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DNA-launched live-attenuated vaccines for biodefense applications

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Summary

A novel vaccine platform uses DNA immunization to launch live-attenuated virus vaccines *in vivo*. This technology has been applied for vaccine development against positive-strand RNA viruses with global public health impact including alphaviruses and flaviviruses. The DNA-launched vaccine represents the recombinant plasmid that encodes the full-length genomic RNA of live-attenuated virus downstream from a eukaryotic promoter. When administered *in vivo*, the genomic RNA of live-attenuated virus is transcribed. The RNA initiates limited replication of a genetically defined, live-attenuated vaccine virus in the tissues of the vaccine recipient, thereby inducing a protective immune response. This platform combines the strengths of reverse genetics, DNA immunization and the advantages of live-attenuated vaccines, resulting in a reduced chance of genetic reversions, increased safety, and improved immunization. With this vaccine technology, the field of DNA vaccines is expanded from those that express subunit antigens to include a novel type of DNA vaccines that launch live-attenuated viruses.

Keywords

DNA vaccine; alphavirus; flavivirus; live-attenuated vaccine; Venezuelan equine encephalitis; chikungunya; yellow fever

1. DNA-launched live-attenuated vaccines as novel DNA vaccines

Among vaccine platforms, DNA vaccination represents one of the most recent immunization technologies [1-3]. DNA vaccines continue to raise considerable interest due to the ease of

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production and administration, genetic stability, no requirement for a cold chain, and activation of humoral, cell-mediated, and innate immunity. However, despite promising results in preclinical trials, the clinical application of traditional DNA vaccines has been limited due to various factors including low DNA uptake, low immunogenicity in humans and the need of repeated booster vaccinations with considerable quantities of DNA, as reviewed elsewhere [1, 4, 5]. Furthermore, traditional DNA vaccines are designed to express a single vaccine-relevant antigen in the tissues of vaccine recipient, essentially serving as a vector for transient expression of the subunit antigen vaccine. However, subunit vaccines themselves in most cases show low immunogenicity and require multiple boosts, advanced adjuvants, or expression as virus-like particles (VLPs) to induce efficient immunity [6-9]. Improvement of DNA vaccination remains a fundamental goal of vaccine research. Methods to improve DNA vaccine-induced immunity include various techniques including prime-boost approach [10, 11], development of advanced vaccine delivery methods such as electroporation [4, 12-14], as well as improvements to plasmid production and plasmid vector design such as addition of plasmid-encoded innate immunity agonists [15-18].

Recently described DNA-launched live-attenuated vaccine platform is a novel technology that combines chemical and genetic stability of DNA with the exceptional efficacy of live-attenuated vaccines (Fig. 1). This platform represents recombinant DNA that can launch live-attenuated virus *in vitro* or *in vivo* [19-25]. DNA-launched live-attenuated vaccines were sometimes called iDNA® vaccines to distinguish them from traditional DNA vaccines [19-21]. Characteristics of DNA-launched (iDNA) live-attenuated vaccines are shown in Table I in comparison with traditional DNA vaccines and traditional live-attenuated virus vaccines. As any DNA vaccine, iDNA plasmids are isolated from bacteria and include a eukaryotic promoter, such as cytomegalovirus (CMV) major immediate-early promoter. However, unlike a traditional DNA vaccine that involves transcription of mRNA for expression of a subunit antigen, the iDNA vaccines transcribe the full-length genomic RNA of the live-attenuated vaccine virus. The full-length viral RNA then initiates limited replication of live-attenuated virus in the tissues of vaccine recipient resulting in efficient immunization (Fig. 1). Essentially, the iDNA plasmid turns a limited number of cells in the vaccine recipient into the cell-scale factories for “manufacturing” of live-attenuated vaccine [19, 20, 25].

Thus, the iDNA technology represents a novel type of DNA vaccine (Table I). With the introduction of DNA-launched iDNA vaccines, DNA-based vaccines can be subdivided into (i) DNA vaccines that express subunit antigens and (ii) DNA vaccines that launch replication-competent, live-attenuated vaccines. Furthermore, the DNA-launched iDNA vaccine represents a novel reverse genetics system that can be used for the rational vaccine design by introducing targeted mutations to improve vaccine characteristics, such as additional attenuating mutations. Finally, the iDNA plasmid can be used as a genetically stable repository seed to prepare live-attenuated virus *in vitro* either for subsequent use as a traditional live-attenuated vaccine or, after virus inactivation, as a traditional inactivated virus vaccine.

2. DNA-launched viruses as improved live-attenuated vaccines

In contrast to DNA vaccines, live-attenuated vaccines represent the oldest known vaccines and are amongst the most successful and cost-effective medical interventions in the history [26]. Because of live-attenuated vaccines, smallpox has been eradicated since 1980 and poliomyelitis is nearing global eradication. Live vaccines constitute approximately half of all currently licensed vaccines. They often provide life-long immunity following a single dose vaccination [26] (Table I). The limitation of live vaccines is that they can be chemically and genetically unstable and vulnerable to genetic reversion mutations, especially vaccines for RNA viruses [27-29]. Furthermore, it is not always possible or practical to prepare live-attenuated viruses for reasons of safety or an inability to efficiently grow the microorganism. The genetic instability, the need for cold chain, the logistics and biosecurity of live virus production, as well as other limitations have constrained the use of many live-attenuated RNA virus vaccines. For example, live-attenuated experimental vaccine 181/25 against chikungunya virus (CHIKV) and TC-83 vaccine against Venezuelan equine encephalitis virus (VEEV) have been developed decades ago and extensively evaluated in the clinical trials. However, both vaccines demonstrated a potential to cause adverse effects, which have been associated with the possibility of genetic reversion mutations. For these reasons, improved vaccines for CHIKV and VEEV are needed, although 181/25 and TC-83 continue to be used under Investigational New Drug (IND) protocols. These IND vaccines with the clinical history represent a better starting point for vaccine development against CHIKV and VEEV than their counterpart wild-type pathogenic viruses [19, 20].

It should be noted that when reversion mutation is found in a patient, it is not always clear if the mutation occurred *in vivo* after immunization, or it has originated in the process of vaccine manufacturing and was present in the vaccine lot before immunization. For all live-attenuated viral vaccines including DNA and RNA viruses, it is expected that reversion mutations may be present as a small fraction of bulk vaccine preparations. However, it is important to maintain reversion mutations at a minimal level to ensure vaccine safety. One of the best examples is the poliovirus vaccine. The World Health Organization (WHO) established Standard Operating Procedure for poliovirus (Sabin) vaccine types 1, 2 or 3 that allows mutant analysis by PCR and restriction enzyme cleavage (MAPREC), as well as calculation of the allowable percentage of revertants [30].

Reduction of reversion mutation rates is an important goal for vaccine development. Improved genetic stability can play a role in preventing reversion mutations and maintaining attenuated phenotype. The *E. coli*-produced iDNA plasmid represents genetically defined molecular clone and is expected to produce the virus with higher homogeneity than a standard viral population. This suggests safety advantage of iDNA for vaccine applications and its usefulness as a reference source for the production of live or killed vaccines with improved safety characteristics. Direct vaccination with iDNA *in vivo* is expected to further minimize the number of replication cycles of vaccine virus, thus further reducing the probability of reversion mutations in comparison with traditional manufacturing. Preliminary data confirmed genetic stability of DNA-launched virus including stability of attenuating mutations [20, 31, 32]. However, additional research is needed *in vitro* and *in vivo*. The cell can be a hostile environment to foreign DNA and RNA [33, 34] and can

potentially affect DNA plasmid integrity and transport to the nucleus, as well as synthesis of viral RNA in the nucleus and transport of intact RNA to the cytoplasm.

Overall, the iDNA plasmid is easier in preparation, storage and use than live-attenuated virus. The production of recombinant DNA in *E. coli* is a well-developed technology [17, 18], and DNA-based vaccines are simpler in production and less expensive logistically in transportation and storage. Therefore, iDNA technology is expected to improve the major current limitations of live-attenuated vaccines. These improvements can include genetic and temperature stability, homogeneity of initial vaccine virus seed, virus banking, production, as well as vaccine transportation and storage. Potentially, DNA can prime innate immune responses before launching live-attenuated virus and improve its immunogenicity. Importantly, DNA-launched iDNA vaccines still provide protective immunity with a single dose similarly to live-attenuated vaccines [19-21] (Table I).

3. DNA-launched vaccines and biodefense

The development of vaccines for potential biological threats is one of the most important biodefense strategies [35]. Many biodefense-relevant viruses are RNA viruses including (+)RNA alphaviruses and flaviviruses [36]. Highly pathogenic viruses can be considered either naturally occurring disease threats or potential biological weapons that can be deployed by bioterrorists. Biodefense activities are focused on protecting civilian and military populations from biodefense pathogens, which include public health, veterinary and agricultural threats. The goal of the biodefense vaccines is to prevent disease outbreaks; and in the case if an outbreak occurs, to effectively contain it. An ideal biodefense vaccine would allow for the quick deployment of vaccines in response to emerging or engineered pathogens [5].

Challenges in biodefense vaccine development include difficulties of working with pathogens, which are often classified as select agents and require the highest level of biological containment (ABSL/BSL-3 and/or ABSL/BSL-4) and appropriate biosecurity precautions [35]. Furthermore, there are hurdles in conducting clinical efficacy studies, because most of the biodefense-relevant diseases are unpredictable and occur sporadically. Animal models are being considered as alternative way to demonstrate efficacy to support vaccine licensing. However, in spite of introduction the FDA “Animal Rule” 15 years ago, the only vaccine licensed using this regulatory pathway is BioThrax (Anthrax Vaccine Adsorbed), approved at the end of 2015. Finally, in contrast to manufacturing of commercially-attractive vaccines, there is no incentive for pharmaceutical industry to invest in biodefense vaccines, which are intended in most cases for either rapid production to respond to a public health emergency, or for stockpiling of the vaccine that may never be used. In the absence of outbreaks or emerging threats, the usual market for biodefense vaccines is small and is comprised mainly of hospital personnel, first responders, travelers, and military and civilian personnel in the endemic areas, who can come in contact with the biodefense-relevant viruses. Therefore, due to various scientific and economic reasons, there are currently no vaccines licensed for general use for nearly all biodefense-related viruses, with only rare exclusions such as smallpox virus vaccine [37].

Preferably, the biodefense vaccine should elicit protective immune responses in a single dose and be unaffected by pre-existing immunity to vaccine components. The iDNA-launched vaccine approach can potentially provide an innovative solution for the biodefense vaccination strategies due to (i) genetic stability, (ii) thermostability, (iii) activation of innate immunity, and (iv) the ability to elicit protection with a single vaccination. As any bacterially-produced plasmid, the iDNA contains CpG motifs and is expected to stimulate innate immunity and to effectively prime adaptive immunity [34, 38]. The presence of DNA in the cytoplasm of mammalian cells is perceived as an alert signal by cGAS and other STING-dependent sensors [39, 40]. In response, the immune system initiates transcription of anti-viral genes such as type I interferons and production of inflammatory cytokines such as IL-1 β . Activation of innate immunity and induction of cytokine response serves as efficient priming for the acquired virus-specific immune responses, and as a result, improvement of immune responses is expected [40].

In the absence of outbreaks, DNA-launched vaccines can be useful for vaccinating personnel at risk of infection with biodefense-related and emerging viruses, such as first responders and hospital personnel. Healthcare personnel need vaccinations due to their proximity to potentially infected patients [41]. A single-dose vaccination with DNA-launched iDNA vaccine may provide much needed protection from emerging or exotic pathogen [19, 20]. As safety and efficacy are confirmed in these individuals, the use of iDNA vaccines can be extended to additional populations. Live-attenuated vaccines have been broadly used in diverse groups of patients. For example, FluMist live-attenuated influenza vaccine was used in healthy persons aged 2-49 years with no serious adverse effects [42]. Potentially, a single, small dose of DNA-launched vaccine may represent for healthy persons a safety advantage over other approaches that involve multiple boosts and potentially harmful impurities. Traditional vaccines often contain contaminants and additives resulting from manufacturing, which can cause adverse effects in some patients [43, 44].

In summary, iDNA technology is uniquely suited to address challenges of biodefense vaccines. Manufacturing of iDNA plasmid requires standard methods of DNA isolation. The plasmid vaccine can be either stockpiled, or rapidly prepared in the case of an outbreak. Chemical stability of DNA makes it more suitable for stockpiling and transportation as compared to live or killed virus vaccines. In addition, DNA has favorable temperature stability profile and can be formulated to be stored and shipped at ambient temperature. Several iDNA vaccines described so far in the literature are based on highly attenuated virus strains, which are exempt from select agent regulations. This facilitates vaccine preparation, because safety of the parent strains has been demonstrated in the numerous clinical trials or by long history of vaccine use in people (such as yellow fever 17D vaccine). The previous clinical parent vaccine background facilitates work with these vaccines and their derivatives. As a reverse genetics system, DNA-launched iDNA plasmids can be rapidly modified if needed to introduce additional mutations to improve safety and immunogenicity.

The summary of published DNA-launched iDNA vaccines is shown in Table II.

4. DNA-launched vaccines for alphaviruses

Published alphavirus DNA-launched vaccines include experimental proof-of-concept iDNA vaccines for CHIKV and VEEV (Table II).

4.1 CHIKV vaccine

CHIKV causes outbreaks of chikungunya fever worldwide and represents an emerging biodefense threat [45, 46] (Fig. 2). The virus belongs to the *Alphavirus* genus of *Togaviridae* family, along with several mosquito-borne pathogenic arboviruses [45, 46]. CHIKV is transmitted to humans primarily by *Aedes aegypti* and *A. albopictus* mosquitoes [47-49] and has a major health impact including severe arthralgia, respiratory failure, cardiovascular disease, hepatitis and central nervous system problems, especially in the elderly and children [50, 51]. CHIKV is found worldwide, with nearly 40 countries reporting endemic or epidemic chikungunya fever, mostly in warm climates in Asia, Africa and recently in Europe and the Americas [52]. Epidemics of CHIKV included the 2005-2006 outbreak in La Reunion islands in the Indian Ocean that caused 284 deaths, epidemic in India with estimated 1.3 million people infected [53, 54], and an ongoing epidemic in the Caribbean and South America. With an increase in global travel, the risk for spreading CHIKV to non-endemic areas has increased [55]. Climate changes and urbanization favor geographical expansion of CHIKV [56-58]. Travel-associated cases have also been recorded in Europe, Australia, and the U.S., and some travelers were viremic [59]. Given the current large epidemics and the worldwide distribution of *A. aegypti* and *A. albopictus*, there is a risk of emerging CHIKV pandemic [60]. Currently there is no approved vaccine or specific therapy for the disease. Two candidate vaccines have recently qualified to enter clinical phase II trials, a chikungunya VLP-based vaccine and a live-attenuated measles vectored vaccine [61]. Live-attenuated vaccines prepared by using reverse genetics have been described [62, 63] and 181/25 vaccine has been tested in Phase I/II clinical trials [64]. A promising therapeutic approach using DNA to launch a monoclonal antibody capable of neutralizing the virus was also reported [65].

Experimental CHIKV DNA-launched vaccines were prepared and evaluated *in vitro* and in mice [19, 32, 66]. In one study, CHIKV vaccine candidates were attenuated by deleting a large part of the gene encoding nsP3 or the entire gene encoding 6K and were administered as viral particles or infectious genomes launched by DNA [66]. The resulting attenuated mutants were genetically stable, elicited neutralizing antibodies and T cell responses after a single immunization and protected C57BL/6 mice from viremia and joint swelling. Other vaccines included proof-of-concept construct derived from 181/25 vaccine (Fig. 2), as well as vaccine constructs that contained additional mutations to improve safety [19]. For example, the prototype iDNA plasmid encoded the full-length infectious genome of live-attenuated CHIKV clone 181/25 downstream from a eukaryotic promoter. Transfection with as little as 10 ng of this iDNA was sufficient to initiate replication of vaccine virus *in vitro*. Furthermore, BALB/c mice were vaccinated by injection-electroporation with a single 10 µg dose of iDNA encoding genomic RNA of CHIKV vaccine 181/25. After vaccination, all mice have seroconverted, developed neutralizing antibody and resisted experimental challenge with pathogenic CHIKV (Table II). Thus, live-attenuated CHIKV vaccine can be

launched *in vitro* or *in vivo* by using iDNA injection-electroporation and appears to represent a promising vaccination strategy for CHIKV [19, 32].

4.2 VEEV Vaccine

VEEV is another alphavirus that shares many genomic and structural characteristics with CHIKV (Fig. 3). VEEV is a veterinary and human pathogen that infects equids and humans *via* many mosquito vectors, including *Culex*, *Mansonia*, *Psorophora*, and *Aedes* species [67]. The population of susceptible mosquitoes is abundant in the U.S. [68-71]. VEEV causes epizootics and outbreaks in the North, Central, and South America including an outbreak in Texas in 1971 [72, 73] (Fig. 3). Climate, ecological changes and international travel increase the risk of VEEV re-emergence [46, 70, 74]. Furthermore, the virus can also be easily prepared in large quantities and aerosolized as a potential biological weapon [74, 75]. The initial VEEV symptoms are similar to influenza and are difficult to diagnose [75]. The potentially threatening effects of the VEEV outbreaks demand an effective VEEV vaccine [76]. Experimental TC-83 live-attenuated vaccine has been characterized [77] and provides protection against many epizootic viruses of the VEEV complex [78] including IAB, IC, and IE. In the TC-83 vaccine virus, the nucleotide 3 in the 5' untranslated region and amino acid 120 within E2 glycoprotein have been associated with attenuation [77]. However, the vaccine causes adverse effects such as headache and fever in approximately 23% of vaccine recipients, while another approximately 18% of recipients do not develop sufficient neutralizing antibody titers [79]. Reversion mutations can explain adverse effects that have been observed with live-attenuated vaccines including TC-83 [80]. Nevertheless, due to its long history of clinical use, the TC-83 represents a logical starting point for proof-of-concept studies, as well as for preparation of improved vaccines against VEEV [80].

Early examples of DNA-launched VEEV-based constructs have focused on the launch of VEEV RNA replicon vectors for expression of heterologous genes of interest [38, 81, 82]. Replicon vector represents genomic self-amplifying VEEV RNA, in which structural polyprotein region is replaced with a heterologous gene of interest. Replicon RNA cannot launch live virus because it does not encode the VEEV structural proteins. However, they have been successfully used as vaccine vectors for vaccinations against cancer [83, 84] and infectious agents including biodefense-relevant viruses [85-89]. In DNA-launched replicon vectors, VEEV replicon RNA was placed under the transcriptional control of RNA polymerase II promoter, such as CMV, resulting in transcription and self-amplification of replicon vector with improved expression of a heterologous gene. For example, DNA-launched replicon plasmid expressed 3- to 15-fold more green fluorescent protein *in vitro* than a traditional DNA vaccine [38]. Inoculation of mice with DNA-launched replicon encoding human immunodeficiency virus type 1 gp160 has increased humoral responses by several orders of magnitude as compared to an equivalent dose of a traditional DNA vaccine. These increases were also observed at 10- and 100-fold-lower doses of the replicon vaccine construct [38]. Studies involving DNA-launched VEEV replicons have demonstrated the possibility of launching VEEV RNA *in vitro* and *in vivo*.

DNA-launched iDNA vaccine expressing live-attenuated VEEV has been described recently [20]. A proof-of-concept DNA-launched VEEV pTC83 vaccine was prepared using the

backbone of live-attenuated experimental TC-83 vaccine [90], which is currently used under IND protocol for immunization of medical research personnel at risk [74, 91, 92]. Additional live-attenuated vaccine candidates have been developed, which included gene rearrangement and additional subgenomic promoter. Gene rearrangement has been shown to provide attenuating effect for several viruses including VEEV [93-95]. The pTC83 iDNA plasmid directed transcription of viral RNA *in vivo* and initiated limited replication of a genetically defined, TC-83-like vaccine virus. Less than 10 ng of experimental pTC83 iDNA initiated replication of the vaccine virus *in vitro* (Table II). The TC-83 antigens were expressed in the cytoplasm of transfected cells. By 48 hr, all CHO cells were positive for TC-83 antigens suggesting virus replication. To evaluate this approach *in vivo*, BALB/c mice were vaccinated with a single dose of pTC83 iDNA i.m. using electroporation. After vaccination, all mice seroconverted with no adverse reactions. Four weeks after immunization, animals were challenged with the lethal epidemic strain of VEEV. All iDNA-vaccinated mice were protected from fatal disease, while all unvaccinated controls succumbed to infection and died [20]. Furthermore, five out of ten challenged animals did not have any detectable viremia after challenge. The remaining five animals had low viremia as compared to unvaccinated control animals. In contrast to iDNA-vaccinated mice, all unvaccinated controls developed high viremia, lost in average 32% of weight, and succumbed to infection and died by day 7 post challenge [20].

The launch of the virus was also confirmed in BALB/c mice using *in vivo* transfection with pTC83 iDNA. Viremia could not be detected by direct plaque assay; however, viremia was detected in plasma samples after amplification in Vero cells. RNA was isolated from the recovered virus, and TC-83 cDNA fragment nt 8559-9850 containing the entire E2 gene was generated by RT-PCR and sequenced. The presence of attenuating mutation E2-120 [96] in DNA-launched virus *in vivo* was confirmed in the virus by cDNA sequencing [20].

5. DNA-launched vaccines for flaviviruses

Flavivirus DNA-launched candidate vaccines included experimental vaccines developed for West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) (Table II).

5.1 WNV vaccines

WNV was discovered in 1937 in the West Nile district of Uganda and has been subsequently classified within the genus *Flavivirus* along with JEV in Asia, St. Louis encephalitis virus in the New World and Murray Valley virus in Australia. Historically, its circulation was limited to Africa and Asia, with occasional cases in southern Europe, possibly introduced by migratory birds. The emergence of this tropical African virus into New York in 1999 was an unexpected event [97]. Since then, WNV has become an important epidemiological concern for public health worldwide (Fig. 4). In the U.S., mostly *Culex* mosquito transmits the virus in nature and to humans. *C. pipiens* mostly transmit WNV in the northern U.S.; *C. quinquefasciatus* mosquitoes are active in the southern U.S., while *C. tarsalis* transmits the WNV in many areas of the central and western U.S. that overlap with the areas of *C. pipiens* and *C. quinquefasciatus*. West Nile fever develops in approximately 25% of WNV- infected

individuals. The disease varies in clinical severity, and symptoms may last for extended period of time. Neuroinvasive disease and neurological manifestations, such as encephalitis, meningitis, acute flaccid paralysis, develops in approximately 1% and carries a fatality rate of ~10%. Encephalitis has a highly variable clinical outcome and is often associated with long-term morbidity. A safe, efficacious and cost-effective vaccine is needed [98].

Experimental DNA-launched WNV vaccine constructs have been described [22, 99] (Table II). Plasmid DNA directing transcription of the infectious full-length RNA genome of Kunjin virus from a mammalian expression promoter was prepared. Kunjin is the indigenous WNV strain found in Australia [100]. The plasmid included attenuating mutation Pro-250 to Leu in the NS1 protein. Mice were vaccinated with the plasmid *via* i.m. route. After vaccination, Kunjin virus was isolated from the blood of immunized mice 3-4 days after DNA injection indicating that the full-length infectious genomic RNA was transcribed and launched the virus *in vivo*. No symptoms of disease were observed. By 19 days after vaccination, neutralizing antibody was detected in the serum. After challenges with lethal doses of the virulent WNV NY99 strain or Kunjin strain i.c. or i.p, mice vaccinated with 0.1-1 µg of plasmid DNA were protected against disease. The results provided evidence that delivery of an attenuated replicating WNV *via* plasmid DNA vector can provide an effective vaccination strategy against virulent WNV.

Other examples of DNA-launched experimental WNV approaches were also reported [24, 99, 101]. Live-attenuated WNV vaccine in the clinic is not available. In the absence of clinically tested live-attenuated WNV vaccine, chimeric attenuated virus W1806 serologically similar to virulent NY99 strain of WNV was used to study the effects of mutations found in Japanese encephalitis virus (JEV) vaccine SA14-14-2. Promising characteristic and strong attenuating effect were described; however, additional attenuated mutations may be needed to further improve safety of the W1806 virus [101].

5.2 JEV vaccine

JEV is transmitted by the mosquito *Culex tritaeniorhynchus* and causes epidemics throughout Asia. For the past decades, killed virus vaccines prepared in tissue culture or in mouse brain have been used to immunize travelers and residents of enzootic countries. Cost, efficacy and safety concerns of these vaccines have led to the development of live-attenuated vaccines SA14-14-2 and chimeric YF-JEV, as well as purified inactivated, tissue culture-derived vaccine [102].

Replication of DNA-launched JEV flavivirus *in vitro* has been described [23, 103]. JEV infectious clone has been converted into a stable DNA-launched construct, and JEV replication was confirmed in cell culture (Table II). However, additional research is needed to demonstrate feasibility of DNA-launched live-attenuated JEV vaccine *in vivo*.

5.3 YFV vaccines

YFV is transmitted by the mosquito *Aedes aegypti* and causes an acute hemorrhagic fever disease in tropical Africa and Latin America (Fig. 5). YFV live-attenuated 17D strain has been developed as highly effective vaccine that was administered to people for almost a century and serving as an example of a successful viral vaccine. The iDNA plasmid that

encoded the full-length RNA genome of 17D vaccine downstream from a cytomegalovirus (CMV) promoter was described [21] (Fig. 5). The vaccine was designed to transcribe the full-length viral RNA and to launch 17D vaccine virus *in vitro* and *in vivo*. Initially, plasmid constructs containing the full-length YFV 17D cDNA were found to produce low yields of DNA in *E. coli*, possibly due to potentially toxic products expressed in bacteria. Therefore, short intron sequences were introduced into the YFV 17D cDNA to disrupt potentially toxic open reading frame (ORF) and to improve the yields of iDNA plasmid. Transfection with 10 ng of YFV 17D iDNA plasmid was sufficient to start replication of vaccine virus *in vitro* (Table II). Safety of the iDNA-derived 17D virus was confirmed in AG129 mice deficient in receptors for IFN- $\alpha/\beta/\gamma$. Finally, direct vaccination of BALB/c mice with a single 20 μ g dose of iDNA plasmid resulted in seroconversion and elicitation of virus-specific neutralizing antibodies in animals (Table II).

Another approach involved the bacterial artificial chromosome (BAC) to prepare DNA-launched YFV vaccine [31]. Similarly to the previous study [21], plasmid containing the full-length YFV 17D cDNA was found to be genetically unstable during propagation in *E. coli*. Therefore, a low-copy number BAC clone pBeloBAC-FLYF was prepared and characterized in cell culture and in mice. High titers of YFV-17D were generated upon transfection of the BAC DNA into cells, whereas a mutant with deletion in the capsid-coding region pBeloBAC-YF/ C was restricted to a single round of infection, with no release of progeny virus. Homologous prime-boost immunization of AAD mice with both pBeloBAC-FLYF and pBeloBAC-YF/ C elicited specific dose-dependent cellular immune response against YFV 17D. Furthermore, vaccination of A129 mice with pBeloBAC-FLYF resulted in the induction of YFV-specific neutralizing antibodies in all vaccinated animals. Thus, both studies have shown that DNA-launched live-attenuated virus immunization approach represents a promising vaccination strategy for YFV [21, 31].

6. Challenges for DNA-launched vaccines

Infectious clone methodology became a valuable tool of experimental virology. Traditionally, the full-length cDNA clones included T7 or SP6 bacteriophage RNA polymerase promoter so that the full-length genomic viral RNA could be prepared by using transcription *in vitro*. The resulting full-length RNA needed to be transfected in the permissive cells to produce live virus that can be harvested for vaccine preparation. The DNA-launched approach, which is based on intracellular transcription of (+)RNA virus genome cDNA cassettes from eukaryotic promoters in transfected cells, became an alternative to the traditional infectious clone methodology.

Challenges for iDNA vaccines include the process of configuring each vaccine for a particular target. Alphaviruses and flaviviruses replicate in the cytoplasm and do not involve nucleus. However, iDNA vaccine uses nucleus for transcription of the full-length infectious RNA genome and requires RNA transport to the cytoplasm. The difficulties include a possibility of degradation or inactivation of the full-length infectious genomic RNA in the nucleus by nucleases or splicing via cryptic splice sites, as well as potential problems in the RNA transport from the nucleus through the nuclear pore to the cytoplasm. Once the live vaccine virus is successfully launched, it is expected to undergo limited replication and

induce an immune response; however, the longevity of virus production from the DNA construct remains to be studied.

Another challenge is that many full-length cDNA clones are unstable in *E. coli*, which makes it very difficult to manufacture. For example, the difficulties in preparation of the full-length YF cDNA are well documented [21, 31, 104]. The use of the full-length clones is often limited by the instability of plasmids due to a transcriptional activity of eukaryotic promoters in *E. coli* resulting in synthesis of products toxic for the bacterial host. Strategies have been designed to circumvent this problem. One strategy involved insertion of introns to disrupt potentially toxic ORFs [21, 103]. Another strategy involved preparation of BAC clones with low copy number with potentially resulted in reduced toxic effects and in improved stability. The completely synthetic methods to produce large plasmid DNA with improved characteristics are also becoming available. A potential possibility to alleviate limitations associated with the DNA format would be using mRNA format, if RNA can be configured for vaccination *in vivo*. Thus, methods can be developed to alleviate constraints posed by the instability of infectious clones during propagation in *E. coli*.

Another potential challenge is the availability of the prototype live-attenuated virus that can serve as a parent for the development of the full-length iDNA clone. For VEEV and CHIKV alphaviruses, live-attenuated vaccines have been developed and evaluated in clinical trials [64, 79]. Likewise, live-attenuated clones with the human clinical history are well known for JEV and YFV flaviviruses. However, for other alphaviruses and flaviviruses, the clinically tested, live-attenuated viruses may not be readily available. Part of the reason is that the process of preparation of traditional live-attenuated vaccines by multiple passages in cell culture or *in vivo* is time-consuming and complex with no guarantee for success. However, reverse genetics of the DNA-launched plasmids provides a powerful tool for the development of rationally designed live-attenuated vaccines. Potential ways to streamline vaccine development can include reverse genetics, as well as bioinformatics, rational design of attenuating mutations, and *in vitro* high throughput screening methods. For example, additional mutations involving duplication of the 26S promoter, insertion of IRES element, and structural gene rearrangement were introduced into the alphaviral DNA-launched vaccines with the aim to improve vaccine safety [19, 20].

7. Conclusion

DNA-launched live-attenuated experimental vaccines have been developed for biodefense-relevant and emerging alphaviruses and flaviviruses including CHIKV, VEEV, WNV, JEV, and YFV. Nearly all human populations on the planet except polar and subpolar regions are at risk of infection with at least one of these viruses (Fig. 3-5). The recent changes in climate, urbanization, trade and migration patterns favor spread of these viruses from endemic regions to new areas, which is exemplified by introduction of WNV in New York in 1999.

Traditionally, DNA vaccines combine remarkable genetic and chemical stability, while live-attenuated virus vaccines have the advantage of inducing rapid, long-term immunity after a single-dose vaccination. In the DNA-launched vaccine approach, a live-attenuated vaccine is

launched from iDNA *in vivo*, with no need for external cell substrates or multiple virus passages for vaccine production. As a result of “manufacturing” of live-attenuated vaccine *in vivo*, the vaccine virus represents a homogenous population, which minimizes reversion mutations and represents a safety advantage. In addition to the genetic stability, the technology allows chemical and thermal stabilization of live-attenuated viruses and avoids cold chain. Because of these features, DNA-launched virus technology has advantages for vaccination. Potentially, the technology can also facilitate preparation of molecular repositories for existing viral vaccines. Essentially, the DNA-launched vaccine technology allows effective conversion of live-attenuated virus vaccine into the DNA vaccine format, as well as preparation of improved vaccines using rational design and reverse genetics methods. Recent data on safety, immunogenicity and efficacy of DNA-launched viral vaccines warrant further research on this promising novel technology [19-21, 31, 32, 101].

8. Expert commentary

Improved vaccines for emerging and re-emerging biodefense-relevant viruses are needed to prevent viral infections and to promptly respond to the increasing number of disease outbreaks and epidemics. During recent years, a significant progress has been achieved in preparation and evaluation of the DNA-launched live-attenuated vaccines. Promising safety, immunogenicity and efficacy characteristics of these vaccines have been reported, which provides a strong foundation for continuing development of DNA-launched live-attenuated viral vaccines.

The DNA-launched vaccine technology extends the advantages of both traditional DNA and live-attenuated vaccines. This platform minimizes the potential for reversions or adverse effects of traditional live-attenuated vaccine, ensures genetic stability, and results in efficient immunization in animal models [19-21, 25, 32]. The technology involves many advantages of a DNA immunization and live-attenuated vaccines and potentially, can enhance not only safety but also immunogenicity and efficacy of live-attenuated vaccine. Recombinant DNA produced in bacterial cells activates multiple pathways of innate immunity signaling, which results in production of a variety of cyto- and chemokines and provides priming effects to stimulate the acquired virus-specific immune responses. However, more research is needed to study DNA-launched vaccines including the longevity of vaccine virus expression from DNA and immunological mechanisms of protective responses such as activation of innate immunity and long term memory responses.

9. Five-year view

Biodefense vaccines for emerging diseases are critically important to protect populations against the emerging pathogens [35, 105]. With the increase of the outbreaks of emerging viral diseases, multiple technologies are being used for the development of preventive countermeasures including recombinant platforms, such of DNA vaccines [14], live viruses [63, 106], viral vectors [86, 87, 107-109] and virus-like particles [8, 110-112]. As discussed above, iDNA-launched live-attenuated virus vaccines have certain advantages to prevent and contain outbreaks of emerging diseases including the simplicity of production of these vaccines, as well as their potential to induce long term protective immunity after a single

dose administration. Feasibility of iDNA vaccine has been confirmed in animal models for several alphaviruses and flaviviruses of the global health impact.

The future developments are expected to include several aspects. First, clinical trials are needed to confirm feasibility of iDNA approach in humans. Alphaviruses and flavivirus infections represent an acute and growing threat in many parts of the world. For example, CHIKV has been listed as a priority along with Ebola filovirus among top priority pathogens for vaccine development [105]. WNV is another priority pathogen for the development of vaccine [105]. Potentially, DNA-launched vaccines can enter Phase I clinical trials within the next five years. For the clinical trials, DNA-launched live-attenuated vaccines can initially be used to vaccinate at-risk healthy adult populations (first responders, hospital and military personnel) with the minimal chances to develop adverse reactions. Thus, iDNA vaccines may provide medical and public health officials with vaccination options against emerging pathogens.

Second, additional preclinical research is needed to determine molecular mechanisms of DNA-launched vaccines including the longevity of virus expression from iDNA, the role of innate, humoral, and cell-mediated immune responses, as well as long term memory and efficacy. Preliminary research supported advantages in safety, immunogenicity and efficacy of this approach including vaccine small dose and its genetic stability [19, 20, 32]; however, additional research is needed.

Third, other alpha- and flaviviruses, as well as other RNA viruses, can be potentially configured into the DNA-launched vaccine format. The reverse genetics methods in the context of iDNA vaccine can provide an effective way for the rational design of vaccines for Western and Eastern equine encephalitis alphaviruses, as well as for multiple flaviviruses including dengue, tick-borne encephalitis and Zika viruses. However, multiple challenges in preparation of DNA-launched vaccines, such as instability and low yields of the full-length clones in *E. coli*, possibility of genomic RNA degradation in the nucleus, and the absence of attenuated strains, require in most cases a customized preparation of the full-length DNA-launched plasmid clones for each target RNA virus. Once prepared, the resulting constructs can be used as a reverse genetics system for the rational design of novel vaccines. Considering supportive preliminary data for multiple DNA-launched live-attenuated virus vaccines, it appears that this novel technology is well positioned for further development.

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Reference annotations

* Of interest

** Of considerable interest

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Key issues

- Nearly all human populations on the planet except polar and subpolar climates are at risk of infection with endemic or/and emerging alpha- and flaviviruses.
- DNA-launched live-attenuated virus experimental iDNA vaccines have been developed for alpha- and flaviviruses including CHIKV, VEEV, WNV, JEV, and YFV.
- The DNA-launched vaccine technology combines and extends the advantages of both traditional DNA vaccines and classic live-attenuated vaccines.
- Supportive preliminary data for multiple DNA-launched live-attenuated virus vaccines warrant further development of this promising technology including conducting human clinical trials.

Fig. 1.

Overview of immunization using DNA-launched virus vaccine approach. The full-length viral cDNA is placed downstream from optimized CMV or other eukaryotic promoter sequences. In the tissues injected with iDNA, transcription from the CMV promoter yields the full-length, infectious genomic RNA capable of initiating limited replication of live-attenuated virus, thus inducing protective immune responses. Electroporation can increase DNA uptake, lower DNA vaccine dose and improve efficiency of DNA vaccination *in vivo* [1, 4, 5].

Chikungunya Virus (CHIKV)

Fig. 2.

Global distribution (in green) of chikungunya virus (CHIKV) (cdc.gov), transmission electron microscopy, and virus 3D image reconstruction (http://viperdbscripps.edu/info_page.php?VDB=3j2w). Bottom panel shows the prototype 181/25 DNA-launched CHIKV vaccine construct including location of CMV promoter, subgenomic 26S promoter, as well as regions corresponding to the 5' and 3' termini of 181/25 CHIKV genomic RNA. Polyproteins of CHIKV are also indicated (adapted from [19]).

Venezuelan Equine Encephalitis Virus (VEEV)

Fig. 3.

Area at risk of infections (in red) with Venezuelan equine encephalitis virus (VEEV), cryo-electron microscopy microphotograph [114], and virus 3D image reconstruction (http://viperd.b.scripps.edu/info_page.php?VDB=3j0c). Bottom panel shows the prototype TC83 DNA-launched VEEV vaccine construct including location of CMV promoter, subgenomic 26S promoter, as well as regions corresponding to the 5' and 3' termini of TC83 genomic RNA. Polyproteins of VEEV are indicated (adapted from [20]).

West Nile Virus (WNV)

Fig. 4.

Global distribution (shown in yellow) of West Nile virus (WNV) (cdc.gov), electron microscopy (<http://hardinmd.lib.uiowa.edu/cdc/2290.html>), and structure of immature WNV (http://viperd.b.scripps.edu/info_page.php?VDB=2of6). Bottom panel shows the prototype WNV DNA-launched vaccine construct including location of CMV promoter and the 5' and 3' termini of WNV genomic RNA. Polyproteins of WNV are indicated.

Yellow Fever Virus (YFV)

Fig. 5.

Countries at risk of infection with yellow fever virus (YFV) (who.int), electron microscopy of YFV (https://www.vaccines.mil/Yellow_Fever), and structure of immature YFV particle (http://viperd.b.scripps.edu/info_page.php?VDB=1na4). Bottom panel shows the prototype YFV 17D DNA-launched YFV vaccine construct including location of CMV promoter and the 5' and 3' termini of 17D genomic RNA. Polyproteins of YFV 17D are indicated (adapted from [21]).

Table I

Comparison of live-attenuated vaccines, traditional DNA vaccines, and DNA-launched (iDNA) live-attenuated vaccines.

Vaccine feature	Live-attenuated	Traditional DNA	DNA-launched live-attenuated
Genetic stability		<i>Yes</i>	<i>Yes</i>
High purity		<i>Yes</i>	<i>Yes</i>
Simplicity of production		<i>Yes</i>	<i>Yes</i>
Avoid cold chain		<i>Yes</i>	<i>Yes</i>
Single-dose vaccine	<i>Yes</i>		<i>Yes</i>
Effective protection	<i>Yes</i>		<i>Yes</i>
Long-lasting immunity	<i>Yes</i>		NT

NT, not tested

Table II

Examples of DNA-launched live-attenuated vaccine viruses.

Virus Family	Vaccine	Dose, <i>In vitro</i>	Dose, <i>In vivo</i>	Vaccine Route	Viremia	Neutralizing Antibody	Protection	Reference
Alpha virus	CHIKV	5 ng	10 µg	IM/EP	+/-	+ (10/10)	+ (10/10)	[19]
	VEEV	8 ng	50 µg	IM/EP	+/-	+ (10/10)	+ (10/10)	[20]
			10 µg	IV	+/-		+	
Flavi virus	WNV		0.1-10 µg	IM	+	+	+	[22, 24, 99, 101]
	JEV	2 µg	nt	n/a	n/a	n/a	n/a	[23, 103]
	YFV	10 ng	20 µg	IM/EP	-	+ (10/10)	nt	[21]
		5 µg	0.1-10 µg	IM/EP	+	+	nt	[31]

CHIKV, Chikungunya virus; VEEV, Venezuelan equine encephalitis virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; YFV, yellow fever virus; IM, intramuscular; EP, intramuscular/electroporation; IV, intravenous; nt, not tested; n/a, not applicable.