Zika Virus Attenuation by Codon Pair Deoptimization Induces Sterilizing Immunity in Mouse Models

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ABSTRACT Zika virus (ZIKV) infection during the large epidemics in the Americas is related to congenital abnormalities or fetal demise. To date, there is no vaccine, antiviral drug, or other modality available to prevent or treat Zika virus infection. Here we designed novel live attenuated ZIKV vaccine candidates using a codon pair deoptimization strategy. Three codon pair-deoptimized ZIKVs (Min E, Min NS1, and Min E+NS1) were *de novo* synthesized and recovered by reverse genetics and contained large amounts of underrepresented codon pairs in the E gene and/or NS1 gene. The amino acid sequence was 100% unchanged. The codon pair-deoptimized variants had decreased replication fitness in Vero cells (Min NS1 $\gg$ Min E > Min E+NS1), replicated more efficiently in insect cells than in mammalian cells, and demonstrated diminished virulence in a mouse model. In particular, Min E+NS1, the most restrictive variant, induced sterilizing immunity with a robust neutralizing antibody titer, and a single immunization achieved complete protection against lethal challenge and vertical ZIKV transmission during pregnancy. More importantly, due to the numerous synonymous substitutions in the codon pair-deoptimized strains, reversion to wild-type virulence through gradual nucleotide sequence mutations is unlikely. Our results collectively demonstrate that ZIKV can be effectively attenuated by codon pair deoptimization, highlighting the potential of Min E+NS1 as a safe vaccine candidate to prevent ZIKV infections.

IMPORTANCE Due to unprecedented epidemics of Zika virus (ZIKV) across the Americas and the unexpected clinical symptoms, including Guillain-Barré syndrome, microcephaly, and other birth defects in humans, there is an urgent need for ZIKV vaccine development. Here we provided the first attenuated versions of ZIKV with two important genes (E and/or NS1) that were subjected to codon pair deoptimization. Compared to parental ZIKV, the codon pair-deoptimized ZIKVs were mammal attenuated and preferred insect to mammalian cells. Min E+NS1, the most restrictive variant, induced sterilizing immunity with a robust neutralizing antibody titer and achieved complete protection against lethal challenge and vertical virus transmission during pregnancy. More importantly, the massive synonymous mutational approach made it impossible for the variant to revert to wild-type virulence. Our results have proven the feasibility of codon pair deoptimization as a strategy to develop live attenuated vaccine candidates against flaviviruses such as ZIKV, Japanese encephalitis virus, and West Nile virus.

KEYWORDS codon pair bias, E protein, NS1 protein, Zika virus, deoptimization, vaccines

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Zika virus (ZIKV) is an enveloped virus that belongs to the *Flaviviridae* family (1). ZIKV was first isolated from the blood of a febrile rhesus macaque in 1947 in the Zika forest of Uganda (2) and has become a major public health risk globally, driven by the current unprecedented epidemics of ZIKV across the Americas (3–5). ZIKV is usually associated with asymptomatic infections or mild febrile illness that is accompanied by rash conjunctivitis in human (6); however, during the large epidemics in the Americas, ZIKV infection has tended to cause more-severe clinical manifestations, including Guillain-Barré syndrome (GBS), meningoencephalitis, microcephaly, and other birth defects (3, 4, 7). The virus is mainly transmitted by *Aedes* mosquitoes, but human-to-human transmission through sexual and vertical routes has also been reported, which was different from most other flaviviruses (8, 9). The efficient transmission and comparatively limited antiviral therapeutic options have aggravated the current panic over ZIKV. To date, there is no effective licensed vaccine or antiviral treatment against ZIKV infection, although several vaccine candidates have been described, including formalin-inactivated vaccines (10, 11), live attenuated vaccines (12), genetic vaccines (11, 13–17), and virus-like particle (VLP) vaccines (18, 19). Therefore, new options for the development of a ZIKV vaccine are needed.

The ZIKV genome is a single plus-strand RNA approximately 11 kb long and contains a single open reading frame (ORF) encoding a polyprotein that is subsequently cleaved by cellular and viral proteases into three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (20, 21). The structural proteins form viral particles and mediate attachment and entry of ZIKV into host cells, while the nonstructural proteins are engaged in viral genome replication, virus assembly, and evasion of the host innate immune response (20, 22, 23). Specifically, the envelope (E) protein is considered a major determinant for ZIKV pathogenesis and is involved in modulating the viral infection cycle (24). Although not being a component of viral particles, NS1 plays an essential role in viral RNA replication as well as in host immune recognition and evasion (25, 26). Hence, the multifunctional roles of E and NS1 gene products were regarded as ideal targets for attenuation to create novel live attenuated ZIKV vaccines.

Codon pair bias is interpreted as an unequal frequency in the usage of synonymous codon pairs in certain species (27–29). Based on the algorithm to quantify codon pair bias, every codon pair harbors a codon pair score (27, 28). Codon pairs with positive codon pair scores are statistically overrepresented, which may indicate that they are preferred by the organism, while others with negative codon pair scores are underrepresented (28, 30). For instance, GCCGAA (codon pair score of -1.717) is strongly underrepresented and is used only one-seventh as frequently as GCAGAG (codon pair score of 0.411), even though it contains GCC, the optimal Ala codon (27). Codon pair deoptimization, also known as synthetic attenuated virus engineering (SAVE), is a novel technique for viral attenuation by increasing the presence of underrepresented codon pairs (27, 31). In contrast, another attenuation strategy, codon deoptimization, has also been generated by introducing the least-preferred codons for the majority of the amino acid residues of the target genes (32, 33). Codon pair-deoptimized strains harbor identical amino acid sequences conserving the same repertoire of epitopes as the wild-type (WT) pathogen, which may provide favorable immunogenicity and protective immunity, and contain numerous synonymous substitutions, which could make the generation of virulent revertants unlikely (34). Remarkably, the processes of codon pair deoptimization and codon deoptimization are often accompanied by increases in CpG and UpA dinucleotide frequencies (28, 35, 36). CpG and UpA dinucleotides are rare in mammalian genes (37, 38) and eukaryotic RNA viruses (39, 40), as are the codon pairs with a central xxCpGxx or xxUpAxx generally (28, 30). An increased xxCpGxx content may induce an innate immune response in certain cells, which could decrease the replicative fitness of intracellular virus (41), while the xxUpAxx abundance is deemed to reduce mRNA stability (42). To date, codon deoptimization has been used to attenuate poliovirus, respiratory syncytial virus, foot-and-mouth disease virus, arenavirus, and influenza virus (32, 33, 41, 43–46), and the codon pair deoptimization strategy has also

TABLE 1 Characteristics of deoptimized ZIKV genome segments

Virus	Deoptimized coding region (positions)	Human codon pair bias of WT segment	Human codon pair bias of deoptimized segment	Mosquito codon pair bias of WT segment	Mosquito codon pair bias of deoptimized segment	No. of silent mutations (total no. of nucleic acids)
Min E	1–1512	0.0336	−0.5741	−0.0099	−0.0729	363 (1,512)
Min NS1	1–1056	0.0059	−0.5162	−0.0077	−0.0809	219 (1,056)
Min E+NS1	1–2568	0.0222	−0.5503	−0.0090	−0.0764	582 (2,568)

been used to generate attenuated poliovirus, respiratory syncytial virus, vesicular stomatitis virus, porcine reproductive and respiratory syndrome virus, dengue virus, and influenza virus (27, 28, 30, 31, 47–49).

In this study, three codon pair-deoptimized ZIKVs were designed, *de novo* synthesized, and recovered by reverse genetics. All the codon pair-deoptimized ZIKVs were attenuated to different extents in Vero cells (a mammalian cell line) but not in C6/36 mosquito cells. Like their phenotype *in vitro*, codon pair-deoptimized ZIKVs were attenuated *in vivo* and were also shown to conserve potent immunogenicity that completely protected vaccinated mice from lethal challenge and vertical virus transmission during pregnancy. These results raise the possibility of using codon pair deoptimization for the generation of novel live attenuated ZIKV vaccine candidates.

RESULTS

Generation of codon pair-deoptimized ZIKVs. Because the codon pair biases between humans and mosquitoes are poorly correlated (28), the codon pair scores of the E and NS1 genes of an Asian-lineage Zika virus, SZ-WIV01, were reduced deeply according to the human codon pair bias table but not according to the mosquito table (28). As is shown in Table 1, a total of 363 synonymous mutations were introduced into the specified E coding region, in which the deoptimized human codon pair score ranged from 0.0336 to −0.5741, whereas the change was minimal for the mosquito codon pair score. The same thing happened in the NS1 coding region, with the average human codon pair score being reduced from 0.0059 to −0.5162 and the change being minimal for the mosquito codon pair score. In consideration of the potential impact on virus attenuation, the increases in the frequencies of xxCpGxx and xxUpAxx were also calculated. As shown in Table 2, all the codon pair-deoptimized segments possess significantly more xxCpGxx (294% to 455% increase) or xxUpAxx (185% to 371% increase) dinucleotides than the WT counterparts.

The full-length wild-type ZIKV (ZIKVwt) cDNA clone was constructed using reverse-genetics methods (see Materials and Methods). ZIKVwt was recovered by transfection in Vero cells. Based on the full-length ZIKVwt cDNA clone, we designed and generated, by reverse genetics, three synthetic codon pair-deoptimized ZIKVs, named Min E, Min NS1, and Min E+NS1, in which various genome regions were subjected to codon pair deoptimization (Fig. 1). This enabled comparisons of the biological properties, pathogenicities, and immunogenicities of the WT and codon pair-deoptimized viruses *in vitro* and *in vivo*.

TABLE 2 Increases in frequencies of C₃G₁ and U₃A₁ in deoptimized ZIKV genome segments

Gene	Encoded segment	Length (nt)	No. of C ₃ G ₁ dinucleotides	ΔC ₃ G ₁ (% increase) ^a	No. of U ₃ A ₁ dinucleotides	ΔU ₃ A ₁ (% increase) ^a
E	WT	1,512	20		14	
E	Deoptimized	1,512	111	+91 (455)	66	+52 (371)
NS1	WT	1,056	17		13	
NS1	Deoptimized	1,056	67	+50 (294)	37	+24 (185)
E+NS1	WT	2,568	37		27	
E+NS1	Deoptimized	2,568	178	+141 (381)	103	+76 (281)

^aC₃G₁ refers to xxCpGxx, a dinucleotide formed between two codons. ΔC₃G₁ refers to the change in xxCpGxx when deoptimized segments are added with respect to WT segments; this is equally applicable to ΔU₃A₁.

FIG 1 Construction of the infectious cDNA clone of ZIKVwt and generation of codon pair-deoptimized ZIKVs. (A) Strategy for constructing the full-length cDNA clone of ZIKVwt. Four cDNA fragments, fragments A to D, that cover the complete ZIKV genome were synthesized from viral RNA using RT-PCR and sequentially cloned into plasmid pACYC177 to form the full-length cDNA clone of ZIKV (ZIKVwt-FL). The CMV promoter, HDVr/SV40 poly(A), and the positions of relevant restriction sites are shown. (B) Gene maps of the codon pair-deoptimized ZIKVs Min E, Min NS1, and Min E+NS1. Codon pair-deoptimized genes are shown as white boxes, the WT E gene is shown as orange boxes, and the WT NS1 gene is shown as blue boxes. Restriction sites (KpnI and ClaI) used for construction are indicated.

Growth properties of codon pair-deoptimized ZIKVs *in vitro*. Vero cells and C6/36 cells were used to analyze the replicative properties of codon pair-deoptimized ZIKVs with each virus at the same multiplicity of infection (MOI) of 0.01. In Vero cells, Min E as well as Min E+NS1 showed poor replication (Fig. 2A and B and 3). As for Min NS1, although its endpoint titers reached levels comparable to those of ZIKVwt, it displayed delayed replication kinetics; Min NS1 had reduced levels of viral RNA at 1 and 2 days postinfection (dpi) ($P < 0.01$) (Fig. 2A) and low infectious titers at 3 and 4 dpi ($P < 0.01$) (Fig. 2B) compared with the WT virus. In addition, the average sizes of infectious foci decreased in the order WT, Min NS1, Min E, and Min E+NS1 in Vero cells (Fig. 2G). These results indicated that the replication of the codon pair-deoptimized variants in Vero cells dramatically decreases (for replication fitness, Min NS1 $\gg$ Min E $>$ Min E+NS1).

In C6/36 cells, all of the codon pair-deoptimized viruses as well as ZIKVwt reached maximum viral loads of $> 2 \times 10^{10}$ copies/ml at 8 dpi (Fig. 2C). Compared with ZIKVwt, Min E+NS1 even displayed enhanced RNA replication kinetics before 4 dpi (Fig. 2C), although there were no significant differences. An immunostaining focus assay was carried out to determine the infectious titers. The maximal titers of Min NS1 and Min E between 7 and 8 dpi were comparable to that of the WT virus; however, they displayed

FIG 2 Replication of WT and codon pair-deoptimized ZIKVs in cell culture. (A to D) Vero cells (A and B) or C6/36 cells (C and D) were infected with viruses at an MOI of 0.01. Viral loads were determined by qRT-PCR (A and C), and virus titers were measured by an immunostaining focus assay on Vero cells (B and D). (E and F) Growth properties of viruses were determined by passing them on Vero cells at an MOI of 0.01. L.O.D., limit of detection. (G) Plaque size phenotypes of virus variants on Vero cells, visualized by immunostaining following incubation for 4 days (WT and Min NS1) and 7 days (Min E and Min E+NS1) under methylcellulose at 37°C. Data shown (A to F) are the means and standard deviations (SD) analyzed by Student's *t* test (two tailed) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

delayed replication kinetics. Min NS1 had lower infectious titers at 1, 3, and 4 dpi ($P < 0.05$) (Fig. 2D), and Min E had reduced infectious titers at 1, 3, 4, 5, and 6 dpi ($P < 0.05$) (Fig. 2D). The infectious titers of Min E+NS1 decreased stepwise at all time points ($P < 0.05$) (Fig. 2D).

FIG 3 IFA of E protein expression in Vero cells infected with WT or codon pair-deoptimized ZIKVs. Vero cells were infected with viruses at an MOI of 0.01. At 2, 3, and 4 dpi, IFA was performed as described in Materials and Methods. All the images were captured at a $\times 10$ magnification. Green represents E protein, and blue represents nuclei (stained with Hoechst 33258).

Next, multipassage analysis was performed to test the passage stability of ZIKVs. Both ZIKVwt and Min NS1 reached high viral loads (Fig. 2E) and were capable of developing infectious foci in Vero cells (Fig. 2F) from passage 1 (P1) to P5. Min E as well as Min E+NS1 were nonviable in Vero cells by the fourth passage and the second passage, respectively (Fig. 2E and F).

Diminished virulence of codon pair-deoptimized ZIKVs in AG6 mice. An AG6 mouse model was used to evaluate the diminished virulence of codon pair-deoptimized ZIKVs. Four-week-old AG6 mice were infected with different doses of ZIKVwt or codon pair-deoptimized ZIKVs by the intraperitoneal (i.p.) route. Under our experimental conditions, ZIKVwt was highly virulent in these AG6 mice, with a 50% lethal dose (LD_{50}) of 1.78 PFU. Min NS1 was slightly attenuated in mice, with an ~ 1.7 -fold increase in the LD_{50} compared with that of ZIKVwt (Table 3). Dramatic attenuations were observed with Min E and Min E+NS1, revealing $\sim 1,000$ -fold and $\sim 2,000$ -fold increases in median 50% lethal dose (MLD_{50}) values, respectively, compared with ZIKVwt (Table 3). The order of attenuations in the animals (Min E+NS1 > Min E $\gg$ Min NS1) was consistent with the order of attenuations in tissue culture cells.

Comparative analysis of pathogenicity. AG6 mice were infected with 100 inclusion-forming units (IFU) of either ZIKVwt or codon pair-deoptimized ZIKVs and monitored for 28 days for weight loss (Fig. 4A) and mortality (Fig. 4B). Mice were also periodically euthanized to perform virus detection for various organs (at 3 and 7 dpi) (Fig. 5 and 6A and B) and sera (at 3 and 6 dpi) (Fig. 6C). As expected, codon pair-deoptimized ZIKVs showed levels of pathogenicity different from those for ZIKVwt. Mice infected with Min E+NS1 shared kinetics of weight gain comparable to those of

TABLE 3 Median lethal dose values for AG6 mice after intraperitoneal inoculation

Virus	MLD_{50} (no. of PFU/mouse)
ZIKVwt	1.78
Min E	1,750.83
Min NS1	2.98
Min E+NS1	3,981.07

FIG 4 Attenuation of codon pair-deoptimized ZIKVs in AG6 mice. Groups of AG6 mice (4 weeks old; $n = 6$) were infected intraperitoneally with 10^2 IFU of WT or codon pair-deoptimized ZIKVs. Body weight loss (A) and survival (B) were monitored daily for 4 weeks. Mice were euthanized when they lost 25% of their initial body weight.

mock-treated mice (Fig. 4A), and all mice survived (Fig. 4B). In contrast, the survival rates of the ZIKVwt, Min NS1, and Min E groups were 0%, 16.7%, and 83.3%, respectively. Immunohistochemistry (IHC) staining of brain tissue sections showed a wide distribution of E protein in mice infected with the WT virus; in contrast, it was difficult to detect the distribution of E protein in mice infected with Min E+NS1 virus (Fig. 5).

The pathogenicity and virus load in sera as well as in infected organs are usually positively correlated (12, 30); thus, viral titers in sera, heart, liver, spleen, lung, kidney, brain, testes, uterus, ovary, eye, intestine, and muscle were measured by quantitative real-time PCR (qRT-PCR) (Fig. 6A and C) and an immunostaining focus assay (Fig. 6B). Min E+NS1 was nonviable in all the tested organs except for spleen. The mean viral load in Min E+NS1-infected spleens was $10^{2.17}$ copies/ μ g total RNA at 3 dpi, which was at least 3 orders of magnitude lower than that in their WT-infected counterparts ($P < 0.05$), and was reduced to $10^{1.28}$ copies/ μ g total RNA at 7 dpi. The supernatants (homogenized Min E+NS1-infected spleens) were not capable of developing infectious foci in Vero cells (Fig. 6B). In contrast, in ZIKVwt-infected animals, viral RNA was widespread in all the tested organs up to 7 dpi (Fig. 6A), which ultimately resulted in the death of all the remaining animals by 9 dpi (Fig. 4B).

Immunogenicity and efficacy of codon pair-deoptimized ZIKVs in AG6 mice. To evaluate whether codon pair-deoptimized ZIKV immunization elicited B cell-mediated humoral immunity, sera were collected 28 days after vaccination through the retro-orbital sinus, and titers of ZIKV-specific neutralizing antibodies (NAb) were measured by a 50% plaque reduction neutralization test (PRNT₅₀). Mice infected with the WT or codon pair-deoptimized viruses were found to have comparable neutralizing antibody titers against the WT virus, whereas the control mice did not develop a detectable

FIG 5 Immunohistochemical staining of E protein in brain sections from infected mice. AG6 mice were infected with 10^2 IFU of viruses ($n = 3$). The brain tissues from mice infected with the WT virus were collected at 7 dpi. The brain tissues from mice infected with Min E+NS1 were collected at 28 dpi.

FIG 6 Viral loads in organs or sera of infected AG6 mice. (A and B) Mice ($n = 3$) were infected with 10^2 IFU of viruses and euthanized at day 3, day 6, or day 7, and organ viral loads were determined at day 3 and day 7 by qRT-PCR (A) and an immunostaining focus assay (spleen) (B). (C) Serum viral loads were determined at day 3 and day 6 by qRT-PCR. Data shown (A to C) are the means $\pm$ SD analyzed by Student's t test (two tailed) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

PRNT₅₀ titer (PRNT₅₀ titer of <10) (Fig. 7A). To evaluate cellular immune responses in AG6 mice, on day 28 postimmunization, ZIKV-specific T cells from the spleen were restimulated with heat-inactivated WT virus *in vitro* and analyzed by a gamma interferon (IFN- γ) enzyme-linked immunosorbent spot (ELISPOT) assay. The results showed

FIG 7 Humoral and cellular immune responses induced by codon pair-deoptimized ZIKVs in mice. (A) Prechallenge neutralization antibody titers were measured on day 28 (day 7 for WT virus) after immunization using a standard PRNT₅₀ assay. (B) Cellular immune responses were assessed on day 28 after immunization by IFN- γ ELISPOT assays. Data shown (A and B) are the means $\pm$ SD analyzed by Student's t test (two tailed) (**, $P < 0.01$; n.s., not significant).

FIG 8 Protection efficacy of codon pair-deoptimized ZIKVs in mice. Mice were immunized with 10^2 IFU of codon pair-deoptimized viruses or mock vaccinated with PBS. At 4 weeks postvaccination, animals were challenged with 10^4 IFU of WT virus (an $\sim 5,500$ -fold-higher MLD_{50}). (A and B) Body weight loss (A) and survival (B) were evaluated for 14 days after challenge. Mice were euthanized when they lost 25% of their initial body weight. (C) Postchallenge viremia was quantified by qRT-PCR on day 3 after challenge. (D) Postchallenge neutralization antibody titers were determined at day 14 after challenge by a standard PRNT₅₀ assay. Data shown (C and D) are the means $\pm$ SD analyzed by Student's *t* test (two tailed) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

that the average IFN- γ levels secreted from the Min E+NS1-immunized group were significantly higher than those for the mock-immunized group ($P < 0.01$) (Fig. 7B).

The mice were then challenged with 10^4 PFU of WT virus intraperitoneally, representing an $\sim 5,500$ -fold-higher MLD_{50} of ZIKVwt. All vaccinated animals survived without detectable peripheral viremia and any signs of disease (weight loss, ruffled fur, hind limb paralysis, hunched posture, or lethargy) through day 14 (Fig. 8A to C), whereas the sham-vaccinated mice produced a mean viremia of $(5.2 \pm 4.0) \times 10^8$ copies/ml on day 3 after challenge (Fig. 8C) and died by 10 days after challenge (Fig. 8B). Furthermore, high titers of neutralizing antibodies in challenged mice were also detected (Fig. 8D). Taken together, single-dose vaccination with the Min E+NS1 virus can elicit a robust immune response that fully protects AG6 mice against a subsequent lethal challenge. On day 28 after challenge, we measured the neutralization titers in the mouse sera again; notably, the postchallenge neutralization titers were equivalent to the prechallenge neutralization titers.

To determine if Min E+NS1 immunization could protect pregnant AG6 mice, we mated immunized AG6 female mice with 8-week-old naive AG6 male mice at day 32 postimmunization and challenged the pregnant mice with 10^4 PFU of WT virus at embryo day 6 (E6) (Fig. 9A). As expected, high titers of neutralizing antibodies in pregnant mice were detected at day 1 before challenge (Fig. 9B). Following WT challenge, Min E+NS1-immunized mice had no signs of disease (weight loss, ruffled fur, hind limb paralysis, hunched posture, or lethargy) throughout the experiment. Phosphate-buffered saline (PBS)-immunized mice developed high levels of maternal viremia; however, Min E+NS1-immunized mice had no detectable maternal viremia (Fig. 9C). All PBS-immunized mice died without delivery; in contrast, all Min E+NS1-vaccinated dams successfully delivered healthy pups at term with normal viability

FIG 9 Min E+NS1 immunization protected AG6 mice during pregnancy. (A) Scheme of immunization of 4-week-old AG6 female mice with 10² IFU of Min E+NS1 or PBS. (B to D) At day 32 postimmunization, vaccinated female mice were mated with AG6 males. Pregnant mice (*n* = 4) were infected with 10⁴ IFU of WT virus on E6. (B) Neutralization antibody titers were measured on day 1 before challenge, using a standard PRNT₅₀ assay. (C) Maternal viremia on day 2 after challenge was quantified by qRT-PCR. (D) Outcome of fetuses from Min E+NS1- or PBS-vaccinated dams. ^a, all PBS-immunized pregnant mice died without delivery; ^b, maternal neutralization antibody titers for pups delivered at term to Min E+NS1-vaccinated dams were measured on the 21st day after birth. Data shown (B and C) are the means ± SD analyzed by Student's *t* test (two tailed) (***, *P* < 0.001).

(Fig. 9D). Modest to low levels of maternal neutralization antibody were detected in the sera of pups even at the 21st day after birth (Fig. 9D). Collectively, preconception maternal immunity induced by Min E+NS1 immunization efficiently protected AG6 mice during pregnancy and prevented viral transmission to the fetus.

DISCUSSION

We have investigated the strategy of codon pair deoptimization as a means to develop novel attenuated versions of ZIKV, a pathogenic virus that has caused GBS, meningoencephalitis, microcephaly, and other birth defects in humans (3, 4, 7). Viruses harboring deoptimized codon pairs in the E gene, NS1 gene, and E+NS1 gene were designed, rescued, and proven to be attenuated to different extents *in vitro* and *in vivo*. It should be pointed out that the most attenuated virus, Min E+NS1, possessed potential for the generic development of live attenuated vaccines that produced robust immunogenicity, provided complete protection against a lethal challenge of ZIKV, protected AG6 mice during pregnancy, and prevented viral transmission to the fetus with a single dose.

As far as we know, several live-attenuated ZIKV vaccines have been reported (12, 50). Shan et al. generated a recombinant live attenuated ZIKV vaccine candidate by the deletion of 10 nucleotides (nt) in the viral 3' untranslated region (UTR) by reverse genetics (12), while Li et al. developed and characterized a recombinant chimeric ZIKV vaccine candidate expressing the prM-E proteins of ZIKV using the licensed Japanese encephalitis virus live attenuated vaccine SA14-14-2 as the genetic backbone (50). Using the strategy of codon pair deoptimization, our attenuated Min viruses express identical whole protein sequences conserving an intact antigenic repertoire. Moreover, due to the large number of mutations introduced, these attenuated Min viruses are unlikely to develop virulent revertants through gradual nucleotide sequence mutations.

Several mechanisms appeared to be related to the attenuation in ZIKV Min variants

caused by rare codon pairs. The major effect of codon pair deoptimization should be a decreased efficiency of translation in a context-dependent manner (27, 30, 32). A string of “rare” codon pairs compounds the difficulties of readthrough by the ribosome, resulting in fewer precursor proteins per mRNA. Other parameters coordinated with the translation elongation rate, such as ribosomal stalling, premature dissociation of the translation initiation complex, protein processing, folding, and/or stability, may also be involved (28, 33, 51). Additionally, the increased frequencies of CpG and UpA dinucleotides can also play important roles in RNA virus attenuation (28, 35, 36, 52, 53). Mammalian genomes and eukaryotic RNA viruses exhibit marked CpG/UpA suppression (37–40). The increased dinucleotide frequency may induce an innate immune response in host cells (41, 54) and/or reduce mRNA stability (42). The recoded segments with rare codon pairs are generally associated with an enrichment of CpG and UpA dinucleotides (28, 30, 41, 43, 44). In agreement, we found that all the codon pair-deoptimized segments possess significantly more xxCpGxx or xxUpAxx dinucleotides than the WT counterparts. Codon pair bias has been suggested to be a direct consequence of CpG/UpA dinucleotide bias (55), and the increased frequency of CpG/UpA dinucleotides may be a key genetic contributor to virus attenuation by codon pair deoptimization (56), although this has been disputed (28, 57). Another finding is that the effect of codon pair usage or dinucleotide frequencies on translation is minor or nonexistent (43, 44, 52, 56), which warrants further investigation. In conclusion, it is difficult to distinguish the two effects (the increased frequencies of CpG and UpA dinucleotides or the increased frequency of disfavored codon pairs) that mediate the attenuation of ZIKV Min variants in this study.

Each of the codon pair-deoptimized ZIKVs replicated less efficiently in Vero cells than the WT virus. Min E+NS1 was the most restricted codon pair-deoptimized mutant and exhibited the smallest infectious foci (Fig. 2G), the slowest replication kinetics, and the lowest peak titer (Fig. 2A and B), informing that it involves an “additive” relationship of the effect of recoded genomes, with significantly more rare codon pairs, as found with poliovirus (27), or significantly more CpG/UpA dinucleotides than in Min E and Min NS1 (41, 42, 54). Min NS1 was less restricted in Vero cells than Min E. The seemingly counterintuitive result may be explained by the amounts of codon pair changes introduced into the corresponding segments or inappropriate molar ratios of proteins relative to mRNAs and implied that suboptimal NS1 could be tolerated to some extent. A key result was the observation that all three codon pair-deoptimized ZIKVs replicated more efficiently in C6/36 cells than in Vero cells (Fig. 2). The alteration of cell tropism was due to the introduction of hundreds of underrepresented human codon pairs. Because there was a poor correlation between human and mosquito codon pair preferences, the accumulation of underrepresented human codon pairs would not drift the cumulative codon pair score too far according to the insect table (28). One of the potential benefits is the practicability of the launching platform for the high-yield production of an attenuated ZIKV vaccine in insect cell systems, such as Sf9 and Sf21 cell lines. Although the increased CpG/UpA dinucleotide composition profoundly reduced the replication ability of RNA viruses in mammalian cells, it is uncertain whether this is also true in mosquito cells. Therefore, the fact that codon pair-deoptimized ZIKVs prefer mosquito cells over mammalian cells may not be associated with differences in sensing CpG/UpA in the deoptimized viruses compared to the WT virus.

Mice deficient in the type I and type II IFN ($\text{IFN-}\alpha/\beta/\gamma$) receptors are extremely susceptible to ZIKV infection and display severe disease signs, including hind limb weakness, paralysis, and death, and provide a platform for identifying determinants of ZIKV virulence and testing the efficacy of antivirals and vaccines (8, 58–60). Thus, AG6 mice lacking the type I and type II IFN ($\text{IFN-}\alpha/\beta/\gamma$) receptors were used in this study. As expected, diminished virulence was clearly identified in the codon pair-deoptimized variants (Table 3), revealing ~ 1.7 -fold (Min NS1), $\sim 1,000$ -fold (Min E), and $\sim 2,000$ -fold (Min E+NS1) increases in MLD_{50} values compared with the WT virus. We hypothesize that this observation is related to viral attenuation in tissue culture cells, although viral attenuation in tissue culture cells does not (necessarily) translate to that in animals (28).

It is known that ZIKV was associated with microcephaly and caused testis damage (leading to male infertility in mice) (7, 61, 62). In the immunocompromised mouse model, ZIKV was widespread in all the tested tissues, including brain and testis (58, 59), which was also found in our work (Fig. 6A). In contrast, Min E+NS1 was nonviable in all the tested organs (the supernatants of homogenized spleens were not capable of developing infectious foci, in spite of low-level detection of viral RNA). This observation suggested that the risk of brain and testis damage is negligible. Despite high attenuation of Min E+NS1 in the host, it induced high levels of neutralizing antibodies and IFN- γ in mice and conferred protection against lethal challenge with the WT virus. In addition, preconception maternal immunity induced by Min E+NS1 immunization is sufficient to protect pregnant AG6 mice and their fetuses (Fig. 9). We hypothesize that the strong protection was ascribed to the fact that our codon pair-deoptimized viruses obtained a repertoire of epitopes identical to that of the WT virus (the amino acid sequence is 100% preserved). To date, Min E+NS1 is the first attenuated version of a flavivirus with two important genes that were subjected to codon pair deoptimization simultaneously, maintaining a balance between efficacy and safety.

In summary, we describe the first large-scale recoding of ZIKV, a flavivirus that belongs to a large family of mosquito-borne human pathogens. Min E+NS1 displayed the potential to develop into a promising live-attenuated vaccine candidate. Results from this study demonstrated the feasibility of the rapid attenuation of ZIKV through the codon pair deoptimization strategy. The unparalleled advantage of the codon pair deoptimization strategy is that reversion to wild-type virulence is unlikely due to numerous synonymous substitutions without changing the amino acid sequence (34, 43). Thus, the codon pair deoptimization strategy would add safety to the features of live attenuated viruses, which has broad application in the development of vaccines for flaviviruses and other important viruses.

MATERIALS AND METHODS

Ethics statement. All experiments involving animals have been reviewed and approved by the Animal Care Committee of the Wuhan Institute of Virology (permit no. WIVA07201603), in accordance with animal ethics guidelines of the Chinese National Health and Medical Research Council (NHMRC).

Cells. African green monkey kidney epithelial cells (Vero; ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Life Technology, Australia), 100 U/ml penicillin, and 100 μ g/ml streptomycin and maintained in 5% CO₂ at 37°C. *Aedes albopictus* C6/36 cells (ATCC CRL-1660) were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA) containing 10% FBS in 5% CO₂ at 28°C.

Design of codon pair-deoptimized sequences. E and NS1 genes were recoded by rearranging existing synonymous codons to minimize the cumulative codon pair scores according to the human codon pair bias table (27). RNAfold software (63) was used to maintain the free energy of the folding of the RNA within a narrow range and to avoid large changes in the secondary structure of the customized RNA as a consequence of codon rearrangement. The mutated viral RNA segments were then synthesized commercially (Beijing Tsingke Biotech Co., Beijing, China).

Construction of ZIKV infectious clones. The Asian-lineage strain SZ-WIV01 was obtained from the China Centre for General Virus Culture Collection (CCGVCC) (64). To generate the infectious cDNA of ZIKVwt, viral RNA was extracted from the parental virus by using TRIzol reagent (TaKaRa, Dalian, China) and reverse transcribed by using a PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the respective manufacturers' instructions. Five PCR fragments covering the complete viral genome of ZIKV were amplified from the reverse-transcribed cDNA. PCR fragment 1 containing nt 1 to 1590 of the genome was fused with a cytomegalovirus (CMV) promoter and cloned into the low-copy-number plasmid pACYC177 at the KpnI and XhoI sites, yielding subclone A. PCR fragment 2 containing nt 1532 to 3129, the beta-globin intron (nt 857 to 989 in HaloTag CMV-neo vector pHTN [GenBank accession no. JF920304]), and PCR fragment 3 containing nt 3130 to 5309 were overlapped and cloned into pACYC177 at the AvrII and XhoI sites, yielding subclone B. A previous study (65) provided some clues for the insertion site of the intron. PCR fragment 4 containing nt 5291 to 8588 was cloned into pACYC177 at the ClaI and XhoI sites, yielding subclone C. PCR fragment 5 containing nt 8545 to 10942, the hepatitis D virus ribozyme (HDVr) sequence, and simian virus 40 (SV40) poly(A) were overlapped and cloned into pACYC177 at the SfiI and XhoI sites, yielding subclone D. The four subclones were assembled step-by-step into a full-length infectious cDNA clone of ZIKVwt-FL, as shown in Fig. 1.

To generate the infectious clones (ICs) of codon pair-deoptimized ZIKVs, the codon pair-deoptimized cassette (see above) was synthesized *de novo*, overlapped with flanking regions at either end, and cloned into ZIKVwt-FL at the KpnI and ClaI sites, yielding ZIKV Min E-FL, ZIKV Min NS1-FL, and ZIKV Min E+NS1-FL, respectively (the designation "Min" signifies that genes were designed with a minimized

human codon pair score in this article). Before their transfection, all the ICs were verified using a restriction map and complete sequencing.

Rescue of infectious viruses and stock production. ICs were transfected into a 35-mm culture dish containing 80 to 90% confluent monolayers of Vero cells by using Lipofectamine 3000 (Life Technologies) in Opti-MEM (Life Technologies). The supernatant was harvested at 7 days posttransfection (dpt) (4 dpt for ZIKVwt), clarified by centrifugation, and stored at -80°C .

Each virus was amplified in C6/36 cells at a multiplicity of infection (MOI) of 1 in a 100-mm culture dish. Viral supernatants were harvested at 7 days postinfection (dpi), clarified by centrifugation, aliquoted, and stored at -80°C . The nucleotide identities were confirmed by sequencing.

Virus growth kinetics. Subconfluent (80%) cells in 100-mm culture dishes were infected at an MOI of 0.01 in a volume of 2 ml. After 1.5 h of incubation at 37°C , cells were washed twice with 4 ml of phosphate-buffered saline (PBS), and 8 ml DMEM with 2% FBS (for Vero cells) or RPMI 1640 with 2% FBS (for C6/36 cells) was added. A total of 800 μl of cell supernatants was sampled at different time points postinfection, clarified by centrifugation, aliquoted, and stored until use. The numbers of virus particles were determined by quantitative real-time PCR (qRT-PCR), and infectious titers of the viruses were quantitatively analyzed using an immunostaining focus assay.

Multipassage analysis. Each virus was passaged on Vero cells for five rounds. The virus derived from ZIKV IC-transfected Vero cells was defined as the parental P0 virus and used for passaging. At each passage, a calculated MOI of 0.01 was used to infect 35-mm culture dishes of subconfluent (80%) cells. After 1.5 h of incubation at 37°C , cells were washed three times with PBS, and 2 ml DMEM with 2% FBS was added. At 4 dpi, viral supernatants were harvested, clarified, and aliquoted; qRT-PCR and an immunostaining focus assay were performed; and supernatants were transferred to new 35-mm culture dishes containing naive Vero cells. It should be noted that 10- μl nondiluted stock solutions of Min E and Min E+NS1 were used for P2 to P5.

Plaque assay and immunostaining focus assay. Virus titrations of ZIKVwt were determined with plaque assays, and values were expressed as PFU (PFU per milliliter). Briefly, Vero cells at 80% confluence in 24-well plates were inoculated with 100 μl of 10-fold serial dilutions of viral samples in serum-free DMEM. After 1.5 h of incubation, 1 ml of 1.25% methylcellulose-containing 2% FBS was added to each well. After incubation for 4 days, cells were fixed with 4% buffered formalin and stained with 0.5% crystal violet. Plaque morphology and numbers were recorded after rinsing the plates with deionized water.

An immunostaining focus assay was carried out according to a previously described protocol (12), with modifications. In brief, Vero cells at 80% confluence in 24-well plates were inoculated with 100 μl of 10-fold serial dilutions of viral samples. After 1.5 h of incubation, 1 ml of 1.25% methylcellulose-containing 2% FBS was added to each well. Cells were incubated at 37°C for 7 days before being fixed in a methanol-acetone (1:1) fixation solution. After fixation, the cells were incubated with ZIKV-specific hyperimmune mouse serum, followed by incubation with goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) as a secondary antibody. Finally, viral foci were detected by the addition of the DAB (3,3-diaminobenzidine) HRP substrate, according to the manufacturer's instructions (Enhanced HRP-DAB kit; Tiangen, China).

Indirect immunofluorescence assays (IFAs). The cells infected with ZIKVs were washed once with PBS and fixed with cold (-20°C) 5% acetic acid in acetone for 15 min at room temperature (RT). The fixed cells were washed with PBS three times and incubated with a 4G2 mouse monoclonal antibody (MAb) that is cross-reactive with flavivirus E protein (ATCC) (diluted 1:200) for 1 h. After three rinses with PBS, the cells were incubated with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Proteintech, Wuhan, China) at a 1:200 dilution with PBS at RT for 1 h. After three rinses with PBS, cell nuclei were stained with Hoechst 33258. The fluorescent signal images were taken with a Nikon fluorescence microscope (Tokyo, Japan).

Animal immunization. AG6 mice deficient in type I and II interferon ($\text{IFN-}\alpha/\beta/\gamma$) receptors were gifts from Qibin Leng (Institute Pasteur of Shanghai, Chinese Academy of Sciences) and were bred under specific-pathogen-free conditions in the Animal Resource Center at the Wuhan Institute of Virology, Chinese Academy of Sciences. Four-week-old AG6 mice were infected with 10^4 , 10^3 , 10^2 , 10^1 , or 10^0 PFU WT or mutant viruses by intraperitoneal (i.p.) injection. PBS was injected into the mock-infected mice by the same route. The clinical course of viral infection was monitored by survival, weight loss, and disease symptoms. The 50% lethal dose (LD_{50}) for each ZIKV was determined by using the method of Reed and Muench (66). At 3 and 6 dpi, to measure viremia, serum samples were collected from anesthetized mice and clarified by centrifugation for 5 min at $3,000 \times g$. At 3, 7, 14, and 28 dpi, heart, liver, spleen, lung, kidney, brain, testes, eye, ovary, uterus, intestine, and muscle of the immunized mice were removed, weighed, and homogenized with zirconia beads in 1 ml of TRIzol reagent. Next, quantification of viral loads in samples was performed using qRT-PCR. At 28 dpi, the immunized mice were challenged by the i.p. route with 10^4 IFU of ZIKVwt, and viremia was measured on day 2 after challenge. On day 14 after challenge, all the mice were anesthetized, bled for the titration of neutralizing antibody, and sacrificed.

Quantitative real-time PCR assays. Total RNA was extracted from cell supernatants, sera, or organs by using TRIzol reagent and reverse transcribed by using the PrimeScript RT reagent kit. A universal pair of primers (67) (forward primer AARTACACATACCARAACAAAGTG and reverse primer TCCRTCCCYCTY TGGTCTTG) was used to amplify the region spanning positions 9378 to 9479 in the NS5 gene, which is preserved in all virus strains. All qRT-PCR assays were performed with SYBR green master mix (Bio-Rad) on the CFX96 touch real-time PCR detection system (Bio-Rad). Cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 65°C for 45 s. The ZIKVwt NS5 gene was utilized as a standard and was cloned into pGEM-T (Promega, WI, USA). Log dilutions of the DNA

standard were included with each RT-PCR assay. The virus concentration was determined by interpolation onto the curve made up of 10-fold serial dilutions of the standards.

Immunohistochemistry. Tissues were fixed in 4% formaldehyde at 4°C for 24 h and embedded in paraffin. For IHC, the paraffin-embedded tissues were sectioned at a thickness of 5 μ m and mounted onto slides. After being heated at 60°C for 1 h, the slides were deparaffinized with xylene and then cleared with alcohol. After antigen retrieval, the sections were incubated with an anti-ZIKV envelope (E) protein MAbs (1:100 dilution; BioFront Technologies, FL, USA) overnight at 4°C. Following incubation with the antibodies overnight, goat anti-mouse IgG conjugated with HRP was applied to each slide. Visualization was performed with the DAB reagent (Envision system kit; Dako). The sections were also stained with hematoxylin and eosin. Images were acquired with the whole-slide digital Panoramic scanner (3D-Histech, Budapest, Hungary).

PRNT₅₀. A 50% plaque reduction neutralization test (PRNT₅₀) was developed for measuring ZIKV-specific neutralizing antibodies according to a previously described protocol (14), with modifications. Briefly, heat-inactivated serum samples were 2-fold serially diluted and incubated with 100 PFU ZIKVwt at 37°C for 1.5 h. Next, the virus-serum mixture (200 μ l) was added to Vero cells at 80% confluence in 12-well plates. After incubation at 37°C for 1.5 h, a 1.25% methylcellulose overlay was added, and plates were incubated for 4 days at 37°C in 5% CO₂. The cells were then fixed with 4% formalin and stained with 0.5% crystal violet. Plaque morphology and numbers were recorded after rinsing the plates with deionized water. The PRNT₅₀ titer was expressed as the reciprocal of the highest dilution of each serum sample that caused a 50% reduction in the plaque number relative to the control samples. Samples with titers of ≥ 10 were considered seropositive.

ELISPOT assay. Splenocytes were isolated by using mouse lymphocyte separation medium (Dakewei, Beijing, China) at 28 dpi and adjusted to a concentration of 5×10^6 cells/ml in complete RPMI 1640 medium. The level of production of IFN- γ was measured using an ELISPOT assay according to the manufacturer's instructions (Dakewei). Briefly, 96-well polyvinylidene difluoride (PVDF) plates (Millipore, Bedford, MA) were precoated with anti-mouse IFN- γ . Next, 100 μ l of lymphocytes was added to the wells in triplicate and stimulated with heat-inactivated ZIKV (10⁶ IFU/well), along with RPMI 1640 medium alone (as a negative control) or concanavalin A (ConA) (5 μ g/ml; Sigma) (positive control). Following 20 h of incubation at 37°C, the lymphocytes were removed, 100 μ l of biotinylated anti-mouse IFN- γ was added, and the plate was incubated at 37°C for 1 h. Following washing, the plate was incubated with a properly diluted streptavidin-HRP conjugate solution at 37°C for 1 h. Finally, 100 μ l of aminoethylcarbazole substrate solution was added, and the mixture was incubated at RT for 25 min in the dark. The reaction was stopped by washing with demineralized water, air dried, and read using an ELISPOT reader (Bioreader 4000; Bio-sys, Germany). The numbers of spot-forming cells (SFC) per 5×10^5 cells were calculated. Medium backgrounds consistently contained <10 SFC per 5×10^5 cells.

Statistical analysis. The Student *t* test and analysis of variance (ANOVA) were used to analyze all the virologic and immunologic data if there were significant differences ($P < 0.05$). Statistical analyses were performed with IBM SPSS Statistics v18.0 (IBM, Chicago, IL, USA).

Accession number(s). The codon pair-deoptimized sequences presented in this paper have been submitted to GenBank under accession no. [MH055376](#) for the WT, [MH055377](#) for Min E, [MH055378](#) for Min NS1, and [MH055379](#) for Min E+NS1.

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