The current leading Zika vaccine candidates in clinical testing are based on live or killed virus platforms, which have safety issues, especially in pregnant women. Zika subunit vaccines, however, have shown poor performance in preclinical studies, most likely because the antigens tested do not display critical quaternary structure epitopes present on Zika E protein homodimers that cover the surface of the virus. Here, we produce stable recombinant E protein homodimers that are recognized by strongly neutralizing Zika specific monoclonal antibodies. In mice, the dimeric antigen stimulate strongly neutralizing antibodies that target epitopes that are similar to epitopes recognized by human antibodies following natural Zika virus infection. The monomer antigen stimulates low levels of E-domain III targeting neutralizing antibodies. In a Zika challenge model, only E dimer antigen stimulates protective antibodies, not the monomer. These results highlight the importance of mimicking the highly structured flavivirus surface when designing subunit vaccines.
Zika virus (ZIKV) is an enveloped single stranded RNA virus that is transmitted by mosquito vectors. As a member of the flavivirus genus, ZIKV shares many structural features with other medically important flaviviruses such as West Nile virus, yellow fever virus, and dengue virus (DENV). The flavivirus envelope (E) protein is a major target of neutralizing and protective human antibodies. E oligomers form complex quaternary structure epitopes on the viral surface which are of particular importance as protective human antibodies bind to these higher order protein structures1.

ZIKV infection during pregnancy can result in neurodevelopmental defects (congenital Zika syndrome). This has stimulated work on subunit vaccines, as live attenuated and other replicating virus vaccines are contraindicated during pregnancy. However, for flaviviruses subunit vaccines based on recombinant E proteins (rE) have performed poorly in preclinical studies compared with virus or virus-like particle (VLP) vaccines2–6. In aqueous solution, flavivirus rE proteins are in a dynamic equilibrium that favors the monomer over the dimer at physiological temperature, which likely explains poor binding by potently neutralizing human antibodies targeting quaternary epitopes and the overall poor immunogenicity of these antigens in preclinical studies2,7,8.

Here, we investigate if ZIKV rE dimers are better subunit vaccine antigens than monomers. Unlike the E monomer, the dimer is recognized by strongly neutralizing mAbs isolated from ZIKV patients and the dimer, not the monomer, stimulates strongly neutralizing and protective antibodies that targeted epitopes that were similar to epitopes recognized by human antibodies following natural Zika virus infection. These results highlight the importance of mimicking the highly structured flavivirus surface when designing subunit vaccines. The flavivirus field has a long history of using E monomers as vaccine antigens with limited success. These results highlight the importance of mimicking the highly structured flavivirus surface when designing subunit vaccines. The flavivirus field has a long history of using E monomers as vaccine antigens with limited success. These results highlight the importance of mimicking the highly structured flavivirus surface when designing subunit vaccines, and are applicable to developing second generation subunit vaccines against Zika as well as other medically important flaviviruses like dengue and yellow fever.

**Results**

**Expression of ZIKV rE monomers and homodimers.** Here, to investigate if ZIKV rE dimers are better subunit vaccine antigens than monomers, we expressed ZIKV rE variants (aa1–402) that are monomers (rEM) or stable dimers (rED) under physiological conditions. We generated stable ZIKV rED proteins as previously described for DENV E protein by introducing a disulfide bridge (A264C) in the E-domain II interactive region of the homodimer8,10 (Fig. 1a). The oligomeric state of the purified rEM and rED proteins were confirmed by protein gel electrophoresis (Fig. 1b). The stable rED, but not the rEM, was efficiently recognized by neutralizing mAbs that have dimer-dependent quaternary footprints, such as ZIKV-specific mAbs A9E, G9E, and ZKA-230 and flavivirus cross-reactive mAbs C8 and C10 (Fig. 1c).

**ZIKV rED, but not rEM, induces neutralizing antibodies.** After confirming that rED was a dimer displaying native quaternary epitopes, we immunized C57BL/6 mice with 5 µg rEM, 5 µg rED, 5 µg rEM+alum, or 5 µg rED+alum and analyzed ZIKV-specific IgG and neutralizing antibody titers over time (Fig. 2a). In the absence of alum, rEM and rED induced minimal IgG titers after the initial immunization, but this response increased upon boosting (Fig. 2b, c). In the alum adjuvanted groups, high IgG titers were observed even after the prime alone, with minimal change upon boosting (Fig. 2b). Although rEM elicited ZIKV-binding antibodies in the absence of alum, these antibodies failed to neutralize ZIKV (Fig. 2d). In contrast, non-adjuvanted rED elicited ZIKV-neutralizing antibodies (Fig. 2d). The addition of alum adjuvant elevated neutralizing antibody levels in both groups, but neutralizing titers were significantly higher after rED immunization compared with rEM. Our results demonstrate that, while both antigens stimulated ZIKV-specific IgG, the rED stimulated antibodies that are more potently neutralizing compared with the rEM stimulated antibodies.

**ZIKV rED stimulates antibodies that target complex epitopes.** We next performed experiments to determine if antibodies stimulated by rEM and rED antigens recognized different epitopes on the ZIKV E protein. Many flavivirus type-specific antibodies recognize relatively simple epitopes on domain III of the E protein (EDIII), whereas other type-specific antibodies recognize more complex quaternary structure epitopes displayed on E protein homodimers or higher order structures that cover the viral surface. Most EDIII-specific neutralizing antibodies have been isolated from mice immunized with inactivated flavivirus antigens11–13. In contrast, most quaternary epitope directed neutralizing antibodies have been isolated from people infected with wild-type flaviviruses2,4,14–17. In fact, recent studies demonstrate that neutralizing antibodies in
people exposed to flavivirus infections or effective live attenuated vaccines predominantly recognize quaternary epitopes, with simple EDIII epitopes accounting for only a minor fraction of neutralizing antibodies. To compare levels of EDIII-directed antibodies in animals immunized with rEM or rED, we used recombinant ZIKV-EDIII conjugated to magnetic beads to deplete mouse immune sera of EDIII-binding antibodies (Fig. 3a). We tested control-depleted and EDIII antibody-depleted immune sera to estimate the levels of binding (Fig. 3b) and neutralizing antibodies (Fig. 3c) in each sample targeting epitopes on EDIII. In mice immunized with rEM, ~70% (rEM group) and ~60% (rEM + alum group) of total ZIKV-specific IgG recognized epitopes on EDIII (Fig. 3b). On the other hand, significantly less EDIII-specific antibodies were induced after immunization with rED (~30% for rED and ~20% for rED + alum). The antibody binding patterns were also reflected in the ZIKV neutralization assays. The majority of neutralizing antibodies in the rEM + alum group were lost following depletion with EDIII antigen (Fig. 3c). In contrast, no loss of neutralization was observed in the animals immunized with rED or rED + alum after the removal of EDIII-binding antibodies (Fig. 3c). We conclude that the rEM antigen induces an EDIII focused antibody response that is poorly neutralizing, whereas rED antigens stimulate robust neutralizing antibody responses that target epitopes distinct from simple EDIII epitopes.

We recently isolated two ZIKV-specific strongly neutralizing human mAbs (A9E and G9E) from a person who was infected with ZIKV in 2016. These antibodies map to complex epitopes on domain I (A9E) and domain II (G9E) of ZIKV E protein. Moreover, antibody blockade assays using these mAbs and human immune sera indicated that the epitopes targeted by these mAbs also are targeted by antibodies in immune serum from Zika patients but not dengue patients. We used a blockade of binding (BOB) assay to test if mice immunized with rEM or rED developed antibodies that recognize A9E or G9E epitopes on ZIKV. We also tested if rEM or rED antigens stimulated antibodies that targeted the epitope of human mAb EDE C10, which binds to a E dimer-dependent epitope that is conserved between DENV and ZIKV. Sera from mice immunized with rEM did not block binding of A9E, G9E, and C10 mAbs to ZIKV (Fig. 3d). In contrast, sera from mice immunized with rED antigen efficiently blocked binding of A9E and G9E (~80%) and partially blocked binding of EDE C10 (~35%) to ZIKV. These results demonstrate that the rED antigen and natural ZIKV infections stimulate antibodies that target similar complex epitopes on the virion. The rEM fails to induce similar antibodies and directs the antibody response to simple epitopes on ZIKV EDIII.

ZIKV rED induces protective antibodies in mice. Next, we investigated if the immune sera of mice vaccinated with rEM + alum and rED + alum could protect mice from lethal ZIKV challenge. We immunized wild-type mice as previously described (Fig. 2), and transfected heat-inactivated immune sera to Ifnar1−/− mice which are highly susceptible to ZIKV. Ifnar1−/− mice were challenged with 1000 FFU of ZIKV H/PF/2013, 1 day post serum transfer. Mice were monitored for weight loss and ZIKV-induced disease, and mice were bled at days 0 (before virus challenge), 4, and 14 (Fig. 4a). The potently neutralizing mAb G9E was used as a positive control for protection against ZIKV infection. Mice that received G9E or serum from rED + alum immunization were completely protected against weight loss and disease signs. In contrast, mice receiving rEM + alum serum exhibited significant weight loss and hindlimb paralysis (Fig. 4b). At 4 days post challenge, viremia levels in the rED + alum group were significantly reduced compared with the rEM + alum and vehicle groups (Fig. 4c), and all groups cleared viremia at day 14. We also measured ZIKV-specific IgG titers (Fig. 4d) and neutralizing antibody titers (Fig. 4e) at days 0 and 14. Mice receiving rEM + alum and rED + alum immune sera showed similar ZIKV-specific IgG titers at day 0, which were
marginally higher for both groups at day 14. On the other hand, neutralizing antibody titers were higher at day 14 in the rEM + alum group, likely caused by an endogenous immune response elicited against replicating virus in these animals (Fig. 4c).

Discussion

The magnitude, sudden onset and severe clinical manifestations of ZIKV epidemics highlight the need for safe and effective vaccines for key patient populations including pregnant women. While effective live attenuated vaccines have been developed for several flaviviruses, they are contraindicated in pregnant women. Live vaccines typically are ineffective during the first year of life because maternal antibodies interfere with vaccine replication. It has been difficult to formulate tetravalent live attenuated DENV vaccines that stimulate balanced and effective responses to each serotype because of differential replication of vaccine virus components. Subunit vaccines may address some of these challenges, but progress has been slow and subunit vaccines have not performed well in non-human primate models24. Our studies offer a potential explanation for the poor performance of subunit vaccines and a strategy for developing more effective flavivirus vaccines. Our results demonstrate that a critical aspect of rE antigen design is to develop E proteins that display complex quaternary epitopes that recapitulate antigen presentation on the surface of infectious virus particles. Here we demonstrate that ZIKV rE-M and rE-D subunit antigens induce antibody responses that differ in functionality and domain focus. ZIKV rE monomers stimulated an EDIII biased, poorly neutralizing IgG response. In contrast, ZIKV rE dimers induced strongly neutralizing and protective antibodies that also blocked the binding of strongly neutralizing human mAbs to quaternary epitopes on ZIKV. While finer mapping studies are required to comprehensively map the specificity of rE-D stimulated antibodies, our studies suggest that by designing the E protein to be a stable homodimer, it is possible to induce strongly neutralizing and protective antibodies that mimic properties of protective antibodies induced by natural infection. It is possible that inactivated whole virus or virus-like particle (VLP) vaccines will also stimulate antibodies to quaternary structure epitopes. An advantage to using rE-D-protein subunits over inactivated virion or VLP vaccines is the absence or prM protein in subunit vaccine but not virus or VLP vaccines. PrM antibodies are non-neutralizing and some studies suggest that enhance the replication of flaviviruses25. Additional studies are needed to compare the immunogenicity, efficacy and safety of ZIKV rE-dimer, inactivated virus and VLP vaccines. The strategy we describe here for ZIKV E protein may also be applicable for developing subunit vaccines against other medically significant flaviviruses.

Methods

Cells and viruses. Vero-81 cells (ATCC CCL-81) were maintained in DMEM medium (Gibco) + 1% non-essential amino acids, 5% fetal bovine serum, streptomycin (100 µg per ml), and penicillin (100 U per ml) at 37 °C with 5% CO₂.
**Fig. 4** ZIKV rE\(^\text{D}\) induce protective antibodies in mice. **a** Wild-type mice were immunized with rE\(^\text{M}\) + alum or rE\(^\text{D}\) + alum. Five hundred microliters of heat-inactivated immune sera (16 weeks post immunization) (or 200 µg of neutralizing mAb G9E) was transferred by intraperitoneal injection to Ifnar\(^{-/-}\) mice 24 h prior to challenge with 1000 FFU of ZIKV by subcutaneous footpad inoculation \((n = 5\) for rE\(^\text{M}\) and rE\(^\text{D}\) and \(n = 3\) for controls). **b** Challenged mice were weighed daily for 14 days; weights are shown percent of starting weight. Data represent the mean +/- SEM of 3-5 mice per group and were censored once one mouse in a group died. **c** Viremia was measured at 4 days post challenge by qRT-PCR. **d**-e ZIKV-specific IgG and neutralizing antibody titers were determined for challenged mice prior to challenge (day 0) and at the end of the experiment (day 14). Statistical differences were determined by one-way ANOVA followed by a Tukey’s test \((^{*}p<0.05)\). Source data are provided as a Source Data file.

Mouse immunizations and challenge. Female C57BL/6 mice were purchased from Jackson Laboratory and used for immunizations at 6–12 weeks of age. Female A129 \([\text{B6.129S2-Ifnar1tm1Agt/Mmjax}}]\) mice were purchased from MMRRRC (Mutant Mouse Resource & Research centers supported by NIH) and used in challenge experiments at 7 weeks of age. All mouse experiments were performed under protocols approved by the University of North Carolina Institutional Animal Care and Use Committee, in compliance with ethical and federal regulations (the Public Health Service Policy on Humane Care and Use of Laboratory Animals, Animal Welfare Act, and the Guide for the Care and Use of Laboratory Animals).

For evaluation of ZIKV rE immunogenicity, mice were immunized subcutaneously in the flank with 5 µg soluble rE\(^\text{M}\) \((\text{Alhydrogel, Invivogen})\) \((n = 5)\), 5 µg rE\(^\text{M}\) + 500 µg alum (Alhydrogel, Invivogen) \((n = 5)\), 5 µg rE\(^\text{D}\) \((n = 3)\), 5 µg rE\(^\text{D}\) + 500 µg alum \((n = 5)\), or PBS \((n = 3)\). All groups received three immunizations (day 0, 21, and 63), and serum samples were collected on day 21, 28, 70, 77, 84, 91, 98, 105, and 112 by submandibular bleed.

To evaluate the protective activity of rE-induced antibodies, heat-inactivated immune sera were passively transferred to ZIKV susceptible A129 mice. Serum samples (days 70 through 112) of mice immunized with rE\(^\text{M}\) + alum and rE\(^\text{D}\) + alum \((days 70–112)\) were pooled and 500 µl of immune sera (per mouse, \(n = 5)\), or vehicle control serum \((n = 3)\) was injected into A129 mice, via intraperitoneal route 1 day prior to ZIKV challenge. The ZIKV-neutralizing mAb G9E\(^{23}\) was used as a positive control \((200 µg/mouse, n = 3)\). Mice were challenged with 1000 FFU of ZIKV \((\text{H/PF/2013})\) by subcutaneous footpad inoculation. Mice were weighed daily for 14 days and monitored for clinical disease signs. Mice losing 30% of their starting weight were humanely euthanized.

ZIKV rE-induced antibody evaluation. ZIKV-specific IgG responses were measured by antigen-capture ELISA. Briefly, ZIKV was captured on IM7 (2 µg per µl) coated ELISA plates and incubated with 1:50 or serially diluted mouse sera 3, 4, 10, and 16 weeks post immunization. Next, plates were incubated with alkaline phosphatase conjugated anti-mouse IgG (1:2500; Sigma A9044) diluted anti-mouse IgG for 1 h at 37 °C. Wells were developed using alkaline phosphatase substrate and absorbance was measured at 405 nm. Endpoint dilution (EPD) titers where mice sera signals reached background (vehicle control) levels was determined using GraphPad Prism software.

Evaluation of neutralizing antibody responses. Neutralizing antibody titers were determined using a flow cytometry based assay. Vero-B1 cells were seeded \((25,000\) cells per well) and incubated at 37 °C overnight. Immunized mouse sera was serially

### Protein analysis

The oligomeric state of purified rE\(^\text{M}\) and rE\(^\text{D}\) proteins was characterized by ELISA and SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. For SDS-PAGE, 1 µg of rE\(^\text{M}\) and rE\(^\text{D}\) were incubated in denaturing gel loading buffer for 10 min at 95 °C. After centrifugation, proteins were separated by SDS-PAGE and gels (original gel in Source Data File) were incubated with CBB stain \((0.1%\) coomassie blue, 10% acetic acid, 50% methanol). Protein confirmations were analyzed by antigen-capture ELISA. ZIKV rE\(^\text{M}\) and rE\(^\text{D}\) were captured on Ni\(^{2+}\) -coated ELISA plates and subjected to binding of ZIKV-specific 2 ng per µl mAbs (A9E, G9E, and ZKA-230)\(^{22}\) and flavivirus cross-reactive mAbs (4G2 and 1M7)\(^{16,29}\)
µl of RNA was added to 18 µl of PCR mastermix (10 µl of iTaq universal probes). Midgley, C. M. et al. An in-depth analysis of original antigenic sin in dengue vaccine stimulates neutralizing and enhancing antibodies in mice. Vaccine 28, 8085–8094 (2010).

5. Williams, K. L., Wahala, W. M., Orozco, S., de Silva, A. M. & Harris, E. Antibodies targeting dengue virus envelope domain III are not required for type-specific protection or prevention of enhancement in vivo. Virology 429, 12–20 (2012).

6. Liu, Y., Liu, J. & Cheng, G. Vaccines and immunization strategies for dengue prevention. Emerg. Microbes Infect. 5, 77–83 (2016).

7. de Alvís, R. et al. Identification of human neutralizing antibodies that bind to multiple epitopes on dengue virions. Proc. Natl Acad. Sci. USA 109, 7439–7444 (2012).

8. Allison, S. L., Stadler, K., Mandl, C. W., Kunz, C. & Heinz, F. X. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J. Virol. 69, 5816–5820 (1995).

9. Slon Campos, J. L. et al. Temperature-dependent folding allows stable dimerization of secretory and virus-associated E proteins of Dengue and Zika viruses in mammalian cells. Sci. Rep. 7, 966 (2017).

10. Rouvinski, A. et al. Covalently linked dengue virus envelope glycoprotein dimers reduce exposure of the immunodominant fusion loop epitope. Nat. Commun. 5, 1841 (2014).

11. Wahala, W. M. et al. Natural strain variation and antibody neutralization of dengue serotype 3 viruses. PLoS Pathog. 6, e1000821 (2010).

12. Pitcher, T. J. et al. Functional analysis of dengue virus (DENV) type 2 envelope protein domain 3 type-specific and DENV complex-reactive critical epitope residues. J. Gen. Virol. 96, 288–293 (2015).

13. Zhou, Y. et al. The mechanism of differential neutralization of dengue virus type 3 strains by monoclonal antibody 8A1. Viruses 4, 57–64 (2013).

14. Fibriansah, G. et al. DENGUE VIRUS. Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. Science 349, 88–91 (2015).

15. Swansström, J. A. et al. Dengue virus envelope e epitope monoclonal antibodies isolated from dengue patients are protective against Zika virus. J. Virol. 88, 1123–1131 (2014).

16. Fibriansah, G. et al. A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. EMBO Mol. Med. 6, 358–371 (2014).

17. Rouvinski, A. et al. Recognition determinants of broadly neutralizing human antibodies against dengue viruses. Nature 520, 109–113 (2015).

18. Fahimi, H., Mohammadipour, M., Haddad Kashani, H., Parvin, F. & Sadjadzadeh, M. Dengue viruses and promising envelope protein domain III-based vaccines. Appl. Microbiol. Biot. 102, 2977–2996 (2018).

19. Guzman, M. G., Hermida, L., Bernardo, L., Ramirez, R. & Guillen, G. Domain III of the envelope protein as a dengue vaccine target. Expert Rev. Vaccines 9, 347–347 (2010).

20. Qu, P. et al. Insect cell-produced recombinant protein subunit vaccines protect against Zika virus infection. Antivir. Res. 154, 97–103 (2018).

21. Yang, M., Dent, M., Lai, H., Sun, H. & Chen, Q. Immunization of Zika virus envelope protein domain III induces specific and neutralizing immune responses against Zika virus. Vaccine 35, 4287–4294 (2017).

22. Elphick, J. & Smit, J. M. The complexity of a dengue virus: a review of the human antibody response. PLoS Negl. Trop. Dis. 9, 3749–3756 (2015).

23. Collins, M. H. et al. Human antibody response to Zika targets type-specific quaternary structure epitopes. JCI Insight 4 https://doi.org/10.1172/jci.insight.124588 (2019).

24. Martin, J. & Hermida, L. Dengue vaccine: an update on recombinant subunit strategies. Acta virológica 60, 3–14 (2016).

25. Luo, Y. Y. et al. Identification of a novel infection-enhancing epitope on dengue prM using a dengue cross-reacting monoclonal antibody. BMC Microbiol. 13, 194 (2013).

26. Baronti, C. et al. Complete coding sequence of zika virus from a French polynesia outbreak in 2013. Genome Announc. 2 https://doi.org/10.1128/genomeA.00500-14 (2014).

27. Brien, J. D., Lazeer, H. M. & Diamond, M. S. Propagation, quantification, detection, and storage of West Nile virus. Curr. Protoc. Microbiol. 31, 15D 13–15D 13 18 (2013).

28. Metz, S. W. et al. In vitro assembly and stabilization of dengue and Zika virus envelope protein homo-dimers. Sci. Rep. 7, 4524 (2017).

29. Henschel, A. E., McCown, J. M., Burke, D. S., Seguin, M. C. & Brandt, W. E. Epitopic analysis of antigenic determinants on the surface of dengue-2 viruses using monoclonal antibodies. Am. J. Trop. Med. Hyg. 34, 162–169 (1985).

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Author contributions
S.W.M. and A.T. performed the majority of experiments. A.B., J.F., and M.J.M. expressed and purified ZIKV monomer and dimer proteins. C.A.L., H.M.L., and S.T. performed the
animal experiments. S.W.M., S.T., and A.M.dS. designed the study. S.W.M. wrote the paper and all authors reviewed the paper.

Competing interests
The authors declare no competing interests.

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