, social environment, educational level, are few examples of factors affecting the vaccine confidence. Education, as well as transparency, would be an important aspect to keep in mind when trying to increase vaccine confidence.

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NA, PG, and MN designed the study. NA, MA-J, IB, GP, KC, MM, MN, and PG performed research. All authors analyzed data, all authors wrote the paper.

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CORRECTION
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Corrigendum: Fake News or Weak Science? Visibility and Characterization of Antivaccine Webpages Returned by Google in Different Languages and Countries

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Keywords: information quality, google, Internet, news, news media, vaccines, autism, public understanding of science

A Corrigendum on

Fake News or Weak Science? Visibility and Characterization of Antivaccine Webpages Returned by Google in Different Languages and Countries

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In the original article, there was a mistake in **Supplementary Material Data Sheet 1** as published. In the tab "Australia" the value of cell M191 should be "0" instead of "1" and that of cell N191 should be "1" instead of "0." The corrected **Supplementary Material Data Sheet 1** has been replaced in the original article.

In addition, there was a mistake in **Table 6** as published. The percentage of vaccine-negative websites for Australia should be "33.3%" instead of "16.7%." The corrected **Table 6** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 6 | Frequency of vaccine-negative webpages in each typology.

	google.com	UK	AUS	FR	IT	Man	Port	Ara
Comm	71.4	60.0	33.3	77.8	0.0	25.0	16.7	0.0
Gov	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HP	0.0	0.0	16.7	0.0	12.5	27.8	7.1	0.0
News	6.8	7.0	2.0	0.0	2.1	12.1	7.3	3.9
NP	10.0	11.5	4.8	30.0	0.0	15.4	33.3	0.0
Other	34.0	40.0	39.7	30.8	30.6	29.8	56.4	15.8
Prof	7.7	7.7	5.0	7.1	0.0	10.0	17.4	0.0
ScJ	0.0	23.1	7.7	0.0	0.0	0.0	0.0	0.0
Average vaccine-negative in the total search engine result page (SERP)								
	16.6	19.7	16.0	19.6	11.5	19.0	24.2	7.5

Data indicate the percentage of vaccine-negative webpages in each typology. Cells are color coded to show difference from "expected" based on the frequency in the total SERP shown in the bottom row (red, above the expected frequency; green, below the expected frequency).

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REVIEW
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The US Military Commitment to Vaccine Development: A Century of Successes and Challenges

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The US military has been a leading proponent of vaccine development since its founding. General George Washington ordered the entire American army to be variolated against smallpox after recognizing the serious threat that it posed to military operations. He did this on the recommendation from Dr. John Morgan, the physician-in-chief of the American army, who wrote a treatise on variolation in 1776. Although cases of smallpox still occurred, they were far fewer than expected, and it is believed that the vaccination program contributed to victory in the War of Independence. Effective military force requires personnel who are healthy and combat ready for worldwide deployment. Given the geography of US military operations, military personnel should also be protected against diseases that are endemic in potential areas of conflict. For this reason, and unknown to many, the US military has strongly supported vaccine research and development. Four categories of communicable infectious diseases threaten military personnel: (1) diseases that spread easily in densely populated areas (respiratory and dysenteric diseases); (2) vector-borne diseases (disease carried by mosquitoes and other insects); (3) sexually transmitted diseases (hepatitis, HIV, and gonorrhea); and (4) diseases associated with biological warfare. For each category, the US military has supported research that has provided the basis for many of the vaccines available today. Although preventive measures and the development of drugs have provided some relief from the burden of malaria, dengue, and HIV, the US military continues to fund research and development of prophylactic vaccines that will contribute to force health protection and global health. In the past few years, newly recognized infections with Zika, severe acute respiratory syndrome, Middle East respiratory syndrome viruses have pushed the US military to fund research and fast track clinical trials to quickly and effectively develop vaccines for emerging diseases. With US military personnel present in every region of the globe, one of the most cost-effective ways to maintain military effectiveness is to develop vaccines against prioritized threats to military members' health.

Keywords: vaccines, military medicine, army, development, history

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INTRODUCTION

Infectious diseases occur worldwide (1, 2). It is therefore no surprise that militaries have throughout history been subject carriers, and vectors of infectious pathogens. Until World War II, the majority of deaths in military units engaged in combat were due to infectious diseases rather than direct combat injuries (3). Personnel lived in close quarters ate common prepared food and were

exposed to poor sanitary conditions in the battlefield. Outcomes of military campaigns were often driven by the health conditions more than military preparedness (4). The threat of malaria was clear in the mind of Gen Douglas McArthur when, in 1943 he remarked to Dr. P. F. Russell: "this will be a long war if for every division I have facing the enemy I must count on a second division in hospital with malaria and a third division convalescing from this debilitating disease!" (5). Military epidemiologists were instrumental in the discovery of vector-borne diseases and mechanisms of transmission of many infectious diseases. Military doctors deployed with troops in the battlefield were able to study the environment and the diseases that affected the soldiers. Their experience informed vaccine development for many infectious diseases (4, 6, 7).

Warfare has changed in the quarter century since the end of the Cold War. Military operations have become smaller, faster, and asymmetric, with "complex operations other than war" (4). Military personnel may be stationed abroad for extended period of times with frequent contact with the local populations, vectors, and animals that increase the risk of exposure to diseases that are not a threat on US soil. For the same reason, monitoring emerging diseases and potential biowarfare pathogens has been an interest of the US military (8).

Developing safe and effective vaccines is a cost-effective solution to prevent infectious diseases and maintain healthy and combat-ready personnel. For this reason, the US Department of Defense (DoD) has funded vaccine research for several infectious diseases affecting people around the world (**Figure 1**). However, it is important to underline how vaccine manufacturing has become one of the most challenging processes because of its complexity and inherited uncertainties of vaccine research and development. The cost of developing safe and effective vaccines has greatly increased, and without innovation and continuous commitment, it will become an unsustainable and unobtainable goal for the US military.

The DoD conducts most of its endemic infectious diseases vaccine research at the Walter Reed Army Institute of Research and at the Naval Medical Research Center. Research and development of biowarfare countermeasures is conducted at the US Army Research Institute for Infectious Diseases at Fort Detrick, Maryland. These institutes operate overseas research units in multiple sites in Africa, Thailand, Georgia, Cambodia, Singapore, and Peru. A central mission of these institutes has been to study, design, and develop safe and effective vaccines that would protect US military personnel. Many young physicians started their careers in vaccinology in the US military and then moved to industry or academia where they continued to make important contributions to the field. Dr. Albert Sabin, the father of oral polio vaccine, was an Army major working in the Pacific Theater during World War II and contributed to the generation of the first Japanese encephalitis (JE) vaccine and to the epidemiology of dengue. In addition, the military recognized the benefit of being able to test vaccines in endemic areas where the epidemiology of the infectious disease of interest is well documented. Since the 1960s, the US Army maintained a collaborative effort with the Royal Thai Army (RTA) by establishing a South East Asia Treaty Organization Medical Research Laboratory in Bangkok, Thailand that became the Armed Force Research Institute of Medical Science (AFRIMS) in 1977. The collaborative effort between the Thai and US Army doctors at AFRIMS, the Ministry of Public Health (MoPH), and Thai academic institutions working in collaboration with the pharmaceutical industry has conducted vaccine efficacy trials for JE, hepatitis A (HepA), dengue, and HIV resulting in the licensure of vaccine for JE, HepA, and dengue (6, 9).

THE EARLY YEARS

Smallpox

The first large-scale smallpox infection prevention campaign was conducted in 1777 by the Continental Army (10). General George Washington knew that the troops were vulnerable

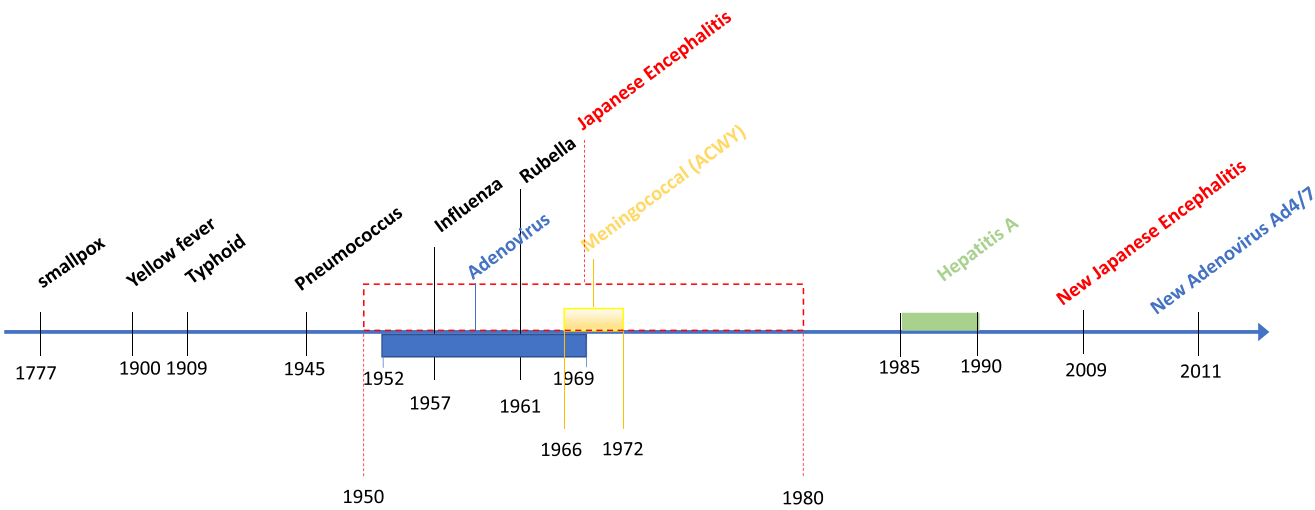

FIGURE 1 | Timeline display of US military involvement in the research and development of major vaccines.

to smallpox and made the strategic decision to have soldiers variolated. This decision may have contributed to the defeat of the British in the Revolutionary War (1776–1783). The Army continued to vaccinate its recruits against smallpox until the early 1990s, 20 years after vaccination stopped in the civilian population and smallpox was considered eradicated (7). After the 2001 terrorist attack on the United States and the use of anthrax spores in a bioterrorism attack, smallpox once again was viewed as a potential threat to US military readiness (11). Smallpox is caused by an orthomyxovirus and poses a high epidemic risk (12, 13). Using smallpox as a biological weapon was unfortunately not new to warfare; sundries, blankets, and handkerchiefs were distributed to Native Americans in 1763 around Fort Pitt, Pennsylvania to decimate the local, native population (3). Restarting smallpox vaccination in a post-eradication world with a live vaccine was not without risk and serious adverse events (AEs) were reported (14, 15). After 2003, the military decided to vaccinate only individuals who were due to be deployed in "high risk" areas. New smallpox vaccine formulations associated with fewer AEs have been developed. Currently, the Strategic National Stockpile has three smallpox vaccines: ACAM2000®, the only licensed smallpox vaccine in the US; Aventis Pasteur Smallpox Vaccine (APSV); and Imvamune (Bavarian Nordic); the Center for Disease Control recommends routine vaccination only for specific populations at high risk of occupational exposure (16).

EARLY TWENTIETH CENTURY

Yellow Fever

The US territorial expansion brought new challenges to the military. With the acquisition of Cuba after the Spanish-American war, US troops stationed on the island were decimated by yellow fever, a debilitating disease with an estimated 20% fatality rate (17). A young group of preventive medicine officers led by Major Walter Reed was able to contain the disease by identifying the route of transmission through mosquitoes and by implementing vector control measures, they were able to control the disease. Ultimately the etiology was identified as a filterable virus transmitted by *Aedes aegypti* mosquito and by the 1930s, a vaccine was developed and is currently still in use (18). Recent yellow fever epidemics in South America and Africa highlight the importance of yellow fever vaccination in endemic areas. The recent epidemics and a recent manufacturing problem of the only US licensed yellow fever vaccine YF-VAX®, produced by Sanofi Pasteur, have caused a vaccine shortage. By mid-2017, worldwide stockpiles were depleted and new vaccine manufacturing will not resume till mid-2018. This event has impacted the US Military and the general population (19). Both the Centers for Disease Control (CDC) and the DoD have developed contingency measures to counteract this threat by fractioning the vaccine dose as it was demonstrated that even lower doses were immunogenic (20, 21). An important message that emerges from this incident is that a closer monitoring of worldwide stockpiles of vaccines for preventable diseases remains key when there is only one (FDA approved) vaccine manufacturer.

Typhoid

More US troops died in military training camps due to enteric fever caused by Gram negative bacillus *Salmonella enterica* serovar Typhi than died on the battlefield during the Spanish-American War. The same scenario unfolded during the Anglo-Boer War where 8,225 British troops died of typhoid compared to 7,582 of wounds (7, 18). A British pathologist, Sir Almroth Wright was the first to develop a typhoid vaccine at the Army Medical School, Netley, England. He pioneered a vaccine preparation method that involved heat inactivation of bacilli taken from an infected patient. After his success, Major Frederick Russell of the US Army modified the vaccine formulation and after establishing the safety and efficacy of the vaccine, typhoid immunization became a requirement for all US troops after 1911. Consequently, the US Army had the lowest typhoid fever incidence of any of the major combatants in World War I. With improvement in sanitation systems enteric fever due to *S.* Typhi has become rare in the developed world but in low- and middle-income countries where clean water and sanitation are still a challenge, it infects 20 million people and kills over 100,000 every year (22).

Pneumococcus

Streptococcus pneumoniae was discovered by Major George Sternberg in 1881, the same year as Louis Pasteur's seminal discovery (23). Upper respiratory diseases were a major problem for troops, which spurred the Army to develop a vaccine against pneumococcal pneumonia. By 1930, polyvalent pneumococcal vaccines were tested at different sites, but the final successful clinical trial was performed in Sioux Falls in 1944–1945 at the Army Air Force Technical School where a high incidence of pneumococcal pneumonia was found (6). The vaccine did not have a great impact because the greater availability of penicillin lessened the need for a vaccine in healthy young adults. Subsequently, multivalent pneumococcal vaccines were introduced for the elderly (24) and ultimately the development of conjugated 10 or 13 valent formulations have drastically reduced the rate of invasive pneumococcal disease in infants, saving millions of lives since their introduction in the early 2000s (24–26).

MID TWENTIETH CENTURY

Influenza

During World War I, the pandemic of Spanish influenza had a devastating impact on the US military, claiming the lives of over 43,000 sailors and soldiers. The fast spread of the disease was aided by transport of troops across the oceans where close quartering contributed to the spread of the virus. The Armed Forces Epidemiological Board (AFEB) which evolved from previous Military Infectious Diseases Boards that were commissioned to study the epidemiology of influenza and other highly occurring infectious diseases was led by Thomas Francis Jr., the first scientist to isolate the influenza virus from an infected human (18). The work of AFEB was instrumental in the preparation of the first whole-inactivated virus vaccine tested in hospitalized inmates, military recruits, and college students. The first inactivate strain was type A influenza and sooner after the first vaccination season

the inactivated B virus strain was added, and the first bivalent influenza vaccine was used to vaccinate troops in 1945. Data on flu vaccine efficacy showed that new strains were appearing in circulation each season leading to changes in vaccine virus composition to match the circulating influenza strains (18, 27). The US military has continued to study the influenza virus and the quest for a more effective vaccine, and epidemiologists have made recommendations on the composition of the yearly flu vaccine through the years (18, 27–31).

Adenovirus Type 4 and 7

As military scientists were investigating influenza infections at Fort Leonard Wood in 1952–1953, they realized that another virus with similar symptomatology was causing an acute respiratory disease (ARD) in recruiting and training camps. Adenovirus 4 and 7 was isolated from these soldiers. A formalin-inactivated adenovirus type 4 and 7 vaccine was introduced in 1956 and was soon replaced by an oral formulation that was 50% effective in reducing hospitalization caused by ARD (18). Wyeth Pharmaceuticals provided the vaccine for the US military until 1996 when production was halted. As a result of stopping vaccinations, the rate of adenovirus infections at the recruiting training centers increased dramatically in the following years (18). The cost of hospitalization and toll on military personnel health and readiness was a deciding factor for the US Army to enter into contract with Barr Pharmaceuticals in 2001 to resume production of the Ad4 and Ad7 vaccines (18). After clinical trials showed high seroconversion rate and safety profile, the Ad4 and Ad7 vaccines were approved by the US FDA and vaccination resumed in 2011 at all military recruiting centers. A follow-up study showed that after 2 years of vaccination, a 100-fold reduction in disease burden was observed in the recruit population (32).

Rubella

The rubella virus was first isolated by three military scientists (Captain Paul Parkman, Captain Malcom Artenstein, and Lieutenant Colonel Edward Buesher) from a recruit hospitalized at Fort Dix during an adenovirus outbreak in 1961 (7). The isolation of the virus prompted the development of the first rubella live-attenuated vaccine, available for the general population in 1969 (18, 33, 34). Several improvements were then brought to the original vaccine (strains, cell substrate) (34). Since then, rubella cases have steadily declined through the years and the occurrence of congenital rubella syndrome (CRS) has been drastically reduced. CRS can affect virtually every organ and the severity increases if the infection occurred early in gestation (34). The measles-mumps-rubella combination vaccine was critical to the reduction of the devastating impact of CRS (35).

Japanese Encephalitis

The Japanese encephalitis virus (JEV) was first isolated in 1935 from the brain of a patient who died of encephalitis in Japan. In early 1940, Major Albert Sabin was assigned the task of developing a vaccine against JEV. He and his team produced a formalin-inactivated vaccine from JEV-infected mouse brain that was administered to more than 250,000 military personnel during World War II. With the continued presence of US military personnel in Asia in the 1950s and during the Korean War, it became apparent that the vaccine was not efficacious enough, so vaccination was halted and research on a better vaccine commenced. In the mid 1980s, the US Army conducted the pivotal study in Thailand that led to the US FDA approval of a JEV vaccine (JE-VAX), a new whole virus formalin-inactivated vaccine (7, 18). This virus was still produced from mouse-infected brains and although it was deemed safe, concerns remained and the two pharmaceutical companies that produced and distributed JE-VAX (BIKEN and Sanofi Pasteur, respectively) stopped production in 2005. Because of the continuous threat of JE together with the need for military personnel to be station throughout Asia, the US military remained engaged in the search for a second-generation JEV vaccine preparation. Promising results with a new JEV vaccine formulation developed by Intercell AG and tested in phase I by WRAIR scientists led to the full development and approval of IXIARO (36–38). IXIARO is a JEV attenuated SA-14-14-2 strain grown in Vero cells, this vaccine was approved for use in children in 2013, and it is registered in several endemic countries. IMOJEV® (JE-CV and previously known as ChimeriVax™-JE) is a novel recombinant chimeric virus vaccine developed by Sanofi Pasteur using the yellow fever virus (YFV) vaccine vector YF-17D, replacing the cDNA encoding the envelope proteins of YFV with that of the attenuated JEV strain SA14-14-2. IMOJEV® single dose was found to be safe, highly immunogenic, and capable of inducing long-lasting immunity in both preclinical and clinical trials. It has been tested in the US military personnel (39).

Meningococcal Vaccine

Meningococcal disease, caused by the bacterium *Neisseria meningitis*, is associated with outbreaks among personnel in highly confined settings such as military training camps and university campuses. Outbreaks were commonly reported in military recruits since the nineteenth century, but during the Vietnam War (1964–1971), an epidemic of serogroup B and C meningococcal meningitis among US army recruits resulted in the closing of Fort Ord in California. The death rate from the epidemics during this period was similar to those due to malaria. The concurrent surge of antimicrobial resistance pushed the US military to accelerate vaccine research. The human immunological response to the bacterium served as the basis for the first polysaccharide vaccine against serogroups A and C. In 1969, four major papers were published by US Army researchers that defined the assay for bactericidal antibody using human complement that was accepted as a correlate of protection in humans and served as the basis of licensure for all existing meningococcal vaccines (40–43).

Phases I–III were conducted by the US military leading to a licensed vaccine to serogroup A followed by a combined serogroup A/C vaccine in 1970 and 1978, respectively (18). Meningococcal serotype B vaccine was harder to develop because its antigens have homology with human neuronal proteins. Although the US military was not involved with the development of this vaccine, it is worth noting that through reverse vaccinology, a method of vaccine design that starts with the prediction of antigens from the

genome sequence of a meningococcal B strain (MenB), two new products are now available (44, 45).

Hepatitis A

Hepatitis A (Hep A) virus causes hepatitis epidemics in military personnel deployed in areas with poor sanitary conditions. The epidemics rarely caused death, but servicemen would develop jaundice, be indisposed, hospitalized, and unable to fight. The US military doctors first demonstrated that immunoglobulin could prevent or attenuate Hep A disease, however, protection was temporary and needed continuous re-injections that were unfeasible for long deployments in endemic areas. Therefore, WRAIR scientists sought to develop an effective vaccine. They discovered the best method of culture of Hep A virus and established in animal models that one serotype could protect against strains from other endemic areas. In 1986, they produced the first formalin-inactivated vaccine tested in humans. The phase III trial of Hep A vaccine commenced in 1991 in Thailand through a collaboration of the Thai MoPH and Smith Kline Beecham Biologics (now GSK). It involved 20,000 children vaccinated with Hep A vaccine and 20,000 with Hep B vaccine as control. The success of this trial brought the Hep A vaccine (HAVRIX) to licensure in 1995 (18).

CURRENT CHALLENGES

Several other debilitating infectious diseases represent a serious public health threat, in particular for the military personnel and for which a preventive vaccine is not yet available.

Malaria

Malaria, a mosquito-borne infectious disease, is derived from the Italian word that comes from the contracted form of *mala aria* or "bad air," referring to the "intermittent fevers that affected people living near marshy districts and attributed to the unwholesome airs that were produced by the stagnant waters." In 1775, the first US Continental Congress appropriated $300 for the first medical acquisition of "Peruvian bark" for the treatment of fever. This was prior to the discovery of the malaria parasite, but it was well recognized at the time that the bark of the cinchona tree, from which quinine is extracted, was effective in treating malarial fever (46). It was not until the 1880s when the French army surgeon, Charles Louis Alphonse Laveran first noticed the appearance of parasites (*Plasmodium* spp.) in the blood of a patient suffering from malaria in Algeria. In 1900, Col. William Crawford Gorgas with other Army colleagues recognized the importance of vector-borne mosquito transmission of infectious diseases to humans and implemented one of the most effective vector control programs in Panama in 1904. Within 3 years the incidence of malaria was reduced from 800 cases/1,000 workers to just 16/1,000 (47).

Until World War II, the military strategy against malaria remained primarily vector control. In 1943, the introduction of dichlorodiphenyltrichloroethane (DDT) greatly aided those efforts. During World War II, quinine, used for both treatment and chemoprophylaxis, was in short supply for Allied troops because the majority of cinchona plantations were located in Java (the Dutch East Indies) which was controlled by the Japanese. It became clear that new drugs, and a vaccine, were needed to maintain effective force protection. A malaria drug development program was started that included academic, government, industry, and military partners in an unprecedented effort to discover new antimalarial drugs. This highly classified program resulted in the discovery of chloroquine and primaquine for the treatment and prophylaxis of both falciparum and vivax malaria (48). After World War II, the US DoD remained a leading investor in malaria drug and vaccine development, which was reinvigorated by the Vietnam war and the spread of chloroquine resistance (49). It was during this time that WRAIR emerged as a lead developer in new antimalarial drugs as well as malaria vaccines (47, 50).

Due to the complexity of the malaria parasite life cycle in humans and mosquitoes, that includes asexual and sexual stages, it has been difficult to develop an effective vaccine. Early clinical experiments done by the University of Maryland and the WRAIR showed that irradiated, infected mosquitoes could transfer attenuated *P. falciparum or P. vivax* sporozoites through multiple infected mosquito feedings. The immune response generated conferred subsequent protection against wild-type falciparum malaria in controlled human malaria infection model (CHMI) (51, 52). These early studies, although crude, demonstrated that a vaccine against malaria was possible. A biopharmaceutical company (Sanaria) has devised a method to purify malaria sporozoites (the infective stage of the parasite) from irradiated, aseptic mosquitoes and store the irradiated sporozoites in a stable, frozen formulation. Irradiated sporozoites (referred to as PfSPZ) are thawed and administered intravenously. The PfSPZ vaccine has been tested in phase I/II clinical trials and demonstrated to protect against clinical malaria using a well-established CHMI model (53, 54). Though recent field trial results were mixed (55), this method of vaccination is being pursued, though production and scale up remain as significant hurdles (56). Another approach spearheaded by WRAIR scientists has been the use of a recombinant protein approach based on the circumsporozoite protein (CSP) of the pre-erythrocytic (sporozoite) stage of the malaria parasite. CSP was one of the first surface-expressed, GPI-anchored proteins cloned (57) from *Plasmodia* and was shown to be a key target for protective immunity-induced by irradiated sporozoites in animal models as well as in clinical malaria (58, 59). This strategy was undertaken in collaboration with Smith Kline Beecham (subsequently GSK), which resulted in the initial testing of the RTS,S malaria vaccine candidate in combination with several novel adjuvants by US Army investigators (60). RTS,S consists of a single fusion protein composed of the CSP central *R*epeat region and *T cell* epitopes with hepatitis B *S*urface antigen. This is co-expressed with free hepatitis B surface antigen in yeast cells, resulting in self-assembling viral like particles (61). Initial promising results led to the clinical development of RTS,S adjuvanted with $AS01_E$ through a pivotal phase III efficacy trial. The vaccine was given a positive scientific opinion after review by the European Medicines Agency for use outside the European Union (62–64). Phase III testing

in children aged 5–17 months, showed efficacy of 51.3% against all episode of clinical malaria over 12 months at all site tested. Efficacy was lower at 18 months and was further reduced after 3 years of follow-up. A fourth vaccination seems to increase slightly efficacy overall at 32 months. WHO recommended this vaccine to be tested in small pilot studies to understand if the data can be replicated in the normal health care delivery system (65). However, this level of efficacy would not be considered sufficient for force health protection [as compared to traditional chemoprophylaxis which is ~90% effective with good adherence (66)]. These observations led to renewed interest in further assessment of the dose and schedule of RTS,S based on the initial results of et al. in 1997 where a regimen of 0, 1, and 7 months with a fractional third dose (1/5th of the first two doses) resulted in six of seven participants (87%) protected from controlled human malaria infection (60). A subsequent phase IIa trial, conducted in 2013, replicated these results, protecting 26 of 30 subjects (87%) using the CHMI model (67), suggesting that further improvements to the efficacy of this approach are feasible and warrant further clinical development (68).

Dengue

Dengue fever is a mosquito-borne disease caused by one of four serotypes of dengue virus (DENV), a flavivirus transmitted by *Aedes aegypti*. DENV causes a febrile illness that can occasionally be fatal, especially if managed poorly. Dengue is more likely to be severe upon infection with a second serotype different from an initial infecting serotype, and can be associated with plasma leakage, severe bleeding, respiratory distress, and organ impairment. An estimated 50–100 million annual symptomatic dengue infections are associated with 500,000 cases of severe dengue and about 20,000 deaths. Overall, the mortality rate is low (<1%) if managed properly. The quest for an effective dengue vaccine started more than 50 years ago, but an effective and safe vaccine has proved elusive. US military personnel have dealt with dengue since the beginning of the twentieth century during the Spanish-American War. In 1906, a dengue epidemic affected troops stationed at Fort McKinley, Manila, Philippines, and the Army Tropical Disease Board made the study of DENV a priority. During World War II in the South Pacific, the rapid expansion of troops and bases permitted DENV to spread from island to island on planes and ships used to supply bases. By 1944, most islands in the South Pacific had identified cases of dengue, and it was estimated that dengue in Melanesia and neighboring islands caused more than 80,000 sick days and infection rates of 12% among US troops (69). During and after World War II, Major Albert Sabin isolated DENV serotypes 1 and 2 from Hawaii and New Guinea, and William M. Hammon identified DENV serotypes 3 and 4 from the Philippines as the cause of hemorrhagic fever (7, 18, 69). There is no current antiviral medication available for DENV infection. Monitoring of dengue cases and judicious fluid replacement have reduced the mortality rate to less than 1% (70, 71). Given the high attack rates and substantial burden of symptomatic illness, the military has focused on the development of a safe and effective dengue vaccine.

Dengue Vaccine Development: Lessons Learned and Current Challenges

Dengue vaccine has been in development for over 50 years and has presented a challenge because of the unique characteristic of the immune responses to the four virus serotypes, lack of immune correlates of protection, and lack of suitable animal models (72). A dengue vaccine sponsored by Sanofi Pasteur has been licensed in multiple dengue endemic countries, and several candidate vaccines are at various stages of development (73). Although the US military has maintained interest in several different candidate vaccine to prevent dengue, Sanofi Pasteur in 2010 initiated the first phase 2b proof of concept trial using their CYD tetravalent dengue vaccine (CYD-TDV). The trial was conducted in Thailand among 4,000 children aged 4–11 years. CYD-TDV is composed of four chimeric live-attenuated viruses (CYD1–4) based on a yellow fever vaccine backbone (YF 17D) with structural DENV proteins (74). Preclinical and clinical studies have shown that a three-dose vaccination regimen induced balanced immune responses to all four serotypes, and pre-existing flavivirus infection seemed to induce a more rapid immune response with no increase in vaccine-derived viremia. Unfortunately, this tetravalent vaccine did not provide equal protection against all four dengue serotypes, with especially low efficacy against DENV-2 (75). Subsequent multi-country phase III trials in Asia and Latin America in 2- to 16-year-old children showed good efficacy against DENV-3 and 4, moderate efficacy against DENV-1 and marginal efficacy against DENV-2. Notably, an increase in relative risk of severe dengue in vaccine recipients aged 2–5 years during the third year of the Asian phase III trial (76, 77) led to the age indication of children 9 years of age and above. CYD-TDV (trademarked as Dengvaxia®) eventually received licensure in 20 dengue endemic countries but has not yet been approved in the US. Based on an assessment of potential overall public health benefit, WHO recommended in July 2016 that vaccination could be carried out in highly endemic areas (>70% dengue seropositive rates) (65, 78). However, in November 2017 Sanofi, who continued to monitor safety of their vaccine, announced the results from a new laboratory test that could infer dengue serostatus prior to vaccination in subjects from their phase III trials and found that seronegative persons of any age (including those >9 years) who receive Dengvaxia® had a higher risk of severe dengue. On April 19, 2018, the WHO Strategic Advisory Group of Experts on Immunization revised their recommendations, emphasizing individual testing before vaccinating in order to minimize the likelihood of seronegative individuals receiving Dengvaxia®.[1]

The US army initially focused on tetravalent live-attenuated vaccine candidates, entering into a partnership with GSK in early 2000. A tetravalent live-attenuated dengue vaccine candidate was eventually evaluated in a phase II clinical trial in Puerto Rico (79). Subsequently, a purified, inactivated whole virus approach was pursued with GSK (80, 81), leveraging GSK's proprietary adjuvants to try to elicit more durable tetravalent immune

[1] http://www.who.int/immunization/diseases/dengue/revised_SAGE_recommendations_dengue_vaccines_apr2018/en/ (Accessed: May 13, 2018).

TABLE 1 | HIV phase I/II/III tested by the US Army.

Vaccine	Trial (# of participants)	Company
Gp120SF2(B)/MF59 (87)	Phase I (n = 52)	Chiron
Gp120SF2(B)/MF59 + gp120CM235/MF59 (88)	Phase II (n = 370)	Chiron
ALVAC-HIV (vCP1521) prime + oligomeric gp160 (92TH023/LAI-DID) or ALVAC-HIV (vCP1521) prime + bivalent gp120 (CM235/SF2) (93)	Phase I/II n = 130	Sanofi Pasteur and Novartis Vaccine and Diagnostics
ALVAC-HIV (vCP1521) prime + AIDSVAX B/E (92)	Phase I/II n = 122	Sanofi Pasteur and VaxGen
ALVAC-HIV (vCP1521) prime + AIDSVAX B/E (94)	Phase III n = 16,402	Sanofi Pasteur and VaxGen
MVA CMDR (CRF01_AE) (95)	Phase I n = 48	LVD/NIAID/WRAIR/MHRP
PENNVAX-G DNA + MVA-CMDR (96)	Phase I n = 88	Innovio Pharmaceutical + LVD/NIAID/WRAIR/MHRP

responses (72). US Navy has primarily pursued a DNA vaccine approach, evaluating a tetravalent DNA vaccine candidate in a phase I trial (82). Takeda has developed a tetravalent recombinant attenuated vaccine, TDV, based on a common, molecularly cloned DENV type 2 called DENVax-2. Serotypes 1, 3, and 4 are represented in the vaccine by substituting prM and E genes of DENVax-2 with those of their respective serotypes. TDV has undergone phase II trials and is currently in the midst of a large multi-country phase III efficacy trial in Asia and Latin America (NCT02747927), that includes a US Army site in the Philippines. US NIH developed their own tetravalent recombinant attenuated vaccine candidates, TV003/TV005, and has sponsored the candidate through phase I and II trials, including a trial in Thailand with the US Army. US NIH provided licenses to various manufacturers for ongoing product development, including Butantan, Vabiotech, Panacea, Serum Institute of India, Indian Immunologicals Inc., Medigen, and Merck. Butantan is currently conducting a large phase III efficacy trial of the vaccine in Brazil (NCT02406729).

Human Immunodeficiency Virus

HIV poses a significant and persistent threat in terms of readiness and force protection and may act as a war-starter by affecting the stability and security of nation-states. In 2001, the Armed Forces Epidemiology Board identified HIV as a disease of military importance; the 2001 DoD Report on Biological Warfare Defense Vaccine Research and Development identified HIV as the fourth greatest infectious disease threat to DoD forces. Department of Army Headquarters designated HIV vaccine development as an Army Technology Objective, a status reserved for the highest priority science and technology efforts.[2]

HIV military relevance has been recognized from the very beginning of the pandemic. In 1985, the US military recognized the emerging HIV-1 epidemic as a new threat to US and allied forces worldwide. The United States Congress mandated the establishment of the US Military HIV Research Program (MHRP) to develop effective preventive measures to include prevention education, vaccine development and implementation of novel anti-viral therapies, and clinical management tools for the US DoD and Allied Forces (83). Much of the early HIV vaccine development in the Army focused on developing a vaccine against strains (subtype B and CRF01_AE) found in Thailand, because significant rates of HIV infection from heterosexual spread were found in RTA recruits from Northern Thailand and because of the strong and successful partnership between the US Army through AFRIMS and the RTA (84). The well-developed health surveillance system developed by the Thai MoPH together with the RTA was instrumental in the early collection of samples that allowed the scientists at AFRIMS and WRAIR to show that the majority of HIV-1 circulating in Thailand was a recombinant form (CRF01_AE) together with the already known North America B serotype (85). Thailand's strong public health infrastructure and the early adoption of standardized HIV testing among the RTA recruits gave detailed information on the prevalence of HIV infection among the different geographical regions and the general Thai population. The collection of data further documented that aggressive education and behavioral interventions that were implemented by the government and non-for-profit organizations were effective in reversing the epidemic (86). The Thai AIDS Vaccine Evaluation Group that was established early on as a way to bring together the RTA, US Army already working together at AFRIMS and the major university research centers in Thailand (84). The first set of phase I trials tested recombinant envelope proteins alone or in combination that were derived from both circulating HIV strains (**Table 1**) (87, 88). The vaccinations were well tolerated and induced strong antibody responses. The addition of a canarypox prime (ALVAC-HIV) improved cellular immune responses. ALVAC-HIV was tested in phase I/II trials in the US and demonstrated good cellular immunogenicity but poor antibody responses (89–91). Prime/boost combinations were tested in the US and Thailand (92, 93) and by early 2000 Sanofi Pasteur and VaxGen entered an agreement to test their products in a prime-boost phase III trial (RV144). This HIV efficacy trial involved 16,402 community risk Thai individuals recruited in Rayong and Chonburi provinces through a partnership between the US Army, NIH, RTA, MoPH and Mahidol University. RV144 was the first and remains the only HIV efficacy trial to show protection, with vaccine efficacy of 31% at 42 months (94).

RV144 also established a correlate (biomarker) associated with decreased risk of HIV infection (antibody to the HIV gp120 V1V2 region) (97). The study led to a series of additional insights regarding potential correlates of risk (98) and had greatly informed the ongoing ALVAC + g120 efficacy trial in the Republic of S. Africa. The US MHRP continues to invest in prime-boost strategies with a variety of immunogens (95, 96) (**Table 1**). The US military has maintained a strong support for HIV vaccine research and development and continuous monitoring of the epidemic.

[2]http://archive.defense.gov/pubs/ReportonBiologicalWarfareDefenseVaccineRDPrgras-July2001.pdf (Accessed: May 13, 2018).

Enteric Diseases

Although personal hygiene, sanitation measures, and antibiotics have greatly improved conditions in military training camps, installations, and combat field sites, enteritis continues to plague military forces during deployments (99, 100). A few studies have tried to understand the days-work lost during military deployment to justify the founding for enteric vaccines (101, 102). Enteric diseases *per se* are not life threatening and although the burden of time lost for soldiers may not be substantial it is imperative to consider the effect that these infections may have on the population at large (101). Vaccines against enteric diseases may benefit deployed soldiers and their families in high-risk areas and there is secondary benefit for leisure travelers as well as populations living in low- and middle-income countries where hundreds of thousands die annually of diarrheal diseases. Besides acute illness, diarrheal diseases may cause chronic debilitating conditions like Guillain–Barre syndrome after infection with *Campylobacter* and reactive arthritis in 5% of individuals after *Shigella* and *Campylobacter* infection. Post-infection irritable bowel syndrome is now recognized as a sequela of infectious diarrhea and occurs in approximately 10% of individuals post-gastroenteritis. These chronic conditions may decrease work hours, quality of life, and increase the health cost burden to society (101).

The US Army has invested heavily on the development of enteric vaccines focusing primarily on three pathogens: Enterotoxigenic *E. coli* (ETEC), *Shigella*, and *Campylobacter* as they are considered the most important threat to troops worldwide. The US military has many different vaccines in the pipeline at various stages of development. Briefly, an attenuated *Shigella* vaccine (WRSS1) is in a phase IIb clinical trial, and one *Shigella* inactivated vaccine is in phase I clinical testing. Also, subunit vaccine like for *Shigella flexneri* 2a (Invaplex) and the bioconjugate Flexin2a are in preclinical development. Subunit protein made of fimbrial tip adhesin of ETEC CF proteins have been also tested by the US Naval Medical Center in phase 2 clinical trials (103).

Rickettsial Diseases and Scrub Typhus

Human rickettsial diseases were grouped as "typhus fever" as they shared common symptoms. As diagnostics improved, it was discovered that the rickettial disease could be divided into three distinct groups: (1) tick typhus group (Rocky Mountain spotted fever as an example), (2) typhus group (louse born or epidemic typhus), and (3) scrub typhus group. The Rickettsiae are proteobacteria and can be transmitted by mites, fleas, flies, ticks, and lice. Mortality rates vary by species but can be high during period of war, famine and social disruption or because of underdiagnoses. The first clear account of Typhus fever occurred during the military siege of Granada in 1489 where 17,000 deaths were reported in the Spanish Army (104). Typhus devastated Napoleon's troops during the invasion (and retreat) of Russia in 1812. Rickettsial diseases were present through World War I and II but since the etiology was discovered and troop's hygiene improved, the incident cases also diminished. The use of the insecticide DDT, various chemical repellents, and the discovery of antibiotics collectively reduced the burden of rickettsial diseases. In addition, military troops on both fronts had access to various effective vaccines and most of the casualties were among the civilian populations (104).

The only reported rickettsial disease cases and deaths during World War II were from Scrub typhus infection (*Orientia tsutsugamushi*) in the Asia-Pacific region. Military scientists from WRAIR and University of Maryland discovered in 1948 that the antibiotic chloromycetin was effective against Scrub typhus but eventually resistance emerged (104). During the Vietnam War, Fever of Unknown Origin was caused mainly by Scrub typhus and had a co-infection incidence of 6% with Malaria. Until World War II, Scrub typhus was considered a sub-tropical disease, but it became apparent during the following year when US troops were stationed in Japan and Korea that seasonal Scrub typhus was present in these areas as well.

New Challenges

Chikungunya Resurging

The Chikungunya virus is transmitted by *Aedes* mosquitoes and was first isolated in 1953 in Tanzania. Symptoms of Chikungunya infections include abrupt onset of fever with acute arthralgia and arthritis that can last for a very long time. In 1962, the US Army isolated a strain of Chikungunya from an individual in Thailand and started the development of an attenuated vaccine. A chikungunya vaccine was eventually developed by a partnership between the Salk Institute-Government Service Division under contract with the DoD in 1984. At the time, a review of the funding priorities for potential disruptive diseases for military operations, ranked Chikungunya low on the scale of threats to the military and as consequence the project was halted. In 2005, a resurgence of Chikungunya infection was observed in Kenya and Reunion Island where tens of thousands of individuals were infected and over 200 fatalities were reported (105). Representatives of the French Ministry of Health contacted the US Secretary of Health and Human Services as they were aware of the US Army's previous work and several pharmaceutical companies also expressed interest (105). Currently, formulations of live-attenuated Chikungunya vaccine similar to the product shelved in the mid 1990 have been tested in a phase II trial, together with other similar strains that were obtained from the US Army laboratories (106). Hopefully the vaccine will find funding through licensure as it is considered a re-emerging infection in low- to middle-income countries.

Zika Virus (ZIKV)

Even though ZIKV has been known since 1947, its spread and consequent illness reached pandemic proportion only in 2013. It is currently present in more than 80 countries and causing millions of infections yearly (107). ZIKV is transmitted by the Aedes mosquito, which is ubiquitous and favors urban areas as breeding ground. It is transmitted from mother to fetuses, *via* sexual intercourse and possibly *via* transfusion and organ transplantation (107). The ability of ZIKV to cause both dengue-like febrile symptoms and neurological conditions (Guillain–Barre syndrome and encephalitis) and to cause marked teratogenicity, makes it a formidable foe for public heath control effort and consequently for military operations. Early reports of infection with ZIKV were out of Africa but the disease presentation was

confounded by co-infection with other diseases. Diagnosis was hampered by cross-reactivity with closely related flaviviruses. It is safe to assume that infection with ZIKV had probably occurred but was unrecognized or misdiagnosed as dengue or JE and never reached epidemic proportions (108). The first epidemic was reported in Yap, Federated States of Micronesia, in 2007, followed by an outbreak in the French Polynesia in 2013. There was subsequent spread throughout the South Pacific. In 2015, a major epidemic of neurological disease in infants occurred in Brazil and rapidly spread through the Americas. Singapore and Vietnam were the sites of two outbreaks, and there was widespread infection in Thailand in 2017 (108). This emerging disease was declared a Public Health Emergency of International Concern by WHO in 2016. More than 40 candidate vaccines are in preclinical stages and 7 are currently being tested in phase I throughout the world. The US military research group at WRAIR in collaboration with the Beth Israel Deaconess Medical Center is testing a ZIKV purified inactivated virus based on their previous experience with JEV vaccines (109). Currently, new Zika infection rates have dramatically plunged in South America, possibly due to "herd immunity." Nevertheless, the quest for an effective vaccine must remain at the forefront to combat this debilitating disease (110).

Hanta Virus

Although only discovered in 1993, Hanta virus can infect humans through exposure to aerosolized rodent's excreta; infection causes hemorrhagic fever with renal syndrome (HFRS, old world rodents) or hemorrhagic fever with pulmonary syndrome (HFP, new world rodents). Most infections occur in China (111). The US military has justified the need for a vaccine by outlining the risk of exposure that troops could face in natural disasters or wars (particularly on the Korean peninsula), where destruction of human environments and stress on population may increase exposure to Hanta virus. A clear example of this risk was brought to light during the conflict in Bosnia Herzegovina; a serosurvey for Hanta virus among soldiers showed elevated rate of exposure (16.1%) compare to the population living in the endemic areas (6.2%) (111).

Because China and Korea have had the greatest number of HFRS, both countries have developed a brain-derived inactivated HFRS vaccine which, together with public health education, have reduced but not contained cases of HFRS. This vaccine is not licensed outside Asia and does not cross-react with the Hanta serotypes circulating in Europe.

The US Army tested an HFRS vaccinia vectored vaccine, but it was poorly immunogenic in humans who were already expose to vaccinia (112). A DNA-based vaccine was subsequently developed. New vaccines, which carry Hantaan and/or Puumala M segments to induce broader immunity, were tested in a phase I clinical trials in three cohorts and showed promising results (113).

BIOTERRORISM

Not only are endemic diseases of concern for the military, so are potential exposures to agents deliberately introduced into the environment through biological warfare (BW) or bioterrorism (114).

TABLE 2 | Vaccines for bioterrorism.

Licensed vaccines	Vaccines in R&D
Smallpox vaccine	Vaccinia (cell culture)
Anthrax	Botulinum toxoids
Plague	Tularemia
	Ricin toxin
	Q fever
	VEE, EEE, WEE

VEE, Venezuelan equine encephalitis; EEE, Eastern equine encephalitis; WEE, Western equine encephalitis.

Although President Richard Nixon terminated the offensive biological weapons program in 1969 and 1970 by executive order, research efforts in biowarfare countermeasures continue (115). During Operation Desert Shield before the Persian Gulf War and after the 9/11 events and the anthrax attacks on US institutions, it has become evident that BW remains a potential threat to US soldiers.

Bioweapon threats could include the deliberate release by attackers of an agent that causes one or more of a variety of different diseases. Public health authorities have developed a system to prioritize biological agents according to their risk to national security. Category A agents are the highest priority, and these are disease agents that pose the greatest risk to national security because they can be transmitted from person to person and/or result in high mortality, and/or have high potential to cause social disruption. These are anthrax, botulism (*via* botulinum toxin, which is not passable from person to person), plague, smallpox, tularemia, and a collection of viruses that cause hemorrhagic fevers, such as Ebola, Marburg, Lassa, and Machupo. These disease agents exist in nature (with the exception of smallpox) and could be manipulated to make them more dangerous. Category B agents are moderately easy to disseminate and result in low mortality. These include brucellosis, glanders, Q fever, ricin toxin, typhus fever, and other agents. Category C agents include emerging disease agents that could be engineered for mass dissemination in the future, such as Nipah virus (CDC index of possible threats).

The use of effective vaccines would likely protect lives and limit disease spread in a biological weapons emergency. Licensed vaccines are currently available for a few threats, such as anthrax and smallpox, and research is underway to develop and produce vaccines for other threats, such as tularemia, Ebola virus, and Marburg virus. Many bioweapon disease threats, however, lack a corresponding vaccine, and for those that do, significant challenges exist to their successful use in an emergency situation.[3]

The DoD Joint Vaccine Acquisition Program has several experimental vaccines in development (**Table 2**). These vaccines will be further developed and tested with the intent of obtaining products licensed by the US Food and Drug Administration (12, 116, 117).

[3] https://www.historyofvaccines.org/content/articles/biological-weapons-bioterrorism-and-vaccines (Accessed: May 13, 2018).

Anthrax

Anthrax is the second threat after smallpox that requires a major research and development effort in order to meet civilian and military needs. The most likely scenario for a bioterrorism attack is probably a covert attack, which exposes an urban population to an anthrax spore aerosol. If the release is detected or the first cases are rapidly diagnosed, rapid action can save many lives (12).

Providing the exposed population with antibiotics followed by vaccination could be lifesaving for exposed persons who would otherwise become ill with untreatable inhalation anthrax in the subsequent few weeks. Prophylactic antibiotics alone will prevent disease in persons exposed to antibiotic-susceptible organisms but incorporating vaccination into the treatment regime can greatly reduce the length of treatment with antibiotics. Without vaccination, antibiotics must be continued for 60 days; if effective vaccination can be provided, this can be reduced to 30 days (12). The current anthrax vaccine manufactured by Bioport (formerly the Michigan Department of Public Health Laboratory) is an alum-adsorbed, partially purified culture filtrate of *Bacillus anthracis* with highly protective antigen content. The schedule for administration is 0, 2, and 4 weeks and 6, 12, and 18 months. This vaccine is safe and efficacious and is being used by the armed forces to protect personnel against the use of anthrax as a weapon.

Immunization of rhesus monkeys followed by a high-dose aerosol challenge has convincingly demonstrated the capability of this vaccine to protect against aerosol challenge with *B. anthracis* spores. The multiple dose requirement, however, is a drawback for civilian use. Studies in progress may find ways to allow modification of the schedule. Vaccine supply is limited, as is production capacity. As a result, at least for the immediate future, the armed forces will require the entire available supply. This vaccine is made by a method developed before the advent of molecular biology and requires dedicated facilities because *B. anthracis* is a spore-forming organism. In addition to having a multiple-dose requirement, the vaccine is not highly purified and contains multiple extraneous proteins. The characteristics of the vaccine and the constraints on the present method of manufacturing argue strongly against procuring large amounts for civilian use when the technology and the science base exist to rapidly develop a second-generation, improved anthrax vaccine.

Anthrax depends on two toxins (lethal factor and edema factor) for virulence. A protein called protective factor is an essential component of both toxins. The protective factor content is the basis for the effectiveness of the current vaccine. A vaccine based on purified protective factor made by recombinant technology has been protective in animals. Use of a modern adjuvant with purified recombinant protective factor should make it possible to have a very effective two-dose vaccine. A recent report of the Institute of Medicine Committee on Research and Development to Improve Civilian Medical Response to Chemical and Biological Terrorism makes a strong case for a major research and development effort leading to an improved second-generation vaccine.

Questions regarding the ability of existing anthrax vaccines to protect against anthrax, strains engineered to contain additional virulence genes have been raised in Russia. Research is needed to address this and related questions about the pathogenesis of anthrax and protective immunity.

The value of vaccinating law-enforcement and emergency response personnel, who must respond to threats (real or otherwise), depends on the nature of their work and the immediacy of the threat. Laboratory personnel who must work with unknown materials and with high concentrations of known infectious materials must be vaccinated. These are additional justifications for moving ahead with a vigorous development program for anthrax and smallpox vaccines.

VACCINE COSTS AND DoD BUDGET

Because it is recognized that some of these same BW or endemic disease agents are also potential threats to civilians, significant funds have been programmed for the Biomedical Advanced Research and Development Authority (BARDA) to stockpile vaccines against a few of the most likely pandemic disease threats or bioterrorism agents, such as pandemic influenza, anthrax, and smallpox. Although there is overlap in the missions of BARDA and DoD, their ultimate goals differ in that BARDA focuses on countermeasures for treating the population after exposure to a bioterrorism agent or in response to a pandemic, whereas the DoD aims to provide protective immunity to the armed forces prior to exposure. Today, however, while vaccination of deployed troops remains a matter of national security, the cost of vaccine development has increased to the point where, without innovation and renewed commitment, the current scope of military vaccine development efforts is not sustainable.

Protecting the health of military personnel is clearly in the best interest of the US, and vaccination is the best way to prevent endemic and BW disease threats. The question, therefore, is how to pay for the numerous vaccines that would need to be developed to accomplish this goal. One answer might be for the military to just fund all of the efforts required. Many comparisons of the cost of medical countermeasures vs. the cost of fighter jets, tanks, etc. have been made, and while it is true that the DoD medical research program is small compared with the acquisition of artillery and vehicles, such comparisons are not meaningful, as the requirement for one does not negate the requirement for the other. Realistically, the chances of major increases in the DoD budget to pay for vaccines are not good. Consequently, it will be necessary to either reduce the scope of the effort to only a few high impact diseases, or to develop novel vaccine platforms and innovative (and shortened) licensing strategies to meet the need to protect deployed troops, and for spillover benefits to the civilian community (114).

AUTHOR CONTRIBUTIONS

SR-K organized, researched, and wrote the main manuscript; I-KY reviewed the manuscript and was the point of contact for dengue vaccine research; RP reviewed the manuscript and was the point of contact for malaria vaccine research; J-LE reviewed

the manuscript and was the point of contact for bioterrorism part; JK reviewed the manuscript and was point of contact for HIV vaccine research; RO reviewed the manuscript and had the most insights in the current vaccine military research program.

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The Challenges Imposed by Dengue, Zika, and Chikungunya to Brazil

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Brazil has a well-established immunization program in which vaccines are provided through the Public Health System free of charge to the whole population, obtaining high coverage and reducing the incidence of important infectious diseases in children and adults. However, the environmental changes and high mobility rates of the population occurring in the last decades have triggered the sequential introduction of a series of vector-borne emerging infectious diseases, such as Dengue, Zika, and Chikungunya, that have imposed a considerable burden on the population, with yet unmet solutions. The first to be introduced in Brazil was the Dengue virus, reaching epidemic levels in 2010, with over 1 million cases annually, maintaining high infection rates until 2016. Brazil has invested in vaccine development. The Zika virus infection, initially assumed to have appeared during the World Cup in 2014, was later shown to have arrived earlier in 2013. Its emergence mobilized the Brazilian scientific community to define priorities and strategies, that rapidly investigated mechanisms of pathogenesis, differential diagnostics, and determined that Zika virus infection *per se* causes relatively mild symptoms, however, in pregnant women can cause microcephaly in the newborns. The diagnostics of Zika infection is confusing given its similar symptoms and cross-reactivity with Dengue, which also hindered the appraisal of the extent of the epidemics, which peaked in 2015 and finished in 2016. Another complicating factor was the overlap with Chikungunya virus infection, which arrived in Brazil in 2014, being prevalent in the same regions, with similar symptoms to both Dengue and Zika. Although Dengue infection can be fatal and Zika infection in pregnant woman can lead to newborns with microcephaly or an array of neurodegenerative manifestations, the Chikungunya infection is a debilitating disease leaving chronic sequelae, which unfortunately has received less attention. Precise differential diagnostics of Dengue, Zika, and Chikungunya will be necessary to evaluate the actual extent of each of these diseases during this overlapping period. Here we review the impact of these emerging infections on public health and how the scientific community was mobilized to deal with them in Brazil.

Keywords: emerging infectious diseases, Dengue virus, Zika virus, Chikungunya virus, Brazil

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INTRODUCTION

Brazil has a well-established immunization program in which vaccines are provided through the Public Health System free of charge to the whole population, obtaining high coverage, and reducing the incidence of important infectious diseases in children and adults.

However, despite the efforts to reduce the burden of vaccine-preventable diseases, the environmental changes and high mobility rates of the population occurring in the last decades have triggered the sequential introduction of a series of vector-borne emerging infectious diseases, such as those caused by Dengue virus (DENV), Zika virus (ZIKV), and Chikungunya virus (CHIKV), and more recently Yellow fever virus (YFV), that have imposed a considerable burden on the population, with yet unmet solutions. These emerging infectious diseases can remain at reduced levels for varying periods, reaching epidemic levels during outbreaks and are cyclic in nature, depending on the presence of the *Aedes aegypti* vectors.

THE ARBOVIRUS

Although all these viruses share a common set of urban vectors, *Aedes aegypti,* and *albopictus* worldwide, they have distinct genomes and evolutionary histories. The genus *Flavivirus* (family *Flaviviridae*), such as the DENV, ZIKV and also YFV, are transmitted by arthropods such as, mosquitoes, ticks, mites, etc. (1). These viruses have a capsid with icosahedral symmetry spherical, surrounded by a viral envelope made of a lipid bilayer, with approximately 50 nanometers (nm) in diameter (1) The *Flavivirus* genome consists of a linear RNA molecule with positive polarity (+ ssRNA) of approximately 11 kilobases (Kb) in length, with a single open reading region (RLA), which encodes a polypeptide of approximately 3,400 amino acids flanked by two non-coding regions (5′ and 3′ UTR) at the ends of the genome (**Figure 1**). The first three proteins are the structural proteins (capsid-C, membrane precursor-prM, and envelope-E), and the last seven are non – structural proteins (NS1-NS5) (1, 4).

On the other hand, the genus *Alphavirus* and *Rubivirus*, both belong to the *Togiviridae* family. The genus *Rubivirus* includes only one species of virus, the Rubella virus, whereas the *Alphavirus* constitute a group of viruses with a more diversified molecular and antigenic classification (3, 5). Viruses belonging to the *Alphavirus* genus are often classified as New or Old World *Alphaviruses*, according to the geographical location in which they were originally isolated (6). Among the New World *Alphaviruses* there are several viruses which typically cause encephalitis in humans and other mammals, while Old World *Alphaviruses*, include viruses that cause fever, rash, arthralgia and rarely cause lethality, such as the Chikungunya virus (CHIKV), Semliki forest virus (SFV), and others (5). *Alphaviruses* also have a + ssRNA approximately 12 Kb in length, but they constitute a completely different group when compared to *Flavivirus* in terms of molecular architecture, although both have small icosahedral, enveloped capsids. The genome of viruses belonging to this genus is organized into two distinct RLAs (7), with genes located in the first RLA responsible for the synthesis of non-structural proteins and those located in the second RLA responsible for the synthesis of structural proteins (**Figure 1**).

A BRIEF ACCOUNT ON THEIR EPIDEMIOLOGY

In the last three decades, DENV was the arbovirus that caused the greatest public health problems in Brazil, with continuous reintroductions that were responsible for the maintenance of the virus in the country and the introduction of new lineages (8). CHIKV was first documented in Brazil in 2014 (9), followed by the ZIKV in 2015 (10). Since then, Brazil has been experiencing the co-circulation of DENV, ZIKV, and CHIKV viruses with hyperendemic (i.e., concomitant) circulation of the four DENV serotypes (8, 11).

The first dengue epidemic reported in Brazil was in 1845, followed by outbreaks in the 1850's and 1920's. The eradication of *Aedes aegypti* in the 1930s through a Program coordinated by PAHO maintained it away until the vector was reintroduced in 1976 from the Caribean. All four serotypes of DENV were systematically reintroduced in Brazil from the Caribean in the 1980s. Initially DENV-1 and DENV-4 in the northern region, followed by a larger outbreak in Rio de Janeiro of DENV-1 in the late 1980's and another in the northeast region in the 1990's (12). It has been suggested that DENV-1 and DENV-4 cause milder disease symptoms than DENV-2 and DENV-3, and the first outbreaks of DENV-2 occurred in the 1990's with increasing cases of severe dengue (SD) and consequent fatalities. DENV-3 was introduced in the 2000s and became the most prevalent serotype, after which it alternated with DENV-2 causing high incidence of SD, spreading to several states and regions. All serotypes of DENV circulated in alternated fashion in distinct localities in Brazil with a pattern of increasing prevalence in time. Epidemic levels occurred since 2010, and eventually in 2013, all four serotypes reached a hyperendemicity status as shown in the State of São Paulo (8). There were over 1 million cases annually, maintaining high infection rates until 2016. Nevertheless, the processes that shape the transmission patterns at urban scales for these emergent viruses are poorly understood, especially the impact of factors such as human population movement and urbanization, all of which are crucial for optimal vaccine development, vaccination strategies, and public health intervention planning.

The Dengue epidemic was a public health concern during the World Cup in 2014 due to the high transmission rates and the mass gatherings occurring when Brazil hosted about 600,000 foreign visitors. Nonetheless, the quantitative risk was considered to be low due to previous exposure of the host population to circulating virus (13). The strategies currently used to contain DENV infections rely on vector control, such as public awareness campaigns, use of insecticides and vector monitoring systems. Nevertheless, new strategies interfering either with the virus infectivity (*Wolbachia* bacteria) or vector viability

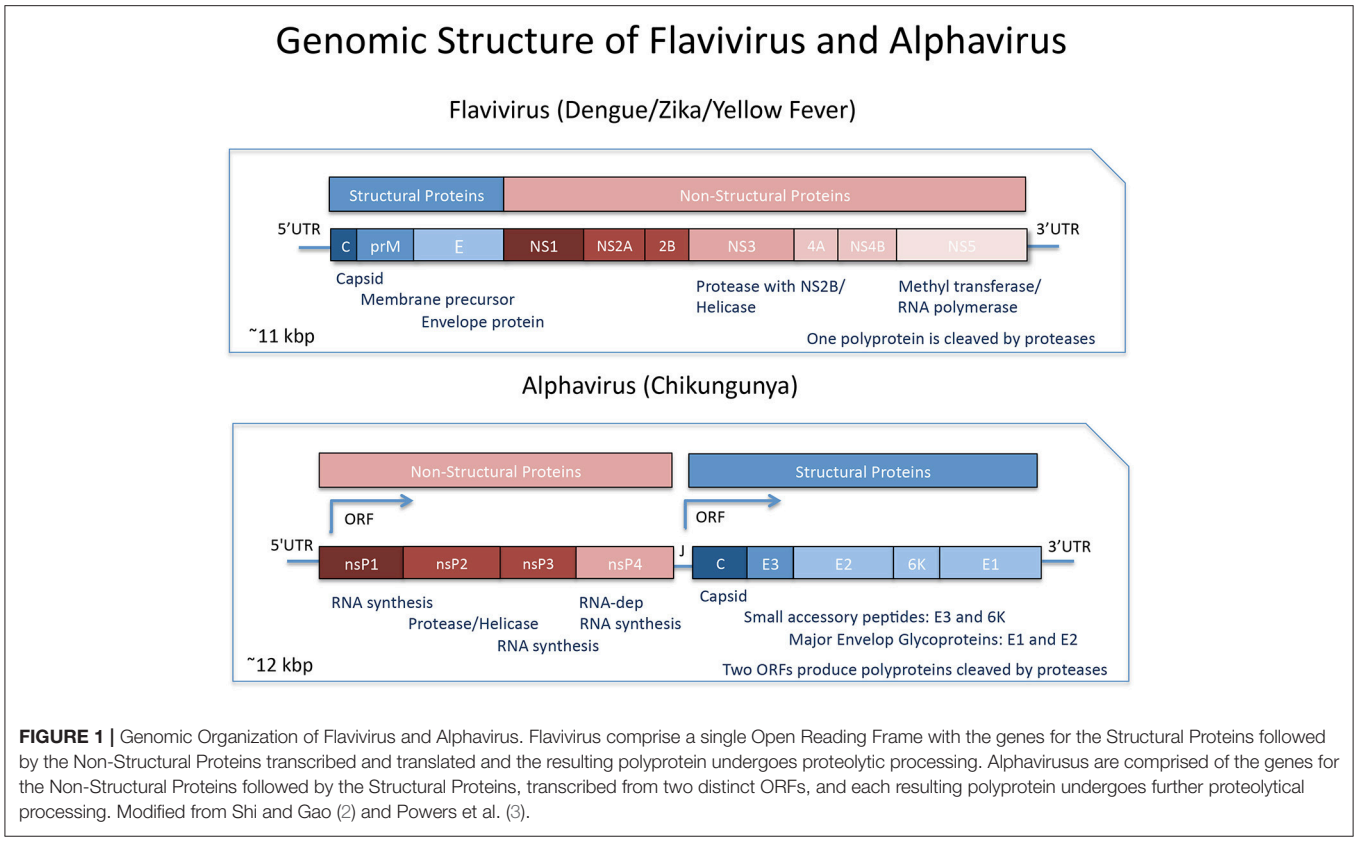

FIGURE 1 | Genomic Organization of Flavivirus and Alphavirus. Flavivirus comprise a single Open Reading Frame with the genes for the Structural Proteins followed by the Non-Structural Proteins transcribed and translated and the resulting polyprotein undergoes proteolytic processing. Alphavirusus are comprised of the genes for the Non-Structural Proteins followed by the Structural Proteins, transcribed from two distinct ORFs, and each resulting polyprotein undergoes further proteolytical processing. Modified from Shi and Gao (2) and Powers et al. (3).

(Transgenic mosquitos—Oxitec) are being developed and tested (12).

As a consequence of the re-circulation of the *Aedes aegypti* vector, other emergent viruses were introduced through different paths. For example, the ECSA genotype of CHIKV came directly into Brazil from Africa, with an outbreak in Bahia, while the Asian CHIKV genotype came from Haiti into the northern region (9). CHIKV entered Brazil in 2014 with a rapid and explosive spread leading the Pan American Health Organization (PAHO) and the Centers for Disease Control and Prevention (CDC) to issue a guide to prevent future CHIKV epidemics in the Americas (14).

The ZIKV infection was first reported in 2015, initially assumed to have arrived during the World Cup in 2014, but later shown to have arrived earlier in 2013 (10, 15). ZIKV was probably introduced from the Pacific (French Polynesia or Easter Island). An outbreak of exanthematous illness was initially associated with DENV and CHIKV in Salvador, Bahia, latter identified as the ZIKV (11, 16). The ZIKV infection was mistakenly diagnosed by its similar symptoms and cross-reactivity with DENV, which also hindered the evaluation of the extent of the epidemics. Another complicating factor was the overlap of ZIKV and CHIKV infections, being prevalent in the same regions, with similar disease presentation (17, 18). Similar co-circulation of ZIKV and CHIKV was previously observed in the Pacific (19, 20). The rapid spread of ZIKV was also a concern for the high population gatherings during the 2016 Olympic Games, which brought together millions of international visitors at risk of further disseminating the outbreak (21). However, the epidemic peaked in 2015 and was finished by 2016.

DISEASES AND SEQUELAE

DENV infections can display varying outcomes, from asymptomatic to relatively mild flu-like symptoms, up to severe dengue leading to a significant proportion of case fatalities (22). On the other hand, CHIKV and ZIKV have emerged worldwide as true highly pathogenic viral pathogens for humans (14, 23). They have experienced significant geographical expansions, which in less than 10 years led to the crossing of the Pacific Ocean, reaching the American continent (9, 15). Although the ZIKV infection *per se* causes relatively mild symptoms, by the end of 2015, the physician Adriana Melo, reported the potential association between microcephaly cases and ZIKV infection during gestation and Celina Turchi coordinated the task-force that established the evidences that confirmed an association.

In November 2015, the Brazilian Ministry of Health (MS) declared a state of national public health emergency because of the ZIKV outbreak, with the objective to provide greater impulse and agility to the investigations. With the continuing increase of the epidemic, in February 1st, 2016 the World Health Organization declared that the ZIKV epidemic was a global public health emergency (http://www.who.int/emergencies/zika-virus/en/). FAPESP, a research funding institution from São Paulo, established a fast-track for Zika projects. These measures

demonstrate the high concern of these governmental entities with this epidemic, which constituted a serious public health threat, with potentially immense economic and social importance. This situation mobilized the Brazilian scientific community to define priorities and strategies that rapidly investigated mechanisms of pathogenesis and differential diagnostics methodologies. Soon, Cugola et al. (23) provided the causal proof of the association between ZIKV and microcephaly by using *in vivo* and *in vitro* systems. More recently, a mouse model of ZIKV teratogeny with early embryo exposure to the virus reproduced the severe malformations and delayed development of the embryos (24). The study of ZIKV infection in discordant twins has brought insights into the role of the susceptibility of neural progenitor cells (25).

Although Dengue infection can be fatal and ZIKV infection in pregnant women can lead to microcephaly in the infant, CHIKV infection is a debilitating disease, leaving chronic sequelae, which unfortunately has received less than necessary attention. A precise differential diagnostics of Dengue, Zika, and Chikungunya at the point of action will be necessary for a much needed evaluation of the actual extent of each of these diseases during this overlapping period (26).

VACCINE DEVELOPMENT, PREPAREDNESS AND RESISTANCE TO VACCINATION

Although the incidence of DENV and ZIKV in Brazil has decreased in 2017, it is still important to develop vaccines for these diseases due to the cyclic nature of the epidemics and its possible spread to other locations. Estimation of the dengue hospitalization costs in Brazil and recent vaccine efficacy trials (27), set the stage for determining the cost effectiveness of new dengue vaccines, even considering their low efficacy levels, once incorporating the effect of herd immunity (28).

There are a few vaccines in development against DENV that have reached clinical trials. The first is a live attenuated tetravalent vaccine composed of the pre-membrane and envelop proteins of DENV of each serotype with the non-structural and capsid proteins of the attenuated yellow fever vaccine virus YF-17D developed by Sanofi. This vaccine has undergone a Phase III trial in Asian-Pacific and Latin American countries, showing efficacy between 47 and 83%, depending on the serotype, higher for children older than 9 years (66%) than for those lower than 9 years (45%) (26, 29), and has been registered for commercialization. However, post-marketing studies have recently determined that this vaccine can increase the risk of severe dengue in individuals susceptible to infection with DENV (i.e., not-previously infected) (30).

Brazil has invested in the development of live attenuated dengue vaccines (31, 32), one of which results from a collaboration between the US National Institutes of Health and Instituto Butantan. Estimated cost of production for this vaccine concluded that it would be affordable for most developing countries (31). This is a tetravalent live attenuated vaccine currently in a multi-center Phase III clinical trial (33). Another live attenuated vaccine is a chimeric construct based on DENV-2 backbone, developed by Takeda (Japan), which will be entering clinical trials (33).

Following the emergence of the ZIKV outbreak in Brazil and its association with microcephaly, a global effort for the development of vaccines was launched, stimulated by WHO's declaration of a public health emergency. The first strategies pursued which showed protection against ZIKV challenge in mice were DNA vaccines and inactivated virus vaccines due to the advantages of these platforms in terms of quick development (34, 35) and these have progressed rapidly (36). Mid 2016, WHO and UNICEF organized a working group for consultation in the development of a ZIKV vaccine Target Product Profile for use in a future emergency outbreak, laying out guidelines for developers and regulators (37). At that time there were over 30 vaccine candidates in development using a large variety of different strategies, including mRNA vaccination or Virus-Like Particles based vaccines, with promising results (38–40) and the more advanced ones had undergone FDA approval for clinical trials. The discussions raised a series of points on the best pathways forward concerning safety and regulatory issues (41). Although the local and global efforts lead to early developments of vaccine candidates, the decline in cases and unforeseen emergent outbreaks may hinder further progress in their development (42).

On the other hand, the decline in ZIKV incidence was closely followed by a devastating outbreak of Yellow Fever (YF) in nonhuman primates, initiating in Minas Gerais, early in 2017, and spreading to Espírito Santo, São Paulo, and Goiás (43). Because of significant spillover into the human population, this alarming outbreak triggered mobilization of public health measures to contain the spread of the wild type (jungle) YF and hinder the onset of urban YF (44). Mass vaccination campaigns were initiated to cope with the increasing number of reported and confirmed human cases. Two important factors took place in dealing with this outbreak. Once the first fatalities due to YF were announced, public alarm initially triggered a rush to the public health system in search for immunization. The sudden increase in demand for vaccine resulted in shortage of vaccine stocks and enormous lines formed by the population at immunization sites. Since the production of vaccine is a long process, the decision was toward vaccine fractionation, previously demonstrated to be efficacious. WHO sent a small emergency stockpile and Biomanguinhos, Fiocruz, expedited vaccine production to meet the plan to achieve the immunization of 20 million individuals in endemic areas.

On the other hand, we believe that mass vaccination can increase the otherwise small level of adverse events occurring due to the vaccine. At the same time the media overreacted prematurely amplifying through social networks concerns on the adverse effect of the vaccine, which found resonance in the incipient but increasing anti-vaccine movement. As a result of general perception, vaccine resistance became an important factor in this outbreak.

On a whole, it is clear that the presence of the vector has facilitated consecutive virus outbreaks and it will be important to invest more efficiently in vector control. On the other

hand, while it is still around, close surveillance has identified early signs of different outbreaks of emerging infections, which has been essential to allow prompt organization of public health measures. The scientific community and government sectors have been mobilized toward the investigation of the different pathogens, bringing insights into their epidemiology and pathogenesis, new vaccine developments or increasing vaccine supply, depending on their respective state of knowledge and development. Considering the severity of these diseases, we will always consider that the whole process can be expedited and improved in order to reduce the burden on public health.

AUTHOR CONTRIBUTIONS

PZ and LL wrote sections of the review. Both authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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New Vaccine Technologies to Combat Outbreak Situations

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Ever since the development of the first vaccine more than 200 years ago, vaccinations have greatly decreased the burden of infectious diseases worldwide, famously leading to the eradication of small pox and allowing the restriction of diseases such as polio, tetanus, diphtheria, and measles. A multitude of research efforts focuses on the improvement of established and the discovery of new vaccines such as the HPV (human papilloma virus) vaccine in 2006. However, radical changes in the density, age distribution and traveling habits of the population worldwide as well as the changing climate favor the emergence of old and new pathogens that bear the risk of becoming pandemic threats. In recent years, the rapid spread of severe infections such as HIV, SARS, Ebola, and Zika have highlighted the dire need for global preparedness for pandemics, which necessitates the extremely rapid development and comprehensive distribution of vaccines against potentially previously unknown pathogens. What is more, the emergence of antibiotic resistant bacteria calls for new approaches to prevent infections. Given these changes, established methods for the identification of new vaccine candidates are no longer sufficient to ensure global protection. Hence, new vaccine technologies able to achieve rapid development as well as large scale production are of pivotal importance. This review will discuss viral vector and nucleic acid-based vaccines (DNA and mRNA vaccines) as new approaches that might be able to tackle these challenges to global health.

Keywords: viral vector vaccine, DNA vaccine, mRNA vaccine, pandemics, vaccine development

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INTRODUCTION

The world population has grown to 7.6 billion people in 2018, more than half of which live in densely populated urban settings. Travel habits have changed radically; the number of people traveling by plane is growing each year and amounted to a total of 3.7 billion in 2016[1]. The high population density, as well as the extreme increase of contact between people from virtually all areas of the world highly favor global spreading of pathogens. This pandemic risk is further increased by the climate change that influences the distribution, abundance, and prevalence of pathogen-bearing vectors, promoting infections with a range of vector-borne diseases. The occurrence of pandemic outbreaks in the past decades has clearly demonstrated the reality of global pandemic threats.

Human immunodeficiency virus (**HIV**), the causative agent of acquired immunodeficiency syndrome (AIDS), represents a zoonosis from non-human primates in West-central Africa and has claimed more than 35 million lives since its discovery in 1983[2]. Despite the development

[1]http://www.iata.org/pressroom/pr/Pages/2017-02-02-01.aspx.
[2]http://www.who.int/en/news-room/fact-sheets/detail/hiv-aids.

of effective highly active anti-retroviral therapy (HAART), drugs are cost intensive and access to therapy remains problematic in resource limited settings in which the majority of infections occur. Development of a direly needed vaccine against HIV has proven extremely difficult and identification of a suitable method for generating such a vaccine remains the focus of research.

Influenza A viruses occur in annual seasonal outbreaks. However, their ability to infect a variety of different species as well as their high genomic variability additionally bears the constant risk of a zoonosis introducing a virus with completely new immunogenic properties into the human population. While the occurrence of a future influenza pandemic is almost certain, it is impossible to predict the characteristics of the virus and the severity of the symptoms it induces. This unpredictability can be illustrated by the "swine flu" (H1N1pdm09) on the one hand, that led to a phase 6 pandemic alert declared by the WHO in 2009 but caused relatively mild symptoms and the 1918 influenza A H1N1 pandemic ("Spanish flu") on the other hand, that resulted in the deaths of around 50 million people (1). Currently licensed seasonal influenza vaccines are specific for pre-defined viral strains and are unable to protect against a future pandemic. Hence, new vaccine technologies able to induce broad protection against influenza A viruses are urgently required.

Severe acute respiratory syndrome (SARS) first occurred in China in 2002 and was caused by a novel coronavirus (CoV) that likely originated in bats (2, 3). SARS CoV caused a global outbreak with 8,000 infected patients, leading to 774 deaths in 26 countries (4). A notable aspect of the SARS epidemic was the efficacy of containment measures that halted the spread of disease. Following this, ongoing efforts to develop a vaccine against SARV-CoV were discontinued (5). In 2012, a new coronavirus appeared in Saudi Arabia causing **Middle East respiratory syndrome (MERS).** Like SARS CoV, the virus originated in bats and likely spread to humans via infected dromedary camels. According to the WHO, there have been 2,143 confirmed cases of MERS, with 750 deaths in 27 countries since 2012.[3] A variety of research activities are currently ongoing to develop a vaccine against MERS CoV. However, a licensed vaccine is not yet available.

Ebolaviruses belong to the family *Filoviridae* (consisting of the two genera Ebolavirus and Marburgvirus) that cause hemorrhagic fever with a high mortality rate and whose natural reservoir is believed to be in bats (6). Since the first documented Ebolavirus outbreaks in 1976, Ebolaviruses have emerged periodically in outbreaks that mostly occurred in Central African countries.[4] During this period, attempts to develop a vaccine against Ebolaviruses were made but remained at research and early development stages. However, when Ebola virus appeared in West Africa in late 2013, it hit a region heavily affected by poverty and armed conflicts, in which many factors, among them a dysfunctional health system, contributed to the inability to control the virus. The 2013–2016 Ebola crisis represented the first epidemic caused by an Ebolavirus with 28,616 cases and 11,310 deaths reported.[5] At late stages of the epidemic, several vaccine candidates were tested in clinical trials, the most advanced of which (rVSV-ZEBOV) showed clinical efficacy in a ring-vaccination clinical trial (7).

The vector borne diseases **Dengue**, **Chikungunya**, and **Zika** are transmitted by species of *Aedes* mosquitoes and induce similar symptoms such as fever and severe joint pain. At present, more than half of the world's population lives in areas where these mosquito species are present. Infection rates for all these viruses have increased dramatically in the last decades: according to the WHO, cases of dengue fever have risen 30-fold in the past 50 years. Zika virus was first identified in non-human primates in Uganda in 1947 (8) and has since caused several outbreaks in different areas with reported mild symptoms such as self-limiting febrile illness. Since 2014, however, outbreaks in Asia and the Americas have been linked to severe clinical manifestations, including Guillain–Barré syndrome in adults and congenital abnormalities, including microcephaly, following infection during pregnancy. A possible explanation for the emergence of these aggravated symptoms could be mutations introduced in the virus that allowed adaptation to the new environment and resulted in changes to pathogenicity. The occurrence of around one million laboratory confirmed cases of Zika in South America, with over 4,000 cases of microcephaly led to the declaration of a Public Health Emergency of International Concern (PHEIC) in February 2016 (9). The Zika crisis has prompted the accelerated development of vaccines against Zika virus, seven of which have entered clinical trials (10). Likewise, several clinical trials are currently ongoing testing different technologies for a vaccine against Chikungunya or Dengue. However, with the exception of a vaccine against Dengue (Dengvaxia® developed by Sanofi Pasteur) no other vaccine has been licensed for these diseases. Of note, Dengvaxia® has recently been associated with increased risk of more severe disease in subjects who had never been exposed to the virus (11). In April 2018, the WHO recommended a pre-vaccination screening strategy, in which Dengvaxia® is only used in dengue-seropositive individuals.[6]

In addition to pandemic threats, the list of **multi drug resistant (MDR) organisms** is ever-growing, favored by the misuse and overuse of antibiotics. This holds true for the use of antibiotics in both humans and, even more problematically, in animals, where antibiotics are routinely used for prevention of disease and promotion of growth in livestock. MDR organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant tuberculosis (MDR-TB) are becoming a serious threat to global public health. According to WHO estimates, 490,000 new cases of MDR-TB were registered in 2016, of which only 54% could be successfully treated. Again, the solution to this growing threat could be the development of efficient vaccines to prevent MDR organisms from further spread.

[3]http://www.who.int/emergencies/mers-cov/en/.

[4]https://www.cdc.gov/vhf/ebola/outbreaks/history/chronology.html.

[5]http://www.who.int/csr/disease/ebola/en/.

[6]http://www.who.int/immunization/diseases/dengue/revised_SAGE_recommendations_dengue_vaccines_apr2018/en//.

THE CHALLENGES OF VACCINE DEVELOPMENT IN OUTBREAK SITUATIONS

Conventional vaccines, developed by attenuating or inactivating the respective pathogen, have successfully decreased the burden of a number of infectious diseases in the past, leading to the eradication of small pox and significantly restricting diseases such as polio, tetanus, diphtheria, and measles. However, established methods may not always be suitable or even feasible in outbreak situations. Live attenuated vaccines generally bear the risk of reversion, rendering this approach unfavorable for highly pathogenic, possibly largely uncharacterized organisms. Inactivation may not induce protective responses, as is the case for Ebola (12) or can even lead to undesired effects, like formalin-inactivated RSV (respiratory syncytial virus) that induced exacerbated disease upon wildtype RSV infection in clinical trials in the 1960s (13). Furthermore, outbreak scenarios may limit conventional vaccine development in terms of producibility. Since these methods require whole pathogen cultivation and propagation, vaccine production may be hampered by factors such as difficult or impossible cultivation of the respective pathogen under *in vitro* conditions or the requirement of a high biosafety level and specialized labs for cultivation. Hence, new and highly versatile approaches that are independent of whole pathogen cultivation are required to effectively and quickly combat outbreak situations.

In order to proof effective against an upcoming pandemic, these new technologies need to overcome a number of challenges. The unpredictable nature of emerging pathogens represents one of the pivotal problems for pandemic preparedness. Zoonoses present a constant threat to introduce a previously uncharacterized pathogen into the population, as was the case for HIV as well as for SARS and MERS CoV. The outbreaks caused by pandemic influenza virus demonstrate the potential of a known pathogen to mutate and adapt to a new host or environment, with unpredictable outcomes for its immunogenic properties and the severity of symptoms it induces. As demonstrated by the recent epidemics and pandemics, the risk of such events is highest for RNA viruses, whose high mutation rates favor adaptability.

Since the vaccine targets remain undefined before an outbreak occurs, time remains one of the major hurdles for effective vaccine development. Currently, the average development time for conventional vaccines from preclinical phase is more than 10 years (14), highlighting the dire need for new approaches that allow extremely fast development and licensing to prevent an emerging outbreak from global spread.

A further major problem is the cost associated with vaccine development and production: using established technologies, development of a new vaccine candidate is estimated to amount to more than 500 million USD, with further expenses to establish facilities and equipment ranging from 50 to 700 million USD (15). While some costs for vaccine development cannot be avoided in order to keep the required safety standards, the need for dedicated production processes and facilities for each vaccine in most conventional vaccine technologies keeps validation and production costs high. Especially considering resource limited settings such as the 2013–2016 Ebola crisis and the fact that outbreak situations represent niche markets, new technologies are required to support more cost effective vaccine production.

A further issue is production capacities of established methods, which are often insufficient to support global vaccination. Even if the potential threat is known and vaccine manufacturing technologies are established, like for pandemic influenza vaccine, production capacity to meet peak demands during a pandemic remains problematic. Thanks to efforts coordinated by the WHO, the potential production capacity for pandemic influenza vaccines in 2015 could in theory support the vaccination of 43% of the population with two doses of vaccine (16). However, the global distribution of vaccine production is far from equal between industrial nations and the developing world: according to a survey made in 2015, only 5% of influenza vaccine doses were distributed among Southeast Asia, Eastern Mediterranean, and Africa WHO regions, which comprise about half of the world's population (17). In addition, most currently licensed vaccines would take 5–3 months between identification of a pandemic influenza and vaccine distribution, which would give a pandemic virus ample time for global spread. Hence, technologies that enable fast production of large amounts of vaccine are direly needed in the face of pandemic threats.

Efforts to meet these challenges are made by monitoring viruses with high pandemic potential and programs, most notably Coalition for Epidemic Preparedness Innovations (CEPI), that finances and develops vaccines against likely pandemic threats.

VACCINE TECHNOLOGIES

The past decades have witnessed the development of a wide array of new vaccination technologies ranging from targeted attenuation techniques of live pathogens to the delivery of biologically engineered protein and peptide antigens as well as viral vector and nucleic acid based antigens. Many of these technologies have yielded highly promising results which are discussed in excellent reviews elsewhere (18–21). Here, we will focus on the discussion of viral vector and nucleic acid based vaccines that have shown promise for offering solutions to the challenges of vaccine development. In order to visualize the time required between the occurrence of recent outbreaks and the onset of clinical trials, **Figure 1** depicts an overview of the most important pandemics in relation to the start of clinical trials using different viral vector and nucleic acid based vaccines.

Viral Vector Based Vaccines

Viral vector based vaccines, that rely on the delivery of one or more antigens encoded in the context of an unrelated, modified virus, represent a highly versatile platform that offers many advantages over more established vaccine technologies. This technology either employs live (replicating but often attenuated) or non-replicating vectors. Research conducted since the 1980s has established a variety of viruses as vaccine vectors by engineering them to encode for heterologous antigens that are shuttled into the host cells by the vector. Upon delivery, antigens

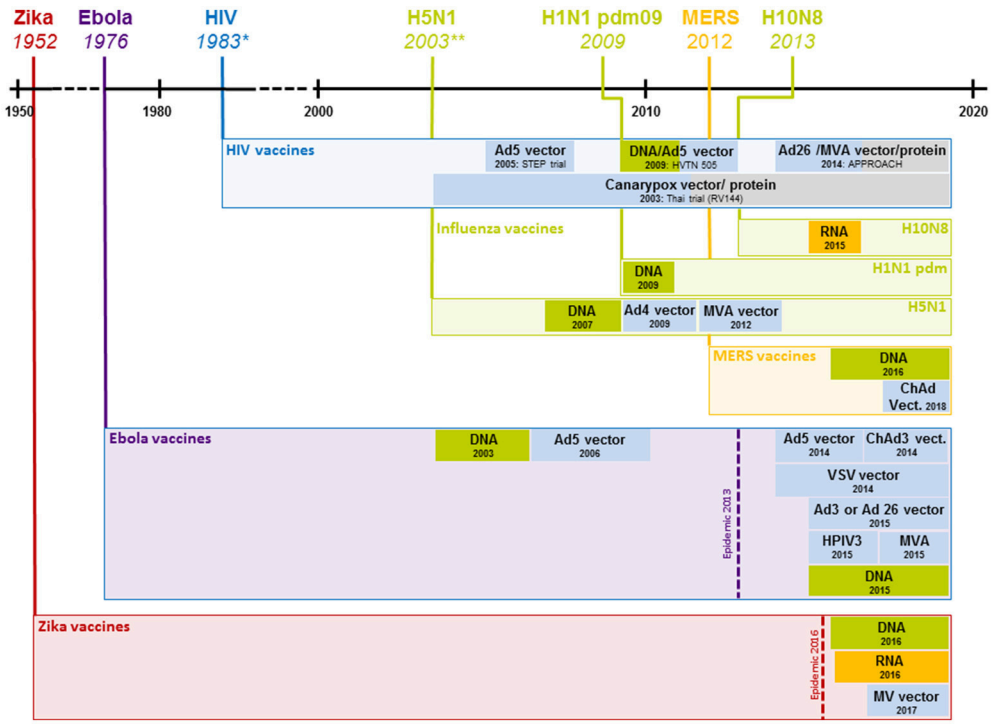

FIGURE 1 | Clinical development of vaccines against recent outbreaks. The timeline above indicates the year a given virus started spreading in the human population; boxes below represent the start of clinical vaccine development and the employed technology (shown exclusively for viral vector and nucleic acid based vaccines). For HIV, only select studies that represent major advances are shown. *1983 represents the year the HI virus was discovered; the virus likely started spreading at the beginning of the twentieth century. **2003 represents the year H5N1 caused rising numbers of infections, the first H5N1 infection in a human was registered in 1997. Ad4, 5, 26, human adenovirus type 4, 5 or 26; ChAd, chimpanzee adenovirus; HIV, human immunodeficiency virus; H5N1, influenza H5N1; H1N1 pdm09, influenza H1N1 2009 "swine flu"; H10N8, influenza H10N8; DNA, deoxyribonucleic acid based vaccine, MVA, modified vaccinia Ankara; RNA, ribonucleic acid based vaccine; VSV, vesicular stomatitis virus; HPIV3, human parainfluenza virus type 3; MV, measles virus.

are expressed and the host is able to induce immune responses against the respective target pathogen (22).

Description and Mode of Action

A wide array of different viruses has been employed as a basis for constructing viral vector based vaccines (23). Among others, these vectors include adenoviruses, parvoviruses (e.g., adeno-associated viruses, AAV), togaviruses (e.g., Semliki Forest virus), paramyxoviruses (e.g., measles virus, Newcastle disease virus or human parainfluenza virus), rhabdoviruses (e.g., vesicular stomatitis virus, VSV), and poxviruses (e.g., Modified vaccinia Ankara, MVA). Since a comprehensive discussion of all these vectors would exceed the scope of this review, we will only describe some commonly used viral vectors, i.e., adenovirus, measles virus, and VSV in some detail, whose use in clinical studies will be discussed below.

Adenovirus (Ad) vectors are among the most commonly employed viral vectors, with vast amounts of both preclinical and clinical studies assessing their protective efficacy against a variety of infectious diseases available. *Adenoviridae* are non-enveloped viruses with an icosahedral capsid and a linear double-stranded DNA genome, whose size ranges from 30 to 40 kb. Next to a multitude of adenoviruses occurring in different animal species, there are 57 identified human adenovirus that are classified into seven species A–G. Adenoviral receptors are expressed on the surface of most human cells, allowing a broad tissue tropism of the virus (24).

Ad based vaccines can be constructed as replication-competent or replication-defective vectors, which are generated by replacing the E1A and E1B (early transcript 1 A and B) genomic region by an antigen expression cassette, thereby abolishing the viral ability to replicate (25). In addition, the viral E3 and E4 genes are frequently deleted to prevent elimination of Ad infected cells by the immune system and leaky expression of the inserted antigen, respectively (25). Since adenoviruses shuttle their genome in the nucleus of the host cell for transcription and replication, the risk of genomic integration exists, however, the vector predominantly remains episomal (24). Adenoviral vectors are able to stably express inserts of up to 8 kb, supporting the expression of most target antigens as well as multivalent or multi-pathogen vaccines (26). The vector is easily manipulated by insertion of a transgene cassette into the viral backbone via homologous recombination or through a direct cloning step *in vitro* (27). Adenoviral vectors can be manufactured in mammalian cell culture systems, most commonly using HEK 293 cells that provide E1 protein in trans to allow viral replication. These production systems support high viral yields at relatively low production costs,

but amplification of viral seed requires biosafety level 2 (BSL2) facilities (23).

Adenoviral vectors are able to induce potent antibody as well as T cell responses with variations in the immune response depending on the serotype employed (28). Replication-deficient Ad5, one of the most widely used adenoviral vectors, is able to induce exceptionally potent $CD8^+$ T cell as well as antibody responses (29). However, the widespread pre-existing immunity to this virus in the human population, that can inhibit transgene expression and inactivate the viral vector, hampers its clinical use (30). This issue has been met by developing adenoviral vectors of non-human origin, such as the chimpanzee virus derived vector ChAd63 (31). An alternative approach is the selection of rare serotypes with low prevalence in humans such as Ad26 or Ad35 (32) which induce enhanced memory and more poly-functional $CD8^+$ T cells compared to Ad5 (28).

Measles virus (MV), a common human pathogen, belongs to the family of *Paramyxoviridae*. MV is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome of ∼16 kb. Measles virus vaccines have been generated by serial passaging of infectious virus through different cell lines resulting in a live attenuated virus that is replication deficient in humans. The introduction of numerous mutations in this process has established a highly stable vaccine for which reversion to pathogenicity has never been observed (33). Moreover, MV is unable to integrate into the host genome and a lyophilization process for MV vaccine has been established, increasing the thermostability of the naturally unstable virus. MV vaccine induces extremely durable responses with both antibodies and $CD8^+$ cell persisting as long as 25 years post vaccination (34).

Due to the helical nature of the ribonucleoprotein (RNP) complex, the viral genome is highly flexible and accepts insertions of up to 6 kb, as long as the total number of nucleotides in the genome can be divided by 6 ("rule of six"). The ability to accept relatively large transgenes offers the opportunity to generate multi-pathogen or multivalent vaccines (26). However, the need to rescue the negative-sense RNA genome by reverse genetics renders manufacturing of the virus and the insertion of the transgene more complex compared to other viral vectors. Several ways to generate transgene expressing MV have been described and transgene cassettes can be inserted at different positions in the viral genome (35). MV vaccines can be grown in chick embryonic fibroblasts or cell lines such as Vero or MRC-5 cells and manufacturing processes for clinical use are well-established. However, the manufacturing and bulk vaccine production requires BSL2 facilities, which might restrict availability of manufacturing facilities in an outbreak setting.

Recombinant measles viruses are able to induce high levels of both humoral and cellular immune responses against the transgene (33). Importantly, MV is able to infect cells of the immune system, including macrophages and dendritic cells, thus supporting delivery of target antigens directly to antigen-presenting cells (36). T cell-mediated responses to MV are dominated by a $CD4^+$ phenotype, unlike the more $CD8^+$ dominated response to adenoviral vectors, which might be a consideration for vaccine development. Since live attenuated MV is routinely used as a vaccine in child immunization programs in many countries, pre-existing immunity to MV as a viral vector has been raised as a concern. However, studies in mice and macaques showed no impact of previous MV exposure on transgene immunity (29). In agreement with animal studies, a clinical study conducted in the context of a MV vaccine against CHIKV likewise demonstrated that anti-vector immunity did not compromise vaccine efficacy (37).

Vesicular Stomatitis Virus (VSV), a member of the *Rhabdoviridae* family, is an enveloped virus containing a single stranded, negative-sense RNA of ∼11 kb. The virus naturally infects livestock with sporadic infections found in humans (38). The resulting low risk of pre-existing immunity and the lack of a DNA intermediate during viral replication makes VSV attractive as a safe vaccine for applications in humans. The establishment of a reverse genetic system for VSV in 1995 has allowed manipulation and propagation of the virus (39). VSV is generally employed as an attenuated vector, which is achieved by different methods, such as introducing mutations in the viral matrix (M) protein, rearranging the order of the viral protein, insertion of non-viral proteins and partial or complete deletion of the viral glycoprotein (G), the determinant for viral infectivity (40). Attenuation is essential for vaccine safety, since neurovirulence of the wild-type VSV has been detected upon intracranial inoculation in animal models (41). Transgenes can be inserted at different positions in the viral genome resulting in varying levels of transgene expression. A common method for transgene insertion replaces the G protein, which alters tissue tropism of the virus (42). The amount of additional genomic material stably accepted in the genome is 4–5 kb (29). VSV can be grown to high titers in most mammalian and insect cell lines. Depending on the way the virus has been manipulated, methods for viral propagation may vary.

VSV induces robust antigen-specific neutralizing antibody responses. Modest $CD8^+$ and $CD4^+$ T cell immunity has been described in several studies, however, the asset of the vaccine is the effective induction of humoral responses (29).

Delivery of Viral Vector Based Vaccines

Administration of viral-vectors can take place by different routes: next to intramuscular vaccination, intranasal (43), intradermal (44, 45), and oral vaccination (46) have been tested for different viruses in clinical studies. Next to the ability of the employed virus to infect certain tissues, the choice of immunization route is dependent on several considerations. The route of administration affects the quality of the induced immune response and the choice of application route thus depends on the target pathogen, i.e., if a mucosal response is required for inducing protection, oral or nasal delivery of the vaccine may be preferable over parenteral applications. In addition, the route of administration needs to be reliable and easy to perform in an outbreak situation, arguing for established routes of vaccination such as oral or intramuscular administration (47).

Since viral-vectors are complex vaccines that induce strong immune responses, the use of additional adjuvants is generally not required. Some clinical studies have tested recombinant viral vaccines in combination with additional immune-stimulating components (48, 49) but found no increase in immunity in the

adjuvanted group (49). Nevertheless, the modification of the immunological compartment introduced by an adjuvant might still prove beneficial in the context of some viral vectors.

Advantages and Disadvantages

Given the large amount of different viral vectors available and the vast knowledge gathered about their manipulation and function as immunogens, viral vector based vaccines represent a valuable and highly versatile platform for vaccine development. Viral genomes can be manipulated to express any antigen of choice and the ability to stably accept relatively large insertions in their genome supports the development of a large variety of vaccines. Delivery of the target antigen as genetic information allows faithful antigen generation, targeting and processing, i.e., correct protein folding, multimerization, modifications such as glycosylation, and specific targeting in the cell are ensured. Of note, this mostly holds true for viral target antigens derived from human pathogens which are expressed in their natural environment, whereas isolated bacterial or parasitic antigens might be localized and processed differently in mammalian cells compared to their natural host. Viral vectors induce stimuli in the target cells that mimic natural infection, thereby inducing potent immune responses. Hence, viral vector based vaccines can be delivered without additional adjuvants and, with variations depending on which vector is employed (see above), strong antigen-specific cellular and humoral immune responses against the target antigen can be induced. Strategies to achieve replication incompetency or attenuation of modern viral vectors generally ensure a good safety profile of viral vector based vaccines. For most commonly employed viral vector based vaccines, high yield production processes with means of upscaling have been established, supporting the use of these technologies for pandemic settings.

Despite many advantages, several aspects have to be considered when developing a viral vector based vaccine. Firstly, viral vectors are genetically modified organisms (GMOs) and are therefore considered a potential risks to human health and environment associated with the release of these organisms. European regulatory agencies require environmental risks assessment (ERA) to evaluate potential environmental and health risks posed by the GMO (50). In the USA, the FDA has published guidelines for Environmental Assessments (EA).[7] What is more, the use of viral vector based vaccines raises safety concerns for use in humans, such as potential integration into the host genome or too high or persistent replication of attenuated vaccines, that need to be carefully assessed before entry into, as well as during clinical development. These concerns are not only important in terms of safety, but might also lead to delays of clinical studies in case of a pandemic.

In terms of vaccine manufacturing, each viral system requires different cellular systems for high yield propagation, necessitating different manufacturing facilities for each viral vector platform. As viruses may undergo recombination during production, great care must be taken to keep cell cultures free of material that can lead to the emergence of recombined and uncharacterized pathogens (51). In general, the presence of adventitious agents, i.e., microorganisms that may have been unintentionally introduced into the manufacturing process, needs to be assessed vigorously during vaccine manufacturing (52). Since production of viral vector based vaccines is a complex process that often requires a multitude of components of human or animal origin, such as cell substrates, porcine trypsin or bovine serum, the need to exclude contaminants requires extensive testing during various steps of the manufacturing process. Indeed, several examples for contaminants in viral vaccines, such as porcine circovirus contaminations in rotavirus vaccines, have highlighted the reality of this risk (53). These factors make production of viral vector based vaccines a highly complex and comparatively cost-intensive process. If the viral vector is derived from a virus able to infect humans, the effect of pre-existing immunity on vector immunogenicity has to be addressed. Depending on the vector, this effect may or may not hamper immune responses, as was the case for Ad5 and MV vectors, respectively (see above). Dampening of immune responses by pre-existing immunity may necessitate time and cost intensive screening procedures before clinical trials and compromise the use of a given vector for further indications in the same vaccinee.

Viral Vector Based Vaccines in Potential Pandemic Settings Using Ebola Virus as an Example

Viral vector based vaccines have been employed for the development of vaccines against many different pathogens in a vast number of preclinical and clinical studies. However, so far only one viral vector based vaccine, i.e., Dengvaxia, which is a recombinant Dengue vaccine based on the yellow fever attenuated strain 17D, has been licensed for human use. More comprehensive summaries of their applications in the context of prophylactic vaccines are published elsewhere (23, 29). In this review, we will focus on two exemplary vector based vaccines developed in the context of the recent Ebola pandemic in order to highlight some of the advantages and disadvantages of this technology for outbreak situations.

First studies employing viral vector based approaches to develop vaccines against **Ebolaviruses** started as early as the 1990s. However, most approaches were still in preclinical stages when the Ebola pandemic emerged in 2014. Viral vector based vaccines against Ebolaviruses have been tested in the context of non-replicative vectors such as modified vaccinia strain Ankara (MVA), human adenovirus (Ad) and replication-defective recombinant chimpanzee adenovirus type3 (ChAd3 vaccine) as well as replication competent vectors including VSV-EBOV, human parainfluenza virus type 3 (HPIV3), recombinant cytomegalovirus (rCMV), and recombinant rabies virus (RABV). Clinical trials were conducted for VSV-EBOV, ChAd3 vaccine, Ad26-EBOV, Ad5-EBOV, HPIV3, and MVA-vector vaccine (54). These vaccines rely on vector based expression of the viral glycprotein (GP), the only surface protein and single target of neutralizing antibodies alone or in combination with additional viral proteins. Here, we will focus on the discussion of two adenoviruses, i.e., Ad5 and ChAd3, and VSV-EBOV vectors as three of the earliest vector based vaccines to enter clinical trials upon the 2014 pandemic.

[7] https://www.fda.gov/downloads/Guidances/UCM439273.pdf.

The first adenovirus based vaccine against Ebola, replication defective Ad5 expressing EBOV GP, was described in 2000 and tested in combination with DNA vector vaccination in non-human primates (NHPs). Vaccination was found to be protective but required long vaccination schedules (55). This vaccine was further developed by generating a vector expressing both GP and the nucleoprotein (NP) to enhance T cell responses. Indeed, vaccination with this vector resulted in complete protection in NHPs upon a single vaccination. Protection was found to correlate with both the generation of specific $CD8^+$ T cell and antibody responses (56). Further studies employed an Ad5 vector developed by Crucell Holland BV that expressed GPs from two Ebolavirus subspecies [Ebola virus (EBOV) and Sudan Ebolavirus (SUDV)] featuring a point mutation that reduced protein cytotoxicity. The vaccine was found to be protective in NHPs while allowing deletion of NP from the construct as well as dose sparing (57). Given these encouraging results, a clinical trial (NCT00374309) was initiated in 2006 (**Table 1**). This study showed safety as well as the induction of antibody and T cell responses, but no significant generation of virus neutralizing titers (58). Importantly, this study also demonstrated that the induction of antibodies was reduced in participants with pre-existing immunity against Ad5. Given the high prevalence of 60–90% of Ad5 in the human population, this finding might compromise the use of Ad5 for the development of human vaccines. Upon the outbreak of the Ebola pandemic in 2014, a new Ad5 based vaccine was developed in a joint effort by the Beijing Institute of Biotechnology and Tianjin CanSino Biotechnology Inc. This vaccine was the first to incorporate the GP of the 2014 epidemic Ebola strain and was produced as a lyophilized powder that facilitated vaccine transport and storage by allowing storage at 2–8°C. A phase I clinical trial initiated at the end of 2014 (NCT02326194), showed no serious adverse events, although higher incidences of injection-site reactions were associated with higher Ad5 doses (**Table 1**). Importantly, this study showed that high doses of Ad5 vector were able to overcome the negative effects of pre-existing immunity, as participants with a high baseline concentration of Ad5 neutralizing antibodies still induced robust GP-specific antibody and T cell responses (59). A phase II clinical study (NCT02575456) testing the Ad5 viral vector was initiated in Sierra Leone in October 2015 (**Table 1**), results are not yet publicly available.

In addition to Ad5 vector based strategies, limitations associated with the high prevalence of this virus in the human population are met in parallel approaches employing the far less prevalent Ad26 and Ad35 or related viruses such as chimpanzee derived adenoviruses (ChAd3). Especially ChAd3 is among the most widely evaluated vectors for the development of a vaccine against Ebola. Two vaccines developed by the NIAID VRC, i.e., replication defective ChAd3 encoding for EBOV GP alone or in combination with SUDV GP, were tested in preclinical studies which demonstrated complete protection in NHPs for both vaccines 5 weeks after single injection, using 10^{10} viral particles. However, immune responses waned several months after prime vaccination which could be prevented by boosting with MVA encoding for GPs from EBOV and SUDV (60). Starting in September 2014, both vaccines were tested in phase I clinical trials (NCT02231866, NCT02240875, and NCT02267109) demonstrating an acceptable safety profile of ChAd3 vectors, the induction of GP specific antibody responses in almost all subjects as well as T cell responses in a subset of study participants (61–63) (**Table 1**). ChAd3 encoding for EBOV GP has been moved on to phase II clinical studies and is licensed by GSK (64). Published results of a phase I/II clinical trial (NCT02289027) report immunogenicity in almost all vaccine recipients and significantly increased antibody responses in the vaccine group compared to the placebo group at 6 months (65) (**Table 1**). Importantly, the PREVAIL study (NCT02344407), a phase II clinical trial that directly compared ChAd3 and rVSV-ZEBOV based vaccines, demonstrated that both vaccines elicited immune responses one month after vaccination that were largely maintained through 12 months (66). In addition, further trials are evaluating a prime-boost regimen of ChAd3 followed by MVA vaccines (64). Overall, ChAd3 based vaccine appears to be a safe and efficacious candidate for Ebola vaccine development.

rVSV-ZEBOV currently represents the most promising candidate for the development of an effective vaccine against Ebolaviruses. This vaccine consists of a live attenuated VSV in which the VSV glycoprotein is removed and replaced with the GP from a 1995 EBOV strain. rVSV-ZEBOV was developed by the Canadian National Microbiology Laboratory and is now licensed to Merck. Preclinical studies published in 2004 and 2005, respectively, demonstrated complete protection from a lethal EBOV challenge infection in mice using a mouse-adapted strain (67) and NHPs with a single injection (68). rVSV-ZEBOV was demonstrated to be fully protective in NPHs when the vaccine was applied only seven days before challenge (69) and showed promise as a post-exposure prophylaxis in NHPs: injection with one or two doses of vaccine 1 or 24 h after EBOV exposure resulted in 33–67% protection (70). The vaccine was tested in ten completed phase I clinical trials with the earliest study having been initiated in October 2014 (71). First results from clinical studies (NCT02283099, NCT02287480, and NCT02296983) published in 2016 (72) showed robust and persistent induction of GP specific antibody responses as well as virus neutralizing titers with higher titers elicited in higher dose groups (**Table 1**). However, these studies also raised safety concerns: doses of 1×10^7 PFU or higher were associated with the development arthritis lasting a median of 8 days. In addition, some participants experiencing arthralgia developed a maculopapular rash indicative of VSV replication and dissemination. Following this, the study was suspended and resumed one month later using a lower dose of 3×10^5 PFU (NCT02287480). Reduction of viral titers employed for vaccination yielded reduced adverse events. However, while the frequency of GP specific antibody induction remained similar to cohorts vaccinated with higher doses (94%), levels of antibody responses were reduced.

Of note, further phase I clinical trials (NCT02269423, NCT02280408) (**Table 1**) employing high doses of rVSV-ZEBOV demonstrated dose-dependent induction of GP reactive antibody titers in all participants but only mild adverse events without further cases of arthritis (73).

TABLE 1 | Exemplary clinical trials employing viral vector based vaccines in the context of Ebola vaccine development.

Study start	*N*	Vaccine and delivery	Outcome
NCT00374309 Sept 2006	31	**Ad5** IM 2×10^9 or 2×10^{10} VP Antigen: GP EBOV and SUDV	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** - Antibody responses in 100% (SUDV GP) and 55% (EBOV GP) of subjects in the higher dose group - No significant induction of VNTs - T cell responses in 82% (SUDV GP) and 64% (EBOV GP) **Of note:** Reduced immunogenicity in participants with pre-existing immunity against Ad5
NCT02326194 Dec 2014	120	**Ad5** IM 4×10^{10} or 1.6×10^{11} VP Antigen: GP EBOV (2014)	**Phase I** **Safety**: No serious adverse events. **Immunogenicity:** - Antibody responses in all but two participants (lower dose) and all (higher dose group) by d28 - Specific T cell responses (by ELISPOT and ICS); **Of note:** high dose of Ad5 vector able to overcome negative effects of pre-existing immunity
NCT02575456 Oct 2015	500	**Ad5** IM 8×10^{10} or 1.6×10^{11} VP Antigen: GP EBOV (2014)	**Phase II** Results not yet publicly available
NCT02269423; NCT02280408 Oct 2014	78	**VSV, attenuated** one or two doses IM 1×10^6, 2×10^7 and 1×10^8 PFU Antigen: GP EBOV (1995)	**Phase I** **Safety:** Mild adverse events, no cases of arthritis **Immunogenicity:** - Antibody titers in all participants by day 28 - Increased levels of total and VNTs upon delivery of higher doses
NCT02231866; NCT02240875*; NCT02267109* Aug 2014–Aug 2017	325	**ChAd3, replication deficient** Single dose IM 1×10^{10}, 2.0×10^{10}, 2.5×10^{10}, 5×10^{10}, 1×10^{11}, 2.0×10^{11} VP Antigen: GP EBOV (1976) ± GP SUDV (1977)	**Phase I** **Safety:** Acceptable safety profile, mild to moderate adverse events. **Immunogenicity:** - Antibody responses in almost all subjects; indications for durability (significant antibody titers detectable up to 48 weeks post vaccination) - VNTs in some subjects - Antigen-specific $CD4^+$ and $CD8^+$ T cells in some subjects - Increased immune responses upon MVA boost
NCT02289027; NCT02344407; (NCT02485301); (NCT02548078)** Oct 2014 - Nov 2015	5244	**ChAd3, replication deficient** Single dose IM 2.5×10^{10}, 5×10^{10}, 1×10^{11} VP Antigen: GP EBOV (1976)	**Phase I/II** **Safety:** NCT02289027: Acceptable safety profile NCT02344407: serious adverse events within 12 months after inj. in 8.0% (40/500) of participants (9.4% in rVSV-ZEBOV) **Immunogenicity:** NCT02289027 - Antibody responses peaked at d28 (51 μg/ml high dose group); still significantly over placebo at d180 (25.5 μg/ml) - $CD4^+$ and $CD8^+$ T cell responses in 57% (28/49) and 67% NCT02344407 - Antibody responses in 70.8 and 63.5% of the participants at 1 and 12 months, respectively (83.7 and 79.5% for VSV-ZEBOV)
NCT02283099; NCT02296983; NCT02287480 Nov 2014	158	**VSV, attenuated** single dose IM 3×10^5, 3×10^6, 1×10^7, 2×10^7, 5×10^7 PFU Antigen: GP EBOV (1995)	**Phase I; Phase I/II** **Safety:** Doses of 1×10^7 PFU or higher: - Arthralgia in 22% (11/51) participants of Geneva cohort; arthritis confirmed in 9/ 11 cases; maculopapular rash in 27% (3/11) of these cases - Self-limiting cases of arthritis in 3.4% (2/60) participants in Germany and Kenya cohort Dose of 3×10^5 PFU: - Reduced adverse events in mild to moderate range with arthralgia in 23% (13/56) participants **Immunogenicity:** - Antibody responses in all subjects; persisted for 6 months - Dose dep. VNTs in 85% (107/126) of vaccinees

(Continued)

TABLE 1 | Continued

Study start	*N*	Vaccine and delivery	Outcome
NCT02378753			**Phase II/III**
March 2015	7651	**VSV, attenuated** single dose IM 2×10^7 PFU Antigen: GP EBOV (1995)	**Safety:** Acceptable, one serious adverse event **Immunogenicity:** - Ring vaccination approach; 48 clusters (4,123 people) and 42 clusters (3528 people) randomly assigned to immediate and delayed vaccination (21 days later) - No cases of Ebola virus disease with symptom onset at least 10 days after randomization (immediate vaccination), 16 cases from seven clusters (delayed vaccination) 100% vaccination efficacy

*This table exclusively lists exemplary clinical trials discussed in the text. Ad5, Adenovirus 5; EBOV, Ebola virus; GP, Glycoprotein; ICS, intracellular staining; IM, intramuscular; N, number of study participants; PFU, Plaque Forming Unit; SUDV, Sudan virus; VNT, virus neutralization titers; VSV, vesicular stomatitis virus; VP, viral particles. *Boost with MVA based vaccine evaluated; **Direct comparison with rVSV-ZEBOV arm.*

A phase II/III clinical trial (NCT02378753) was initiated in Guinea in March 2015 assessing vaccine efficacy upon vaccination using one dose of 2×10^7 PFU in a cluster randomization design with a ring vaccination approach (**Table 1**). Participants, including individuals at high risk, were assigned to clusters that were randomly subjected to immediate and delayed vaccination (21 days later). The study report demonstrated promising results (74, 75). No cases of Ebola virus disease with symptom onset at least 10 days after randomization were detectable in the immediate vaccination group, while 16 cases of Ebola virus disease from seven clusters occurred in the delayed vaccination group, demonstrating 100% vaccination efficacy. Of 43 serious events registered upon vaccination, only one was judged to be causally related to vaccination. Given these results, rVSV ZEBOV is currently the most promising candidate for a licensed vaccine against Ebola virus.

Nucleic Acid Vaccines

Nucleic acid based technologies employ either antigen encoding plasmid DNA or RNA, as messenger RNA or viral replicons. Upon their cellular uptake and expression, nucleic acid encoded antigens can elicit humoral as well as cell-mediated immune responses. Both technologies are extremely versatile due to the ease of antigen manipulation they allow. The production of antigens in the target cells offers the advantage of mimicking protein synthesis during an infection, i.e., protein localizations such as presence in the plasma membrane and modifications such as glycosylation patterns can be formed with a high degree of faithfulness. Importantly, they support the delivery of any antigen of choice, regardless of whether it was derived from a virus, bacterium or parasite, supporting vaccine development against a wide array of pathogens. Since vaccine characteristics are independent of the encoded proteins, development of different vaccines can take place without the need to establish new production, purification and validation methods as well as manufacturing facilities. Hence, nucleic acid based technologies support fast and flexible vaccine development and production. Since all vaccines can be produced using the same basic components, manufacturing of several vaccines can take place in one established facility cutting both costs and time of vaccine production dramatically. Lastly, their synthesis mostly relies on chemically synthesized material, supporting large-scale production with relative ease.

DNA Vaccines

Description

DNA vaccines are generated by insertion of a eukaryotic expression cassette encoding for the antigen(s) of choice into a bacteria-derived plasmid. The plasmid backbone generally contains elements that permit propagation and selection of the vector in *Escherichia coli*, i.e., an origin of replication that supports high yields of the plasmid during bacterial growth and a selectable marker, mostly the bacterial antibiotic resistance gene against Kanamycin, which allows stable inheritance of the vector. Since regulatory safety concerns have been raised against the presence of non-functional sequences, especially the antibiotic resistance marker, for human use, the marker has been replaced or removed in new generations of DNA vaccines (76). In addition, minimal DNA constructs devoid of a bacterial backbone, such as the semi-synthetic minicircle DNA (77) and the fully synthetic Doggybone™ (78), have been developed. The eukaryotic expression cassette is comprised of a 5′ promotor, typically derived from cytomegalovirus (CMV) that supports high transcription levels, the gene of interest and a 3′ polyadenylation (poly A) signal, required for nuclear export, translation and stability of the transcript mRNA, that is usually obtained from rabbit β-globin or bovine growth hormone genes (76).

Delivery of DNA vaccines

Research on DNA vaccines has started as early as the 1990s, where the most common route of administration was intramuscular (IM) or intradermal (ID) injection using a conventional needle. However, vaccination with a DNA vector alone generally leads to relatively low immunogenicity, especially in large animal models and humans. A factor that may play a role is the need for DNA vaccines to cross two cellular membranes, i.e., the plasma, as well as the nuclear membrane, in order to achieve protein expression. Of note, this does not hold true for RNA vaccines, which are translated upon crossing the plasma or endosomal membrane, respectively. Hence, additional methods have been developed that are able to enhance DNA uptake, expression and immunogenicity. These include

various delivery devices such as gene gun, needle free injection devices (jet injection) and *in vivo* electroporation, which is among the most widely used and has been shown to yield promising results in both preclinical and clinical trials (79, 80). Furthermore, different formulations of DNA have been tested, i.e., encapsulation in lipid nanoparticles, containing cationic lipids and cholesterol, adsorption to polymers such as polyethyleneimine and adsorption or encapsulation in biodegradable nanoparticles, such as poly(lactic-co-glycolic acid) (PLGA) or chitosan (81). These methods are largely directed at improving the uptake of the DNA molecule into the cell and thus enhancing antigen expression. In addition, different approaches to modify and improve DNA mediated immune responses have been developed. For this, "molecular adjuvants" such as pattern recognition receptor (PRR) ligands and different cytokines, most commonly IL-12, are co-delivered with the encoded antigen and strategies to direct the antigen to certain cellular compartments or specifically target antigen presenting cells (APCs) to enhance immune responses have been established (82). In addition, DNA vaccines have successfully been employed for prime-boost regimen in combination with other vaccine technologies such as protein- or viral vector based vaccines.

Mode of action

Although a multitude of studies show that DNA vaccination is able to elicit both humoral and cellular immune responses, through activation of $CD8^+$ cytotoxic and $CD4^+$ helper T cells, respectively, the exact mechanism of action remains to be evaluated. Upon entry in the cell, DNA vaccines are sensed by a variety of innate immune receptors. While TLR9 is not critical for DNA vaccine efficacy, the STING/TBK1/IRF3 pathways and the AIM2 inflammasome are involved in DNA vaccine mode of action and other factors might additionally be involved (82). Early experiments testing bombardment with DNA coated gold particles delivered ID demonstrated transfection of both keratinocytes and professional APCs, i.e., Langerhans cells, explaining the source of both MHCI and MHCII restricted antigen recognition by $CD8^+$ cytotoxic and $CD4^+$ helper T cells, respectively (83). However, IM vaccination with DNA vectors mostly results in transfection of myocytes (84). Since several studies have established a role for bone marrow derived APCs in the activation of MHCI restricted $CD8^+$ T cells upon DNA vaccination (85–87), the most likely mechanism in this scenario seems to be cross-priming and presentation of both MHCI and MHCII restricted antigens by professional APC upon phagocytosis of transfected somatic cells.

Advantages and disadvantages

As specified above, the use of nucleic acid based vaccines offers a number of advantages in different aspects of vaccine development and production. However, employing DNA as a basis for vaccination also implicates some disadvantages. A concern in this context is the long-term persistence of DNA plasmids upon injection. Indeed, DNA persistence was shown in various preclinical studies that demonstrated the presence of plasmid DNA for up to 2 years upon IM injection with low but detectable expression and immunogenicity in a mouse model (88). According to the FDA, DNA persistence is not generally evident at ectopic sites in biodistribution and persistence studies, but remains detectable at the injection sites for periods exceeding 60 days[8]. Especially in the context of this long-term persistence, the presence of foreign genetic information in the nucleus of transfected cells poses the additional risk of genomic integration into the host's chromosomes and the resulting threat of mutagenesis and oncogenesis. Despite negative results in several studies focusing on detection of DNA integration events upon IM injection in small animal models, genomic integration events were detectable following electroporation in mice (89, 90) demonstrating that integration represents a small risk that nevertheless needs to be considered in systems with enhanced DNA uptake. The FDA recommends integration studies to be included whenever plasmid DNA exceeding 30,000 copies per μg of host DNA persists in any tissue by study termination. The WHO advises integration studies as part of the preclinical safety program of DNA vaccines[9]. In addition, injection of bacterial DNA, sensed by the presence of unmethylated CpG motifs, has been associated with safety concerns, such as the generation of antibodies against the injected DNA. However, no anti-DNA antibodies have been detectable in mice, rats, rabbits or non-human primates (90). Potential expression of the antibiotic resistance marker in vaccinated organisms has likewise raised safety concerns that are met by the replacement of these markers in next generation DNA vaccines. Lastly, expression of cytokines or co-stimulatory molecules that are used to enhance DNA immunogenicity might lead to unintended adverse effects upon cytokine expression and release such as generalized immune suppression, chronic inflammation or autoimmunity. The WHO recommends monitoring the persistence of a cytokine expressing plasmid as well as appropriate preclinical models, such as animal models responsive to the respective human cytokine to ensure vaccine safety.

DNA vaccines in potential pandemic settings

Since the first experiments in the 1990 (91), DNA vaccines have been employed for vaccine development up to clinical trials against a large variety of human pathogens such as HIV, influenza virus, malaria, hepatitis B virus, respiratory syncytial and herpes simplex virus. No DNA based vaccine is licensed for human use as yet, but several DNA based vaccines have been licensed for veterinary applications, such as an equine vaccine against West Nile Virus. Given their high degree of versatility, DNA vaccines have been tested for their efficacy to protect against recent pandemic threats including HIV, MERS, Ebola, and Zika, some of which will be discussed in more detail below.

The first effective vaccines against **Ebolaviruses** developed in preclinical experiments employed DNA vector based antigen expression. These approaches relied on expression of the viral glycoprotein (GP), to induce neutralizing antibodies as well as nucleoprotein (NP) as a target for antibody as well as

[8]https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm091968.pdf.

[9]http://www.who.int/biologicals/publications/trs/areas/vaccines/dna/Annex%201_DNA%20vaccines.pdf?ua=1.

T cell responses. Induction of both humoral and T cell-mediated immunity as well as protective efficacy against rodent adapted viral strains was demonstrated in guinea pigs and mice, upon vaccination with DNA encoding for GP and NP using intramuscular injection or intradermal delivery using a gene gun, respectively (92, 93). Later studies established protection induced by a trivalent DNA vaccine encoding for GP of two Ebolaviruses and a Marburgvirus (94) and protection from lethal challenge against an Ebolavirus [Ebola virus (EBOV)] upon DNA vaccination in combination with adenoviral vectors in non-human primates (55). Having a set of promising preclinical data established, the first phase I clinical trial (NCT00072605) using a DNA vaccine against Ebola was started in 2003, well before the Ebola crisis in 2014 (95) (**Table 2**). This study employed a trivalent DNA vaccine consisting of plasmids encoding for transmembrane-deleted forms of GP derived from two Ebolaviruses as well as NP produced by Vical Inc.. Results demonstrated safety and tolerability of this vaccine as well as specific antibody responses to at least one of the three antigens in all subjects. However, no detectable virus neutralizing responses were elicited in this trial. A further phase I clinical trial (NCT00605514) conducted in 2008–2009 (96) employed wildtype GP constructs that had been found to elicit superior responses over transmembrane deletions of GP in the context of adenoviral delivery in NHPs (57) (**Table 2**). Two different DNA vaccines encoding for GPs of two species of Ebolavirus (produced by the VRC/NIAID Vaccine Pilot Plant, operated by Leidos) or Marburg Marburgvirus (MARV) GP (produced by Althea Technologies), respectively, were administered. This study confirmed safety of both DNA vaccines. 80% of subjects were found to elicit specific antibody responses against one of the GPs. Given the reassuring safety profile, a phase Ib study (NCT00997607) was conducted in Uganda in 2009 (97) (**Table 2**). Both vaccines were well tolerated but immune responses remained poor with around 50% and 30% of the subjects eliciting antibody responses against the Ebolavirus and MARV components, respectively. Overall, results of these early generations of DNA based vaccines were somewhat discouraging. However, efforts were renewed using improved DNA technologies, upon the outbreak in 2014. Inovio is developing and testing their GP encoding DNA vaccine candidate INO-4212 (a combination of two DNA vaccines, i.e., INO-4201 and INO-4202, encoding for GP derived from a pre-2013 and a current viral isolate, respectively). Proving the versatility and speed of the approach, a clinical trial was initiated in early 2015 (NCT02464670) (**Table 2**). The study assesses vaccine safety, tolerability, and immunogenicity of the components with and without an IL-12 encoding plasmid (INO-9012). Preliminary results have shown a favorable safety profile; ∼90% of the participants generated an Ebola-specific antibody immune response.

A large number of preclinical and clinical studies have assessed the ability of DNA vaccines to mediated protection against **influenza** viruses, either alone or as part of prime boost strategies. These vaccines mainly rely on plasmid based expression of hemagglutinin (HA), one of the viral surface antigens and the main target for neutralizing antibodies against influenza. In terms of pandemic preparedness in DNA only vaccination strategies, Vical Inc. has developed and tested a vaccine that targets the highly pathogenic avian H5N1 influenza endemic in poultry. Its ability to cross the species barrier, that was first discovered in 1997 and caused rising numbers of human infections between 2003 and 2008, renders this virus a high pathogenic risk. So far, the virus is not able to spread efficiently and sustainably from human to human but H5N1 bird to human infections have caused the death of 453 people worldwide until 2017.[10] DNA vaccines expressing HA of the viral strain A/Vietnam/1203/04 were either employed alone or in combination with the conserved nucleoprotein (NP) and ion channel protein (M2) derived from different subtypes as targets of T cell responses. NP and M2 had previously been shown to protect mice against lethal challenge in the absence of an HA component (98). Clinical trials testing DNA vaccines in combination with the lipid-based adjuvant Vaxfectin® were initiated in 2007 after protective efficacy was demonstrated in preclinical studies in mice and ferrets (99) (NCT00709800 and NCT00694213) (**Table 2**). Vaccines were found to be well tolerated and HI titers $\geq$40, the correlate of protection, were elicited in a maximum of 67 and 20% in HA only and trivalent groups, respectively.

Upon emergence of a novel H1N1 influenza that originated in pigs and became pandemic in humans in spring 2009 (100), efforts were made for the accelerated development of a vaccine. A clinical trial (NCT00973895) was initiated by August 2009 using a DNA based approach encoding hemagglutinin protein of A/California/04/2009(H1N1pdm09) whose GMP production was finalized 2 months before licensed monovalent influenza vaccines became available (101) (**Table 2**). However, 4 weeks after the last vaccination, only 30% of subjects had developed positive HI responses that increased to 72%, 4 weeks after boosting with a licensed monovalent influenza vaccine. Based on results gained at this point, the ability for fast manufacturing of a large number of doses could support the use of DNA-based vaccines for controlling a potential influenza pandemic by employing DNA as an initial priming agent, followed by boosting with conventional influenza vaccines upon availability.

DNA based vaccines were among the first to proceed to clinical trials upon the **Zika** crisis in 2016. Leveraging knowledge generated in the context of other flaviviruses, these approaches rely on the expression of the precursor membrane and envelope (Env) (prM-E) proteins which are known to form subviral particles with Env being the target of virus neutralizing antibodies. The first approach developed by Inovio employed a consensus prM-E derived from African and more recent Asian and American strains modified to contain an IgE signal peptide with a putative glycosylation site removed (GLS-5700) (102). This vaccine was shown to be immunogenic and protective in a mouse model upon IM vaccination followed by electroporation. Passive transfer experiments of vaccine-induced sera in an interferon (IFN) α/β receptor knockout mice demonstrated correlation of antibody levels with protection. Furthermore, the induction of virus antibodies and T cell responses upon ID vaccination followed by electroporation was shown in NHPs. Based on these results, two phase I clinical studies were initiated, one

[10]http://www.who.int/influenza/human_animal_interface/2017_07_25_tableH5N1.pdf.

TABLE 2 | Clinical trials employing DNA vaccines in pandemic settings.

Study start	*N*	Vaccine and delivery	Outcome
NCT00072605 Oct 2003	27	**EBOLA** **DNA,** trivalent; NF inj.dev. IM 2–8 mg in week 0, 4, and 8 Antigens: - GPΔTM EBOV - GPΔTM SUDV - NP	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** - Specific antibody response to at least 1/3 antigens in all subjects - Specific $CD8^+$ T cell responses in 30% (6/20) subjects. - No detectable virus neutralizing responses
NCT00605514 Jan 2008	20	**EBOLA** **DNA,** mono or bivalent; NF inj.dev. IM 4 mg in week 0, 4, 8; (32) Antigens: - GP MARV - GP EBOV + GP SUDV	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** - Specific antibody responses against one of the GPs at week 12 in 80% of subjects - $CD8^+$ T cell responses in some of the subjects
NCT00997607 Feb 2010	108	**EBOLA** **DNA,** mono or bivalent; NF inj.dev. IM 4 mg in week 0, 4, 8 Antigens: - GP MARV - GP EBOV + GP SUDV	**Phase Ib** **Safety:** Acceptable safety profile **Immunogenicity:** Specific antibody responses in 30% (MARV) and 50% (EBOV or SUDV) of subjects Antibody titers to near baseline levels by w 44 post vaccination
NCT02464670 May 2015	240	**EBOLA** **DNA,** mono-, bi- or trivalent; IM or ID + EP in 2 or 3 doses 0.8–4 mg GP; 0.2–1 mg IL12 Antigen: - GP EBOV pre 2013 - and/or GP EBOV 2014 - and IL-12 in trivalent vaccine	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** Specific antibody responses in 88% (50/57) (IM) and 95% (119/122) (ID) of participants
NCT00709800 and NCT00694213 Aug 2007	103	**INFLUENZA H5N1** **DNA,** mono- or trivalent; needle or NF inj.dev. IM 0.1–1 mg in week 0, 3 Antigen: - HA of A/Vietnam/1203/04 - HA + NP + M2	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** - HI titers ≥40, in 47- 67% (HA only) and 0- 20% (HA + NP + M2) of participants, peak at d56 - H5-specific T cell responses in 75–100% (HA only) and 50–57% % (HA + NP + M2) of subjects - Responses against HA unaffected by injection method
NCT00973895 Aug 2009	20	**INFLUENZA H1N1** **DNA,** monovalent; NF inj.dev. IM 4 mg in week 0, 4, 8 Antigen: HA of A/California/04/2009	**Phase I** **Safety:** Acceptable safety profile **Immunogenicity:** - HI titers ≥40 in 30% (6/20) of DNA vaccinated subjects - DNA + licensed vaccine HI titers ≥40 in 72% (13/18) - T cell responses in 25% (5/20) of subjects
NCT02809443 (NCT02887482) July 2016 (Aug 2016)	40 (160)	**ZIKA** **DNA,** monovalent; ID + EP 1 or 2 mg in week 0, 4, 12 Antigen: Consensus prM-E; IgE SP; removed glycosylation site	**Phase I** **Safety:** Acceptable safety profile (NCT02809443) **Immunogenicity (preliminary results NCT02809443):** - VNTs in 62% of the participants (Vero cell assay) - Protection of 92% (103/112) of mice by passive serum transfer in challenge model (IFN α/β receptor knockout)
NCT02840487; NCT02996461 Aug 2016 Dec 2016	125	**ZIKA** **DNA,** monovalent; needle or NF inj.dev. IM 4 mg in 2 or 3 doses Antigen: - prM-E; JEV SP (VRC5283) - prM-E; JEV SP and S/TM (VRC5288)	**Phase I/Ib** **Safety:** Acceptable safety profile **Immunogenicity:** - Humoral and T cell responses induced - VNTs in 60%−100% of subjects 4 w after the final vaccination - Best responses in VRC5283: Antibody responses in 100% (14/14) of participants in NF inj, in split doses group; best VNT and T cell responses

(Continued)

TABLE 2 | Continued

Study start	*N*	Vaccine and delivery	Outcome
NCT03110770 Mar 2017	2500	**ZIKA** **DNA,** monovalent; NF inj.dev. IM in 3 doses 4 mg or 8 mg in 2 or 4 inj. Antigen: - prM-E; JEV SP (VRC5283)	**Phase II** Results pending, estimated study completion date Jan 2020

This table exclusively lists clinical trials discussed in the text. EBOV, Ebola virus; EP, electroporation; GP, glycoprotein; GPΔTM, glycoprotein delta transmembrane domain; HA, hemagglutinin influenza; HI, hemagglutination inhibition; ID, intradermal; IL-12, interleukin 12; IM, intramuscular; JEV, Japanese encephalitis virus; M2, ion channel protein influenza; MARV, Marburg virus; N, number of study participants; NF inj.dev, needle free injection device; NP, nucleoprotein influenza; prM-E, preMembrane-Envelope; SUDV, Sudan virus; VNT, virus neutralization titer; SP, signal peptide; S/TM, stem and transmembrane regions.

in flavivirus-naive individuals (NCT02809443) that was started in July 2016 and the other one in dengue virus seropositive subjects (NCT02887482) which began in August 2016 (**Table 2**). Preliminary results from NCT02809443 (103) demonstrated that the vaccine was well-tolerated and induced neutralizing antibodies in 62% of the participants.

A preclinical study published in October 2016 demonstrated the induction of neutralizing antibodies and protection from challenge infection in 17 of 18 NHPs upon two IM vaccinations using a needle free injection device. This study employed two different prM-E constructs based on the sequence of French Polynesian and early Brazilian ZIKV isolates in which the Zika prM signal sequence alone (VRC5283) or in combination with the stem and transmembrane regions (VRC5288) were exchanged with the corresponding sequences from Japanese encephalitis virus (JEV). Both vaccine candidates are evaluated in clinical studies by The Vaccine Research Center (VRC), National Institute of Allergy and Infectious Diseases (NIAID) (**Table 2**). Clinical trials testing VRC5288 (NCT 02840487) and VRC5283 (NCT02996461) were initiated in August 2016 and December 2016, respectively. The results of these phase I studies were published in the Lancet in December 2017 (104). Both trials showed that vaccinations were safe and well tolerated and induced both humoral and T cell responses. Positive neutralizing antibody responses ranging from 60 to 100% were detected 4 weeks after the final vaccination; VRC5283, in agreement with preclinical studies, yielded better responses than VRC5288.

Both DNA based approaches for the development of an effective Zika vaccine appeared safe for human use and yielded promising results. Importantly, they were initiated within months after sequences became available, highlighting the versatility and speed provided by DNA vaccine platforms.

RNA Vaccines

Description

mRNA is an intermediate carrier of genetic information used as template for endogenous protein production in the vaccinated subject. Two major types of RNA have been utilized as prophylactic vaccines against pathogens that cause infectious diseases:

1) Non-replicating mRNA
2) Self-amplifying mRNA

Non-replicating mRNA contains the sequence of the antigen of choice flanked by 5′ and 3′ untranslated regions (UTRs). The advantages of using non-replicating mRNA vaccines compared to self-amplifying mRNA are rooted in the simplicity of the construct, the small size of the RNA, and the absence of any additional encoded proteins that could induce unintended immune responses (105). The design of optimized, efficiently translated mRNA for use as a vaccine has been reviewed previously (105–107). Briefly, conventional non-replicating mRNA is obtained by *in vitro* transcription of a cDNA template, typically plasmid DNA (pDNA) produced in *E. coli*. The pDNA template is linearized using restriction enzymes and is transcribed *in vitro* into mRNA in a mixture containing recombinant phage DNA-dependent RNA polymerase (typically derived from T7 or T3 or Sp6 phage) and nucleoside triphosphates (NTPs) (108). Upon purification, usually via FPLC or HPLC to remove any remaining product related impurities such as reaction components (i.e., enzymes, free NTPs, residual pDNA) or abortive transcriptional byproducts, a pure single mRNA product is obtained (109). Notably, purification of *in vitro* transcribed mRNA seems to be crucial for the amount of immunogen produced in target cells as demonstrated by up to 1,000-fold increased protein production in primary human DCs transfected with HPLC purified compared to unpurified mRNA (110). The *in vitro* transcribed mRNA product contains a protein-encoding open reading frame (ORF) flanked by elements essential for the function of mature eukaryotic mRNA: a cap structure, joined to the 5′ and a poly(A) tail at the 3′ end, as well as 5′ and a 3′ untranslated regions (UTR) (111–113). The 5′ cap is vital for the creation of stable mature mRNA and increases protein translation via binding to eukaryotic translation initiation factor 4E (111, 114). The 5′ cap can be added either during the transcription by inclusion of a cap analog or anti-reverse cap (ARCA) in the reaction (115), or subsequently, using the vaccinia virus capping complex (116). The UTRs, which can be of eukaryotic or viral origin, increase the half-life, and stability of the mRNA, resulting in higher expression of the protein (117–120). The poly A tail of an optimal length is an essential regulatory element to enhance translation and can be either encoded into the DNA template or alternatively added enzymatically post transcription (111, 121, 122). The sequence of the ORF can be optimized using either enrichment of the GC content (123–125) or by replacement of rare codons

by frequently used synonymous codons leading to increased protein production from mRNA (126). Utilization of chemically modified nucleosides can decrease innate immune activation and increase translation of the mRNA (127).

Self-ampifying mRNA vaccines are most commonly based on the alphavirus genome [reviewed in detail in (128–130)], from which the genes encoding the structural protein have been replaced with the antigen of choice. Despite these gene deletions, the viral RNA is replicated and transcribed by the viral RNA polymerase. The full length mRNA of the self-amplifying mRNA vaccines is substantially larger (∼9–10 kb for alphavirus systems) than in non-replicating mRNA vaccines, but contains the same essential elements such as a cap, 5′ and 3′ UTRs, and poly A tail (128). Of note, lower yields and increased occurrence of abortive constructs as a consequence of the large size of these vaccines pose challenges to vaccine production, that make manufacturing processes more difficult compared to non-replicating mRNA vaccines. The additional mRNA contains a sub-genomic promoter and a large ORF encoding for non-structural proteins which, following delivery of the vaccine into the cytosol, are transcribed in four functional components (nsP1, nsP2, nsP3, and nsp4) by the encoded RNA-dependent RNA polymerase (RDRP) (131). RDRP than produces a negative-sense copy of the genome which serves as a template for two positive-strand RNA molecules: the genomic mRNA and a shorter sub-genomic mRNA. This sub-genomic mRNA is transcribed at very high levels, allowing the amplification of mRNA encoding the antigen of choice. Hence, any genetic information encoded by the self-amplifying mRNA vaccine will be amplified many times, resulting in high levels of antigen expression from relatively low doses of the vaccine, which is an appealing attribute of self-amplifying mRNA vaccines compared to non-replicating mRNA vaccines (132). Upon injection in mice, LNP-formulated self-amplifying mRNA encoding firefly luciferase induced protein expression lasting almost two months upon IM delivery (130), while luciferase expression from protamine-formulated, non-replicating mRNA administered ID was usually only detected for several days (133). However, potential interactions between the host and the encoded alphaviral non-structural proteins necessitate further investigation.

Self-amplifying mRNA is most commonly delivered with synthetic delivery vehicles as discussed below. An alternative method is packaging and delivery in virus-like replicon particles (VRPs) produced by a helper cell line that provides the capsid and glycoprotein genes in trans (134). While the lack of structural protein genes contained in VRPs prevents production of further viral particles and cell-to-cell spread, VRPs are capable of infecting cells and expressing the antigen of choice *in vitro* and *in vivo*. Although both preclinical and clinical data for the VRPs are promising, this technology requires the use of electroporation of the genetic material into cell culture cells during the manufacturing process. Although electroporation has been successfully employed under GMP conditions at a scale sufficient to provide material for a phase I study, cost-effective production at industrial scale may be challenging. In addition, there are some safety concerns associated with VRPs, since recombination or co-packaging of replicon and helper RNAs VRPs during their production in cells containing both replicon and helper RNAs could lead to the generation of infectious viruses.

Delivery of mRNA vaccines

In order to act as a vaccine, exogenous mRNA has to enter the cytoplasm where protein expression can take place. In this step, the plasma or endosomal lipid membrane represents a barrier the mRNA vaccine has to cross as efficiently as possible. In addition, the induction of an effective immune response requires stimulation of the innate immune system by the mRNA vaccine. While mRNA has some intrinsic innate stimulation function (see below), this effect can be increased by different ways of mRNA formulation. Hence, several methods to increase both cell delivery and adjuvanticity of mRNA vaccines have been developed.

Immunization can take place via direct injection of **naked mRNA**, especially via routes which lead to effective targeting of APCs, such as intradermal (135–137) and intranodal (138–140) administration. However, when delivered IM, humoral and cellular immune responses induced by naked mRNA remain low compared to LNP-formulated mRNA (141).

Physical delivery methods of mRNA vaccines that likely increase vaccine release into the cytoplasm have been shown to induce immune responses in mice upon administration of non-replicating mRNA and self-amplifying mRNA using a gene gun and *in vivo* electroporation, respectively (142–146).

A more commonly used strategy to increase expression and immunogenicity is the delivery of mRNA in complex with additional components. Among the first approaches was a format, whose two components, free and **protamine-complexed** mRNA (a small arginine-rich nuclear protein that stabilizes nucleic acids), provide both strong antigen expression and immunostimulation (147–150). This vaccine format has proved to be immunogenic and capable of inducting protection against lethal challenge infections with influenza or rabies virus in several animal models (124, 151). Using this format, CV7201, a candidate vaccine against rabies, was investigated as the first ever prophylactic mRNA-based vaccine in healthy human volunteers. The subjects received 80–640 μg of the mRNA vaccine three times by conventional needle-based injection or needle-free injection devices via the intradermal (ID) or intramuscular (IM) route. The vaccine was generally safe with a reasonable tolerability profile and led to the induction of neutralizing antibody titers at levels of 0·5 IU/mL or higher (as the correlate of protection) in 71% of subjects who had received ID injections of 80 or 160 μg mRNA vaccines by needle-free intradermal injection, while needle-based injection was ineffective (152). Antibody responses waned one year after first vaccination but could be boosted to 0·5 IU/mL or higher in 57% of subjects using 80 μg of mRNA delivered ID with a needle free injection device, indicating the induction of B cell memory responses. Although the mRNA vaccine candidate was able to induce antibody responses, further improvements to increase the magnitude and longevity of the immune responses are imperative for the development of an effective vaccine.

The efficacy of mRNA vaccines can benefit significantly from complexing agents such as **lipid- and polymer-based nanoparticles** which enhance uptake by cells and improve delivery to the translation machinery in the cytoplasm. Although commercially available cationic lipids and polymers [e.g., TransIT-mRNA (Micrus Bio LLC) or Lipofectamine (Invitrogen)] are efficient transfection reagents for mRNA in cell lines and primary cells (110, 127) their use for *in vivo* mRNA delivery is limited due to high toxicity and low efficacy of transfection. Safer and more effective complexing reagents which were discussed in detail in some recent reviews (153–156) have been designed in the past few years, leading to the expansion of the field for prophylactic use and the development of more potent and versatile mRNA vaccines. Currently, lipid nanoparticles (LNPs) are the most promising and frequently used class of agents for *in vivo* delivery of mRNA vaccines. LNPs have been intensively studies in the context of siRNA (157) and are well tolerated compared to other non-viral delivery system. Most LNPs rely on ionizable amino lipids which complex the negatively charged mRNA, support assembly into 70–100 nm sized particles and promote escape of the mRNA from endosomal compartments into the cytoplasm where the mRNA can be translated. In addition to ionizable amino lipids, phospholipids, cholesterol and lipid-anchored polyethylene glycol (PEG) are the most commonly used components for LNP formulations. Cholesterol acts as a stabilizing element and plays an important role in the transfection of cells. Lipid-anchored PEG preferentially deposits on the LNP surface, where it can act as a barrier which sterically stabilizes the LNP and reduces non-specific binding to proteins increasing the half-life of the LNPs. Furthermore, the surface of an LNP can be decorated with specific targeting entities which direct the vaccine to certain tissues or cells, such as professional APCs, thereby facilitating the uptake of the mRNA vaccine by the desired type of immune cell and eventually leading to an enhanced immune response against the antigen of choice. Several studies demonstrated that LNPs are effective agents for *in vivo* delivery of non-replicating and self-amplifying mRNA vaccines (130, 141, 158, 159).

In addition to formulation, the **route of mRNA administration** has a crucial impact on the quality and strength of the induced immune response. LNP-mRNA delivered intravenously (IV) primarily targets the liver (160), while ID and IM delivery generally show more prolonged expression of the antigen of choice at the injection site (141, 159, 161). A study comparing different routes of administration of LNP-formulated mRNA coding for luciferase showed that the total amount of protein produced was largest for IV administration, while duration of luciferase expression was the longest for ID followed by IM injection (161). **Intradermal (ID) injection** delivers mRNA vaccines directly into the skin, an organ densely populated with professional APCs such as Langerhans cells in the epidermis and various dendritic cells (DC) subtypes in the dermis. The ID route of administration has been shown to effectively induce a balanced immune response including antibodies as well as Th1 type and cytotoxic T cells for mRNA vaccines formulated in protamine or LNP (124, 150, 158).

The intramuscular (IM) injection of vaccines is the most often practiced route of administration in humans. Since this route of vaccination is simple to carry out and does not require much training for its implementation, it may be the preferred route of administration by the physicians carrying out immunization in regions affected by a pandemic. However, the need for educated personnel to vaccinate people might represent a limiting factor in the face of a pandemic. The induction of strong immune responses after IM injection of mRNA represents a high hurdle, due to lack of co-stimulatory molecules and optimal antigen presentation on muscle cells and low infiltration of the muscle tissue by immune cells. Thus, potent IM mRNA vaccines must allow high antigen expression and presentation and simultaneously induce strong immunostimulatory signals to recruit immune cells to the injection site. The IM administration of non-replicating nucleoside-modified mRNA-LNP vaccines against the Zika virus, as well as influenza A H10N8 and H7N9 viruses proved to be immunogenic and provide protection in preclinical studies in mice, ferrets and NHPs (159, 162, 163). Single IM immunization of NHPs with LNP-formulated mRNAs encoding rabies or influenza antigens induced protective antibody titers, which could be boosted and remained stable during an observation period of up to one year (141).

Mode of action

Exogenous mRNA is immunostimulatory, as it is recognized by a variety of cell surface, endosomal and cytosolic innate immune receptors. Mammalian cells can sense foreign RNA via PRRs such as TLR3, TLR7 and TLR8 located in the endosomes and RIG-I, MDA-5 and PKR located in the cytoplasm as well as NLRP3 and NOD2 (164). Activation of the PRRs by mRNA vaccines results in a robust innate immune response including production of chemokines and cytokines such as IL-12 and TNF at the inoculation site (165), which are innate factors crucial for the induction of an effective adaptive immune response against the encoded antigen. ID immunization with mRNA vaccines upregulates the expression of chemokines including the CXCR3-ligands CXCL9, CXCL10, and CXCL11, that recruit innate immune cells such as DCs and macrophages, to the site of injection (165). Kowalczyk et al. showed that the in the skin, protamine-formulated non-replicating sequence optimized mRNA vaccines are taken up by both non-leukocytic and leukocytic cells, the latter being mostly represented by APCs (150). mRNA was then transported to the draining lymph nodes (dLNs) by migratory dendritic cells. Moreover, the encoded protein was expressed and efficiently presented by APCs within the dLNs as shown by T cell proliferation and immune cell activation, followed by the induction of the adaptive immunity. Importantly, the immunostimulation was limited to the injection site and lymphoid organs as no proinflammatory cytokines were detected in the serum of the immunized mice. Lazzaro et al. demonstrated that $CD8^+$ T-cell priming is restricted to bone-marrow-derived APCs and may involve antigen transfer from myocytes suggesting cross-priming as the prevalent mechanism upon IM injection of self-amplifying mRNA vaccines in mice (166). In a recent publication, Lutz et al. provided first mechanistic insights into the mode of

action of LNP-formulated non-replicating sequence optimized mRNA vaccines, demonstrating a strong activation of the innate immune response at the injection site and in the dLNs in mice. IM injection of LNP-formulated mRNA vaccine resulted in spontaneous uptake of the mRNA by cells surrounding the injection site and strong expression inside transiently transfected cells, including resident professional APCs, neutrophils and non-leukocytic cells (141). Interestingly, similar observations were published using LNP-formulated non-replicating mRNA vaccines containing modified nucleotides which induced rapid and local infiltration of neutrophils, monocytes, and DCs to the site of administration and the dLNs in injected NHPs (167). While these cells efficiently internalized LNPs, mainly monocytes and DCs translated the mRNA and up-regulated key co-stimulatory receptors (CD80 and CD86). This coincided with upregulation of type I IFN-inducible genes, including Mx1 and CXCL10. The innate immune activation was transient and resulted in priming of antigen-specific $CD4^+$ T cells exclusively in the vaccine-draining LNs. The data demonstrate that mRNA-based vaccines induce type-I IFN-polarized innate immunity and, when combined with antigen production by APCs, lead to generation of potent vaccine-specific responses. Professional APCs, with DCs likely being the most relevant cell type for mRNA vaccines, play a critical role in antigen processing and presentation to elicit an immune response against specific antigens. The transfected DCs express the mRNA-encoded antigen in the native form. Expressed proteins are subsequently processed into antigenic peptides and are presented on MHC class I and MHC class II molecules along with co-stimulatory signals to $CD8^+$ and $CD4^+$ T cells, respectively. Antigen expressed in the correctly folded native form can be recognized by B cells that in response produce antibodies against the antigen. A study in NHPs investigating the immunological events leading to antibody responses elicited by a modified non-replicating mRNA encoding influenza A H10 HA encapsulated in LNPs showed that, while both ID and IM administration induced titers considered to be protective, ID delivery generated this response more rapidly (168). Circulating influenza H10-specific memory B cells expanded after each of the two immunizations, along with a transient appearance of plasmablasts. The memory B cell pool waned over time but remained detectable throughout the 25-week study. Following immunization, H10-specific plasma cells (PCs) were detected in the bone marrow and persisted throughout the 25 week observation period with a more profound decline detected in IM group compared to the ID group by the end of the study. Germinal centers were formed in vaccine-draining lymph nodes along with an increase in circulating H10-specific $ICOS^+$ $PD\text{-}1^+$ $CXCR3^+$ T follicular helper cells, a population shown to correlate with high avidity antibody responses after seasonal influenza vaccination in humans. In addition, a non-replicating sequence optimized mRNA vaccine induced long-lived functional antibody responses against HA of influenza A H1N1pdm in NHPs which persisted for one year (141). These results indicate that non-replicating mRNA vaccines potently induce an immunological repertoire associated with the generation of high magnitude long-lived antibodies.

Advantages and disadvantages

Although injection of naked mRNA via the ID or intranodal (135–140) route has been reported to induce immune responses, mRNA alone is not applicable for broad use as a prophylactic vaccine. Because of the omnipresence of extracellular ribonucleases which catalytically hydrolyze RNA, unprotected "naked" mRNA is highly unstable under physiological conditions and due to the hydrophilicity and strong net negative charge of RNA not taken up efficiently by cells after application *in vivo*. However, this challenge has been overcome by complexing of mRNA with highly efficient carriers such as new generations of LNP described above, which protect the mRNA from ribonucleases and allow prolonged *in vivo* expression of the antigen of choice leading to the generation of potent humoral and cellular immune responses following *in vivo* administration.

Activation of the innate immune response by RNA vaccines is potentially a double-edged sword. While systemic type I IFN produced in response to the activation of PRRs can facilitate the adaptive immune response, it can lead to phosphorylation of eukaryotic translation initiation factor 2α (eiF2α) which results in a slowdown and eventually inhibition of protein translation. Pepini et al. report that a self-amplifying mRNA vaccine elicits an inflammatory response within a few hours indicated by the upregulation of several IFN-stimulated genes and that antigen expression and immunogenicity were both enhanced in the absence of IFN-α/β signaling, suggesting that reduction of early type I IFN responses could improve RNA vaccine potency (169). Several approaches have been described which aim at overcoming the stalled translation and increased degradation of mRNA induced by the activation of the type I interferon pathway. One such approach is the use of naturally occurring modified nucleotides to suppress activation of the innate receptor-mediated responses. Kariko and others found that, compared to unmodified mRNA, nucleoside-modified mRNA was translated more efficiently *in vitro* in primary DCs and *in vivo* in mice (127, 170). The second approach developed by CureVac AG is based on the optimization of the nucleotide sequence, and hence the codon usage, relying exclusively on unmodified nucleotides which affects both mRNA stability and immunogenicity. As shown by Thess and colleagues, sequence-optimized, unmodified mRNA led to higher protein expression *in vitro* in HeLa cells and *in vivo* in mice than the respective mRNA containing modified nucleosides (123). However, it remains to be determined which approach, modified or unmodified mRNA, provides a better basis for prophylactic vaccines in humans.

In recent human clinical studies, mild to moderate and in rare cases severe local and systemic reactions were reported for different mRNA platforms (152, 159). Future studies in suitable animal models should carefully evaluate the distribution of the mRNA, expression of the encoded antigen in distant organs, potential safety risks, including local and systemic effects, toxic effects of new delivery systems, as well as the induction of self-reactive antibodies in humans.

mRNA vaccines, like DNA vaccines, are able to induce both humoral and cellular immune responses, encode any antigen

of choice and allow a high degree of adaptability. In terms of manufacturing, both platforms allow production of different vaccines using the same established production process and facility. However, since the production process of mRNA is based on *in vitro* systems and does not require amplification in bacteria or cell cultures, manufacturing of mRNA vaccines is a comparably short and simple to monitor process. As mRNA vaccines do not interact with the host-cell DNA, they avoid the potential risk of genomic integration posed by DNA-based vaccines. Since mRNA vaccines represent a minimal vector containing the ORF encoding the antigen of choice flanked by specific regulatory elements, they do not induce anti-vector immunity as observed for certain viral vector-based platforms (171, 172) and therefore can be administered multiple times. Furthermore, mRNA vaccines can be administered by different routes using conventional needle-based injections and, unlike DNA vaccines, they do not require any additional administration device such as gene gun or electroporation. Therefore, mRNA vaccines offer a flexible one-for-all large-scale, rapid and cost-effective manufacturing process with fast turnaround time. This is vital when facing a pandemic threat requiring a rapid response platform capable of producing protective vaccines in the short time-frame necessary to protect at-risk populations and have an early impact on the progression of an outbreak.

RNA vaccines in potential pandemic settings

An increasing number of preclinical studies have shown promising results for both self-amplifying and non-replicating mRNA vaccines to confer protection against various pathogens, including those with pandemic potential (162, 173–176).

Self-amplifying mRNA vaccines encoding various influenza antigens complexed with LNP or oil-in-water cationic nanoemulsions (CNE) were immunogenic in ferrets, facilitating containment of viral replication in the upper respiratory tract upon influenza infection and conferred protection against homologous and heterosubtypic viral challenge in mice (173, 177, 178). A self-amplifying mRNA vaccine encoding an HIV-1 clade C envelope glycoprotein formulated in CNE, induced potent cellular as well as binding and neutralizing antibody responses in NHPs (179). RNA replicons encoding the glycoprotein complex of the Lassa virus encapsulated into VRP particles were immunogenic and protective in mice and resulted in induction of cross-reactive multifunctional T cell responses (176). Chahal et al. demonstrated in a mouse model that a modified dendrimer nanoparticle (MDNP)-based RNA replicon vaccine platform provides protection against lethal influenza and Ebola virus infections and elicits antibody and $CD8^+$ T cell responses against Zika virus (180, 181). However, so far, self-amplifying mRNA vaccines have not been tested in clinical studies and their safety, tolerability and efficacy in humans has yet to be proven.

A variety of preclinical studies have demonstrated the ability of **non-replicating mRNA** vaccines to induce immune responses and confer protection against pathogens with pandemic potential such as ZIKV, EBOV and influenza. Importantly, some of these approaches are currently being tested in clinical trials. Pardi et al. demonstrated that ID immunization with LNP-encapsulated modified mRNA encoding the prME glycoproteins of **ZIKV** elicited potent and durable neutralizing antibody responses that were protective in mice and NHPs (158). A subsequent study by Richner et al. showed that IM administration of

TABLE 3 | Clinical trials employing RNA vaccines in pandemic settings.

Study start	*N*	Vaccine and delivery	Outcome
NCT03014089		**ZIKA**	**Phase I/II**
Dec 2016	90	**mRNA 1325,** modified nucleotides; LNP-formulated, Antigen: prM-E polyprotein	Results pending; estimated primary completion date in Sept 2018
NCT03076385		**INFLUENZA H10N8**	**Phase I**
Dec 2015	201	**mRNA 1851,** modified nucleotides; LNP-formulated, Antigen: HA of H10N8 A/Jiangxi-Donghu/346/2013	Interim results published for 100 μg IM ($N = 23$) vs. placebo ($N = 8$) **Safety:** acceptable safety profile **Immunogenicity:** - HI titers ≥40 in 100% (23/23) of subjects at day 43 - MN ≥20 in 87% (20/23) at day 43
NCT03345043		**INFLUENZA H7N9**	**Phase I**
May 2016	156	**mRNA 1440,** modified nucleotides; LNP-formulated, Antigen: HA of H7N9 A/Anhui/1/2013	Results pending; estimated primary completion date in Sept 2018
NCT03325075		**CHIKUNGUNYA**	**Phase I**
Aug 2017	60	**mRNA 1388,** modified nucleotides; LNP-formulated Antigen: structural polyprotein	Results pending; estimated primary completion date in Sept 2019

This table exclusively lists clinical trials discussed in the text; prM-E, preMembrane-Envelope; HA, Hemagglutinin; HI, hemagglutination inhibition; MN, microneutralization titers; N, number of study participants; IM, intramuscular; ID, intradermal; LNP, lipid nanoparticle.

a similarly designed ZIKV vaccine resulted in high levels of neutralizing antibody titers that were protective, conferred sterilizing immunity and restricted *in utero* transmission of ZIKV in mice (162, 163). A Phase I/II, randomized, placebo-controlled, dose-ranging study of this ZIKV mRNA vaccine (mRNA-1325) was initiated in December 2016 with an estimated primary completion date in September 2018 (NCT03014089) (**Table 3**).

In the context of **Ebolavirus** vaccines, LNP-encapsulated modified mRNA encoding EBOV GP delivered IM was shown to induce EBOV-specific IgG and neutralizing antibody responses and protected guinea pigs against lethal infection and signs of clinical illness (175). However, no clinical studies employing mRNA vaccines in the context of Ebola virus have been initiated.

Several studies have demonstrated to ability of mRNA vaccines to elicit protective immune responses against **influenza**. Petsch et al. were the first to demonstrate that ID administration of protamine-complexed non-replicating sequence-optimized mRNA vaccines encoding influenza HA was protective in mice upon homologous challenge with influenza H1N1, H3N2, and H5N1 and was immunogenic in ferrets and pigs (124). Furthermore, 10 μg of a comparable HA encoding vaccine delivered IM as LNP formulation elicited functional antibody responses in NHPs, that remained stable over a duration of one year, with HI titer remaining above 1:40 as the surrogate measure of protection in humans (141). A recently published study evaluated the efficacy of LNP-formulated, mRNA vaccines featuring modified nucleotides, that encoded for HA proteins of the potentially pandemic influenza A subtypes H10N8 or H7N9 (159). A single low dose (0.4–10 μg) of H7N9 mRNA vaccine applied ID or IM protected mice from a lethal homologous challenge and reduced lung viral titers were observed upon single-dose ID immunization of ferrets using 10–200 μg. In NHPs, both H10 and H7 mRNA vaccines tested at doses ranging from 200 to 400 μg generated robust HI titers after a single IM or ID immunization which were boosted following the second vaccination. However, upon both H10 and H7 immunization, NHPs that received the 400 μg dose experienced some systemic symptoms (e.g., warm to touch pain at the injection site, injection site irritation, and, in some cases, decreased food consumption) which resolved within 2–3 days. Interim results from a phase I first-in-human, randomized, double-blind, placebo-controlled, dose-ranging study of the H10N8 mRNA vaccine administered IM at a dose of 100 μg in healthy adult subjects (NCT03076385) showed high seroconversion rates, demonstrating robust prophylactic immunity in humans (**Table 3**). Adverse events were mild or moderate with only few severe and non-serious events. Of note, further clinical studies testing the efficacy of a comparable mRNA vaccine format against H7N9 (NCT03345043) and Chikungunya (NCT03325075) are currently ongoing with an estimated primary completion date in September 2018 and 2019, respectively. However, no details of these studies are available as yet.

Overall, these data show that non-replicating LNP-encapsulated mRNA vaccines can induce functional antibody titers at levels associated with protection with acceptable tolerability profiles upon parenteral administration. Future studies that employ LNPs for encapsulation of non-replicating mRNA targeting diverse and more complex antigens are required to demonstrate the broad applicability of this vaccine platform against pathogens posing potential pandemic threats.

CONCLUSIONS

Pandemics such as HIV, Ebola, and Zika have raised the awareness of global threats to human health posed by known as well as newly emerging pathogens and can provide the impetus to prepare against future pandemics by promoting the development of vaccine platforms that can tackle the challenges of outbreak situations. New platforms, such as viral vector and nucleic acid based vaccines meet the prerequisites to provide solutions for some of these challenges by representing highly versatile technologies that allow fast vaccine manufacturing. Each vaccine technology has its own advantages and disadvantages related to its ability to induce certain immune responses, manufacturing capacity and safety for human use (**Table 4**). Viral vector based vaccines are able to induce potent immune responses against the encoded target antigen. Indeed, a number of clinical trials have demonstrated that viral vector based vaccines such as VSV-ZEBOV show great promise for inducing protective responses in humans. However, antigen delivery in the context of an unrelated virus renders this technology relatively complex in terms of manufacturing. Furthermore, the presence of immune targets other than the target antigen can lead to unfavorable effects such as pre-existing immunity hampering immune responses, as seen for Ad5 vectors, or the inability to use the same technology for repeated vaccinations. In addition, delivery of

TABLE 4 | Summarized properties of discussed vaccine technologies.

	Viral vector based vaccines	DNA vaccines	RNA vaccines
Platform versatility	+	+	+
Induction of cellular and humoral immune responses	+	+	+
Fully synthetic vaccine production possible	−	+	+
Delivery as minimal vaccine construct possible*	−	±	+
Repeated vaccine applications possible	±	+	+
Vaccine safety	±	+	++
Immunogenicity demonstrated in clinical studies	+	±	±

**Minimal construct: the vaccine exclusively encodes the target antigen.*

attenuated viral vectors raises safety concerns due to the risk of adverse events and residual viral replication upon delivery, as detected in a small number of subjects in a clinical trial testing VSV-ZEBOV. DNA based vaccines offer the advantage of allowing a relatively simple, fully synthetic production process. While the presence of non-functional sequences in original DNA vectors raised regulatory safety concerns, newer developments allow minimal constructs that exclusively encode for the target antigen. Several studies have demonstrated the safety of DNA vaccines for human use and clinical trials testing vaccines against influenza and Zika have furthermore highlighted the speed of vaccine development supported by this technology. However, the potential for long term persistence and genomic integration and the dependence on injection devices or electroporation represent some important disadvantages of this technology. Some, especially early, clinical studies testing DNA based vaccines have yielded somewhat discouraging results in terms of immunogenicity, while newer trials, such as studies testing DNA vaccines against Zika virus, have demonstrated that this technology is able to induce promising immune responses. Like DNA vaccines, RNA based vaccine technologies support a comparably simple, fully synthetic manufacturing process that allows production of different vaccines using the same established production process and facility. Their inability for genomic integration and lack of persistence in the cells of the vaccinee offers important advantages in terms of vaccine safety. However, since RNA vaccines represent the most recently developed technology described here, their use in humans is less well characterized than for viral vector or DNA based vaccines. Although further studies will be required to fully characterize this technology in humans, clinical studies conducted so far have yielded overall encouraging results in terms of safety and immunogenicity and provide support for further clinical exploration.

While it seems unlikely that a single technology will be able to provide a solution for each future outbreak situation, the combination of present knowledge, ongoing development and the growing understanding of human immunology can provide tools to successfully combat emerging global threats.

AUTHOR CONTRIBUTIONS

SR responsible for writing and coordination of the manuscript. EJ wrote part of the manuscript. KS wrote part of the manuscript. BP discussed the manuscript and reviewed the document.

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REVIEW
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Non-specific Effects of Vaccines Illustrated Through the BCG Example: From Observations to Demonstrations

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Epidemiological studies regarding many successful vaccines suggest that vaccination may lead to a reduction in child mortality and morbidity worldwide, on a grander scale than is attributable to protection against the specific target diseases of these vaccines. These non-specific effects (NSEs) of the Bacille Calmette-Guérin (BCG) vaccine, for instance, implicate adaptive and innate immune mechanisms, with recent evidence suggesting that trained immunity might be a key instrument at play. Collectively referring to the memory-like characteristics of innate immune cells, trained immunity stems from epigenetic reprogramming that these innate immune cells undergo following exposure to a primary stimulus like BCG. The epigenetic changes subsequently regulate cytokine production and cell metabolism and in turn, epigenetic changes are regulated by these effects. Novel -omics technologies, combined with *in vitro* models for trained immunity and other immunological techniques, identify the biological pathways within innate cells that enable training by BCG. Future research should aim to identify biomarkers for vaccine heterologous effects, such that they can be applied to epidemiological studies. Linking biological mechanisms to the reduction in all-cause mortality observed in epidemiological studies will strengthen the evidence in favor of vaccine NSEs. The universal acceptance of these NSEs would demand a re-evaluation of current vaccination policies, such as the childhood vaccination recommendations by the World Health Organization, in order to produce the maximum impact on childhood mortality.

Keywords: BCG, vaccines, epidemiology, non-specific effects, trained immunity, epigenetics

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INTRODUCTION

For over two centuries, vaccines have risen to their place amongst the most significant public health interventions in human history; the introduction of notable vaccines such as the smallpox vaccine, Bacille Calmette-Guérin (BCG), Oral Polio Vaccine (OPV), and measles-containing vaccines have reduced morbidity and mortality worldwide. Smallpox in humans and rinderpest in cattle have been eradicated, and polio is next in line (1).

For many of these vaccines, the exact correlates of protection are still unknown and science has only recently begun to elucidate their biological interactions with the human immune system

(2). There are numerous observations suggesting that the significant public health impact of these vaccines goes beyond simply offering protection against their respective target diseases, especially noted in live vaccines. For example, implementation of measles-containing vaccines in various studies reduces all-cause mortality ranging from 30 to 86%; this depression far exceeds the mortality that is induced by measles illness alone, and is postulated to also reduce bacterial carriage related to sepsis and pneumonia (3). Similarly, the OPV vaccination campaign that was launched in 1998 in Guinea-Bissau was linked to a decreased mortality rate ratio in children under the age of 5, independent of the vaccine efficacy against polio (4). Different heterologous effects due to vaccination, such as protection against unrelated pathogens, anti-tumor properties, and an all-around drop in child mortality have been coined as "off-target" or non-specific effects (NSEs). The NSEs of BCG have been explored for over half of a century now, and BCG has thus become a model of interest for studying such heterologous effects.

Use of the live attenuated BCG vaccine to protect children against severe forms of tuberculosis (TB) became widespread since 1924; a virulent *Mycobacterium bovis* strain was passaged 230 times from 1908 to 1921 on a glycerin, beef bile, and potato medium to obtain BCG (5). The vaccine strain was distributed to different producers (each implementing their own manufacturing procedures) such that many BCG vaccine strains exist today, with varying degrees of attenuation and efficacy against TB (5). The different strains of BCG, and their corresponding diverse target populations, make it difficult to extrapolate epidemiological observations made in one setting toward a more global perspective. Nevertheless, there is growing evidence that supports the presence of NSEs, in both clinical and animal studies.

While pioneer epidemiological studies observe a remarkable reduction in all-cause mortality, the diversity in study designs, inconsistent results, and an inability to firmly correlate BCG NSEs with these results decreases their utility. Similarly, *in vitro* models and animal studies couple possible mechanisms to these puzzling NSEs but fail to demonstrate relevancy in a clinical setting. With novel technologies emerges new scientific evidence regarding NSEs; it is important to strengthen this knowledge and identify new biomarkers that can be used in the clinical setting. The union between molecular biology techniques and epidemiological observations represents the future of vaccine NSE research. Together, these two methodologies could give rise to concrete data that can influence current vaccination policies and optimize existing vaccination schedules.

REVIEW OF THE PAST: EPIDEMIOLOGICAL OBSERVATIONS OF BCG HETEROLOGOUS EFFECTS

Until recent years, the strongest evidence supporting BCG NSEs in humans came from the randomized trials in Guinea-Bissau (2002–2008) (**Table 1**). Due to faulty randomization, the early trial with 105 participants ended in 2004, and then restarted as the main Guinea-Bissau study (18). The main study, like the early study, compared low-birth-weight children who were vaccinated at birth to those who delayed vaccination until 6 weeks of age (19). The investigation, with a sample size of 2,320 infants, revealed that BCG administration as early as possible to newborns of low birth weight corresponds to a reduction in the neonatal mortality rate by over 40% in the first month following immunization, most probably linked to fortifying neonatal defense mechanisms against general septicemia and pneumonia (19, 20).

Prior to this, quasi-randomized studies were confined only to the United States and Canada. A 1933 to 1947 study in Canada examined an indigenous population with high tuberculosis (TB) incidence (**Table 1**) (6). Six hundred and one individuals were observed over 6–14 years after being either vaccinated or not vaccinated within 10 days of birth. This study mainly explored the incidence of death from TB in vaccinated vs. unvaccinated individuals, but also measured death from all causes. There was an observed 6% reduction in the risk ratio (95% CI: −32–33) of all-cause mortality in vaccinated indigenous children vs. unvaccinated, a seemingly small yet significant impact (6). The risk ratio in this case pertains to the cumulative incidence of death occurring in a vaccinated group of individuals vs. an unvaccinated group; the smaller the ratio, the more beneficial vaccination is at reducing the risk of mortality (21). Likewise, from 1935 to 1938, over 3,000 indigenous peoples in the United States took part in a BCG clinical trial which demonstrated a 9% reduction in the risk ratio of death between the vaccinated vs. unvaccinated within a 2-year follow-up period (95% CI: −99–59) (**Table 1**) (7). The decline in mortality in both these cases was not large, and thus, difficult to draw conclusions from. In addition, observations in indigenous communities do not reflect the true diversity of the North American population. In the urban setting of Chicago 451 newborns from households with a TB history were monitored and a 58% reduction in the risk ratio in BCG recipients was observed compared to non-recipients when children were followed up to 13 years (95% CI: −35–87) (**Table 1**) (8). This percentage is much larger than those observed in the indigenous communities. However, some parameters varied between studies: the sample size, the follow-up period used and the prevalence of TB-related deaths.

Aside from these randomized trials, various observational studies were made from the mid-1900s to early 2000s, most notably in Guinea-Bissau, India, Malawi, Papua New Guinea, and Senegal (**Table 1**). These studies generally indicated a beneficial effect of BCG, though the demonstrated reduction in relative risks of all-cause mortality were extremely variable, ranging from 2 to 95%, not to mention that approximately half of these studies occurred in Guinea-Bissau (**Table 1**) (9–17). Despite some studies adjusting for age and gender, such as the Guinea-Bissau studies in the 1980s−1990s, it is difficult to base conclusions off of non-randomized conditions where too many confounders may be unaccounted for. Lastly, although these data suggest an effect in low income settings, no such observations are seen in high income settings (11).

None of the studies showed a difference in protective effects between males and females. The mean age of infants receiving the BCG vaccine also differed between studies, as young as 2 days

TABLE 1 | Summary of epidemiological studies investigating BCG NSEs.

Country (study period)	Sample size	Subject follow-up period	% Reduction in all-cause mortality	Risk of bias	References
Canada (1933–1945)	609	6–14 years	6% (−32; 33)	Moderate	(6)
USA (1935)	3008	2 years	9% (−99; 59)	Moderate	(7)
USA (1941–1960)	451	Up to 13 years	58% (−35; 87)	Moderate	(8)
Benin (1983–1987)	294	4-36 months	32% (−23; 62)	High	(9)
Guinea-Bissau (1984–1996)	1657 + 695 + 4418	6-8 months	37% (−33; 70) 95% (54; 99) 44% (16; 63)	High	(10, 11)
India (1987–1989)	3072	12 months	40% (−97; 82)	High	(12)
Papua New Guinea (1989–1994)	3937	1-6 months	83% (66; 91)	High	(13)
Malawi (1995–1997)	751	8 months	55% (−23; 84)	High	(14)
Senegal (1996–1999)	4421	2 years	2% (−90; 50)	High	(15, 16)
India (1998–2002)	10274	6 months	56% (34; 71)	High	(17)
Guinea-Bissau (early: 2002–2004)	105	1 month	72% (−37; 94)	Low	(18, 19)
(main: 2004–2008)	2343	1 month	45% (11; 66)		

The studies systematically reviewed by the SAGE were determined to be inconclusive, as they did not link the reduction in all-cause mortality with mechanisms of NSEs. Studies assessed as having a very high risk of bias are not included. The percentage of reduction in all-cause mortality consists in a 95% Confidence Interval (CI) further specified by (1-risk ratio). The classification of the risk of bias is as reviewed by the SAGE Working Group.

old to as old as a year (11). Vaccination at earlier time points was associated to a more positive impact by NSEs and a greater reduction in all-cause mortality in non-randomized studies in Guinea-Bissau (1980s—1990s) and Bangladesh (1986–2001). The Guinea-Bissau randomized studies (2002–2008) also supported these observations by comparing low-birth-weight infants of two groups: those vaccinated at birth and those vaccinated at 6 weeks as part of the regular vaccination schedule. In the main study, the risk ratio of death for those vaccinated at birth vs. at 6 weeks was 55% (95% CI: 11–66) (19). Repeated studies, in diverse populations, are required to demonstrate reproducibility and increase confidence in the observations.

Due to the indirectness of many studies in measuring non-TB-related deaths and the large variability among study designs (such as patient follow-up period and national vaccination schedules), the Strategic Advisory Group of Experts on Immunization (SAGE) within the World Health Organization (WHO) has declared in their report that studies about NSEs of BCG (as well as all vaccines in general) are inconclusive (11, 22). After assessing bias using the Cochrane Collaboration tool for randomized studies and a similar tool called ROBINS-I for non-randomized studies, most of the studies supporting BCG NSEs had a high risk of bias, with many confounding factors such as gender, age, child nutritional status, socioeconomic status, and co-interventions or -morbidities (i.e. malaria treatment, impact of other vaccines) (**Table 1**) (11, 23)Despite the verdict, they do acknowledge that the potential for NSEs is present. Work done by immunologists and epidemiologists increasingly support the existence of vaccine heterologous effects. Compared to the inconclusive studies made to investigate NSEs in humans, studies in mice demonstrate biological plausibility for BCG-induced NSEs.

PAINTING THE PRESENT: DEMONSTRATION OF BCG NSES BIOLOGICAL PLAUSIBILITY

Challenge Studies in Mice

As a benchmark model to investigate NSEs, the use of murine models to study BCG allowed the observations of heterologous protection against unrelated pathogens. For example, immunization of specific-pathogen free mice with BCG, or a purified protein derivative of the Mycobacterial antigen tuberculin, displayed an activated macrophage phenotype that produced elevated levels of reactive oxygen species to induce acquired immunity against systemic *Candida albicans* infection. Alleviation of the infection was seen through the reduced load of *Candida* in the spleen, kidneys, and liver, and over a 50% reduction of invasive *Candida* germ tubes (24, 25). Similarly, nude mice were protected from pulmonary schistosomules when challenged with the cercaria of *Schistosoma mansoni* (26). Challenge with other pathogens, including *Babesia* and

Plasmodium infections, also demonstrated protective BCG NSEs in mice (27). These studies suggest that it is indeed possible to obtain immune protection against one pathogen due to prior insult from a dissimilar microbe. Presuming that BCG possesses a hidden capacity to induce broad immune responses, many mechanisms were proposed to support the biological plausibility of these observations and the quest for knowledge extended to human studies.

Possible Mechanisms of Action

For many decades, NSEs have been demonstrated in murine models through primary stimulation with BCG, subsequent challenge with unrelated pathogens and comparing outcomes between vaccinated and unvaccinated animals. *In vivo* murine challenge models are versatile; monitoring immune responses and disease progression can be carried out not only via blood collection, but also via biopsies, bypassing the ethical limitations that human studies face. Only in the last few years have attempts been made to demonstrate mechanistic events responsible for NSEs in humans; with these efforts came a plethora of methods to identify human biomarkers, from cytokine responses to epigenetic indications.

One possible NSE mechanism investigated is the cross-reactivity between vaccine antigens and antigens from unrelated vaccines or pathogens (**Figure 1A**). Despite different origins, some T- and B-cell epitopes may be shared between pathogens (1, 28). However, it is difficult for a relatively rare phenomenon like cross-reactivity to solely account for the extremely diverse heterologous effects seen with BCG. In addition, no particular BCG epitope can yet be connected to protection against non-Mycobacterial species.

An alternative hypothesis proposes that BCG enhances T helper 1 and 17 (Th1, Th17) cell polarization, generation of memory CD4+ T cells, Natural Killer (NK) cell memory, and the corresponding cytokine induction (1, 29, 30). A Dutch study on a small group of BCG-vaccinated young adults demonstrated that non-Mycobacterial stimuli were able to induce heterologous Th1 and Th17 cytokines, such as IFN-γ and IL-17, up to 1 year after receiving the vaccine. Enzyme-linked immunosorbent assays (ELISAs) were used to measure cytokine levels in the plasma (29). It seems that even these antigen-specific memory cells (i.e., memory T and NK cells) can undergo heterologous or "bystander" lymphocyte activation, since memory cells require less signals to be activated upon a second stimulus (**Figure 1A**). Observations in the Dutch study could be attributed to the bystander effect, where BCG components stimulate cytokine production and create a specific cytokine milieu that subsequently leads to existing polyclonal effector T cell (or NK cell) activation or production of antibodies (Abs) by memory B cells (**Figure 1A**). More recently, studies show that a general enhancement of antibody responses could be attributed to the special ability of live attenuated vaccines to promote the production of cytokines by innate cells that favor T follicular helper (Tfh) cell polarization in the germinal center of lymph nodes, promoting affinity maturation of B cells and B cell memory formation (31). In the case of BCG, RNA pathogen-associated molecular patterns (PAMPs), act as markers of microbial viability, and are sensed by Toll-like receptor 8 (TLR8) on monocytes and dendritic cells. Downstream signaling of this receptor-ligand interaction could lead to selective induction of the IL-12p40 component of the IL-12 cytokine and subsequent development of Tfh cells in the lymph node via upregulated IL-12-receptor signaling; killed vaccines do not produce this effect (31). To complement these *in vitro* findings, epidemiological studies identified that hypermorphic TLR8 polymorphisms enhance the protection induced by BCG. Thus, TLR8 could have a central role in the action of live attenuated vaccines in individuals to promote Tfh cell development and subsequent antibody responses to a broad range of pathogens or vaccines. Aside from changes in the cytokine milieu, other mechanisms to nonspecifically activate T cells include the ability of some PAMPs to act as super-antigens, able to activate T cells via non-specific binding on the Vβ chain of TCRs. The rabies vaccine, whose nucleocapsid component possesses super-antigenic properties, could operate in this manner to produce NSEs (32). However, NSE mechanisms such as the bystander effect are unlikely to be the main mode of action in early life because infants lack pre-existing immunity to several microbes, not to mention their humoral responses are still not optimal (22, 33).

Besides bystander activation, the general elevation of cytokine levels, like the increased IFN-γ production by T cells during a primary cell-mediated immune response to BCG, could activate macrophages to boost phagocytic capabilities and thereby protect against secondary bacterial (i.e., pneumococcal) infections (**Figure 1A**) (34). Macrophage phenotyping, using Fluorescence Activated Cell Sorting, demonstrated in young adults that the activation status of macrophages can persist up to a year (29). While this classical cross-protective immune response may play an important role in the reduction of all-cause mortality in infants, it is a short-lived protection that wanes soon after the primary BCG stimulus is cleared from the body. Moreover, BCG-exposed nude mice were protected against heterologous pathogens like *S. mansoni*, indicating that there are also other T-independent, non-classical mechanisms at play.

Trained Immunity

Indeed, despite decades of research indicating the benefits of BCG, the biological processes behind both NSEs and *Mycobacterium*-specific protection are still an enigma (22). A recent immunological paradigm shift sheds light on the black box that is responsible for BCG NSEs, and supports the notion of a T- and Ab-independent mode of action for protection. The dogma has always been that innate immunity lacked immunological memory, while the opposite was true for adaptive immune responses. Recent discoveries in plants, invertebrates and mammals support the notion that innate immune cells do indeed display intrinsic memory characteristics. Such traits have been identified so far in macrophages, monocytes, NK, and other innate lymphoid cells (ILCs) like ILC2s (35). It is of interest to note that NK cells display both antigen-specific memory (as mentioned above) and non-specific memory traits more typical to innate immune cells. Meanwhile, allergen-primed ILC2s, unlike their Th2 cell counterpart, do not have antigen-specific receptors, but respond more strongly to changes in the cytokine

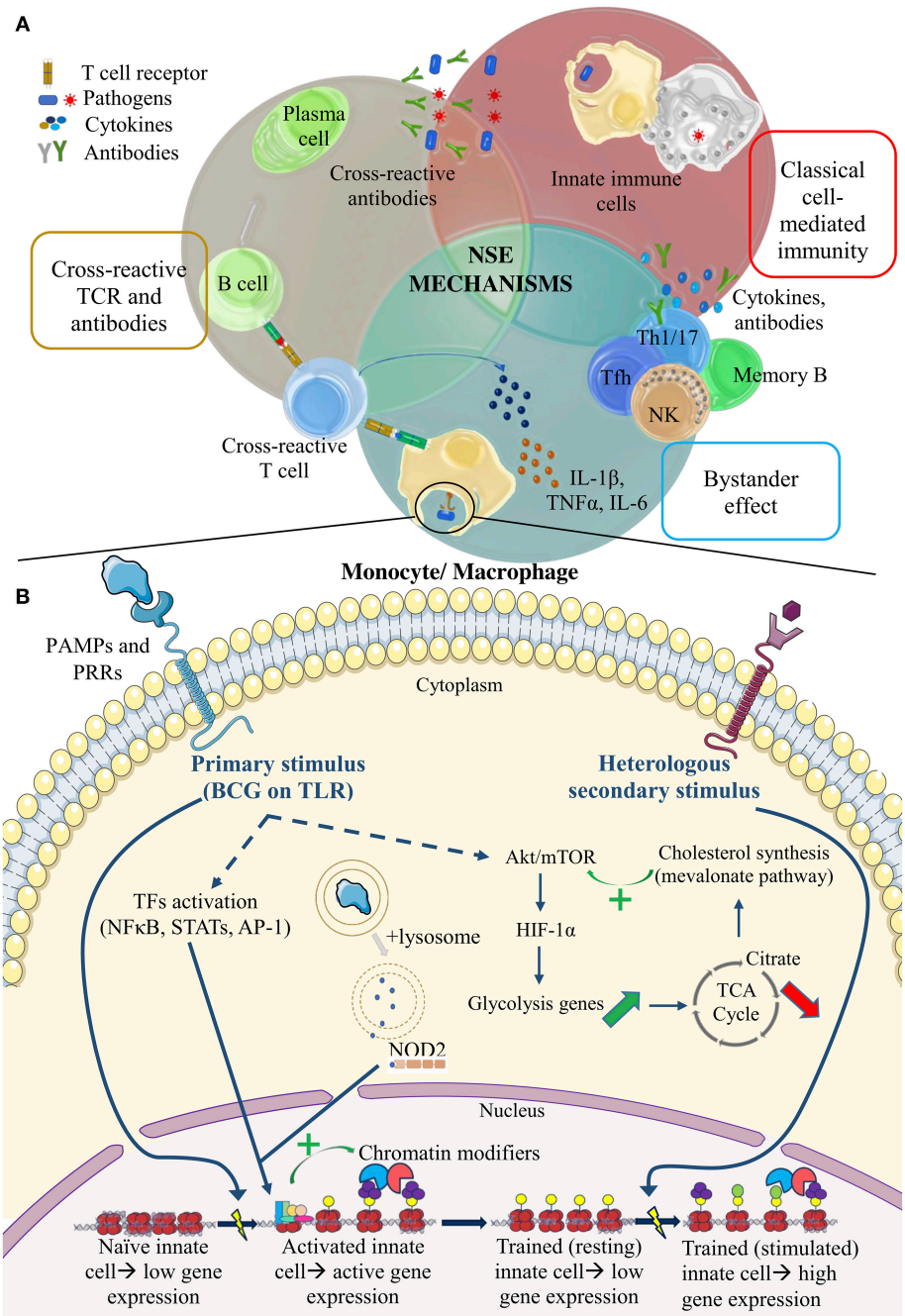

FIGURE 1 | Summary of the potential mechanisms of vaccine NSEs. **(A)** The top half illustrates adaptive mechanisms. Cross-reactive TCRs and antibodies. Lymphocyte antigen receptors recognize similar epitopes from different antigens. Bystander effect. Bystander activation of pre-existing effector or memory cells occurs via changes in the cytokine environment. Classical cell-mediated immunity. Adaptive immune cells potentiate the non-specific activity of innate cells in classical cell-mediated immunity. **(B)** The bottom half illustrates pathways of trained immunity. Primary stimulus of BCG. PRR signaling leads to TF activation, which then recruits chromatin modifiers to genes of interest. This stimulus also activate the autophagy and NOD2 signaling pathway. Upregulation of the Akt/mTOR pathway alters metabolite levels that regulate chromatin-modifying enzymes. Heterologous secondary stimulus. Epigenetic changes within innate cells after training act as de novo enhancers to boost the immune response against a secondary challenge.

environment upon secondary stimulation (like the bystander effect). ILCs demonstrate innate-like memory by responding to IL-33 induction rather than to a specific antigen, resulting in increased proliferation, and IL-5 and IL-13 production (36).

This memory-like phenomenon of innate cells, also referred to as trained immunity, received its namesake from the concept that innate cells encountering a vaccine or microbial component can be influenced or "trained" by a primary stimulus to

improve responsiveness to an unrelated secondary stimulus. The development of an *in vitro* experimental model for trained immunity in primary monocytes paved the way to explore this groundbreaking concept in human cells. The technique has been optimized to investigate the ability of commonly studied stimuli, like BCG, to induce innate immune memory-like response in human monocytes (37). The protocol involves a primary stimulation with BCG to "train" monocytes isolated from healthy donor blood, followed by a resting period for the cells, and then re-stimulation with a stimulus like lipopolysaccharide (LPS), which mimics a secondary heterologous challenge (37). BCG immunization prior to collection of primary cells could allow for *ex vivo* studies as well. At the end of the protocol, cells from either *in vitro* or *ex vivo* approaches can then be harvested and examined for changes in epigenetic traits, proliferation, cytokine production, and metabolic characteristics such as glycolysis or induction of autophagy.

Currently, the proposed mechanism for trained immunity involves the epigenetic reprogramming of innate immune cells, like monocytes, that have been activated by a microbial or vaccine component, and subsequently induced to produce broadly acting cytokines (i.e., TNFα, IL-6, IL-1β, IL-10). Depending on the nature of the primary stimulus, it can allow for the activation of transcription factors (TFs) like NFκB, AP-1, and STATs in myeloid cells, that bind to enhancers and promoters of pro-inflammatory or anti-inflammatory genes, downregulating some of them while upregulating others (38). The TFs allow for the recruitment of RNA polymerase and chromatin modifiers to regulate gene expression (**Figure 1B**). This phenomenon could lead to the creation of latent or "*de novo*" enhancers, which are genetic regulatory elements that acquire enhancer-like epigenetic modifications in response to specific stimuli (**Figure 1B**). Even, when the immune response clears the primary stimulus, some of these more stable modifications (i.e., methylation or acetylation) remain. As a result of their upgraded epigenetic status, innate immune cells are able to respond more vigorously to a secondary stimulus (**Figure 1B**). Immunization with BCG has also been shown to induce epigenetic changes in human monocytes *ex vivo* (35).

Another way to explore human trained immunity by BCG was recently tested, where both primary and secondary stimulation of the immune system are done *in vivo* with BCG and the yellow fever vaccine (YFV). Yellow fever vaccine is a rare opportunity to perform "challenge studies" in humans, since it is a live attenuated vaccine already in human use with detectable, post-vaccination viremia (39). While BCG-induced trained immunity was able to reduce YFV viremia, it did not seem to interfere with the efficacy of the second vaccine, as YFV-induced humoral responses were unaltered by BCG. These results thus support the clinical relevance of BCG trained immunity; it protects against a secondary viral stimulus without interfering with adaptive mechanisms that are also in play (39).

In the case of the YFV "challenge" model, IL-1β was noted to be an especially important cytokine to achieve a more robust innate immune response against the secondary YFV insult following BCG vaccination (39). Its role as a mediator in monocyte trained immunity was supported by both epigenetics and immunological methods. Chromatin immunoprecipitation sequencing (ChIP-seq) was used to detect genome-wide changes in the distribution of histone markers denoting enhancers or active promoters, particularly acetylation on histone 3 lysine 27 (H3K27ac); these activation marks were upregulated in the regulatory elements of pro-inflammatory cytokine genes, such as IL-1β, IL-6, and TNFα. In line with previous findings, genes for Akt/mTOR (mammalian target of rapamycin) kinases and NOD2 (nucleotide-binding oligomerization domain-containing 2) were also epigenetically marked and corresponded to the upregulation in cytokine production *ex vivo*, measured via ELISA. With viral challenge upon administration of YFV, individuals vaccinated with BCG a month earlier exhibited lower YFV viremia. Increased IL-1β levels, in particular, correlated with reduced YFV viremia (25, 39). This challenge model paves the way for future studies that must further investigate mechanisms of trained immunity, which were first identified using *in vitro* and *ex vivo* approaches, in a clinical context and confirm these results.

These findings also demonstrate a classic case of how "-omics" technologies have revolutionized the study of vaccine NSEs, providing a "global" view of changes that cells undergo upon receiving external signals from their environment, whether it be at the genomic, epigenomic, transcriptomic, metabolomic, or proteomic level (**Figure 2**) (1). In this case, genome-wide epigenetic studies have greatly expanded the capacity to identify molecular pathways that may have a key role in monocyte training after BCG immunization, such as proinflammatory cytokine induction, NOD2 pattern recognition receptor (PRR) signaling, and upregulation of the Akt/mTOR signaling pathway that regulates glycolysis (39). Performing -omics studies on cells trained *in vitro* provides insight on potential biomarkers associated to protection.

Other studies also employed epigenetics to elucidate the role of immunometabolism in trained immunity (40, 41). ChIP analysis of BCG-stimulated monocytes revealed more H3K4me3 and less H3K9me3, markers of active euchromatin and suppressive heterochromatin respectively, on promoters of glycolysis genes. Downstream effects of these epigenetic changes on transcription were confirmed via quantitative reverse transcription polymerase chain reaction (RT-qPCR). A similar pattern was seen on the *IL6* and *TNFA* promoters. Notably, the marks on these pro-inflammatory cytokine genes returned to baseline when inhibitors of glycolysis were used, like rapamycin. Therefore, while BCG-induced training is required to alter cell metabolism, these metabolic changes in turn seem to be necessary to maintain a trained phenotype (41).

Metabolic characterization of trained cells was thus performed as follow-up. Glycolytic rate was determined by assessing the glucose consumption from cell culture supernatant, lactate production (the end-product of glycolysis) was measured as a function of the extracellular acidification rate and oxidative phosphorylation was detected via the cells' oxygen consumption rate using the Seahorse Cell Mito Stress Test Kit and other fluorometric assays (41). Pro-inflammatory (M1) macrophages have a tendency toward increased glycolysis and lactate production, while anti-inflammatory (M2) macrophages appear to depend more on oxidative phosphorylation and lipid

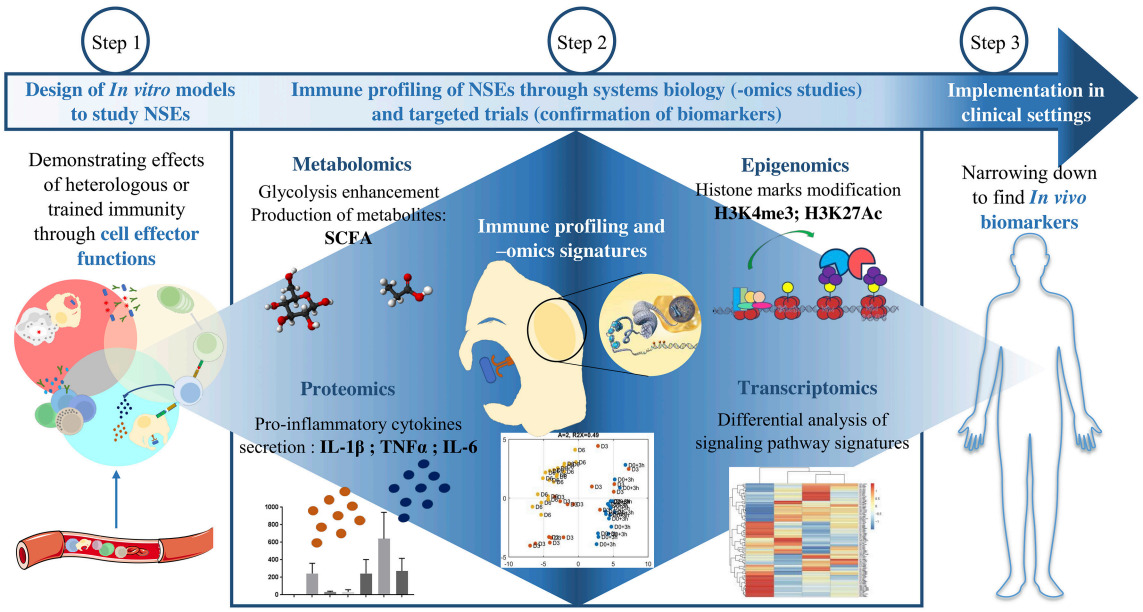

FIGURE 2 | Approaches to investigate BCG NSEs. After designing a model to study trained innate immunity *in vitro*, -omics studies help to identify the global cellular pathways involved. The variations in gene expression are assessed both at the transcriptomic level and at the epigenetic level and then confirmed at the protein level. The modifications of cell metabolism and effector functions are also various angles by which to grant NSEs biological plausibility. The last step consists in narrowing down these modifications in order to find protective biomarkers which can be applied *in vivo* in clinical settings.

metabolism. Similarly, monocytes and macrophages trained with BCG shift to an M1-like phenotype (**Figure 1B**). Glucose consumption increased upon secondary stimulation of these cells, mirrored by a proportional increase in lactate. Notably, the ensuing induction in pro-inflammatory cytokines upon a secondary stimulus was abrogated when blocking the Akt/mTOR pathway during BCG training.

Growing evidence proposes that BCG training and subsequent metabolic changes fuel each other in a regulatory loop. Mevalonate (an intermediate in the cholesterol synthesis pathway) and fumarate (involved in glutamine metabolism), are measurable metabolites that regulate trained immunity through enhanced Akt/mTOR signaling by promoting signaling through the insulin-like growth factor 1 (IGF1) receptor and regulating chromatin methylation, respectively (**Figure 1B**) (42). The exact links connecting metabolic changes to trained immunity are still under investigation. It has been described that the enhancement of glycolysis led to an accumulation of Krebs cycle substrates such as fumarate or succinate. These metabolites were shown to stabilize HIF-1α (hypoxia-inducible factor 1α), a TF downstream of Akt/mTOR signaling that upregulates glycolytic genes and IL-1β production (**Figure 1B**). Fumarate has also been described in the inhibition of histone demethylases, namely KDM5 (43).Lactate, which is the end product of glycolysis has also been demonstrated to inhibit histone deacetylase (HDAC). Together these two observations suggest that shifting the metabolic balance toward glycolysis will increase the proportion of some metabolites which in turn will bring the necessary chemical groups to chromatin-modifying enzymes and thereby enable epigenetic modifications associated with trained immune features. Glycolysis inhibition studies were also performed and resulted in an impairment of trained immunity induced by BCG or other stimulants like β-glucans. Furthermore, direct fumarate supplementation led to β-glucan-like transcriptional profiles of monocytes although fumarate alone was not able to reproduce all the modifications implicated in the trained immunity profile. Moreover, increasing glycolytic flux will also fuels lipogenic pathways such as cholesterol biosynthesis of which mevalonate is an intermediate metabolite. Mevalonate, an intermediate metabolite in the cholesterol biosynthesis pathway, has been described as a key regulatory factor in BCG-stimulated trained immunity as the inhibition of its biosynthesis through statins impaired training of monocytes *in vitro* (44).The extreme intricacy of metabolic shifts and cellular functional responses is due to the high number of interconnected pathways regulated by positive and negative feedback loops (itaconate pathway for instance) (45). The precise role and causality of these molecular and cellular networks will be further investigated in the coming years in order to better understand immunometabolism in the context of trained innate immunity.

At least in the case of BCG, *in vitro* models also demonstrated that autophagy of these cells is a vital component of trained immunity. Independently, NOD2 stimulation with PAMPs was also shown to be required (25). Autophagosomes may thus help to process PAMPs, such as muramyl dipeptide from BCG, to be recognized by the NOD2 intracellular PRR (**Figure 1B**). This event would lead to a downstream signaling cascade and induction of epigenetic changes, such as the H3K4me3 that

was previously described to upregulate pro-inflammatory genes (**Figure 1B**) (25, 46). Suboptimal induction of autophagy via drug inhibitors and certain autophagy-related gene polymorphisms led to the absence of epigenetic modifications seen after BCG stimulation (47). Autophagy activity was assessed through measuring levels of mediators such as autophagy-related proteins LC3A and LC3B, that are involved in autophagosome formation and may be useful as biomarkers of trained immunity by BCG (47).

It was recently demonstrated that BCG can act even in the early stages of myelopoiesis and instruct hematopoietic stem and progenitor cells (HSPCs) in the bone marrow to develop into monocytes and macrophages with a specific epigenetic program. Training of HSPCs may be a vital part of vaccine NSEs, influencing mature myeloid cell function in peripheral organs (48, 49). Both epidemiological and proof-of-concept *in vitro* studies demonstrated that the trained phenotype persists in human monocytes from 3 to 12 months following BCG immunization, in contrast to the usual lifespan of circulating monocytes of up to a day (29, 35).

BCG-Induced Immune Responses in Favor of NSEs

Overall, *in vitro* models and *ex vivo* studies of human immune cells help conceive a general scheme of the immune response in individuals vaccinated with BCG and how it relates to trained immunity, as well as other NSE mechanisms. On the innate arm of the immune response, NK cells, and monocytes undergo epigenetic reprogramming, with an upregulation of permissive H3K4me3 and downregulation of inhibitory H3K9me3 histone marks on the genes of pro-inflammatory cytokines and regulators of glycolysis, among other targets in the genome. Muramyl dipeptide of BCG may have a vital role to play in inducing these changes, acting as a ligand for NOD2-dependent PRR signaling in monocytes and NK cells. Using an *in vitro* model of trained immunity, it was demonstrated that human peripheral blood mononuclear cells (PBMCs) trained with BCG resulted in only minor changes in cell size and morphology, but produced a 4-5-fold increase in IL-6 and TNF-α in response to secondary challenges with LPS or the synthetic lipopeptide Pam3Cys, 6 days later (37, 50). PBMCs isolated from BCG-immunized adults also produced elevated TNF-α and IL-1β levels after secondary challenge 3 months post-vaccination with sonicated *Mycobacterium tuberculosis* or heterologous pathogens such as heat-killed *S. aureus* or *C. albicans* (29).

The heightened capacity to produce innate pro-inflammatory cytokines was even observed after secondary challenge with *E. coli* LPS in monocytes isolated 1 year after immunization. These one-year post-vaccination monocytes expressed elevated levels of activation markers such as CD14, CD11b, TLR4, and mannose receptors, which suggest that BCG-mediated NSEs may be the result of long-term changes in innate immune cell phenotype that allow for non-specific protection against heterologous pathogens (29, 50). In contrast, the heightened expression of activation markers and pro-inflammatory cytokines in response to heterologous challenge was not observed in monocytes isolated from unvaccinated individuals (29, 50).

Maintenance of long-term NSEs induced by BCG may also depend on adaptive mechanisms, in addition to trained immunity. Although, innate immune activation markers were elevated against LPS secondary challenge 1 year post-vaccination, this was not the case for other non-Mycobacterial challenges, such as *C. albicans* and *S. aureus*, where the immune response resembled that which would be mounted against a primary infection. BCG-induced bystander activation of CD4+ T cells can complement trained immunity to prolong NSEs. In the same study by Kleinnijenhuis et al. Th1 and Th17 cytokine (IFN-γ, IL-17, IL-22) levels remained elevated in PBMCs 1 year after BCG immunization, regardless of which secondary non-Mycobacterial stimuli was provided. Unlike these heterologous cell-mediated responses, non-specific antibody responses induced by BCG vaccination are underexamined. Some studies suggest that BCG immunization at birth contributes to humoral responses from subsequent vaccination with *Haemophilus Influenzae* type b, pertussis, hepatitis B and pneumococcal antigens, but the duration and extent of protection remain controversial due to differences in countries' vaccination schedules and the BCG strain used (51–53). Therefore, further investigation into Ab-mediated NSEs with comparable study designs need to be conducted, especially considering that other studies demonstrate a different outcome, where BCG immunization has no impact or a negative effect on Ab production to other vaccines (34, 53).

In addition, it is also important to consider that trained immunity has mainly been characterized in adult monocytes. As in adult *ex vivo* studies, CD4+ T cell proliferation and cytokine responses appear to be enhanced in infant monocytes *ex vivo*, as infants vaccinated with BCG at early time points (0–2 months old) demonstrated elevated levels of lymphocyte proliferation and Th1/Th2 cytokines compared to unvaccinated infants or those vaccinated at later time points (20, 51, 53, 54). As with potential BCG-induced humoral NSEs, the extent of BCG-induced trained immunity in infants remains controversial and varies with each study's host country, their extended programs of immunization and the immunogenicity of the strain of BCG used. For example, elevated monocyte cell surface activation markers were not observed after heterologous challenge with LPS, synthetic lipopeptide Pam3CSK4, *C. albicans* or *S. aureus* on whole blood isolated from infants immunized with BCG at 6 weeks (30). However, in this study, challenge with Pam3CSK4 did indeed result in a significant upregulation of the CD69 activation marker on NK cells, suggesting NK cells may play a role in BCG NSEs, as in adults. Another study closely resembled adult studies, where whole blood samples from low-birth-weight infants vaccinated with BCG at birth in Guinea-Bissau were challenged with Pam3CSK4 a month later. Elevated levels of IL-1β, TNF-α, IL-6, and IFN-γ were observed (20).

One could therefore observe some promising results of innate immune response potentiation and elevated CD4+ T cell responses in favor of BCG NSEs in both infants and adults. A complex interplay between epigenetic reprogramming, cell metabolism and innate immune machinery enables BCG-trained cells to respond more robustly against a second heterologous

insult. Complementing innate immune cell potentiation is the potential for BCG to induce heterologous T cell responses in vaccinated individuals. The interplay between innate and adaptive immune mechanisms could be responsible for the long-term NSEs that in some cases have persisted years after vaccination; while trained immunity may play a larger role in NSEs in early life, adaptive mechanisms can maintain NSEs even when training immunity starts waning (53). However, the duration of BCG-induced trained immunity in infants is still unclear and requires further investigation. To better characterize BCG NSEs, it is important to identify which of the described cellular changes can be used as biomarkers of protection, and then employ them in clinical studies that supplement epidemiological studies monitoring all-cause mortality (**Figure 2**). Finally, biomarkers should be standardized and thresholds should be made, so that results between studies can be compared. Following the systematic review done by the SAGE Working Group, improved study designs to investigate BCG NSEs are being used as a means to achieve these objectives.

TOWARD THE FUTURE: NEW AND IMPROVED STUDIES

As basic science uncovers the mysteries behind BCG's mechanisms of action and proposes how the vaccine can induce non-specific protective effects, it is now important to apply these findings to explain the reduction in all-cause mortality observed in many epidemiological studies. Given the laboratory findings thus far, trained immunity, in combination with adaptive mechanisms, likely have a role to play in BCG NSEs. In order to produce stronger evidence of the heterologous benefits of BCG at a population level, more recent epidemiological studies aim to characterize differences in the immune response of BCG-vaccinated vs. unvaccinated individuals.

Prior to a decade ago, the only randomized trials, as well as a large portion of observational cohort studies, were conducted in Guinea-Bissau and other areas of West Africa. Meanwhile, the quasi-randomized studies performed in North America over 50 years ago were the only studies done in high income countries that were reviewed by the SAGE NSE Working Group from 2012 to 2014, when they conducted a systematic review of epidemiological data that may support the impact of BCG and other vaccines on infant all-cause mortality. The Working Group acknowledged that high mortality settings like Guinea-Bissau are often required for mortality studies, in order to obtain an adequate study power and detect relative effects. On the other hand, in these countries it is difficult to draw inferences due to the difficulty of controlling for all of the confounders affecting mortality (11). Recent studies conducted in both high income and low mortality, as well as low income and high mortality, settings aimed to counter this issue raised by the SAGE (**Table 2**).

For example, the Danish Calmette study, running from 2012 to 2015, randomized over 4,000 infants into those vaccinated with BCG or those who followed the normal Danish vaccination schedule and did not receive BCG. However, this study did not show any added effect of BCG on all-cause hospitalization (55, 56). Meanwhile, a retrospective cohort study looking back to hospitalization rates in Spain from 1997 to 2011, using the Official Spanish Registry of Hospitalizations, favored the opposite outcome; a randomized clinical trial in Uganda monitored clinical illness in 560 (either BCG-vaccinated or non-vaccinated) neonates and demonstrated similar results to those in Spain (**Table 2**) (57, 59). Varying results may be due to study design, variable strains of BCG, the sample population, and other unknowns. To eliminate as many confounders as possible that may influence the ability to detect BCG NSEs, it is no wonder that the SAGE demands that studies be done in more diverse populations, yet in more controlled settings.

Moreover, the Working Group's evaluation from 2012 to 2014 ascertained that although epidemiological studies up until then examined all-cause mortality in relation to BCG, they failed to examine in any depth the mechanisms behind the reduction in mortality. However, they admit that death by TB is secondary in infants compared to sepsis, pneumonia, and diarrhea, so the significant impact that BCG has on all-cause mortality is unlikely to stem only from TB-specific protection (11). Studies are now trying to identify immunological endpoints during clinical studies that may explain NSEs in the population. A more recent randomized trial in Guinea-Bissau not only monitored all-cause mortality of low-birth-weight infants, but also performed whole-blood assays to measure cytokine levels in response to heterologous secondary challenge after BCG vaccination (**Table 2**). Results were promising; elevated levels of IL-1β, IL-6, TNF-α, and IFN-γ following *ex vivo* secondary heterologous challenges of whole blood from BCG-vaccinated infants, compared to unvaccinated infants mirrored a 30% reduction in the neonatal mortality rate (20, 58).

NSES FROM OTHER VACCINES

After discussing the underlying mechanisms of BCG NSEs, it is important to note that NSEs have been studied in other vaccines as well. As mentioned earlier, the epidemiology of measles-containing vaccines and OPV have also been analyzed for the presence of NSEs. Measles-based vaccines have been observed to greatly reduce child mortality in low income communities in Haiti (3, 60). In the case of a vaccine targeting measles, a reduction in all-cause mortality would seem logical, given that the measles virus itself induces transient immunosuppression in infected individuals; immunization is postulated to prevent ablation of the previously existing memory T and B cell repertoire within an individual caused by measles virus infection (61). However, certain observations suggest that other mechanisms besides protection from measles virus-induced immunosuppression are responsible for these NSEs. For instance, a reduction in morbidity is observed in regions where the measles virus has already been eliminated, not to mention an increase in NSEs is observed even in the presence of maternally derived antibodies that could interfere with the efficacy of the live vaccine (62, 63).

While live attenuated vaccines in humans appear to have non-specific protective effects, the same has yet to be observed

TABLE 2 | Recent studies on BCG NSEs following the SAGE working group's systematic review on vaccine NSEs to associate the reduction in all-cause mortality with immunological outcomes.

Country (study period)	Type of study	Subject follow-up period	% Reduction in all-cause mortality or morbidity (95% CI)	Testing for NSEs	References
Denmark (2012-2015)	Randomized	15 months	No significant reduction	Potentiating effect of BCG on cytokine production (TNF-α, IL-6, IL-10)	(55, 56)
Uganda (2015-)	Randomized	10 weeks	Unpublished	IL-1β, IL-6, IL-10, TNF-α, and IFN-γ cytokine levels after secondary stimulation. H3K4me3 on cytokine genes in peripheral blood monocytes.	(57) (results on mortality unpublished)
Guinea-Bissau (2008-2013)	Randomized	12 months	30% (−4; 53)	Measure increases in responses to heterologous stimulation (elevated IL-1β, IL-6, TNF-α, and IFN-γ response)	(20, 58)
Spain (2015)	Retrospective cohort	N/A	41.4% (40.3; 42.5)	None	(59)

with killed or subunit vaccines. In fact, the opposite effect could be induced; Diphtheria-Tetanus-Pertussis (DTP)-vaccination of children, especially girls, has resulted in an increase in all-cause mortality, notably when administered simultaneously or before live attenuated vaccines such as measles-containing vaccines (64). The global analysis conducted by the SAGE Working Group, which excluded studies with poorly defined controls, led to firmer evidence that there may indeed by an increase in all-cause mortality in DTP-vaccinated children, mostly observed in Guinea-Bissau (1, 11). Therefore, while vaccines like BCG and measles exhibit potentially beneficial NSEs that reduce all-cause mortality, the reverse can also be true, where vaccine NSEs could pose as a threat to public health. There is thus a dire need to continue with randomized trials, resolve contradictions between the various studies of vaccine NSEs and produce more convincing evidence for their existence, such that public health authorities can confidently act on the information.

Moreover, the role of NSEs on public health could be expanded further beyond its impact on child mortality, which was the focus of the BCG, measles and DTP epidemiological studies presented above. In fact, NSEs could even exist outside the scope of human vaccines, as observations have also been noted in the veterinary field. The rabies vaccine, like BCG in humans, was also observed to reduce all-cause mortality in dogs via non-specific protection (32). In a low-income region of South Africa, an observational study was conducted from 2012 to 2015 in owned, free-roaming dogs to study the impact of rabies vaccination. Vaccination against rabies is provided free annually in South Africa as a disease control measure, rendering the region a good setting to study the impact of the vaccine; public vaccination campaigns in part reduce the bias that the NSEs observed in rabies-vaccinated dogs may be attributed to a generally better quality of life with more nutrition and visits to the vet compared to unvaccinated dogs. Overall, these observations must still be validated with an improved study design, through the implementation of randomized controlled clinical studies, the elimination of confounding factors such as owner behavior and accessibility of veterinary care, and segregating reduction in mortality due to rabies and similar lyssaviruses from heterologous etiological agents. Unlike the case with human vaccines, rabies is a killed vaccine that appears to have protective NSEs.

CONCLUSION

In conclusion, heterologous effects of vaccines are coming to be realized as a real phenomenon by immunologists and epidemiologists, with mounting evidence suggesting biological plausibility. While the WHO's current position on the matter asserts that there is poor and biased evidence supporting vaccine NSEs, they do acknowledge the high plausibility that NSEs may be one of the puppeteers behind the triumphant story of vaccines. Thus, they call for the need to further solidify a biological basis and better understand the public health impact, such as through determining a specific vaccination schedule and the resulting proportion of deaths that would actually be prevented in each population (11).

As future vaccine studies evolve to meet these expectations, NSEs have the potential to influence the current WHO recommendations on childhood vaccination. Policy changes would not be made based on the question of whether vaccines should continue to be used universally or not, but to influence the timing, sequence or co-administration of these vaccines according to the existence of both protective and harmful NSEs affecting general childhood death rates (11). Recommendations would therefore also address the use of vaccines whose target disease is already eliminated, but may confer non-specific protection to other diseases (46).

The universal acceptance of vaccine NSEs would advance more easily once supranational organizations like the WHO move in favor of their existence. In the case that a new, more efficacious vaccine against TB is developed, it will become important to determine whether this vaccine can also confer NSEs, and whether BCG should still remain in use due to its reduction in all-cause mortality in early life. Vaccination policies need be restructured to provide the maximum benefit, both specific and non-specific. For example, observational studies regarding DTP NSEs clash; some groups suggest

DTP is beneficial while others demonstrate it has deleterious heterologous effects on all-cause mortality, such as through increased incidence of rotavirus infection in girls (11, 65). Although, the SAGE judged it as insufficient evidence to endorse a policy change, results from Bangladesh, India, and Senegal contradict the current WHO recommended schedule of administering DTP after BCG, and instead imply that simultaneous administration of these vaccines may reduce deleterious effects and lower mortality (12).

The incorporation of NSEs into vaccinology would also greatly impact vaccine design; new vaccine technologies can aim to prime both non-specific and pathogen-specific immunity. Many adjuvants already have innate immune targets, and perhaps NSEs can provide enlightenment on their mode of action. Primary endpoints for vaccine efficacy trials may now include innate immune markers as correlates of protection, in addition to adaptive markers like antibody titers or IFN-γ. In addition, the trained immunity concept adds a new chapter to macrophage biology. Previously, depending on the immunological context, such as during infection or in the tumor microenvironment, macrophages are delineated into subsets like "M1" and "M2," or myeloid-derived suppressor cells, without any universal classification for these cells that can span across various disciplines. More recently, a collective framework to describe activated macrophages was proposed, based on cell markers, how these cells were isolated, and the immunostimulants used to activate them (66). For example, "M1" and "M2" could now be referred to as M(IFN-γ) and M(IL-4), based on their activators. Once the trained immunity concept gains ground, it would be interesting to see how it fits into this grander scheme of macrophage nomenclature, taking into account how trained immunity can integrate the various terminologies used across studies. Trained immunity may also have important implications in countering immuno-senescence, where traditional adaptive immunity wanes in the elderly (46). As is the case with BCG being an effective therapy against bladder cancer, NSEs of vaccines can extend beyond protection against infectious diseases; by broadly manipulating the immune system, these vaccines can serve as therapeutics against other ailments, such as cancer and immunodeficiency. The reverse is also true, though; while NSEs can ameliorate immunodeficiency, they can also potentiate autoimmune conditions.

Innovative vaccine designs, that take advantage of NSEs, can also be applied to the "One World One Health" initiative. Protecting animal companions from maladies is an improvement to the overall public health of society, both by reducing health care costs for animals and by decreasing the incidence of zoonotic disease transmission as is the case with rabies (67). The use of such public health measures could also serve as an alternative to antibiotic use to protect production animals against infectious diseases (68, 69). Furthermore, vaccines that can provide early and broad protection via trained immunity would greatly benefit the agricultural industry, where for instance many chicks, piglets and calves are lost each year due to the inefficacy of many vaccines in very early life. Factors such as maternal antibody interference, an immature adaptive immune system (which in mammals becomes apparent after weaning), or even the diversity of farming practices and environmental conditions are at play in this period of an animal's development.

Overall, the vaccine NSE concept has the potential to improve both human and veterinary vaccinology and provide new means to answer both immunological and public health questions. NSEs can provide new concepts to assess what were previously invisible influences on mortality, and also combat challenging diseases. Addressing the NSE question will provide new insights on mechanisms through which existing vaccines work and may influence future vaccine design. The knowledge gained throughout the investigation of NSEs could lead to modifications of global vaccination policies to optimize the benefits of vaccination in reducing childhood mortality and morbidity.

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DU and KD conceived the outline of the manuscript. DU wrote the manuscript. DU, SP, and KD edited the figures of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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HLA Class II Genes *HLA-DRB1*, *HLA-DPB1*, and *HLA-DQB1* Are Associated With the Antibody Response to Inactivated Japanese Encephalitis Vaccine

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Aim: The objective of this study was to evaluate the association of the human leukocyte antigen (HLA) class II genes *HLA-DRB1*, *HLA-DPB1*, and *HLA-DQB1* with the humoral immune response elicited by inactivated Japanese encephalitis (JE) vaccine (IJEV).

Methods: A total of 373 individuals aged 3–12 years in the Inner Mongolia Autonomous Region in China, who received two doses of IJEV at 0 and 7 days, were enrolled in the current study. Based on the individuals' specific JE virus (JEV)-neutralizing antibodies (NAbs), they were divided into a seropositive and a seronegative group. *HLA-DRB1*, *HLA-DPB1*, and *HLA-DQB1* were genotyped using a sequencing-based typing method. Next, the association of the HLA class II genes and their haplotypes with antibody response was evaluated.

Results: Based on NAbs, a total of 161 individuals were classified as seropositive and 212 as seronegative. *DQB1*02:01* was significantly associated with JEV seropositivity ($P < 0.001$, OR = 0.364, 95% CI: 0.221–0.600), while *DQB1*02:02* was significantly associated with JEV seronegativity ($P = 5.03 \times 10^{-6}$, OR = 7.341, 95% CI: 2.876–18.736). The haplotypes *DRB1*07:01-DPB1*04:01-DQB1*02:01*, *DRB1*15:01-DPB1*02:01-DQB1*06:02*, *DRB1*07:01-DQB1*02:01*, and *DPB1*02:01-DQB1*06:02* were very frequent in the seropositive group, while *DRB1*07:01-DPB1*17:01-DQB1*02:02*, *DRB1*07:01-DQB1*02:02*, and *DPB1*17:01-DQB1*02:02* were very frequent in the seronegative group. The presence of *DRB1*01:01*, *DRB1*04:05*, *DRB1*09:01*, *DRB1*12:02*, *DRB1*13:02*, and *DRB1*14:01* was associated with a higher geometric mean titer (GMT) of NAbs than that of *DRB1*11:01* at the *DRB1* locus ($P < 0.05$). At the *DPB1* locus, the presence of *DPB1*05:01* was associated with higher GMTs than that of *DPB1*02:01*

and *DPB1*13:01* ($P < 0.05$), and the presence of *DPB1*04:01* and *DPB1*09:01* was associated with higher GMTs than that of *DPB1*13:01* ($P < 0.05$).

Conclusions: The present study suggests that HLA class II genes may influence the antibody response to IJEV.

Keywords: human leukocyte antigen class II genes, HLA haplotype, inactivated Japanese encephalitis virus vaccine, antibody immune response, association

INTRODUCTION

Japanese encephalitis (JE) is one of the most serious mosquito-borne infectious diseases, with approximately 67,900 individuals being infected by the JE virus (JEV) annually (1). Approximately 75% of these individuals are under 14 years of age, and 50% of the infections occur in China (2, 3).

Vaccination is an efficient method of controlling JEV infection. Four different types of JE vaccine are available in affected countries, namely inactivated mouse brain-derived, live attenuated cell culture-derived, inactivated cell culture-derived, and genetically engineered live attenuated chimeric vaccine. The Vero cell-derived inactivated JE vaccine (IJEV) has been widely used in China, Japan, the US, Europe, Canada, Australia, Hong Kong, Switzerland, and India (4–6). A JEV-neutralizing antibody (NAb) titer of at least 10 has been established as a correlate for protection against JEV, while positive serum conversion rate and geometric mean titer (GMT) have been used as alternative markers of efficacy of JE vaccines (7, 8). After immunization with the JE vaccine, the positive serum conversion rate ranges from 60 to 100% (1, 9). Vaccine efficacy may be influenced by factors such as the type of vaccine and the vaccinated person's age, gender, and nutritional status (6, 10). Several studies have reported that the efficacy of attenuated JE vaccine has reached 85–99.26% in Chinese, South Korean, and Nepalese children; however, it exhibited only 67.2% efficacy in Indians after primary immunization (11–14). These results indicate that different genetic backgrounds of hosts could play an important role in the efficacy of JE vaccines.

As one of the key immune gene complexes, the human leukocyte antigen (HLA) genes play an important role in the adaptive immune response to viruses and vaccines. HLA molecules are divided into three classes: class I, II, and III. Among them, HLA class II molecules (HLA-DR, -DQ, and -DP) bind to extracellular viral antigen peptides and display them on the surface of antigen-presenting cells to $CD4^+$ cells to stimulate their multiplication, which, in turn, stimulate antibody-producing B cells to produce specific antibodies (15, 16). HLA genes exhibit extraordinary polymorphisms, and different alleles can affect the peptide-binding properties of the HLA molecular pocket, which subsequently influences the immune response to a vaccine. In 2005 and 2006, Ovsyannikova et al. (17, 18) observed that *HLA-DPB1*03:01*, *HLA-DPB1*04:01*, and *HLA-DPB1*15:01* are associated with rubella vaccine-induced antibodies. On the other hand, the *HLA-DRB1*15/16-DQB1*06-DPB1*13* haplotype has been associated with high levels of measles antibody response, but low levels of rubella antibody response.

In order to evaluate the association of HLA class II genes *HLA-DRB1*, *HLA-DPB1*, and *HLA-DQB1* and JEV-NAbs with the humoral immune response to IJEV, this study examined Mongolian Chinese individuals who had been administered IJEV.

MATERIALS AND METHODS

Subjects and Vaccination

A randomized, double-blinded, positive-control, non-inferiority IJEV trial was implemented in the Inner Mongolia Autonomous Region of China from August 2012 to September 2013. The IJEV (lot: 20101201) was manufactured in a GMP-accredited facility of the Institute of Medical Biology at the Chinese Academy of Medical Sciences (IMBCAMS) and verified by the National Institute for Food and Drug Control (China, approval no. 2010L02035). Briefly, JEV P3 strains were grown on Vero cell microcarriers in a 75 L bioreactor. The virus suspension was harvested, inactivated with ultra-concentrated formalin, and purified by Sepharose 6FF and DEAE Sepharose FF. The resulting vaccine contained 0.5 mL per dose with $\geq$ 0.6 IU/mL JEV antigens. The clinical study procedure was approved by the Ethics Committee of the Inner Mongolia Autonomous Region Center for Disease Control and Prevention. The IJEV control (lot: 201012B02-1) was manufactured by Liaoning Chengda Biotechnology (Shenyang, China), containing the same concentration of antigens as the vaccine made by IMBCAMS. A total of 1,200 individuals aged 8 months−12 years in the Inner Mongolia Autonomous Region were enrolled to receive two doses of IJEV at 0 and 7 days. They were vaccinated with either the IJEV made by IMBCAMS or the IJEV control at a 1:1 ratio. The inclusion criteria were that the individual was in good health, was not infected by JEV, had not been inoculated with other vaccines within 7 days, and had not been inoculated with attenuated JE vaccine within 1 month. The peripheral blood samples were collected before vaccine administration and 30 days after the second dose received for the detection of neutralization antibody. Considering the limited blood sample volume and the consistency of the test, only individuals of 3–12 years of age, who were negative for NAbs before vaccination, were selected for further HLA genotyping. Finally, after vaccination, 212 individuals negative for NAbs were included in the seronegative group, and 161 individuals positive for NAbs were randomly selected and included in the seropositive group.

Japanese Encephalitis Vaccine Neutralization Antibody Detection

JEV-specific NAbs were determined by the National Institute for Food and Drug Control using the 50% plaque-reduction neutralization test according to the requirement of the Pharmacopeia of the People's Republic of China (19). Briefly, BHK-21 cells were initially inoculated at 10^6 cells/well in 24-well tissue culture plates and propagated for 48 h at 37°C in a CO_2 incubator. The serum samples were inactivated for 30 min in a 56°C water bath, diluted 10-fold, and then serially diluted 2-fold from 1:10 to 1:1280 in Minimum Essential Medium (GIBCO, Grand Island, NY, USA) containing 2% fetal bovine serum and 1% penicillin/streptomycin. The diluted serum and the positive conference serum were mixed with an equal volume of diluted challenge virus (P3 strain, lot 20151102, 500 PFU/mL). The suspensions were kept in a 37°C water bath for 30 min. Afterwards, 0.1 mL aliquots of the virus-serum mixtures were dispensed separately into each well of the 24-well microplates with the BHK-21 cells. The cells were overlaid with medium containing 1% methylcellulose. The cells in the wells were stained after inoculation at 37°C for 5 days in a 5% CO_2 incubator, and the plaques were counted. The NAb titer was defined as the reciprocal value of the last serum dilution that showed 50% or greater plaque reduction compared with the plaque counts in the virus-only control wells. NAbs at 50% plaque reduction neutralization titer (PRNT50) <10 or $PRNT_{50}$ increased <4-fold after vaccination were considered as negative seroconversion, while $PRNT_{50}$ >10, or at least a 4-fold increase after vaccination, was considered to be positive seroconversion. The antibody titers were determined by calculating the GMT as follows: $GMT = Log{-}1\ [(LogX_1 + LogX_2 + \ldots\ LogX_n)/n]$.

HLA-DRB1, *HLA-DPB1*, and *HLA-DQB1* Genotyping

Genomic DNA was extracted from peripheral lymphocytes using the QIAamp Blood Kit (Qiagen, Hilden, Germany). *HLA-DRB1*, *HLA-DPB1*, and *HLA-DQB1* were genotyped using a high-resolution sequencing-based typing method (Applied Biosystems, Foster City, CA, USA). Briefly, exons 2 and 3 of *DRB1* and *DQB1* as well as all exons of *DPB1* were amplified, and the PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Finally, the sequence was analyzed with the 3730xl DNA Analyzer (Applied Biosystems), and the HLA alleles were identified using the SBTengine (Applied Biosystems).

Statistical Analysis

The differences in age and sex between the seropositive and seronegative group were determined using Student's *t*-test or a χ^2 test. The *HLA-DRB1*, *-DPB1* and *-DQB1* allele frequencies were calculated using the PyPop or PyHLA software based on the genotyping results (20–22). The Hardy-Weinberg equilibrium was assessed using the Guo and Thompson method (23). The haplotypes were constructed based on the genotyping results using the expectation-maximization algorithm (20–22). The χ^2 test was used to determine differences in allele and haplotype frequencies between the seropositive and seronegative group. The odds ratios (ORs) and associated 95% confidence intervals (CIs) were also calculated for allele-specific risks. False discovery rate (FDR) correction was used for the multiple comparisons (20). For each gene, the amino acid sequences for all alleles were aligned together. If there was more than one amino acid at one position, a test was performed for each amino acid to examine whether it is distributed differently in the seropositive and seronegative group using PyHLA software (20). Fisher's exact test was used to analyze the association, and the odds ratio was calculated with Haldane's correction of Woolf's method (20). The association between *HLA-DRB1*, *-DPB1*, *-DQB1* alleles and antibody levels was analyzed through the analysis of variance using GraphPad Prism 7.0. *P*-values of < 0.05 were considered statistically significant.

RESULTS

Characteristics of Subjects

Table 1 lists the characteristics of the enrolled subjects. They were randomly selected from the two vaccination groups, which had no difference in seroconversion or GMT ($P > 0.05$). All subjects were negative for NAbs before vaccination. After vaccination, 161 individuals with $PRNT_{50} > 10$ were included in the seropositive group, while 212 individuals with $PRNT_{50} < 10$ were included in the seronegative group. **Table 2** shows that there were no age or gender differences between the seropositive and seronegative group ($P > 0.05$). In addition, there was no difference in NAbs titers according to age and gender ($P > 0.05$) in the seropositive group (data not shown). Moreover, there was no difference in HLA allele distribution according to gender ($P > 0.05$).

Association of HLA Alleles With Neutralizing Antibody Seroconversion of Inactivated Japanese Encephalitis Vaccine

The frequencies of *HLA-DRB1*, *-DPB1,* and *-DQB1* were in Hardy-Weinberg equilibrium in both the seropositive and seronegative groups ($P > 0.05$). At the *HLA-DRB1* locus, the frequencies of *DRB1*01:01* and *DRB1*16:02* were different

TABLE 1 | Demographic characteristics of the IJEV NAb seropositive and seronegative group.

	Seropositive group	Seronegative group	*P*-value
Male	75	109	0.355
Female	86	103	
Age	8.068 ± 0.201	7.611 ± 0.167	0.080

TABLE 2 | Age, gender, and GMTs in the seropositive group.

	Male	Female	*P*-value
n	75	86	
Anti-JEV (Log10)	1.300 ± 0.040	1.237 ± 0.035	0.237
Age	7.713 ± 0.309	8.378 ± 0.260	0.099

between the seropositive and seronegative group; however, after FDR correction, the difference was not considered significant (**Table 3**). At the *HLA-DPB1* locus, there was no significant difference between the seropositive and seronegative group. At the *HLA-DQB1* locus, the frequency of *DQB1*02:01* was higher in the seropositive group (0.152) than in the seronegative group (0.061) ($P < 0.001$; OR = 0.364; 95% CI: 0.221–0.600), while the frequency of *DQB1*02:02* was lower in the seropositive group (0.016) than in the seronegative group (0.104) ($P = 5.03 \times 10^{-6}$; OR = 7.341; 95% CI: 2.876–18.736) (**Table 3**). *DQB1*05:01* and *DQB1*05:02* frequencies were also different between the groups, but the difference was not significant after FDR correction (**Table 3**).

Further analysis of HLA residue levels showed that some HLA residues were associated with JEV antibody seroconversion (**Supplementary Table 1**). At the *HLA-DPB1* locus, residues A56, R96, T170, and V265 were associated with seronegative JEV-NAbs. At the *HLA-DQB1* locus, the residues S57, V116, A125, G135, and P146 were associated with seronegative NAbs, while V89 was associated with seropositive NAbs. At the *HLA-DRB1* locus, D28 and Y30 were associated with seronegative, while L11, K12, F13, L26, C30, I31, and Y32 were associated with seropositive NAbs. Residues containing *HLA-DQB1*02:02* were associated with seropositive JEV-NAbs.

Association of HLA-DRB1, -DPB1, and -DQB1 Haplotypes With Neutralizing Antibody Seroconversion of Inactivated Japanese Encephalitis Vaccine

The *HLA-DRB1*, *-DPB1*, and *-DQB1* alleles with frequencies higher than 0.020 in either the seropositive or seronegative group are listed in **Table 4**. At the level of the three loci, the frequency of the haplotypes *DRB1*07:01-DPB1*04:01-DQB1*02:01* and *DRB1*15:01-DPB1*02:01-DQB1*06:02* was higher in the seropositive group than in the seronegative group ($P < 0.05$), while the frequency of the haplotype *DRB1*07:01-DPB1*17:01-DQB1*02:02* was higher in the seronegative group than in the seropositive group ($P < 0.05$). At the level of the two loci, the frequency of the haplotypes *DRB1*07:01-DQB1*02:01* and *DPB1*02:01-DQB1*06:02* was higher in the seropositive group ($P < 0.05$), while that of the haplotypes *DRB1*07:01-DQB1*02:02* and *DPB1*17:01-DQB1*02:02* was higher in the seronegative group ($P < 0.05$).

Association of HLA Alleles With Neutralizing Antibody GMTs of Inactivated Japanese Encephalitis Vaccine in Seropositive Group

To analyze the association of HLA alleles with JEV-specific NAb GMTs, the 161 individuals in the seropositive group were examined. At the *DRB1* locus, the highest GMTs were in subjects with the *DRB1*14:03* (1.452 ± 0.174), *DRB1*14:01* (1.430 ± 0.383), and *DRB1*13:02* (1.410 ± 0.410) alleles, while the lowest were in those with *DRB1*01:01* (1.000 ± 0), *DRB1*11:04* (1.060 ± 0.135), and *DRB1*11:01* (1.084 ± 0.226) alleles. At the *DPB1* locus, subjects with *DPB1*09:01* (1.376 ± 0.343), *DPB1*05:01* (1.315 ± 0.369), and *DPB1*04:01* (1.313 ± 0.347) alleles had higher GMTs than those with *DPB1*13:01* (1.067 ± 0.133), *DPB1*19:01* (1.151 ± 0.213), and *DPB1*02:01* (1.198 ± 0.289) alleles ($P < 0.05$). At the *DQB1* locus, the highest GMTs were in subjects with the *DQB1*05:03* (1.473 ± 0.383), *DQB1*06:09* (1.452 ± 0.369), and *DQB1*06:03* (1.401 ± 0.460) alleles, while the lowest were in those with *DQB1*05:04* (1.000 ± 0), *DQB1*04:02* (1.151 ± 0.301), and *DQB1*06:04* (1.181 ± 0.404) alleles (**Figure 1**).

When the alleles were compared one by one, some were associated with higher GMTs (**Table 5**). The presence of *DRB1*01:01*, *DRB1*04:05*, *DRB1*09:01*, *DRB1*12:02*, *DRB1*13:02*, and *DRB1*14:01* was associated with higher NAb GMTs than the presence of *DRB1*11:01* ($P < 0.05$) at the *DRB1* locus. At the *DPB1* locus, the presence of *DPB1*05:01* was associated with higher GMTs than the presence of *DPB1*02:01* and *DPB1*13:01* ($P < 0.05$), and the presence of *DPB1*04:01* and *DPB1*09:01* was associated with higher GMTs than the presence of *DPB1*13:01* ($P < 0.05$). There was no significant difference in the GMT between the different *DQB1* alleles ($P > 0.05$).

DISCUSSION

Vaccines are one of the greatest advances in controlling infectious diseases in the past 300 years. The humoral immune response induced by a vaccine produces NAbs, so the seroconversion rate and GMT are widely used to evaluate vaccine efficacy. In the present study, we examined the association of HLA class II genes with the IJEV antibody response to reveal the role of the genetic variation in the HLA class II genes in the IJEV immune response.

HLA class II molecules present viral antigens in the form of peptides derived from the extracellular processing of vaccine peptides, which plays an important role in the humoral immune response to vaccines (24, 25). In an inactivated vaccine, the extracellular vaccine antigens are degraded into smaller peptides and integrated with the HLA class II molecule to constitute the HLA class II peptide complex, which plays a major role in stimulating the differentiation of $CD4^+$ T cells into Th1 and Th2 cells; in turn, the Th2 cells can interact with B cells to promote differentiation into antibody-secreting plasma cells, thus secreting a specific antibody against the vaccine antigen (18).

To date, many studies have reported that HLA class II genes are associated with the vaccine antibody response (17, 26–30). In 1999, McDermott et al. reported that *DQB1*02:02* was associated with a negative antibody response to hepatitis B virus (HBV) vaccination in a population in England (31). In 2005, Ovsyannikova et al. performed a study of the association between HLA and the humoral immune response to measles-mumps-rubella vaccination, finding that *DQB1*02:02* was negatively associated with rubella-specific lymphoproliferation (17). In the present study, *DQB1*02:02* was significantly negatively associated with the IJEV response ($P = 5.03 \times 10^{-6}$; OR = 7.341; 95% CI: 2.876–18.736). However, contrary to previous studies on the HBV, measles, rubella, influenza, and serogroup C meningococcus vaccines, which showed that *DQB1*02:01* was negatively associated with vaccine-induced

TABLE 3 | Frequencies of HLA alleles in the IJEV NAb seropositive and seronegative group.

Allele	P group	N group	P_FET	OR	95% CI	*P*
DRB1*01:01	**0.047**	**0.019**	**0.034**	0.394	0.165–0.940	0.489
DRB1*03:01	0.065	0.061	0.880	0.936	0.517–1.696	1.000
DRB1*04:01	0.025	0.043	0.230	1.740	0.747–4.054	0.925
DRB1*04:02	0.009	0.005	0.657	0.504	0.084–3.034	1.000
DRB1*04:03	0.003	0.017	0.147	5.389	0.660–44.020	0.925
DRB1*04:04	0.019	0.009	0.342	0.502	0.140–1.793	0.925
DRB1*04:05	0.044	0.043	1.000	0.975	0.478–1.992	1.000
DRB1*04:06	0.003	0.012	0.243	3.831	0.445–32.950	0.925
DRB1*04:07	0.003	0.005	1.000	1.521	0.137–16.852	1.000
DRB1*07:01	0.124	0.125	1.000	1.007	0.649–1.562	1.000
DRB1*08:01	0.003	0.002	1.000	0.759	0.047–12.179	1.000
DRB1*08:02	0.006	0.002	0.581	0.378	0.034–4.190	1.000
DRB1*08:03	0.031	0.050	0.267	1.626	0.755–3.502	0.925
DRB1*09:01	0.130	0.123	0.824	0.932	0.603–1.440	1.000
DRB1*10:01	0.025	0.019	0.617	0.755	0.280–2.033	1.000
DRB1*11:01	0.056	0.057	1.000	1.013	0.540–1.901	1.000
DRB1*11:04	0.016	0.007	0.301	0.452	0.107–1.905	0.925
DRB1*12:01	0.075	0.057	0.367	0.745	0.415–1.338	0.925
DRB1*12:02	0.056	0.054	1.000	0.969	0.514–1.827	1.000
DRB1*13:01	0.009	0.019	0.366	2.045	0.538–7.770	0.925
DRB1*13:02	0.034	0.028	0.674	0.824	0.359–1.891	1.000
DRB1*13:03	0.003	0.012	0.243	3.831	0.445–32.950	0.925
DRB1*14:01	0.022	0.035	0.383	1.650	0.665–4.096	0.925
DRB1*14:03	0.012	0.009	0.732	0.757	0.188–3.051	1.000
DRB1*14:05	0.009	0.014	0.739	1.526	0.379–6.150	1.000
DRB1*15:01	0.099	0.101	1.000	1.023	0.631–1.657	1.000
DRB1*15:02	0.034	0.024	0.504	0.683	0.286–1.628	1.000
DRB1*15:04	0.003	0.002	1.000	0.759	0.047–12.179	1.000
DRB1*16:02	**0.003**	**0.026**	**0.016**	8.550	1.098–66.568	0.469
DPB1*02:01	0.227	0.205	0.529	0.881	0.620–1.252	0.992
DPB1*02:02	0.044	0.071	0.157	1.675	0.873–3.214	0.681
DPB1*03:01	0.065	0.045	0.252	0.672	0.355–1.273	0.818
DPB1*04:01	0.152	0.160	0.839	1.064	0.714–1.587	0.992
DPB1*04:02	0.109	0.101	0.809	0.926	0.577–1.483	0.992
DPB1*05:01	0.267	0.276	0.804	1.046	0.755–1.449	0.992
DPB1*09:01	0.037	0.014	0.053	0.371	0.138–0.999	0.681
DPB1*13:01	0.028	0.033	0.832	1.188	0.508–2.779	0.992
DPB1*14:01	0.025	0.014	0.415	0.563	0.194–1.640	0.992
DPB1*17:01	0.031	0.054	0.151	1.790	0.839–3.815	0.681
DPB1*19:01	0.006	0.007	1.000	1.140	0.189–6.864	1.000
DPB1*21:01	0.003	0.007	0.638	2.287	0.237–22.094	0.992
DPB1*41:01	0.003	0.002	1.000	0.759	0.047–12.179	1.000
DQB1*02:01	**0.152**	**0.061**	**6.67E-05**	**0.364**	**0.221–0.600**	**< 0.001**
DQB1*02:02	**0.016**	**0.104**	**3.36E-07**	**7.341**	**2.876–18.736**	**5.03E-06**
DQB1*03:01	0.245	0.248	1.000	1.013	0.723–1.417	1.000
DQB1*03:02	0.044	0.057	0.502	1.320	0.672–2.594	0.845
DQB1*03:03	0.149	0.139	0.752	0.923	0.611–1.393	0.901
DQB1*04:01	0.047	0.040	0.717	0.855	0.420–1.739	0.901
DQB1*04:02	0.012	0.005	0.411	0.377	0.069–2.070	0.845
DQB1*05:01	**0.081**	**0.040**	**0.025**	0.476	0.253–0.892	0.095

(Continued)

TABLE 3 | Continued

Allele	P group	N group	P_FET	OR	95% CI	*P*
DQB1*05:02	**0.022**	**0.057**	**0.025**	2.700	1.149–6.347	0.095
DQB1*05:03	0.022	0.040	0.209	1.880	0.770–4.588	0.627
DQB1*06:01	0.071	0.078	0.781	1.097	0.631–1.908	0.901
DQB1*06:02	0.093	0.078	0.507	0.822	0.490–1.378	0.845
DQB1*06:03	0.009	0.019	0.366	2.045	0.538–7.770	0.845
DQB1*06:04	0.016	0.014	1.000	0.910	0.275–3.009	1.000
DQB1*06:09	0.019	0.014	0.771	0.756	0.242–2.366	0.901

P group, seropositive group; N group, seronegative group. Bold value indicated the alleles showed difference between P and N group before and after FDR correction.

antibody response (32–34), in the present study, *DQB1*02:01* had a significantly positive association with IJEV seropositivity ($P < 0.05$; OR = 0.364; 95% CI: 0.221–0.600). Interestingly, *DQB1*02:01* is reportedly associated with high Th1 IFN-γ secretion, while *DQB1*02:02* is associated with a low measles-specific Th2 cytokine response (35). There is only one amino-acid difference between *DQB1*02:01* and *DQB1*02:02*, namely at position 135 in the peptide binding groove, where *DQB1*02:01* contains aspartic acid and *DQB1*02:02* contains glycine. In 2018, Yang et al. predicted the 3D ribbon models of the HLA proteins and indicated that the amino acid position 135 of HLA-DQB1 was located on the junction point of two β-sheet structures and lies on the β2 domain of protein belonging to Ig protein superfamily (36). The domain is expressed on the extracellular part of the antigen presenting cells and could integrate with CD4+ T cells during the antigen presenting process (36). Thus, we deduced that the amino acid change from a negatively charged Asp in DQB1*02:01 to an uncharged polar Gly in DQB1*02:02 could influence the JEV antigen presentation process, in consequence, the inducing of JEV-specific NAb. The further studies on the exact role of how HLA-DQB1*02:01 and HLA-DQB1*02:02 in the progression of JEV-antibody needs to be elucidated in the future.

We compared the previously reported *HLA-DQA1* and *-DQB1* haplotypes to assess whether there is any preference for *DQB1*02:01* or *DQB1*02:02* over *DQA1* and found that *DQB1*02:01* and *DQB1*02:02* are either in linkage disequilibrium with the same *DQA1* alleles, namely *DQA1*02:01*, *DQA1*05:01,* and *DQA1*03:01* (http://www.allelefrequencies.net/). We then predicted *DQB1*02:01* and *DQB1*02:02* heterodimers with *DQA1* using the E protein sequence of JEV by NetMHCIIpan (http://www.cbs.dtu.dk/services/NetMHCIIpan/logos.php). The predicted peptides of *DQA1*03:01-DQB1*02:01* and *DQA1*03:01-DQB1*02:02* showed no difference. The identification of the actual *DQA1-DQB1* haplotypes existing in the Mongolian population would help for validation of the *DQA1-DQB1* molecular binding with specific JEV epitopes in the future.

In 2012, Schillie et al. found that *DRB1*13:01* and *DRB1*13:02,* with an allele difference at position 86, showed contrary roles in the HBV antibody response (37). In the present study, *DQB1*05:01* showed a positive response association, and *DQB1*05:02* showed a negative response association with IJEV, though the association was not significant after FDR correction. Further HLA residue association study indicated that the *DQB1* residue S57, present in *DQB1*05:02* and *DQB1*05:04*, showed an opposite JEV-NAb response from residue V57, present in *DQB1*05:01*, *DQB1*06:04*, and *DQB1*06:09*. The DQ peptide prediction suggested that the peptide FLVHREWFHDLALPW showed strong binding in both *DQA1*01:01-DQB1*05:01* and *DQA1*01:01-DQB1*05:02*, while the peptides HREWFHDLALPWTPP and RNRELLMEFEEAHAT showed strong binding in subjects with *DQA1*01:01-DQB1*05:02*, but not in *DQA1*01:01-DQB1*05:01* (**Supplementary Table 2**). This finding indicates that *DQB1*05:01* and *DQB1*05:02* may produce different JEV peptides. Moreover, these data indicate that allele differences may change the binding groove of the antigen-HLA complex, in turn influencing T cell receptors expressed on inactivated JEV-specific $CD4^+$ T cells and, finally, playing different roles in the antibody response (27, 37).

In addition to *DQB1*02:01* and *DQB1*02:02*, other HLA class II genes are reportedly associated with the antibody response to vaccines. For example, Jafarzadeh et al. (27) reported that *DRB1*01:01*, *DRB1*13:01*, *DRB1:15:01*, and *DQB1*04:01* were positively associated with HBV antibody response, while *DRB1*03:01*, *DRB1*07:01*, and *DQB1*02:01* were negatively associated. However, other than *DQB1*02:01*, no HLA alleles have been associated with IJEV in the present study. One of the reasons for different HLA alleles being associated with antibody responses could be a distinct immune response to different vaccines or pathogens. Most previous association studies have been performed with attenuated vaccines (mumps, measles, rubella vaccine, etc.) or virus-like particle-based vaccines (HBV), which could induce both HLA class I- and class II-mediated immune response to generate an immune response. However, in an inactivated vaccine like IJEV, the humoral immune response mediated by HLA class II molecules would be key in generating JEV-NAbs. Thus, the difference in the immune response mechanism between the inactivated vaccine and the attenuated vaccine may be caused by the association with different HLA alleles (18, 38). Another reason could be a population-specific difference in HLA distribution, as with HLA genes and their motifs, even if the populations were administered

TABLE 4 | Frequencies of HLA haplotypes in the IJEV NAb seropositive and seronegative group.

Haplotype	P group	N group	*P*	OR	95% CI	*P*
HLA DRB1-DPB1-DQB1						
DRB1*03:01-DPB1*02:01-DQB1*02:01	0.030	0.010	0.053	0.341	0.109–1.065	>0.05
DRB1*03:01-DPB1*04:01-DQB1*02:01	0.013	0.032	0.098	2.448	0.820–7.313	>0.05
DRB1*04:05-DPB1*05:01-DQB1*04:01	0.030	0.024	0.610	0.794	0.326–1.935	>0.05
DRB1*07:01-DPB1*04:01-DQB1*02:01	**0.046**	**0.000**	**< 0.001**	**2.380**	**2.186–2.292**	**<0.05**
DRB1*07:01-DPB1*04:01-DQB1*02:02	0.000	0.025	0.004	1.779	1.669–1.897	0.056
DRB1*07:01-DPB1*17:01-DQB1*02:02	**0.000**	**0.034**	**0.001**	**1.786**	**1.675–1.905**	**0.015**
DRB1*09:01-DPB1*02:01-DQB1*03:03	0.031	0.031	0.956	1.024	0.442–2.370	>0.05
DRB1*09:01-DPB1*04:02-DQB1*03:03	0.019	0.020	0.921	1.055	0.372–2.992	>0.05
DRB1*09:01-DPB1*05:01-DQB1*03:03	0.062	0.034	0.075	0.538	0.270–1.075	>0.05
DRB1*11:01-DPB1*02:01-DQB1*03:01	0.028	0.009	0.059	0.335	0.102–1.100	>0.05
DRB1*11:01-DPB1*04:02-DQB1*03:01	0.013	0.024	0.313	1.785	0.571–5.578	>0.05
DRB1*12:01-DPB1*05:01-DQB1*03:01	0.036	0.029	0.619	0.813	0.360–1.839	>0.05
DRB1*12:02-DPB1*05:01-DQB1*03:01	0.026	0.032	0.625	1.241	0.521–2.953	>0.05
DRB1*15:01-DPB1*02:01-DQB1*06:02	**0.029**	**0.000**	**< 0.001**	**2.355**	**2.166–2.562**	**<0.05**
DRB1*15:01-DPB1*04:01-DQB1*06:02	0.015	0.028	0.222	1.925	0.661–1.405	>0.05
DRB1*15:01-DPB1*05:01-DQB1*06:02	0.036	0.032	0.723	0.866	0.390–1.922	>0.05
DRB1*15:02-DPB1*04:01-DQB1*06:01	0.025	0.011	0.142	0.430	0.135–1.368	>0.05
HLA DRB1-DPB1						
DRB1*03:01-DPB1*02:01	0.029	0.017	0.252	0.566	0.211–1.519	>0.05
DRB1*03:01-DPB1*04:01	0.017	0.031	0.207	1.901	0.689–5.241	>0.05
DRB1*04:05-DPB1*05:01	0.027	0.028	0.888	1.066	0.439–2.587	>0.05
DRB1*07:01-DPB1*04:01	0.049	0.029	0.168	0.588	0.275–1.260	>0.05
DRB1*07:01-DPB1*17:01	0.012	0.036	0.037	3.109	1.012–9.549	0.629
DRB1*09:01-DPB1*02:01	0.034	0.028	0.694	0.845	0.365–1.955	>0.05
DRB1*09:01-DPB1*04:02	0.016	0.020	0.670	1.268	0.425–3.779	>0.05
DRB1*09:01-DPB1*05:01	0.064	0.040	0.172	0.634	0.328–1.225	>0.05
DRB1*11:01-DPB1*02:01	0.028	0.011	0.091	0.387	0.123–1.213	>0.05
DRB1*11:01-DPB1*04:02	0.013	0.024	0.266	1.885	0.606–5.864	>0.05
DRB1*12:01-DPB1*02:01	0.024	0.022	0.936	0.961	0.365–2.529	>0.05
DRB1*12:01-DPB1*05:01	0.034	0.023	0.407	0.692	0.288–1.661	>0.05
DRB1*12:02-DPB1*05:01	0.026	0.036	0.407	1.433	0.610–3.367	>0.05
DRB1*15:01-DPB1*02:01	0.028	0.022	0.651	0.808	0.320–2.039	>0.05
DRB1*15:01-DPB1*04:01	0.019	0.023	0.662	1.256	0.452–3.487	>0.05
DRB1*15:01-DPB1*05:01	0.040	0.041	0.873	1.062	0.507–2.225	>0.05
DRB1*15:02-DPB1*04:01	0.024	0.016	0.405	0.644	0.227–1.830	>0.05
HLA DRB1-DQB1						
DRB1*01:01-DQB1*05:01	0.037	0.019	0.137	0.509	0.206–1.260	>0.05
DRB1*03:01-DQB1*02:01	0.062	0.057	0.814	0.929	0.504–1.455	>0.05
DRB1*04:01-DQB1*03:01	0.022	0.035	0.253	1.692	0.681–4.200	>0.05
DRB1*04:05-DQB1*04:01	0.040	0.035	0.771	0.894	0.419–1.906	>0.05
DRB1*07:01-DQB1*02:01	**0.087**	**0.000**	**< 0.001**	**2.408**	**2.207–2.628**	**<0.05**
DRB1*07:01-DQB1*02:02	**0.016**	**0.099**	**< 0.001**	**7.158**	**2.798–18.311**	**<0.05**
DRB1*07:01-DQB1*03:03	0.016	0.021	0.541	1.409	0.468–4.245	>0.05
DRB1*08:03-DQB1*06:01	0.028	0.045	0.207	1.673	0.747–3.749	>0.05
DRB1*09:01-DQB1*03:03	0.127	0.118	0.789	0.941	0.606–1.464	>0.05
DRB1*10:01-DQB1*05:01	0.025	0.019	0.610	0.773	0.287–2.083	>0.05
DRB1*11:01-DQB1*03:01	0.053	0.052	0.983	1.007	0.525–1.929	>0.05
DRB1*12:01-DQB1*03:01	0.068	0.054	0.473	0.802	0.439–1.467	>0.05
DRB1*12:02-DQB1*03:01	0.050	0.054	0.725	1.125	0.584–2.167	>0.05

(Continued)

TABLE 4 | Continued

Haplotype	P group	N group	*P*	OR	95% CI	*P*
DRB1*14:01-DQB1*05:02	0.009	0.026	0.089	2.902	0.803–10.491	>0.05
DRB1*15:01-DQB1*06:02	0.090	0.073	0.455	0.818	0.482–1.388	>0.05
DRB1*15:02-DQB1*06:01	0.031	0.021	0.429	0.693	0.278–1.727	>0.05
DRB1*16:02-DQB1*05:02	0.003	0.023	0.022	7.701	0.978–60.651	>0.05
HLA DPB1-DQB1						
DPB1*02:01-DQB1*02:01	0.039	0.013	0.024	0.327	0.118–0.908	>0.05
DPB1*02:01-DQB1*03:01	0.063	0.046	0.342	0.735	0.388–1.390	>0.05
DPB1*02:01-DQB1*03:02	0.023	0.036	0.277	1.631	0.670–3.973	>0.05
DPB1*02:01-DQB1*03:03	0.027	0.042	0.229	1.657	0.722–3.802	>0.05
DPB1*02:01-DQB1*05:01	0.027	0.018	0.444	0.681	0.254–1.828	>0.05
DPB1*02:01-DQB1*06:02	**0.032**	**0.000**	**< 0.001**	**2.329**	**2.142–2.533**	**<0.05**
DPB1*02:02-DQB1*03:01	0.020	0.016	0.685	0.798	0.269–2.372	>0.05
DPB1*04:01-DQB1*02:01	0.058	0.032	0.097	0.551	0.270–1.125	>0.05
DPB1*04:01-DQB1*02:02	0.000	0.026	0.003	1.799	1.686–1.920	0.060
DPB1*04:01-DQB1*03:01	0.022	0.032	0.394	1.485	0.595–3.709	>0.05
DPB1*04:01-DQB1*06:01	0.027	0.018	0.399	0.657	0.245–1.759	>0.05
DPB1*04:01-DQB1*06:02	0.015	0.025	0.330	1.700	0.577–5.011	>0.05
DPB1*04:02-DQB1*03:01	0.038	0.041	0.785	1.109	0.528–2.327	>0.05
DPB1*04:02-DQB1*03:03	0.032	0.022	0.453	0.710	0.289–1.744	>0.05
DPB1*05:01-DQB1*03:01	0.077	0.089	0.488	1.206	0.710–2.048	>0.05
DPB1*05:01-DQB1*03:03	0.072	0.032	0.016	0.441	0.222–0.874	0.288
DPB1*05:01-DQB1*04:01	0.038	0.020	0.139	0.515	0.211–1.258	>0.05
DPB1*05:01-DQB1*05:02	0.000	0.026	0.003	1.799	1.686–1.920	0.057
DPB1*05:01-DQB1*05:03	0.010	0.026	0.120	2.558	0.751–8.707	>0.05
DPB1*05:01-DQB1*06:02	0.035	0.037	0.822	1.094	0.501–2.388	>0.05
DPB1*13:01-DQB1*03:03	0.012	0.020	0.411	1.646	0.496–5.465	>0.05
DPB1*17:01-DQB1*02:02	**0.000**	**0.035**	**0.001**	**1.807**	**1.692–1.929**	**0.021**

P group, seropositive group; N group, seronegative group. Bold value indicated the haplotypes showed difference between P and N group before and after FDR correction.

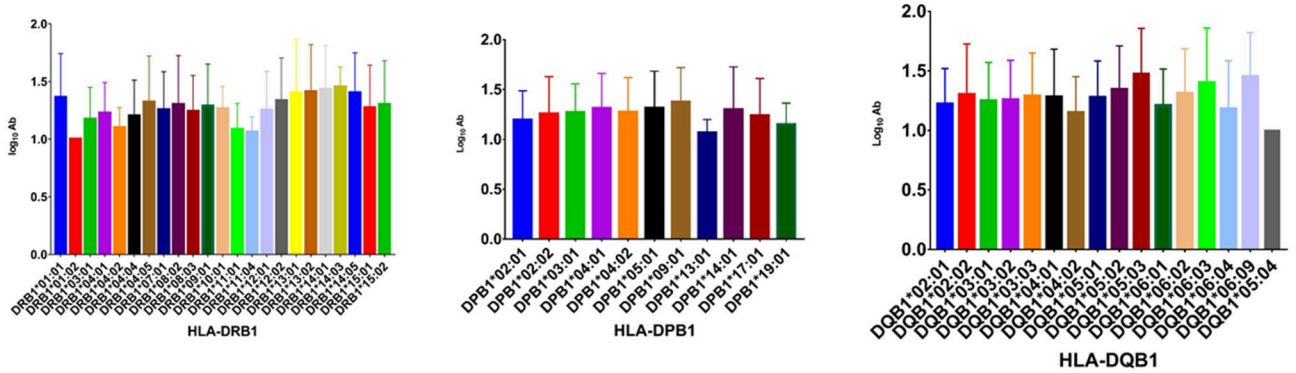

FIGURE 1 | GMTs associated with different *HLA-DRB1*, *-DPB1*, and *-DQB1* alleles in the IJEV NAb-positive group.

the same vaccine. For example, in 2015, Jafarzadeh et al. (27) reported that non-responsiveness to HBV is associated with *HLA-A1*, *-B15*, and *-B40* in Indians, *HLA-A1*, *-A2*, and *-B8* in Caucasians, *HLA-B54* in Chinese, and *HLA-A10* and *-Cw4* in Turkish people. In addition, *DQA1*05:01-DQB1*02:01* is predominant in Europe, Southwest Asia, and North Africa with frequencies of 19.1, 17.2, and 17.0%, respectively, while its frequency is only 2.7% in North America, and it has not been identified in South America. *DQA1*02:01-DQB1*02:01* is predominant in North Africa, common in European and

TABLE 5 | GMTs of JEV NAbs associated with HLA alleles.

Alleles		Allele No.	GMTs	*P*-value
DRB1*01:01 vs. DRB1*11:01	DRB1*01:01	15	1.361 ± 0.098	0.020
	DRB1*11:01	18	1.084 ± 0.053	
DRB1*04:05 vs. DRB1*11:01	DRB1*04:05	14	1.323 ± 0.107	0.049
	DRB1*11:01	18	1.084 ± 0.053	
DRB1*09:01 vs. DRB1*11:01	DRB1*09:01	12	1.287 ± 0.056	0.034
	DRB1*11:01	18	1.084 ± 0.053	
DRB1*11:01 vs. DRB1*12:02	DRB1*11:01	18	1.084 ± 0.053	0.027
	DRB1*12:02	18	1.334 ± 0.087	
DRB1*11:01 vs. DRB1*13:02	DRB1*11:01	18	1.084 ± 0.053	0.012
	DRB1*13:02	11	1.410 ± 0.124	
DRB1*11:01 vs. DRB1*14:01	DRB1*11:01	18	1.084 ± 0.053	0.022
	DRB1*14:01	7	1.430 ± 0.145	
DPB1*02:01 vs. DPB1*05:01	DPB1*02:01	73	1.198 ± 0.034	0.029
	DPB1*05:01	86	1.315 ± 0.040	
DPB1*04:01 vs. DPB1*13:01	DPB1*04:01	49	1.313 ± 0.050	0.044
	DPB1*13:01	9	1.067 ± 0.044	
DPB1*05:01 vs. DPB1*13:01	DPB1*05:01	86	1.315 ± 0.040	0.036
	DPB1*13:01	9	1.067 ± 0.044	
DPB1*09:01 vs. DPB1*13:01	DPB1*09:01	12	1.376 ± 0.099	0.038
	DPB1*13:01	9	1.067 ± 0.044	

Southwest Asia, and rare in North Africa and South America (39). To the best of our knowledge, this HLA allele diversity was generated in the long evolutionary interaction between hosts and pathogens, which makes it encode adequate products to generate immune responses against different pathogens. Thus, different HLA alleles were formed as an outcome of specific pathogen infections and are therefore associated with different infectious diseases (40–42). As such, the mechanism of the immune response to different vaccine antigens could vary based on different vaccines.

In addition to the seroconversion rate, GMT is an important factor in evaluating vaccine efficacy. In the present study, we evaluated the relationship between GMTs and HLA alleles. Interestingly, we found that the HLA alleles associated with an antibody response were different from the HLA alleles associated with GMTs. These results indicate that HLA alleles have different roles in the host immune response.

In summary, we investigated the association between HLA class II genes and antibody response after IJEV administration, determining that *HLA-DQB1*02:01* and *HLA-DQB1*02:02* were associated with NAb seroconversion. Furthermore, certain *HLA-DRB1* and *-DPB1* alleles were associated with higher GMTs than others. The present study suggests that HLA class II genes may influence the antibody response to IJEV. However, as only 161 individuals were examined in the present study, future studies should comprehensively analyze larger samples.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethical standards of the Responsible Committee on Human Experimentation of the Ethics Committee of the Guangxi Centre for Disease Control and Prevention, with written informed consent obtained from all subjects in accordance with the Declaration of Helsinki. The protocol was approved by the Inner Mongolia Autonomous Region Center for Disease Control and Prevention.

AUTHOR CONTRIBUTIONS

MS and LiS: conceived and designed the experiments. YY, HY, LeS, SL, and CL: performed the experiments. YY, JC, and ZZ: data analysis. MS and LiS: manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00428/full#supplementary-material

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Booster Vaccination With GVGH *Shigella sonnei* 1790GAHB GMMA Vaccine Compared to Single Vaccination in Unvaccinated Healthy European Adults: Results From a Phase 1 Clinical Trial

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The investigational *Shigella sonnei* vaccine (1790GAHB) based on GMMA (generalized modules for membrane antigens) is immunogenic, with an acceptable safety profile in adults. However, pre-vaccination anti-*S. sonnei* lipopolysaccharide (LPS) antibody levels seemed to impact vaccine-related immune responses. This phase 1, open-label, non-randomized extension study (ClinicalTrials.gov: NCT03089879) evaluated immunogenicity of a 1790GAHB booster dose in seven adults with undetectable antibodies prior to priming with three 1790GAHB vaccinations 2–3 years earlier (boosted group), compared to one dose in 28 vaccine-naïve individuals (vaccine-naïve group). Anti-*S. sonnei* LPS serum IgG geometric mean concentrations and seroresponse (increase of ≥25 EU or ≥50% from baseline antibody ≤50 EU and ≥50 EU, respectively) rates were calculated at vaccination (day [D]1), D8, D15, D29, D85. Safety was assessed. Geometric mean concentrations at D8 were 168 EU (boosted group) and 32 EU (vaccine-naïve group). Response peaked at D15 (883 EU) and D29 (100 EU) for the boosted and vaccine-naïve groups. Seroresponse rates at D8 were 86% (boosted group) and 24% (vaccine-naïve group) and increased at subsequent time points. Across both groups, pain (local) and fatigue (systemic) were the most frequent solicited adverse events (AEs). Unsolicited AEs were reported by 57% of boosted and 25% of vaccine-naïve participants. No deaths, serious AEs, or AEs of special interest (except one mild neutropenia case, possibly vaccination-related) were reported. One 1790GAHB dose induced a significant booster response in previously-primed adults, regardless of priming dose, and strong immune response in vaccine-naïve individuals. Vaccination was well tolerated.

Keywords: *Shigella sonnei*, 1790GAHB, GMMA (generalized modules for membrane antigen), booster response, antibody persistence, safety

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INTRODUCTION

Diarrheal diseases continue to represent a major cause of death worldwide, with more than 1.6 million fatalities estimated in 2016 (1). Among the three pathogens causing the majority of diarrhea deaths, *Shigella* accounted for 212,438 estimated deaths in all ages and 37,034 in children under 5 years of age, the majority in low-middle income countries (2). The *Shigella* genus encompasses four species and 50 serotypes, differentiated on the basis of the variability of their O antigen (OAg), part of the lipopolysaccharide (LPS) in the outer membrane of the bacteria (3). The global epidemiology of *Shigella* is changing constantly, but recently, the single serotype of *S. sonnei* has shown a significant increase in prevalence in several parts of the world (4–8). Early identification and antibiotic treatment are key factors in the management of shigellosis (9), but *Shigella* species have developed substantial antibiotic resistance (10–13). Therefore, the development of an effective vaccine against *Shigella* remains an important unmet medical need. Several OAg-based conjugate or live-attenuated vaccines are currently under development, but no licensed *Shigella* vaccine is widely available (14–16).

The GSK Vaccines Institute for Global Health (GVGH) investigational *S. sonnei* vaccine 1790GAHB, using GMMA (generalized modules for membrane antigens) as a delivery system for O antigen (OAg), has already been shown to be highly immunogenic and to have an acceptable safety profile in European (17) and Kenyan (18) adults. In a phase 1 study conducted in 50 French adults, five different GMMA OAg/protein doses of 1790GAHB (0.059/1 μg, 0.29/5 μg, 1.5/25 μg, 2.9/50 μg or 5.9/100 μg), administered at each of three intramuscular vaccinations 1 month apart, were compared to placebo administration (17). While the antibody response observed across all vaccine groups peaked with the 1.5/25 μg dose, no substantial difference was seen in the response of participants receiving the three highest vaccine doses (1.5/25, 2.9/50 or 5.9/100 μg) (17). Moreover, *post-hoc* analyses showed that pre-existing anti-*S. sonnei* LPS antibody levels potentially impact response to vaccination. More specifically, participants with detectable antibodies at baseline had higher antibody levels following the first vaccination and a less pronounced decline of antibody levels up to 168 days post-last vaccination than those with undetectable antibody levels at baseline (17).

Long-lived antibody is desired for an effective public health vaccine, as is the ability to boost the response, either through revaccination or infection, especially in young children not previously exposed to *Shigella*. Therefore, this extension study aimed to further characterize the immunogenicity profile of the *S. sonnei* 1790GAHB vaccine in participants with undetectable pre-vaccination antibodies. The study compared a fourth vaccination, 2–3 years after the third vaccination in the parent study, to a single vaccination in vaccine-naïve adults. Based on safety and immunogenicity results obtained in the parent trial (17, 18), a dose of 1.5/25 μg OAg/protein was selected for use in the extension trial.

A summary contextualizing the results and potential clinical relevance and impact of the research is displayed in the Focus on Patient Section (**Figure 1**), for the benefit of healthcare professionals.

MATERIALS AND METHODS

Study Design and Participants

This open label, non-randomized, single center, phase 1, extension study (NCT03089879) was conducted in France between March and August 2017. The extension trial enrolled healthy adults from the parent study, who received three vaccinations with 1790GAHB 2–3 years earlier (boosted group) or who received placebo (17). All participants enrolled from the previous study had undetectable anti-*S. sonnei* LPS antibody levels before first vaccination in the parent study. The extension study further recruited adults with or without detectable anti-*S. sonnei* LPS antibody levels at baseline. The placebo recipients from the parent study and the newly-recruited volunteers were enrolled in the vaccine-naïve group. Individuals aged 22–50 years were eligible for participation in the extension study if they were affiliated with a social security regimen and, for women of child-bearing potential, if they had a negative urinary pregnancy test before vaccination and agreed to use acceptable birth control measures throughout the study. The full list of inclusion/exclusion criteria is provided in the **Supplementary Text S1**.

All participants received 0.5 mL of the *S. sonnei* 1790GAHB vaccine, by intramuscular route. The vaccine was provided as a preservative-free formulation (single vial of 0.7 mL) of *S. sonnei* 1790-GMMA (12 μg/mL measured by OAg and 200 μg/mL measured by protein content) adsorbed to Alhydrogel (0.7 mg Al^{3+}/mL) in Tris-buffered saline. The 0.5 mL dose containing 1.5/25 μg of OAg/protein was obtained by dilution with Alhydrogel in Tris-buffered saline (0.7 mg Al^{3+}/mL), immediately prior to vaccination. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was obtained from each participant prior to conducting any study-specific procedure. The protocol was approved by a National Ethic Committee (CPP EST1), assigned according to the pilot phase of the European Union Regulation No. 536/2014 for clinical trial applications in France. The study was registered at www.clinicaltrials.gov (NCT03089879) and a protocol summary is available at http://www.gsk-clinicalstudyregister.com (study ID 205905).

Study Objectives

The primary objective was to evaluate the memory response elicited by a booster dose of 1790GAHB in primed individuals following three vaccinations with 1790GAHB in the parent study and having undetectable antibody levels prior to the primary vaccination series, as measured by enzyme-linked immunosorbent assay (ELISA). Anti-*S. sonnei* LPS serum immunoglobulin G (IgG) at seven days post-booster vaccination were compared to the administration of a single vaccine dose to vaccine-naïve participants (including placebo recipients from

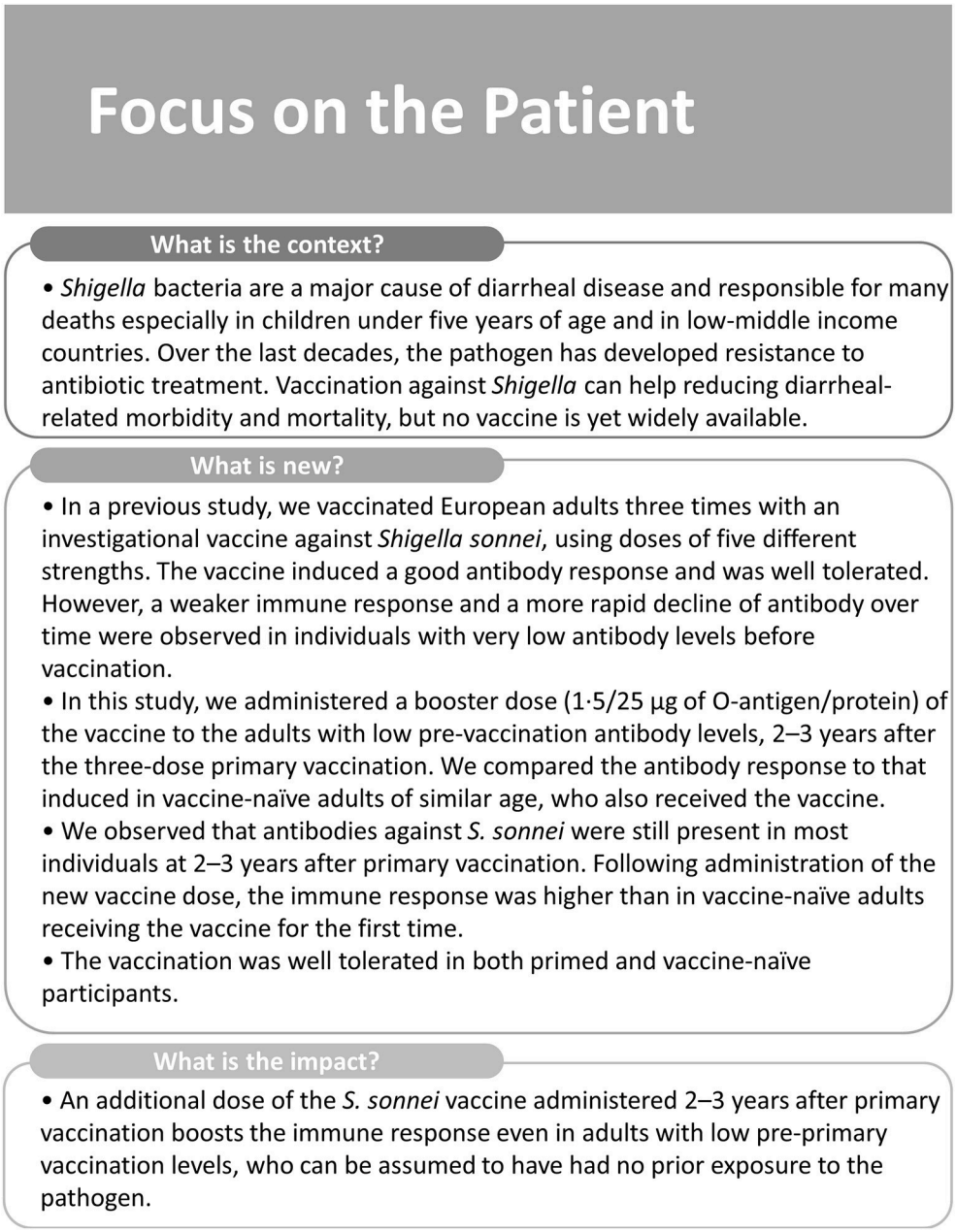

FIGURE 1 | Focus on patient section.

the parent trial and individuals enrolled in the extension trial). Secondary objectives assessed the safety and immunogenicity of 1790GAHB in all study participants, including the antibody profile of the boosted group compared to the vaccine-naïve group at baseline and 7, 14, 28, and 84 days post-vaccination, the antibody profile of the vaccine-naïve individuals with detectable antibody at baseline and at 7, 14, 28, and 84 days post-vaccination, and the persistence of anti-*S. sonnei* LPS antibody levels, at the start of the extension study, in participants primed with 1790GAHB in the parent study ∼2–3 years earlier.

Immunogenicity and Safety Assessments

Blood samples were collected as follows: ∼15 mL were drawn for hematological and 25 mL for serological testing from all participants as part of the initial screening. For serological analyses, further samples of 20 mL were collected from each participant before vaccination and 7, 14, 28, and 84 days post-vaccination. At 28 days post-vaccination, an additional blood sample of 20 mL was drawn to allow the creation of a standard reference serum for subsequent studies. Additional samples of 6 mL were drawn for hematological tests at 7 and 84 days post-vaccination.

Serum was kept frozen below −20°C and transported to GSK (Marburg, Germany). Serologic testing was performed on one aliquot, while the others were stored for future analyses. Anti-*S. sonnei* LPS serum IgG was measured by ELISA using *S. sonnei* LPS as plate coating antigen (19). A dilution series of standard reference serum pool generated during the parent study was included on each ELISA plate. The standard reference serum was calibrated such that 1 ELISA unit (EU) equals the reciprocal of the dilution giving an optical density at 405 −490 nm of 1 in the standard assay. The ELISA detection limit varied from plate to plate, ranging between 5.5 and 7·4 EU.

Antibody responses were assessed by anti-*S. sonnei* LPS serum IgG geometric mean concentrations (GMCs) and seroresponse rates, calculated at each time point. Seroresponse was defined as a post-vaccination increase of at least 25 EU and at least 50% of anti-*S. sonnei* LPS IgG ≤50 EU and ≥50 EU, respectively, at baseline. A level of anti-*S. sonnei* LPS serum IgG of 121 EU was also used as a threshold for the assessment of immune response, similarly to the parent study (17). Post-vaccination levels of 121 EU were found to correspond to the median titer of 1:800 measured in the sera of convalescent individuals previously infected with *S. sonnei*, using the ELISA method by Cohen et al. (20).

After receiving 1790GAHB, participants were monitored at the study site for 4 h. Occurrence of solicited local (pain, erythema, and induration) and systemic (headache, arthralgia, chills, fatigue, malaise, myalgia, and orally-measured fever) adverse events (AEs) during the 7 days post-vaccination period were documented by the participants on diary cards. Unsolicited AEs occurring within 84 days after vaccination were collected by study staff during scheduled (at 7, 14, 28, and 84 days post-vaccination) and unscheduled clinic visits. Solicited AEs continuing beyond 7 days post-vaccination were reported as unsolicited events. Serious AEs (SAEs), AEs of special interests (AESIs; reactive arthritis and neutropenia), and AEs leading to withdrawal from the study were collected throughout the study period and assessed by the investigator as being either probably-, possibly- or not-related to vaccination.

Statistical Analysis

No formal statistical sample size was calculated, as all analyses were descriptive. Serological assessments were carried out on the full analysis set at each time point, which included participants with at least one evaluable serum sample. For each group, GMCs were calculated with their associated two-sided 95% confidence intervals (CIs) by exponentiating the mean and 95% CIs of the logarithmically-transformed (base 10) EU. Geometric mean ratios (GMRs) and associated 95% CIs were computed for GMC at post-vaccination time points vs. pre-vaccination levels, by exponentiating the mean within-subject differences in log-transformed concentrations and the corresponding 95% CIs. For statistical analysis of ELISA data, antibody levels below the limit of detection were set to half that limit.

The number and percentage of participants with seroresponse and post-vaccination antibody level ≥121 EU for anti-*S. sonnei* LPS serum IgG was computed with 95% Clopper-Pearson CIs.

Safety analyses were performed on any solicited or unsolicited AE data collected from participants who received 1790GAHB. All solicited AEs were evaluated on a 3-grade scale as mild, moderate, or severe. The number and percentage of participants with AEs, SAEs, AESIs, new onset of chronic disease, potential immune-mediated disease, medically attended AEs, AEs leading to withdrawal, and clinically significant deviations in hematology test values were summarized.

RESULTS

Demographics

A total of 35 adults participated in the study. Seven adults vaccinated with 1790GAHB in the parent study were re-enrolled in the boosted group. The vaccine-naïve group included two adults receiving placebo in the parent study and 26 newly-enrolled individuals. All participants received the study vaccination and completed the study (**Figure 2**). Demographic characteristics at enrolment in the extension trial are presented in **Table 1**.

Immunogenicity

At seven days post-vaccination, anti-*S. sonnei* LPS IgG GMCs were 168 EU (95% CI: 32–889) in the boosted group compared to 32 EU (95% CI: 17–61) in the vaccine-naïve group (**Figure 3**; **Supplementary Table 1**). Seroresponse rates were 86% (95% CI: 42.1–99.64) and 24% (95% CI: 9.4–45.1) in the boosted and vaccine-naïve groups, respectively (**Figure 4A**; **Supplementary Table 2**). The percentage of individuals with anti-*S. sonnei* LPS IgG ≥121 EU was 71% (95% CI: 29.0–96.3) in the boosted group and 28% (95% CI: 12.1–49.4) in the vaccine-naïve group (**Figure 4B**; **Supplementary Table 2**).

Anti-*S. sonnei* LPS IgG GMCs increased substantially until 14 days post-vaccination in the boosted group, reaching a peak GMC of 883 EU (95% CI: 249-3126), with 100 and 86% of participants achieving seroresponse and antibody levels >121 EU, respectively. Antibody levels then declined at subsequent time points, dropping to about half the peak (GMC of 451 [95% CI: 113–1797]), at 84 days post-vaccination (**Figure 4**; **Supplementary Tables 1**, **2**). Antibody responses in the vaccine-naïve group also increased following vaccination, but more slowly, and showed a broad but much lower peak, with GMCs of 97 EU (95% CI: 51–187) and 100 EU (95% CI: 54–187) at 14 and 28 days post-vaccination, respectively; then declined to 89 EU (95% CI: 48–166) at 84 days post-vaccination (**Figure 4**; **Supplementary Table 1**). At all-time points except baseline, anti-*S. sonnei* LPS IgG GMCs in the boosted group were ≥5-fold higher compared to those in vaccine-naïve participants (**Figure 4**; **Supplementary Table 1**).

The kinetics of antibody response in the vaccine-naïve group depended on the antibody levels at the time of vaccination. Those with antibody levels higher than the detection limit at baseline had antibody kinetics that more closely resembled the boosted group, albeit with a much lower peak in antibody levels. GMCs peaked at 14 days post-vaccination (137 [95% CI: 65–289]),

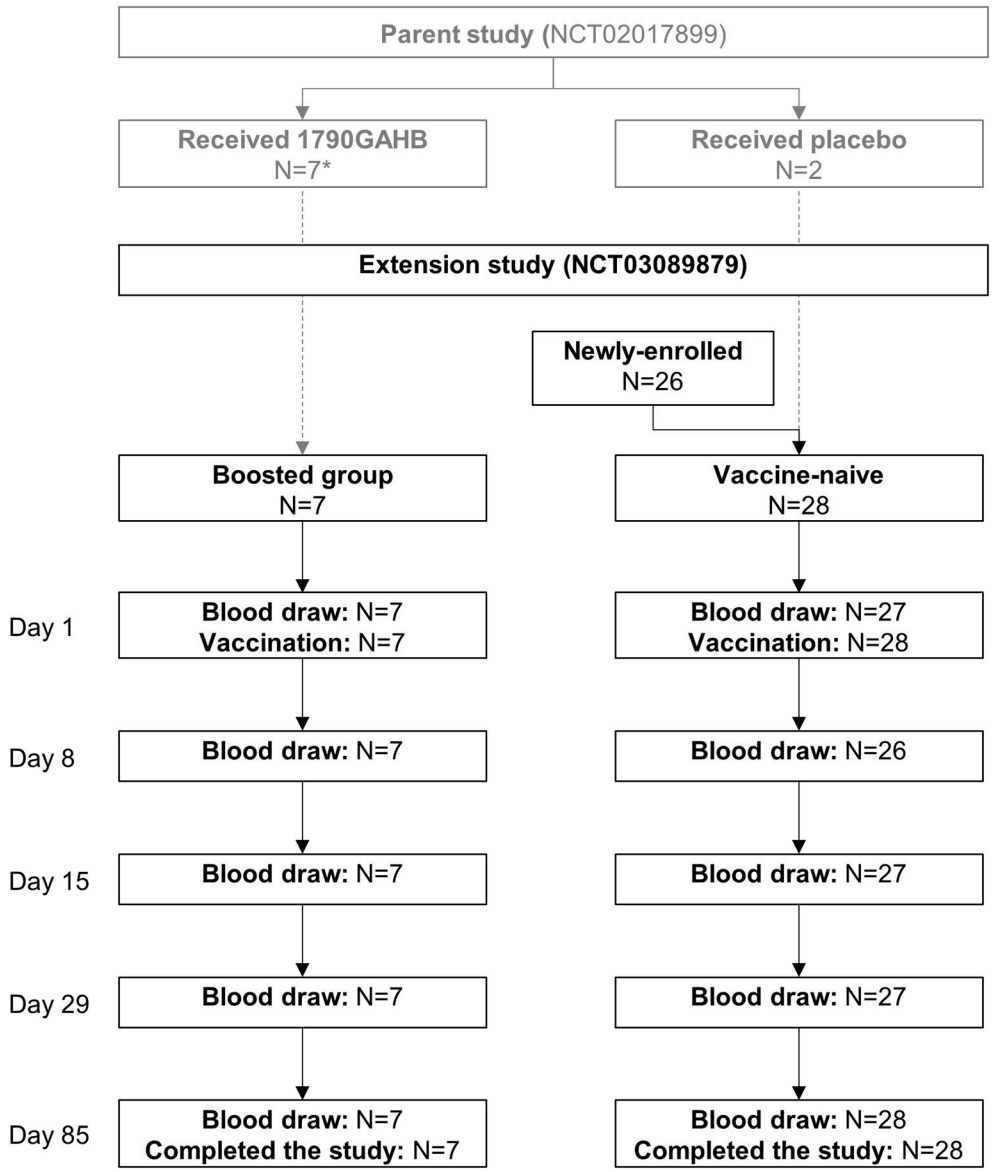

FIGURE 2 | Participant flowchart. N, number of participants. *Received doses of 1790GAHB with an O-antigen/protein content of 0·059/1 μg (4 participants), 0·29/5 μg (1 participant), and 2·9/50 μg (2 participants) in the parent study.

subsequently falling to a GMC of 110 (95%CI: 51–233) at 84 post-vaccination with 1790GAHB. By contrast, adults in the vaccine-naïve group with no detectable antibody at baseline had a slower and smaller raise in antibody; GMCs were 43 (95% CI: 11–175) and 55 (95% CI: 17–174) at 14 and 28 days post-vaccination, with only a small further decrease to a GMC value of 54 (14–208) at 84 days post-vaccination.

When considering anti-*S. sonnei* LPS IgG GMCs of participants in the boosted group across both the parent and extension trials, baseline antibody levels in the extension trial (GMC of 24 [95% CI: 4.12–145]) had decreased by ∼17% compared to those at 6 months after the three-dose primary vaccination series (GMC of 29 [3.15–261]) (**Supplementary Table 3**). A significant individual anamnestic response was observed for each participant in the boosted group, including those primed with only 0.059/1 μg of 1790GAHB in the parent trial (**Figure 5**). Of note, two of the participants, primed with 0.059/1 μg or 0.29/5 μg formulations, respectively, had low anti-*S. sonnei* LPS IgG at 28 days after the third primary dose in the parent study and undetectable levels both at 6 months post-primary vaccination and at the time of the booster dose. These two participants showed post-booster antibody levels peaking at 94 and 282 EU, respectively, at 14 days post-boosting with 1790GAHB in the extension trial. One individual, for whom anti-*S. sonnei* LPS IgG of 1,099 was observed at 28 days post-third primary vaccination, maintained high antibody levels

TABLE 1 | Participant characteristics at enrollment in extension study.

	Boosted group (*N* = 7)	Vaccine-naïve group (*N* = 28)	Total (*N* = 35)
Age (mean ± *SD*), years	37.7 ± 7.9	34.3 ± 8.5	34.9 ± 8.4
Male, *n* (%)	3 (42·9)	17 (60.7)	20 (57)
Race, *n* (%)			
Black	1 (14.3)	1 (3.6)	2 (6)
White	6 (85.7)	26 (92.9)	32 (91)
Other	0 (0.0)	1 (3.6)	1 (3)
Weight (mean ± *SD*), kg	63 ± 15.5	74.6 ± 11.4	72.3 ± 12.9
Height (mean ± *SD*), cm	168.6 ± 11.6	173.3 ± 10.5	172.3 ± 10.7
BMI (mean ± *SD*), kg/m^2	21.9 ± 2.8	24.8 ± 3.0	24.2 ± 3.1

N, number of enrolled participants in each group; SD, standard deviation; n (%), number (percentage) of participants in each category; BMI, body mass index.

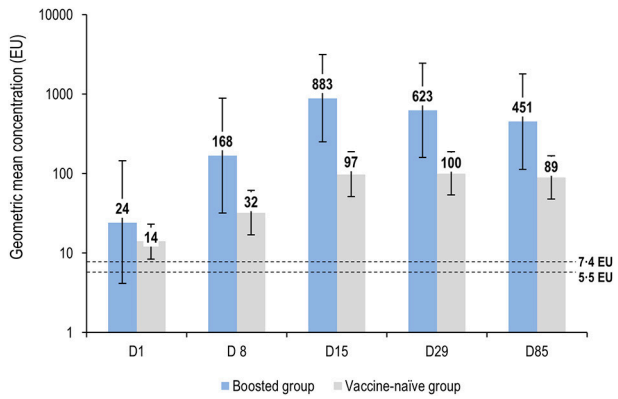

FIGURE 3 | Anti-*S. sonnei* LPS IgG geometric mean concentrations by time point (full analysis set for immunogenicity). LPS, lipopolysaccharide; IgG, immunoglobulin G; EU, enzyme-linked immunosorbent assay units; D, day. Error bars represent 95% confidence intervals. Dashed lines represent the limit of detection of the enzyme-linked immunosorbent assay, which varied from plate to plate, from 5·5 to 7·4 EU.

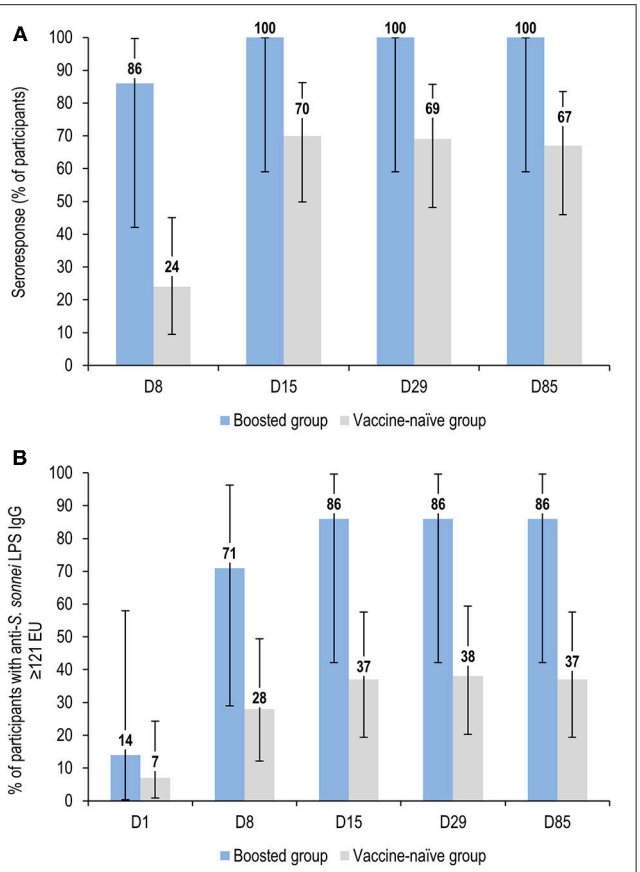

FIGURE 4 | Percentage of participants with seroresponse **(A)** and anti-*S. sonnei* LPS IgG ≥121 EU **(B)** by time point (full analysis set for immunogenicity). LPS, lipopolysaccharide; IgG, immunoglobulin G; EU, enzyme-linked immunosorbent assay units; D, day. Seroresponse to vaccination was defined as an increase in the anti-*S. sonnei* LPS serum IgG level of ≥50% for participants with baseline (D1) levels >50 EU or an increase of ≥25 EU for participants with pre-vaccination (D1) levels ≤50 EU. Error bars represent 95% confidence intervals.

up to re-enrolment in the extension study (908 EU), which further increased following the booster dose and peaked at 14 days post-boosting (4465 EU).

Safety

The most commonly reported solicited local AE was injection site pain after vaccination, reported by 86% of participants in either the boosted and vaccine-naïve groups. Erythema and induration were each reported by one participant in the boosted and the vaccine-naïve groups, respectively. Most of the local AEs were mild to moderate in severity, had the onset within 6 h post-vaccination and resolved within 4 days. Severe pain was reported by a single participant in the vaccine-naïve group (**Table 2**).

The most commonly reported solicited systemic AEs were fatigue, myalgia, headache, and arthralgia, reported by ≥39, 29, ≥14, and ≥11% of participants in either group. Overall, no considerable differences were observed between the two groups in the percentage of participants reporting each of the systemic AEs. The majority of solicited systemic AEs were mild to moderate in severity, had onset within 6 h post-vaccination and were resolved within 4 days post-vaccination. None of the study participants experienced fever. One vaccine-naïve participant reported severe fatigue, 6 h after vaccination (**Table 2**). Three adults (43%) in the boosted group and five (18%) in the vaccine-naïve group were administered analgesics/antipyretics for the treatment of pain/fever occurring within seven days post-vaccination.

Unsolicited AEs were reported by four (57%) participants in the boosted group, compared with seven (25%) in the vaccine-naïve group. The most commonly reported categories of unsolicited AEs were classified by MedDRA system organ class as nervous system disorders (headache in two [29%] individuals in the boosted group and two [7%] participants in the naïve group), or musculoskeletal and connective tissue disorders (arthralgia and musculoskeletal pain in the boosted group and coccydynia

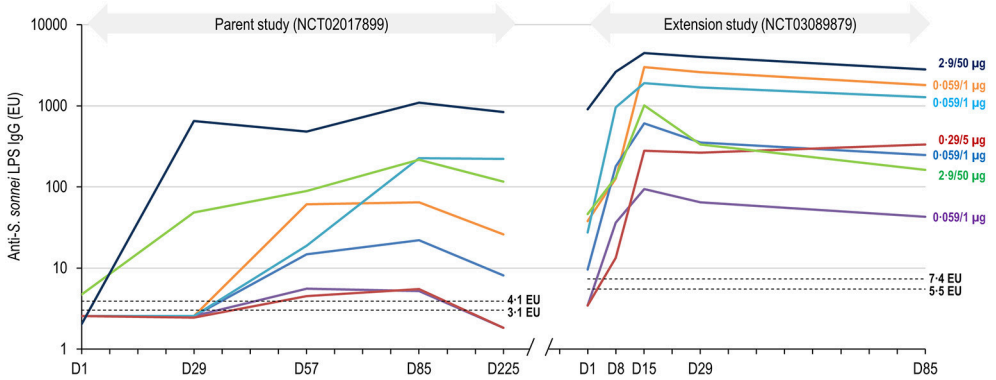

FIGURE 5 | Individual anti-S. sonnei LPS IgG responses of participants in the boosted group throughout the parent and extension studies. LPS, lipopolysaccharide; IgG, immunoglobulin G; EU, enzyme-linked immunosorbent assay units; D, day. The interval from D225 in the parent study and D1 in the extension study is not represented to scale. Dashed lines represent the limit of detection of the enzyme-linked immunosorbent assay, which varied from plate to plate, from 3·1 to 4·1 EU in the parent study (17) and from 5·5 to 7·4 EU in the extension study.

in the vaccine-naïve group, each reported by one participant). All other classes of unsolicited AEs were reported by <2 participants. All reported unsolicited AEs were mild or moderate in severity and were resolved at the time of study termination, with the exception of hemorrhoids in one participant in the vaccine-naïve group. One individual in the vaccine-naïve group reported an AESI (neutropenia), which was assessed by the investigator as at least possibly-related to the study vaccine. The episode was asymptomatic and mild in nature and was resolved by study end. No deaths or SAEs were reported in the study.

DISCUSSION

This study had some limitations due to the small sample size of the boosted group and to the different dose levels received by boosted volunteers in the primary vaccination; additionally, the immunological analyses, which were descriptive in nature, did not include functional assays or assessment of the cell-mediated immunity.

However, this was the first study to assess the longevity of the anti-*S. sonnei* LPS IgG response and the immunogenicity and safety of a booster dose of 1790GAHB, an investigational GMMA vaccine against *S. sonnei*.

A dose of 1790GAHB, administered to adults with anti-*S. sonnei* LPS antibody levels at or below the limit of detection before priming, induced a clear booster response 2–3 years after the completion of a three-dose primary series. A single dose of 1790GAHB administered to vaccine-naïve adults also elicited a robust increase in anti-*S. sonnei* LPS IgG. However, the response elicited by 1790GAHB was consistently higher in participants previously primed with three vaccine doses than in vaccine-naïve adults. A similar conclusion can be drawn by comparing the 1790GAHB responses at 28 days post-vaccination in the boosted group in the extension trial (GMC = 623 EU) with the response of the same participants after their first injection in the parent trial (GMC = 8.56 EU) or in participants with undetectable antibody at baseline and vaccinated with a high dose vaccine (GMC = 143 EU).

The use of alternate ways of assessing magnitude of antibody response (i.e., seroresponse rate and percentages of participants with ≥121 EU) was consistent with the observations made based on GMCs. A substantially higher response in the boosted group compared to the vaccine-naïve group was observed, indicating a clear booster response in previously vaccine-primed participants, even those receiving primary doses of OAg/protein content as low as 0.059/1 µg. The data also suggested that individuals with no pre-existing antibodies at the time of first vaccination can further benefit from the administration of a booster dose of 1790GAHB at 2–3 years post-primary vaccination.

It has been previously reported that there is a significant correlation between serotype-specific anti-LPS IgG antibodies in serum and resistance to shigellosis (20–22). Furthermore, episodes of *Shigella* diarrhea confer protection against future illness due to infection with the same, but not other serotypes (23). It is therefore likely that an effective public health vaccine will need to include antigens from most of the epidemiologically relevant *Shigella* serotypes and induce long-lived high specific anti-*Shigella* LPS antibody levels in immunologically-naïve individuals such as young children, in whom the burden of shigellosis is the highest. This study was conducted in adults with very low antibody levels prior to initial vaccination, emulating populations with no previous exposure to natural infection, and assessed long-term persistence of antibody levels. By evaluating volunteers from the parent trial with low pre-vaccination anti-LPS levels, we found that antibodies against *S. sonnei* persisted up to 3 years following primary vaccination with 1790GAHB and increased considerably after a booster dose. All participants with measurable antibodies at 6 months after the primary vaccination series still had substantial antibody levels at the time of boosting 2–3 years later. Three of them, including the individual with the highest antibody level of about 1,000 EU at 1 month post-third primary vaccination, maintained

TABLE 2 | Summary of local and systemic solicited adverse events (full analysis set).

AE	Severity	Boosted group (*N* = 7)	Vaccine-naïve group (*N* = 28)
SOLICITED LOCAL ADVERSE EVENTS, *n* (%)			
Pain	Any	6 (86)	24 (86)
	Severe	0	1 (4)
Erythema	Any	1 (14)	0
	Severe	0	0
Induration	Any	0	1 (4)
	Severe	0	0
SOLICITED SYSTEMIC ADVERSE EVENTS, *n* (%)			
Arthralgia	Any	1 (14)	3 (11)
	Severe	0	0
Chills	Any	1 (14)	1 (4)
	Severe	0	0
Fatigue	Any	3 (43)	11 (39)
	Severe	0	1 (4)
Headache	Any	1 (14)	5 (18)
	Severe	0	0
Malaise	Any	1 (14)	2 (7)
	Severe	0	0
Myalgia	Any	2 (29)	8 (29)
	Severe	0	0
Fever	≥38.0	0	0

AE, adverse event; N, number of participants included in the analyses; n (%), number (percentage) of participants in each group. Severe solicited adverse events were defined as >100 mm (erythema, induration), or as preventing normal daily activities (pain, headache, arthralgia, chills, fatigue, malaise, myalgia).

their antibody levels without considerable change throughout the entire duration of the studies. Over a period of ∼3 years, between the 1 month post-primary and the pre-booster time points, there was a 2.4-fold decrease in the GMC. Most of this drop occurred in the 6 months following primary vaccination, with just a 1.2-fold drop over the remaining period. Although the comparison is limited by the small number of participants enrolled in this extension study, we observed antibody decay rates very different from those previously reported following vaccination with *Shigella* OAg-specific conjugate vaccine. In one study conducted among Israeli adults, who received a single vaccination of a conjugate vaccine composed of the O-specific polysaccharides of *S. sonnei* covalently bound to *Pseudomonas aeruginosa* recombinant exoprotein A (*S. sonnei*-rEPA), IgG levels declined 2.3-fold over a time period of 6 months after vaccination and another 2.2-fold over the next 18 months, compared with levels at 2 weeks post-vaccination (24). In a second Israeli adult study, antibody levels elicited by the same vaccine decayed 3.4-fold over 4 months post-vaccination in participants not infected with *S. sonnei* (25), while in a study in 4 to 7-year-old Israeli children, vaccinated twice with *S. sonnei*-rEPA, antibody levels declined 4.3-fold over 20 weeks following the second injection (26). Moreover, in our study, an additional vaccination with 1790GAHB elicited an anamnestic response in all participants of the boosted group, regardless of the OAg/protein content of 1790GAHB received during priming 2–3 years earlier.

The incidence of solicited AEs was similar between the boosted and vaccine-naive groups, showing that no increased reactogenicity is expected following a fourth administration of 1790GAHB vaccine at 2–3 years post-primary vaccination. As in previous studies assessing the reactogenicity and safety of the 1790GAHB vaccine, pain at injection site was the most common solicited AE (17, 18). A lower frequency of both local and systemic reactions was reported compared with that following the first dose with the same OAg/protein content of vaccine (1.5/25 μg) administered to Kenyan adults (18). Neutropenia was collected as an AESI due to the occurrence of such episodes in previous studies, including the parent trial (17), but only one mild and asymptomatic neutropenia episode was reported in the current extension study, in a previously unprimed participant. Overall, the safety results of this trial confirmed the acceptable safety profile of 1790GAHB shown in previous clinical trials (17, 18).

CONCLUSIONS

A single administration of the 1790GAHB vaccine elicited a booster response in healthy European adults receiving a three-dose primary schedule 2–3 years earlier and having undetectable anti-*S. sonnei* LPS IgG prior to primary vaccination. A strong immune response was also induced in vaccine-naïve participants. 1790GAHB was well tolerated in all vaccine-naïve study participants, with no increased reactogenicity observed in boosted individuals. These results support further studies investigating the administration of GMMA-based *Shigella* vaccine using primary and booster vaccination schedules in adults and children. As cross protection against other *Shigella* serotypes is unlikely for this monovalent *S. sonnei* vaccine, further development will be based on a multicomponent vaccine including GMMA from other epidemiologically relevant *Shigella* serotypes.

DATA SHARING

Anonymized individual participant data and study documents can be requested for further research from www.clinicalstudydatarequest.com.

AUTHOR CONTRIBUTIONS

AS, LM, AP, JA, ASS, EM, PL, AN, OL, NN, PF, and AL were involved in the study conception and design. OL, AS, LM, AP, EM, PL, AN, LS, NN, VC, and AL were involved in acquisition and generation of data. OL, AP, EM, PL, NN, ASS, VC, and AL performed the study. OL, AS, LM, AP, ASS, PL, AN, VC, and PF were involved in data analysis and data interpretation. All authors contributed substantially to the development of the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00335/full#supplementary-material

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SA, and a patent US2015202274 pending to GlaxoSmithKline Biologicals SA. LM reports grants from Bill & Melinda Gates Foundation, outside the submitted work. In addition, LM has a patent WO2016202872 issued. AN and PF report grants from BMGF, outside the submitted work.

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REVIEW
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Infectious Disease Threats in the Twenty-First Century: Strengthening the Global Response

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The world has developed an elaborate global health system as a bulwark against known and unknown infectious disease threats. The system consists of various formal and informal networks of organizations that serve different stakeholders; have varying goals, modalities, resources, and accountability; operate at different regional levels (i.e., local, national, regional, or global); and cut across the public, private-for-profit, and private-not-for-profit sectors. The evolving global health system has done much to protect and promote human health. However, the world continues to be confronted by longstanding, emerging, and reemerging infectious disease threats. These threats differ widely in terms of severity and probability. They also have varying consequences for morbidity and mortality, as well as for a complex set of social and economic outcomes. To various degrees, they are also amenable to alternative responses, ranging from clean water provision to regulation to biomedical countermeasures. Whether the global health system as currently constituted can provide effective protection against a dynamic array of infectious disease threats has been called into question by recent outbreaks of Ebola, Zika, dengue, Middle East respiratory syndrome, severe acute respiratory syndrome, and influenza and by the looming threat of rising antimicrobial resistance. The concern is magnified by rapid population growth in areas with weak health systems, urbanization, globalization, climate change, civil conflict, and the changing nature of pathogen transmission between human and animal populations. There is also potential for human-originated outbreaks emanating from laboratory accidents or intentional biological attacks. This paper discusses these issues, along with the need for a (possibly self-standing) multi-disciplinary Global Technical Council on Infectious Disease Threats to address emerging global challenges with regard to infectious disease and associated social and economic risks. This Council would strengthen the global health system by improving collaboration and coordination across organizations (e.g., the WHO, Gavi, CEPI, national centers for disease control, pharmaceutical manufacturers, etc.); filling in knowledge gaps with respect to (for example) infectious disease surveillance, research and development needs, financing models, supply chain logistics, and the social and economic impacts of potential threats; and making high-level, evidence-based recommendations for managing global risks associated with infectious disease.

Keywords: global health, global health systems, infectious disease, outbreak, epidemic, pandemic, antimicrobial resistance (AMR), pandemic preparedness and response

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INTRODUCTION

In 1918, as the First World War was winding to a close, a mysterious disease that left victims blue in the face and gasping for air tore through the trenches crisscrossing Europe and traversed the oceans, stowed away on war ships. By the time the so-called Spanish flu had run its course in 1920, the pandemic had infected more than a quarter of the world's population and resulted in some 30 million to 100 million deaths (1, 2). In comparison, the two World Wars are estimated to have killed roughly 77 million combined (3). By any measure, the 1918 flu pandemic was one of the worst catastrophes of the twentieth century.

In the 100 years that have passed since the Spanish flu first besieged the world, no pandemic has approached its magnitude of fatality over such a short period. Humanity's relative good fortune with respect to infectious disease can be attributed, in part, to the elaborate global health system the world has gradually developed as a bulwark against infectious disease threats, both known and unknown. This system consists of various formal and informal networks of organizations that serve different stakeholders; have varying goals, modalities, resources, and accountability; operate at different territorial levels (i.e., local, national, regional, or global); and cut across the public, private-for-profit, and private-not-for-profit sectors.

Despite its track record, whether the global health system as currently constituted can provide effective protection against an expanding and evolving array of infectious disease threats has been called into question by recent outbreaks of Ebola, Zika, dengue, Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), and influenza, as well as the looming specter of rising antimicrobial resistance (AMR). Taken together, these diseases—along with a slew of other known and unknown pathogens—jeopardize not only human health, but also various forms of social and economic well-being. Of particular concern is the lack of a single entity that has a sufficiently high-level and comprehensive view of the full range of potential threats—whether naturally occurring, accidental, or due to intentional biological attack—and of the network of organizations tasked with their surveillance, prevention, and mitigation.

To address emerging global challenges with regard to infectious disease and associated social and economic risks, we propose the formation of a multidisciplinary Global Technical Council on Infectious Disease Threats. The Council, which may be self-standing or housed within an existing organization, would strengthen the global health system by doing the following: (1) improving collaboration and coordination across relevant organizations; (2) filling in knowledge gaps with respect to (for example) infectious disease surveillance, research and development (R&D) needs, financing models, supply chain logistics, and the social and economic impacts of potential threats; and (3) making high-level, evidence-based recommendations for managing global risks associated with infectious disease.

BACKGROUND

Increased longevity is among the most remarkable aspects of human progress. Global life expectancy has increased by 24 years since 1950 (4). Large numbers of people are now living into their eighth and ninth decades (4), and life expectancy is projected to exceed 85 in several countries (and 80 in many more) in the second half of this century (5). These advances reflect precipitous declines in infectious disease mortality, for which we can thank improvements in sanitation, hygiene, the availability of clean water, nutrition, vaccination, antibiotics, medical practices, and health systems, as well as income growth.

While infectious diseases and associated mortality have abated, they remain a significant threat throughout the world. In the twenty-first century, we continue to fight both old pathogens—like the plague—that have afflicted humanity for millennia, and new pathogens—like human immunodeficiency virus (HIV)—that have mutated or have spilled over from animal reservoirs. Some infectious diseases—like tuberculosis (TB) and malaria—are endemic to many areas, imposing substantial but steady burdens. Others—like influenza—fluctuate in pervasiveness and intensity, wreaking havoc in the developing and developed worlds alike when an outbreak (a sharp increase in prevalence in a relatively limited area or population), an epidemic (a sharp increase covering a larger area or population), or a pandemic (an epidemic covering multiple countries or continents) occurs. **Table 1** details some of these most prominent cases of the last 100 years.

Perhaps the greatest challenge of anticipating and responding to epidemics is the vast array of possible causes, including pathogens that are currently unknown. In May 2016, the World Health Organization (WHO) published a list of epidemic-potential disease priorities requiring urgent R&D attention (26). That list has since been updated twice, most recently in February 2018 (see **Table 2**) (40). The Blueprint list of priority diseases "focuses on severe emerging diseases with potential to generate a public health emergency, and for which no, or insufficient, preventive and curative solutions exist" (41). It was developed through an expert consultation involving both the Delphi method and multi-criteria decision analysis. The top prioritization criteria considered were (in order) potential for human transmission, the availability of medical countermeasures, the severity or case fatality rate, the human/animal interface, other factors (not defined), the public health context of the affected area, potential societal impacts, and the evolutionary potential.

Beyond the included pathogens, diseases that are currently endemic in some areas, but could spread without proper control to others, represent another category of threat. Tuberculosis, malaria, and dengue are examples, as well as HIV. Pandemic influenza also merits special attention; indeed, the WHO has developed a separate Pandemic Influenza Preparedness Framework (42).

Meanwhile, the very drugs that helped produce miraculous declines in infectious disease mortality over the second half of the twentieth century are now beginning to lose their effectiveness. AMR is on the rise throughout much of the world, and

TABLE 1 | Prominent outbreaks, epidemics, and pandemics of the last century.

Year(s)	Pathogen	Geographic location	Cases/mortality	Other notes	References
1918–1920	Influenza (Spanish flu)	Worldwide	500 million cases and 30 to 100 million deaths	The Spanish flu claimed the lives of 2–5% of world's population, far exceeding the death toll of WWI.	(1, 2, 6)
1957–1958	Influenza (Asian flu)	Worldwide	1 to 2 million deaths	Accelerated development of a vaccine limited the spread of the responsible influenza strain.	(7)
1968–1969	Influenza (Hong Kong flu)	Worldwide	500,000 to 2 million deaths	The Hong Kong flu was the first virus to spread extensively due to air travel.	(7)
1960-present	HIV/AIDS	Worldwide, primarily Africa	70 million cases and 35 million deaths	HIV was first identified in 1983. The earliest known case came from a blood sample collected in 1959.	(8–10)
1961-present	Cholera	Worldwide	1.4 to 4 million annual cases and 21,000 to 143,000 annual deaths	The seventh cholera pandemic began in South Asia in 1961. Recent notable outbreaks include those in Zimbabwe from 2008 to 2009, Haiti from 2010-present, and Yemen from 2016-present.	(11, 12)
1974	Smallpox	India	130,000 cases and 26,000 deaths	One of the worst smallpox epidemics of the twentieth century occurred just 3 years before the disease was eradicated.	(13)
1994	Plague	India	693 suspected cases and 56 deaths	The outbreak originated in Surat, India. Within days, hundreds of thousands of the city's 1.6 million residents fled, spreading the disease across five states.	(14, 15)
2002–2003	SARS	Originated in China, spread to 37 countries	8,098 cases and 774 deaths	International business travel allowed the SARS virus to spread quickly across continents.	(16, 17)
2009	Influenza (Swine flu)	Worldwide	284,000 deaths	Many public and private facilities in Mexico closed in an attempt to prevent the spread of "swine flu" during the early days of the epidemic. The pork industry also suffered losses, even though eating pork products posed no risk.	(18–20)
2014–2016	Ebola	West Africa, primarily Guinea, Liberia, and Sierra Leone	28,600 cases and 11,325 deaths reported (likely underestimates)	300,000 doses of an experimental Ebola vaccine were subsequently stockpiled.	(21, 22)
2015-present	Zika	The Americas, primarily Brazil	Unknown number of cases and 0 deaths reported	The Zika epidemic has resulted in few, if any, deaths. However, birth defects resulting from infection in pregnant women occurred frequently, which prompted some governments to encourage delaying pregnancy for as long as 2 years.	(23)
2016	Dengue	Worldwide	100 million cases and 38,000 deaths	Dengue outbreaks occur periodically in affected regions. 2016 was notable for the unusual scale of outbreaks across the globe.	(24)
2017	Plague	Madagascar	2,417 cases and 209 deaths	Plague is endemic in Madagascar, but an increase in pneumonic plague, which can be transmitted from human to human, was associated with the recent spike in cases.	(25)

widespread pan-resistant "superbugs" could pose yet another threat if we fail to act (43). While rapid transmission of resistant pathogens is unlikely to occur in the same way it may with pandemic threats, the proliferation of superbugs is making the world an increasingly risky place. AMR threats also differ from epidemic threats in a number of other respects: Most of the top AMR threats are bacterial, and many are typically contracted as nosocomial infections; pathogens of epidemic potential tend to be viral and often emerge from zoonotic reservoirs to cause outbreaks in human populations.

Table 3 documents the WHO's list of priority pathogens for R&D of new antibiotics (44). The list was selected through a multi-criteria decision analysis incorporating both quantifiable evidence and the input of 70 experts with different backgrounds and from a variety of geographies. Notably, the list was not developed to prioritize the top public health threats with respect to AMR, but rather to identify the pathogens for which R&D needs are greatest, considering both health burden and availability of treatment. The WHO explicitly excluded TB from the list and included only bacterial pathogens.

Beyond the pathogens on this list, mounting resistance against the drugs used to treat TB, HIV, and malaria is especially concerning. Resistant TB, for instance, is already responsible for 240,000 deaths globally per year (out of 700,000 total AMR-related deaths, which is likely an underestimate) (43, 45).

TABLE 2 | WHO's Blueprint list of priority diseases requiring urgent R&D attention, 2018.

Disease	Description	Availability of biomedical countermeasures	References
Crimean-Congo hemorrhagic fever (CCHF)	Hemorrhagic fever caused by virus transmitted primarily through ticks and livestock, with case-fatality rate of up to 40%. Human-to-human transmission possible.	No vaccine available; Ribavirin (antiviral) provides some treatment benefit	(27)
Ebola virus disease	Hemorrhagic fever caused by virus transmitted from wild animals, with case-fatality rate of up to 90% (50% is average). Human-to-human transmission is possible.	Experimental vaccine and treatments available	(28)
Marburg virus disease	Hemorrhagic fever caused by virus transmitted by fruit bats, with case-fatality rate of up to 88% (50% is average). Human-to-human transmission is possible.	No vaccine available	(29)
Lassa fever	Hemorrhagic fever caused by virus transmitted from items that have contacted rodent urine or feces, with case-fatality rate of 15% in severe cases (1% overall). Human-to-human transmission is possible.	No vaccine available; Vaccine development funded by CEPI	(30, 31)
Middle East respiratory syndrome coronavirus (MERS-CoV)	Respiratory disease caused by a coronavirus transmitted by camels and humans, with case-fatality rate of 35%.	No vaccine available; Vaccine development funded by CEPI	(31, 32)
Severe acute respiratory syndrome (SARS)	Respiratory disease caused by a coronavirus transmitted from human to human and from an unknown animal reservoir (possibly bats), with a case-fatality rate of 10%.	No vaccine available; experimental vaccines are under development	(33, 34)
Nipah and henipaviral diseases	Disease caused by a virus transmitted by fruit bats, pigs, and humans; can manifest as an acute respiratory syndrome or encephalitis. The case-fatality rate is estimated at 40 to 75% and depends on local capabilities.	Vaccine development funded by CEPI	(31, 35)
Rift Valley fever	Disease caused by a virus transmitted by contact with the blood or organs of infected animals, or by mosquitos. In severe cases, can manifest in an ocular infection, as meningoencephalitis, or as a hemorrhagic fever. Up to 50% case-fatality rate in patients with hemorrhagic fever. No human-to-human transmission reported.	An experimental, unlicensed vaccine exists but is not commercially available; CEPI has an open call for proposals for development of a new vaccine	(31, 36)
Zika	Disease caused by a flavivirus transmitted by *Aedes aegypti* mosquitoes. Can result in microcephaly in infants born by infected mothers and in Guillain-Barré syndrome. Human-to-human transmission is possible.	No vaccine available	(37)
Disease X (representing pathogens currently unknown to cause human disease and requiring cross-cutting preparedness)	N/A	CEPI is funding the development of institutional and technical platforms that allow for rapid R&D in response to outbreaks of any number of pathogens for which vaccines do not yet exist.	(38, 39)

Finally, the global health community must also acknowledge the real threat posed by the possibility of a human-caused infectious disease outbreak, whether from the accidental release of infectious agents from a research facility or from an intentional biological attack. Over the past half-century, several alarming (but thankfully contained) events of this sort have occurred. In 1993, the Japanese doomsday cult Aum Shinrikyo sprayed anthrax spores from the top of a cooling tower in Tokyo in a failed attempt to start an epidemic (46) [In 1995, the same group used a chemical weapon similar to sarin in an attack on the Tokyo subway system that caused 13 deaths and many injuries (47)]. In 2001, an attacker with unknown motives caused terror and chaos in the United States by mailing letters laced with anthrax to the offices of two senators and multiple members of the news media, resulting in five deaths (48). And in 2014, an accident involving live anthrax bacteria at the U.S. Centers for Disease Control and Prevention potentially exposed dozens of workers to the pathogen (49). As long as stores of dangerous pathogens, such as anthrax and smallpox, are maintained (for research purposes), the potential for a damaging accident or intentional attack will remain. Advancements in gene editing and the end of a U.S. government-imposed moratorium on funding potentially risky research involving the editing of deadly viruses may amplify the threat. As early as 2002, researchers demonstrated the feasibility of chemically synthesizing highly infectious agents such as poliovirus (50). More recently, another team of researchers synthesized horsepox, a relative of smallpox not known to harm humans (51). The success of this latter experiment suggests that with rudimentary scientific knowledge and a relatively small amount of money, a group with nefarious intent could synthesize smallpox without significant difficulty and in a short amount of time (52).

INFECTIOUS DISEASE THREATS POSE ECONOMIC AND SOCIAL RISKS

Infectious disease threats—and the fear and panic that may accompany them—map to various economic and social risks. With respect to outbreaks and epidemics (whether naturally occurring or human-initiated), there are obvious costs to the health system in terms of medical treatment and outbreak

TABLE 3 | WHO priority pathogens list for R&D of new antibiotics.

Pathogen	Resistance
PRIORITY 1: CRITICAL	
Acinetobacter baumannii	Carbapenem-resistant
Pseudomonas aeruginosa	Carbapenem-resistant
Enterobacteriaceae	Carbapenem-resistant, 3rd generation cephalosporin-resistant
PRIORITY 2: HIGH	
Enterococcus faecium	Vancomycin-resistant
Staphylococcus aureus	Methicillin-resistant, vancomycin intermediate and resistant
Helicobacter pylori	Clarithromycin-resistant
Campylobacter	Fluoroquinolone-resistant
Salmonella species	Fluoroquinolone-resistant
Neisseria gonorrhoeae	3rd generation cephalosporin-resistant, fluoroquinolone-resistant
PRIORITY 3: MEDIUM	
Streptococcus pneumoniae	Penicillin-non-susceptible
Haemophilus influenzae	Ampicillin-resistant
Shigella species	Fluoroquinolone-resistant

Source: Tacconelli et al. (44).

control. A sizable outbreak can overwhelm the health system, limiting the capacity to deal with other routine health issues and thereby compounding the stress on the system. Beyond shocks to the health sector, epidemics force those who are ill and their caretakers to miss work or be less effective at their jobs, disrupting productivity. When critical human resources like engineers, scientists, and physicians are affected, productivity impacts can be magnified.

Fear of infection can result in social distancing or the closing of schools, enterprises, commercial establishments, transportation, and public services—all of which disrupt economic and other socially valuable activity. Concern over the spread of even a relatively contained outbreak can lead to decreased trade. For example, a ban imposed by the European Union on the export of British beef lasted for 10 years following the identification of a mad cow disease outbreak in the United Kingdom, despite relatively low (hypothesized) transmission to humans (53, 54). Travel and tourism to regions affected by outbreaks are also likely to decline, as has happened in Brazil and several southeast Asian countries when dengue incidence spiked (55–58). In the case of some long-running epidemics, such as HIV and malaria, foreign direct investment can be deterred as well (59, 60).

The economic risks of epidemics are not trivial. A recent study estimated the expected per annum cost of pandemic influenza at roughly $500 billion (0.6% of global income), inclusive of both the cost of lost income and the intrinsic cost of elevated mortality (61). The World Bank similarly estimated that a flu pandemic causing 28 million or more excess deaths could result in a loss of as much as 5% of global GDP (62, 63). The large projected economic impact of an influenza pandemic stems primarily from the anticipated high mortality and morbidity. However, even when the health impact of an outbreak is relatively limited, its economic consequences can quickly become magnified. Liberia, for example, saw GDP growth decline 8 percentage points from 2013 to 2014 during the recent Ebola outbreak in West Africa, even as the country's overall death rate fell over the same period (4, 64).

As with outbreaks and epidemics, the economic risks of AMR begin with increased costs to the health system. Resistant infections demand the use of more expensive second- and third-line treatments and are sometimes associated with prolonged hospital stays (65–67). As incidence of resistant infections grows, the cumulative magnitude of these costs will grow as well.

Perhaps the biggest fear with AMR is that it will progress to the point where a significant number of infections are entirely untreatable. Absent that calamity, we can nonetheless envision a world in which contracting infectious diseases will carry an increased risk of mortality or severe morbidity. As broad-spectrum antibiotics lose their effectiveness, certain procedures (including some common surgeries) that rely on prophylactic antibiotic use may be deemed too risky to administer, resulting in additional morbidity. Some level of decreased productivity is almost certain to be a consequence of AMR's health impact, as excess morbidity and mortality will remove people from the labor force or otherwise diminish their capacity to work. In some economies, reductions in livestock output due to the spread of disease in animal populations could have major repercussions. In a high-impact scenario, AMR may also lead to notable reductions in international trade.

Projections of AMR's potential economic impact vary significantly, as the magnitude of AMR's eventual health burden is difficult to predict for a variety of reasons. The upper bounds of existing estimates are alarming. According to the World Bank, AMR could reduce global GDP by 3.8% by 2050 in a worst-case scenario, with developing economies bearing a disproportionate burden (68). And a 2014 report by the Review on Antimicrobial Resistance, which was commissioned by David Cameron and chaired by Jim O'Neill, projected a cumulative cost of $100 trillion by the mid-century mark if resistance in a number of pathogens, including TB, malaria, and HIV, were to progress unchecked (43). While the likelihood of these extreme scenarios is debatable, it is certain that AMR poses a sizeable economic risk.

Infectious disease threats pose additional social risks beyond those that are strictly economic. Outbreaks and epidemics have the potential to induce geopolitical instability. Fear of an outbreak could lead people to flee their homes [as occurred following an outbreak of plague in Surat, India in 1994 (15)], potentially causing an international migration crisis. Epidemics could also increase the vulnerability of a weak government—especially one with an accompanying weak health system—leading to state fragility.

CHALLENGES

There are a number of complicating factors when it comes to managing the risk of infectious disease. Several ongoing demographic trends point toward an increased potential for transmission of pathogens. While the populations of many

developed countries are stabilizing or even declining in size, rapid population growth continues in regions where infectious disease outbreaks are likely to originate and where many countries have weak health systems that may struggle to cope with epidemics. The population of Sub-Saharan Africa, for instance, is increasing at a rate of 2.65% per year—more than twice the highest rate of population growth experienced by high-income countries since the 1950s (4). 2007 marked the first time in history in which a greater proportion of the world's population lived in urban than in rural areas (69). Urbanization means more humans living in close quarters with each other, amplifying the transmissibility of contagious disease. In areas experiencing rapid urbanization, housing shortages can lead to the growth of slums, which forces more people to live in conditions with substandard sanitation and poor access to clean water, compounding the problem. Finally, with the share of older adults increasing in every country (4), global population aging could further exacerbate the potential for widespread transmission of infectious disease, as immunosenescence leaves the elderly more vulnerable to infection (70).

Climate change may also play a role in driving pathogen transmission, as the habitats of various common disease-carrying vectors—such as the *Aedes aegypti* mosquito, which can spread dengue, chikungunya, Zika, and yellow fever, among other pathogens—expand (71). Human interactions with animal populations have always carried a risk of producing pathogen spillovers (72), and the changing nature of these interactions—as factory farming increases to meet food demand and humans continue encroaching on natural habitats, for example—could promote additional zoonoses. Civil conflict often results in new disease outbreaks or the exacerbation of ongoing ones, especially when populations are displaced, public health infrastructure is affected, or the provision of basic care and immunizations is interrupted (73–76).

The phenomenon of globalization compounds the risks posed by the aforementioned challenges. Many diseases with epidemic potential can be transmitted rapidly, both within and across countries. The proliferation and ease of international air travel and trade increase the difficulty and importance of containing outbreaks in their early phases. Globalization also has implications for AMR: The movement of people makes populations with low rates of circulating resistance vulnerable to transmission of resistant strains from other areas of the globe.

Perhaps the chief challenge for managing AMR is that the use of antimicrobials constitutes the most powerful driver of resistance. Each dose of antimicrobials consumed places evolutionary pressure on target and bystander pathogen populations to develop and proliferate mechanisms of resistance. The baked-in nature of the problem is compounded by the fact that there is currently tremendous need for increased access to antimicrobials in low- and middle-income countries (LMICs), where many continue to die every year from infectious diseases that are easily treated in the developed world (77). As the international community strives to close this access gap, national and global AMR response plans should be carefully designed to avoid exacerbating the unmet need for antimicrobials in LMICs and its consequences for human health.

Several factors complicate the management of the risk for biological accidents and attacks. With respect to accidents, there is a complicated tradeoff between enabling socially valuable research on dangerous pathogens (in order to better understand their spread or contribute to the development of countermeasures, for example) and imposing necessary safeguards to limit any potential danger. Removing the barriers to research on deadly pathogens (including through the manipulation of their genetic makeup) may allow us to be better prepared for naturally occurring outbreaks and attacks, but some specialists worry about the possibility of human error leading to catastrophe (78). Experts cite the relative ease and low cost of producing certain biological agents as a concern when it comes to intentional biological attack, which could come at the hands of a terrorist organization (79, 80). In addition, some biological agents that may be used in an attack (such as anthrax) have lengthy incubation periods, which could make it difficult for national governments to locate and apprehend attackers or otherwise organize a response (81).

There are numerous economic and political challenges to implementing the measures needed to prepare for and respond to infectious disease threats. First, the likelihood of any single infectious agent sparking an epidemic (including via an accident or attack) is relatively low, even if the aggregate risk is high. The diffuse nature of these threats can make it difficult to both prioritize available responses and summon the necessary political will to invest in prevention and preparedness. Similarly, the magnitude of AMR's consequences is not immediately obvious to many policymakers nor to the general public. Currently, AMR is a slow-burning problem that directly affects the lives of a relatively small portion of the global population. If left unchecked, however, that problem could grow exponentially.

Another political challenge involves the lack of a reliable mechanisms for incentivizing international collaboration in the development of new biomedical countermeasures. Manufacturers from high-income countries must sometimes rely on LMICs to provide biological samples needed for R&D, but LMICs have legitimate concerns that they may not receive an equitable share of any benefits resulting from their contributions, including access to vaccines, drugs, and other products. In 2007 these concerns prompted Indonesia to refuse sharing influenza samples needed for vaccine development with the WHO (82). The Nagoya Protocol, which came into effect in 92 countries in 2010, was intended to help address this problem by creating an enforceable system to ensure the sharing of benefits resulting from research based on genetic resources shared between countries. However, some feel that the requirements imposed by the Nagoya Protocol are too cumbersome and that potential jail sentences for scientists who are found to be in violation of its provisions could suppress important research (83). The global community must continue working to find the right balance between ensuring that manufacturers intent on developing critical products for global health can access needed resources expeditiously and promoting an equitable distribution of benefits resulting from those products.

There are established financing issues for global public goods, such as vaccines, to fight epidemics. While the social

value of these vaccines and similar products may be very high, the expected private value to the companies most likely to manufacture them is often quite low (84). For-profit pharmaceutical companies are unlikely to invest in R&D of a product unless it promises a substantial return on investment. Social investment has also suffered, at times, when no immediate crisis spurs public and political interest. For example, U.S. government investments to contend with outbreaks have fallen 50% from their peak during the 2014 Ebola outbreak (85). This cycle of panic and neglect makes it difficult for the global health community to make long-term commitments to necessary epidemic preparedness programs.

There are also scientific and economic barriers specific to the development of effective responses to AMR. Scientifically, bacteria have developed numerous mechanisms for evading antibiotics, and finding new points of attack is becoming increasingly challenging. Economically, there is a misalignment of interests between the public (which has an interest in limiting the use of novel antimicrobials as much as possible to protect their effectiveness, while ensuring their availability at low cost to those who most need them) and pharmaceutical companies (which have an interest in producing products that will be used widely and yield substantial profits). These barriers have conspired to produce no truly novel class of antibiotics in over three decades (86).

Beyond the demographic, social, and economic challenges we have enumerated, the world faces a number of organizational challenges to its ability to manage infectious disease threats. The global system for monitoring, preventing, and responding to infectious diseases is massively complex. Key elements of this system include local and national governments, supranational governmental organizations (e.g., the United Nations and the WHO), international legal agreements (e.g., the International Health Regulations and the Nagoya Protocol), international coalitions and alliances (e.g., the Global Health Security Agenda and CEPI), financing facilities (e.g., the Pandemic Emergency Financing Facility), donors (e.g., the Bill & Melinda Gates Foundation and the Wellcome Trust), and non-governmental organizations (e.g., Gavi, the Vaccine Alliance; the Red Cross; and Médecins Sans Frontières).

The good news is that a number of organizations and entities are in place to help protect the world from calamity. The bad news is that deficiencies exist within this complex system, especially when it comes to coordinating activities among all the players. The 2014 Ebola crisis in West Africa highlighted significant gaps between the WHO's intended functions and its real-world effectiveness as a protector of global health security, as well as more general gaps within the global health system (87–91). Multiple post-mortem reports on the crisis explicitly called for the establishment of a new Center for Health Emergency Preparedness and Response within the WHO to ensure that the organization would better manage epidemic risks moving forward (87–89, 92). The WHO answered these calls by instituting a new Health Emergencies Programme in 2016 to streamline its activities related to health emergencies and create better internal alignment. While the establishment of this Programme represents a step in the right direction, and while the WHO appears to be faring relatively better with the ongoing Ebola outbreak in the Democratic Republic of Congo in difficult circumstances, a vacuum still remains when it comes to the critical role of coordination.

The establishment in 2018 of the Global Preparedness Monitoring Board (GPMB), which is co-convened by the WHO and World Bank, represents another positive step in terms of bolstering the WHO's reach and effectiveness in the area of outbreak and epidemic preparedness and response (93). While the GPMB is intended to take on some portion of the coordinating role that is dearly needed, the Board has an initial term of only 5 years without expectation of continuation, and members will only meet twice per year. This lack of a sustainable organizational plan and lack of dedicated resources (especially human resources) calls into question whether creation of the GPMB represents sufficient change.

National governments have also taken it upon themselves to address the shortcomings revealed by the 2014 Ebola crisis. The Global Health Security Agenda (GHSA), which was started by the United States and launched in 2014, is now a partnership of over 64 countries, international organizations, and non-governmental stakeholders. The GHSA has similar aims to the International Health Regulations (IHR), with a focus on helping participating countries build core capacities for outbreak detection, preparedness, and response. The GHSA is a welcome addition to the global health landscape. However, the GHSA is yet another entity focused only on a portion of epidemic disease management, neglecting, for example, R&D of relevant biomedical countermeasures. It also adds another layer of complexity to the global health system, as its responsibilities overlap with those assigned to the WHO under the IHR. Finally, the GHSA, GPMB, and the Health Emergencies Program all appear to ignore the challenge of AMR.

In addition to improved coordination, more organizational support for funding R&D of technologies to deal with infectious disease threats is dearly needed. For example, while the Coalition for Epidemic Preparedness Innovation (CEPI) is, in principle, filling an important gap by supporting the early development of vaccines for diseases of epidemic potential, there are reasons to question whether current levels of investment are adequate. CEPI's initial business plan proposed investing $600 million to $1 billion in vaccine R&D (94). However, a recent analysis conducted by the organization determined that funding the early development of vaccine candidates against all 11 diseases originally included on the WHO's R&D Blueprint priority list in 2015 would likely cost between $2.8 billion and $3.7 billion (95). This does not account for the cost of scaling up vaccine production and delivery in the event of an outbreak, nor does it cover all of the potential epidemic threats.

The recently launched CARB-X is fulfilling a similar role to CEPI with respect to promoting early R&D of biomedical countermeasures for resistant pathogens (96). CARB-X provides financial, scientific, and business support for antibiotics, vaccines, rapid diagnostics, and other products for resistant bacterial infections. As with CEPI, there is reason to question whether CARB-X, which plans to invest up to $500 million between 2016 and 2021, has enough funding to make a meaningful

impact on the anticipated global AMR burden. In addition, CARB-X may be unnecessarily excluding potential high-impact AMR interventions from consideration for financial support. To qualify for funding through CARB-X, research must target pathogens on the AMR priority pathogen lists established by the WHO and the U.S. Centers for Disease Control and Prevention. Based on this criterion, some products that could have a significant AMR impact, such as a universal (or improved seasonal) influenza vaccine, are ineligible. In general, CARB-X may do well to diversify its investment portfolio, which currently contains only one vaccine (97).

In the wake of Ebola, the world reactively added several new elements to an already complex global system for managing infectious disease threats. There is reasonable justification for each of these elements and a role for them to play. However, given the massive risks associated with infectious disease threats in terms of human health and other forms of social and economic well-being, more resources and proactive reforms are needed. Having evolved in a piecemeal, somewhat *ad hoc* fashion over the course of more than half a century, the current global system lacks coherence. Insufficient coordination among stakeholder organizations leads to inefficiency and missed opportunities. Many responses are available and required to proactively reduce the risk posed by infectious disease threats and prepare for inevitable outbreaks (see **Table 4**). While many organizations are currently engaging in one or more of these activities to tackle a piece of the problem, the world remains in need of a reliable, well-staffed, and well-resourced global entity to put all of the pieces together.

TABLE 4 | Selected responses to infectious disease threats.

Responses
• Health systems strengthening
• Improved (sustainable) urban infrastructure
• Improved public health infrastructure, including clean water and sanitation
• Increased routine immunization
• Mass vaccination following detection of outbreak-prone diseases (e.g., yellow fever)
• Surveillance of infectious disease in human and animal populations, including rates of resistance
○ Building local (laboratory and epidemiological) capacity to diagnose and report cases of infectious disease
○ Leveraging opportunities for informal surveillance (e.g., Google Flu Trends (no longer operating publicly), ProMED)
• Surveillance of possible terrorist organizations and activities
• Monitoring of biocontainment procedures and capabilities in microbiology laboratories
• Regular monitoring of preparedness for outbreaks and biosecurity incidents at national and supranational levels (e.g., Joint External Evaluations)
• Regulation of access to antimicrobials for both humans and livestock
• Investment in R&D of biomedical countermeasures
○ Vaccines
○ Antimicrobials
○ Diagnostics
○ Monoclonal antibodies and other novel treatments
○ Platform technologies
• Supply chain strengthening and improved systems for rapid distribution of countermeasures in the event of an emergency
• Coordination of efforts

TOWARD A UNIFIED APPROACH

In order to better protect the world from infectious disease and the myriad attendant social and economic consequences, we propose the formation of a standing multidisciplinary Global Technical Council on Infectious Disease Threats. The Council would focus explicitly on volatile infectious disease threats as opposed to more stable and predictable global health challenges (e.g., endemic disease). Its mission would be to reduce the health, social, and economic risks emanating from diseases of epidemic potential, AMR, and biosecurity threats. The Council would have three principal aims: (1) to improve collaboration and coordination within the global health system, (2) to fill in critical knowledge gaps, and (3) to advise existing organizations. The Council could be either freestanding or subsumed within another entity. The Council is intended to support and enhance efforts already being made by the WHO, the World Bank, CEPI, Gavi, the GHSA, national governments, global non-profits, and other organizations.

As indicated by its name, the focus of the Global Technical Council on Infectious Disease Threats would be technical. In other words, the Council's outputs would be based on rigorous reviews of the available evidence, and it would operate apolitically. To that end, it would be staffed by a multidisciplinary team of experts working full time. While it would likely be beneficial to keep the size of the Council relatively small, it should encompass—at a minimum—the following areas of expertise: epidemiology, economics, finance, outbreak response, public health, health systems science, R&D, international law, politics, biostatistics and modeling, supply chain management, and clinical trial design.

In service of its mission and to fulfill its aims, the Council would take on a variety of activities. It would identify gaps in disease surveillance, outbreak readiness, basic research on pathogens, R&D of biomedical countermeasures, supply chain and delivery systems, and financing. Council experts would fill in knowledge gaps in these areas, where possible, through active research, and solicit and sometimes fund additional needed research from external experts and entities. The Council would also make high-level, evidence-based recommendations to organizations operating in the domain of infectious disease threats; these recommendations would be based on the technical knowledge of its experts and literature reviews. For example, the Council would regularly carry out health technology assessments, considering the full health, social, and economic benefits of potential interventions for responding to priority infectious disease threats (98), as well as the degree to which alternative interventions may be complementary or substitutable (99). Economic evaluations of potential investments in interventions for specific infectious disease risks (e.g., a vaccine against Marburg) would be conducted in such a way to account for the opportunity cost of foregoing a similar level of investment in horizontal programs such as health systems strengthening or improved infectious disease surveillance. The

Council would issue technical communications through a public forum such as an online bulletin, and it would publish an annual report.

The Council would also foster coordination and collaboration among existing organizations—seeking to reduce duplication of effort, promote integration of ongoing activities, encourage partnerships (including between the public and private sectors), and discourage the use of public funds for the R&D of products for which there are already reasonable market incentives. This coordinating role may be especially impactful with regard to an established but fragmented network of pandemic preparedness funds that appear to overlap in remit, while leaving substantial funding gaps unaddressed (100). The Council may advocate for innovative financing collaborations like the recently established partnership between CEPI, Gavi, the Government of Norway, and the International Finance Facility for Immunization to help fund CEPI's vaccine development portfolio (101). The Council would also seek to develop innovative mechanisms for facilitating the sharing between countries of biological samples critical to the development of novel biomedical countermeasures.

The Council would function much like an independent think tank, and its authority would derive from the credibility of its experts and the evidence and advice they produce. Funding could come from national governments and major donors (similar to the CEPI model). Accountability would come, principally, from the transparent nature of the Council's activities and the publicity of its results. In addition, oversight could be provided by an external review board composed of the leadership from organizations such as the WHO, CEPI, Gavi, Médecins Sans Frontières, and the World Bank. This review board would operate in consultation with representatives of other interested parties, such as private industry, national governments, and patient advocacy groups.

The formation and operation of the Council would result in greater efficiency within the global health system; increased mitigation of health, social, and economic risks due to infectious disease; and the improved protection of at-risk populations.

The preceding enumeration of Council activities and attributes is not intended to be exhaustive. Ideally, before the Council's formation, a rigorous landscape analysis of existing global health organizations and the activities they perform would be conducted in order to: (1) identify the most significant shortcomings of the current system, including redundancies; (2) confirm the need for the Technical Council; and (3) establish a comprehensive strategy for the Council's funding, structure, and initial plan of action.

As stated above, the proposed Council could potentially be housed within the WHO (or another body), or it could be established as a free-standing entity. If housed within the WHO, the purely technical and apolitical nature of the body would bolster the legitimacy of WHO recommendations and activities with regard to infectious disease threats. In this vein, it would be important for Council experts to be granted the autonomy to make their assessments and recommendations independently of any political influence from WHO leadership. At the same time, the Council would work collaboratively with existing WHO programs and advisory committees, such as the Health Emergencies Programme and the Strategic Advisory Group of Experts on Immunization. It may be possible to essentially convert the GPMB into the Technical Council by dedicating sufficient resources to employ a full-time expert staff and ensuring that the GPMB/Council will remain in existence beyond 5 years.

On the other hand, if the Council were established as a separate entity, any resultant competition that emerged between the Council and the WHO would likely represent a boon for the global community, as it would force both the Council and the WHO to step up their games in order to remain relevant in the space of infectious disease threats. Indeed, experts have previously cited the benefits of competition in other domains of global health and international development (102–105).

CONCLUSION

Uncertainty abounds with respect to infectious disease threats and their consequences. Nevertheless, outbreaks and epidemics are virtually guaranteed to continue, AMR will remain a threat as long as we rely on standard antimicrobial therapies, and biosecurity risks are an inherent consequence of pathogen research and of human conflict. Fortunately, responses exist to all these forms of infectious disease threats. The world currently lacks a unified system for developing and implementing these responses in an efficient, coordinated fashion. The establishment of a multidisciplinary Global Technical Council on Infectious Disease Threats would go a long way to reduce unnecessary waste within the global health system, redirect resources where needed, and mitigate the risks posed by infectious disease.

AUTHOR'S NOTE

Tables 1–3, along with small portions of this article, have been adapted, expanded, and updated from an earlier article by Bloom et al. (106).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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