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Leading Edge
Review

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Zika Virus: Immunity and Vaccine Development

Theodore C. Pierson[1,*] and Barney S. Graham[2]
[1]Viral Pathogenesis Section, Laboratory of Viral Diseases
[2]Viral Pathogenesis Laboratory, Vaccine Research Center
National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
*Correspondence: piersontc@mail.nih.gov
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The emergence of Zika virus in the Americas and Caribbean created an urgent need for vaccines to reduce transmission and prevent disease, particularly the devastating neurodevelopmental defects that occur in utero. Rapid advances in Zika immunity and the development of vaccine candidates provide cautious optimism that preventive measures are possible.

Zika virus (ZIKV) is a mosquito-transmitted flavivirus of African origin discovered in 1947 from a febrile Rhesus macaque caged in the Zika forest in Uganda (Dick et al., 1952). Human ZIKV infections were documented rarely, despite serological evidence suggesting widespread exposure (Wikan and Smith, 2016). The potential for significant ZIKV transmission and disease was first appreciated during a 2007 outbreak on Yap Island of the Federated States of Micronesia that affected roughly three-quarters of the population. ZIKV-associated illness was described as a self-limiting mild illness characterized by rash, fever, conjunctivitis, arthralgia, and arthritis (Duffy et al., 2009; Lanciotti et al., 2008). The next significant outbreak did not occur until 2013 in the islands of French Polynesia (Aubry et al., 2015; Cao-Lormeau et al., 2014), despite documented circulation of ZIKV in Southeast Asia during the intervening 6 years (Alera et al., 2015; Heang et al., 2012). This outbreak also had a high attack rate and revealed an association between ZIKV infection and the development of Guillain-Barré syndrome (Cao-Lormeau et al., 2016). ZIKV transmission continued thereafter on multiple islands of the Pacific throughout 2014, at which time it was introduced into South America (Campos et al., 2015; Lednicky et al., 2016; Zanluca et al., 2015). By the summer of 2016, more than 40 countries in the Western Hemisphere had reported locally acquired infections spread by the invasive mosquito species, *Aedes aegypti*, following a path through the Americas similar to that of the chikungunya virus only a few years earlier (Lessler et al., 2016). Unexpectedly, in addition to Guillain-Barré syndrome, the ZIKV epidemic in the Americas has been linked to devastating neurodevelopmental defects in infants of women infected while pregnant, including microcephaly (Johansson et al., 2016; Kleber de Oliveira et al., 2016). A causal link between ZIKV infection and microcephaly has been established by epidemiological evidence and the isolation of virus from the fetal brain (Rasmussen et al., 2016); some important features of this pathobiology have been recapitulated in murine models (Mysorekar and Diamond, 2016). Sexual transmission and an ability to replicate in immune-privileged sites, such as the eyes and testes, are also unique properties of ZIKV that complicate the control and treatment of infection (Brooks et al., 2016; D'Ortenzio et al., 2016; Jampol and Goldstein, 2016; Musso et al., 2015; Ventura et al., 2016). Locally acquired ZIKV infection has now been documented in the United States (http://www.cdc.gov/zika/geo/united-states.html).

Flaviviruses are spherical virions that package a positive-strand RNA genome within a host-derived lipid envelope (Heinz and Stiasny, 2012). ZIKV entry into cells is facilitated by a number of cellular factors shown previously to promote infection of other flaviviruses, including molecules of the TIM/TAM family and the C-type lectin DC-SIGN (Hamel et al., 2015); gene expression studies reveal that many of these molecules are expressed on relevant cell and tissue types in vivo (Nowakowski et al., 2016; Tabata et al., 2016). Flavivirus internalization is typically clathrin dependent and provides virions access to acidic compartments of the endosome, where low pH-dependent membrane fusion occurs. The viral genome is translated in the cytoplasm as a single open reading frame that is cleaved subsequently by viral and host proteases to yield three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven non-structural proteins. New virions are assembled on membranes derived from the endoplasmic reticulum as non-infectious immature virus particles on which prM and E associate as heterodimers organized as trimeric spikes with icosahedral symmetry. During transit through the Golgi, prM is cleaved by a host furin-like protease to become an infectious mature particle covered by E and membrane (M) proteins (Pierson and Diamond, 2012). The structure of mature ZIKV has been solved at high resolution, revealing 90 antiparallel E dimers arranged in a herringbone pattern (Kostyuchenko et al., 2016; Sirohi et al., 2016), similar to the structure of both West Nile (WNV) and dengue (DENV) viruses (Kuhn et al., 2002; Mukhopadhyay et al., 2003; Zhang et al., 2013). The E protein is a three-domain class II viral fusion protein that has critical roles in virus entry and assembly. E protein domain III (E-DIII) is an immunoglobulin-like fold hypothesized to contribute to viral attachment because it protrudes furthest from the surface of the virion, and some mutations in this domain result in increased binding to heparin sulfate on target cells (Chen et al., 1997; Rey et al., 1995). E-DII is an elongated oligomerization domain that contains a highly conserved fusion loop (DII-FL) at the distal end. Domain I E-DI is a β-barrel domain connected to both E-DII and E-DIII by flexible hinges. A single asparagine (N)-linked carbohydrate is attached to E-DI at residue 154; in this position, the N-linked carbohydrate may function to shield the DII-FL. The E protein is

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anchored into the viral membrane by a helical stem and two antiparallel transmembrane domains. The structure of ZIKV prM and its orientation on the immature virion are unknown but is likely to be relatively similar to other flaviviruses, such as DENV, for which structural information is available (Li et al., 2008).

The Innate Immune Response to ZIKV Infection

The innate immune response to flavivirus infection has a key role in orchestrating protection, as evidenced by the enhanced susceptibility of mice lacking innate immune sensors, signaling pathways, and effector molecules, as well as the numerous strategies flaviviruses use to circumvent this control (Lazear et al., 2016; Quicke and Suthar, 2013). ZIKV infection stimulates the production of type I (α, β), type II (γ), and type III (λ) interferon (IFN) and numerous IFN-stimulated genes (ISGs) that limit infection (Bayer et al., 2016; Hamel et al., 2015; Quicke et al., 2016). While the ISGs IFITM3 and, to a lesser degree, IFITM1 have been shown to inhibit ZIKV at an early stage of the replication cycle (Savidis et al., 2016), the activity and mechanism of the repertoire of ISGs stimulated by ZIKV have not yet been cataloged. The NS5 polymerase of ZIKV was shown to degrade STAT2 in a proteasome-dependent manner (Grant et al., 2016), and while the mechanisms differ in some respects, a similar process to limit IFN signaling has been described for DENV. The species-specific nature of this immune evasion mechanism likely contributes to the inability of ZIKV to replicate robustly and to cause disease in immunocompetent mice (Mysorekar and Diamond, 2016).

T Cell Responses to Flavivirus Infection

The cellular immune response to flavivirus infection may contribute to both protection and pathogenesis, as supported by the linkage of HLA alleles and susceptibility to disease in humans (Screaton et al., 2015; Weiskopf et al., 2013). Very little has been published about cellular immunity to ZIKV; the role of this arm of the immune response can only be inferred from studies of related flaviviruses. Studies in mouse models of several flaviviruses defined a protective role for $CD8^+$ T cells; CD8- or MHC-class-I-deficient animals have a reduced capacity for viral clearance, and adoptively transferred cells may be protective (Shrestha and Diamond, 2004). $CD8^+$ T cell responses in humans are readily detectable after flavivirus infection and target both virus type-specific (TS) and cross-reactive (CR) determinants (Bukowski et al., 1989; Mongkolsapaya et al., 2003; Screaton et al., 2015). Multiple lineages of $CD4^+$ T cells have been shown to contribute to protection via their capacity to produce pro-inflammatory cytokines and support the maturation of the antibody response. DENV-reactive cytotoxic $CD4^+$ T cells were recently demonstrated in humans, particularly those with a history of multiple infections (Weiskopf et al., 2015). That these cells were commonly restricted to a protective HLA allele suggests a role for effector $CD4^+$ T cells in protection that merits further study. A role for γδ T cells and NK cells in flavivirus immunity has also been proposed (Wang and Welte, 2013). A recent study of four ZIKV-immune subjects detected ZIKV NS1- and E-reactive memory $CD4^+$ T cells with little capacity to cross-react with DENV (Stettler et al., 2016), but the extent of cross-reactivity with other flavivirus T cell epitopes and their potential role in pathogenesis will require more extensive studies.

The Complex Serology of Flaviviruses

The antigenic structure of ZIKV is similar to other flaviviruses, as predicted by the considerable conservation of the E protein at the amino acid level and early serological studies (Fagbami et al., 1987). Studies of other flaviviruses demonstrate that the structural proteins of the virion (prM and E) and the secreted non-structural protein 1 (NS1) are targeted frequently by antibodies (Heinz and Stiasny, 2012; Muller and Young, 2013). Analysis of monoclonal antibodies (mAbs) from ZIKV-infected humans (Stettler et al., 2016) and mice (Zhao et al., 2016) indicates that this is also true for ZIKV, although additional mapping and structural studies are required. ZIKV-specific antibodies bind TS epitopes unique to ZIKV or to CR epitopes shared among flaviviruses and may contribute to protection via their capacity to directly neutralize infection or via effector functions mediated by the Fc portion of the heavy chain. While the functional properties of antibodies may vary considerably, both TS and CR antibodies may potently neutralize infection (Stettler et al., 2016). Epitope accessibility on the intact virion is a key determinant of neutralization potency (Dowd and Pierson, 2011). The neutralization potency of recently described E-DIII-reactive murine mAbs correlated well with the predicted exposure of their epitopes on the mature virion. As observed with WNV-specific mAbs (Nybakken et al., 2005), antibodies that recognize an accessible epitope on the lateral ridge of E-DIII potently neutralize infection in vitro at a post-attachment step and are protective in vivo (Zhao et al., 2016). In contrast, antibodies that bind the highly conserved E-DII fusion loop have incomplete neutralizing activity consistent with the limited accessibility of this structure on the mature virion (Barba-Spaeth et al., 2016; Dai et al., 2016; Swanstrom et al., 2016; Zhao et al., 2016).

Flavivirus infection also elicits antibodies that bind complex epitopes, which require interactions with more than a single E protein; these antibodies bind to the E proteins arrayed on virions, but not to recombinant monomeric E proteins (Fibriansah et al., 2015; Kaufmann et al., 2010). The recognition of a quaternary epitope on ZIKV was suggested by experiments with neutralizing mAbs shown previously to bind highly conserved E protein dimer-dependent epitope (EDE) on DENV (Dejnirattisai et al., 2015, 2016; Rouvinski et al., 2015). The footprint of EDE antibodies has been defined and includes surfaces on both sides of the dimer interface, including the DII-FL. DENV EDE mAbs were shown to be capable of binding and neutralizing ZIKV to varying degrees depending in part on their reliance on interactions with the E protein loop containing the N-linked carbohydrate; thus, the neutralization potency of CR EDE antibodies against ZIKV is expected to vary (Barba-Spaeth et al., 2016; Dejnirattisai et al., 2016). The EDE antibody C10 was shown to protect against lethal ZIKV infection in the AG129 mouse model (Swanstrom et al., 2016). The structural basis for cross-reactive EDE recognition of ZIKV has been defined and provides a rationale for the development of epitope-based vaccine candidates that may protect against multiple flaviviruses (Barba-Spaeth et al., 2016). The frequency and functional significance of antibodies that bind quaternary epitopes like EDE have not yet been established for ZIKV. Insight into the epitopes that are most frequently targeted by neutralizing antibodies, and how

this correlates with antibody potency, will inform vaccine development efforts.

Binding and neutralization studies of flavivirus-immune sera and mAbs reveal that CR patterns of recognition are common (Crill and Chang, 2004; Lai et al., 2013). However, the relative frequency of TS and CR antibodies may vary as a function of infection history and timing. Analysis of sera from individuals with acute DENV infection revealed a considerable capacity to bind and neutralize ZIKV (Priyamvada et al., 2016), consistent with studies identifying a transient capacity to protect against heterologous infection immediately after flavivirus infection (Sabin, 1952). Similar studies of convalescent sera from subjects recovered from primary DENV infection revealed lower levels of cross-reactive ZIKV neutralizing activity. Convalescent sera or plasma from individuals that experienced sequential DENV infections also had a limited capacity to neutralize ZIKV (Dejnirattisai et al., 2016; Swanstrom et al., 2016).

Antibody-Dependent Enhancement of Infection

Flavivirus-immune sera and mAbs have been shown to be capable of markedly increasing the efficiency of infection of cells expressing Fcγ-receptors (FcR). This process, called antibody-dependent enhancement (ADE) of infection, has been hypothesized to contribute mechanistically to severe clinical manifestations associated with a subset of secondary DENV infections (Halstead, 2007). ADE occurs when virions are bound by antibody molecules with a stoichiometry insufficient to neutralize virus infectivity, promoting more efficient attachment of the viral-immune complex to cells via antibody-FcR interactions (Dowd and Pierson, 2011). Beyond increasing viral burden, viral entry via ADE may modulate the innate response to viral infection and trigger the release of pro-inflammatory cytokines (Halstead et al., 2010). ADE has been demonstrated in vitro with both neutralizing and weakly or non-neutralizing antibodies using many flaviviruses, even those for which ADE has no established role in disease (e.g., WNV) (Pierson et al., 2007). Almost all antibodies capable of binding the virion support ADE. Not unexpectedly, both CR- and ZIKV TS-specific antibodies have recently been shown capable of enhancing ZIKV infection in vitro (Dejnirattisai et al., 2016; Fagbami et al., 1987; Stettler et al., 2016). CR mAbs elicited by ZIKV-infection also have been shown to enhance infection of DENV in the AG129 model of infection, as shown previously with CR mAbs elicited by WNV (Stettler et al., 2016). The observed association of microcephaly with ZIKV following the introduction of ZIKV into the DENV-experienced population of South America led to the hypothesis that CR antibodies might contribute to pathogenesis following ZIKV infection of DENV-immune individuals. While insight into a role for ADE of ZIKV infection may be obtained in vitro and from studies with animal models, a definitive evaluation of this hypothesis awaits the results of ongoing epidemiological studies designed to identify virological, immunological, and genetic factors contributing to neurodevelopmental disease in infants.

ZIKV Vaccine Development

Vaccines are used to protect humans from flaviviruses, including yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV), and DENV (Beck and Barrett, 2015; Guy and Jackson, 2016; Halstead and Thomas, 2011; Jarmer et al., 2014). These precedents suggest that the development of a safe and protective ZIKV vaccine is feasible. While the goal of ZIKV vaccine development is to prevent disease in all recipients, the unique pathogenesis and tropism of ZIKV highlights a distinctive requirement to elicit an immune response capable of protecting both mother and fetus (Rasmussen et al., 2016; Tabata et al., 2016). Whether sterilizing protection will be required to achieve this requirement is unknown and merits study in recently developed animal models. The elicitation of neutralizing antibodies correlates with protection by vaccination for most existing flavivirus vaccines (Pierson and Diamond, 2014). For DENV, this relationship has not been established clearly and may be complicated by the existence of multiple viral serotypes and the potential for ADE. That ZIKV exists as a single serotype simplifies both strain selection for inclusion in vaccine candidates and methods to evaluate vaccine immunogenicity (Dowd et al., 2016). ZIKV vaccine development is being accelerated using multiple antigen-delivery approaches, including nucleic acid vaccines, inactivated virions, live-attenuated ZIKV, and other viral vectors for ZIKV antigen expression (Figure 1). The primary immunological goal of these efforts has been to elicit neutralizing antibodies against the E protein present on the surface of the virion. The rapid progress to date has been remarkable.

The co-expression of flavivirus prM and E proteins in mammalian cells results in the release of subviral particles (SVP) that share structural, antigenic, and functional characteristics with infectious virions (Allison et al., 1995). DNA vaccine candidates expressing prM-E have been developed and studied in humans for multiple flaviviruses, including WNV and DENV (Beckett et al., 2011; Martin et al., 2007). Because DNA constructs have an excellent safety profile and can be manufactured rapidly, this vaccine platform was among the first to be employed by research groups in the public and private sectors. Two phase I studies of prM-E vaccine candidates are underway with plasmids that encode the prM-E genes required for the production and release of ZIKV SVPs. Recent preclinical studies of a ZIKV DNA candidate (originally identified as prM-E in the manuscripts) that expresses ZIKV M and E proteins (without "pr") highlight the possibilities of other approaches. Immunization with a single dose of a codon-optimized DNA expression construct encoding the M and E proteins protected wild-type mice from viremia (Larocca et al., 2016). Passive transfer of antibody, combined with $CD4^+$ and $CD8^+$ T cell depletion studies, indicates that the antibody was sufficient for protection. These single-dose vaccination studies suggest that protection is possible with very low titers of neutralizing antibody (a 1/20 serum dilution) in this semi-permissive model. Intradermal immunization with two doses of M-E completely protected Rhesus macaques from ZIKV challenge (Abbink et al., 2016). In this study, it appeared that a reciprocal EC_{50} titer in a microneutralization (MN) assay of $\sim$100 was near the threshold for passive protection from viremia in nonhuman primates (NHPs). A curious feature of this experimental vaccine construct is the absence of the "pr" portion of prM in the vaccine construct (the plasmid encodes a protein that begins just after the furin cleavage site in prM). The function of prM is to facilitate folding of E and to prevent

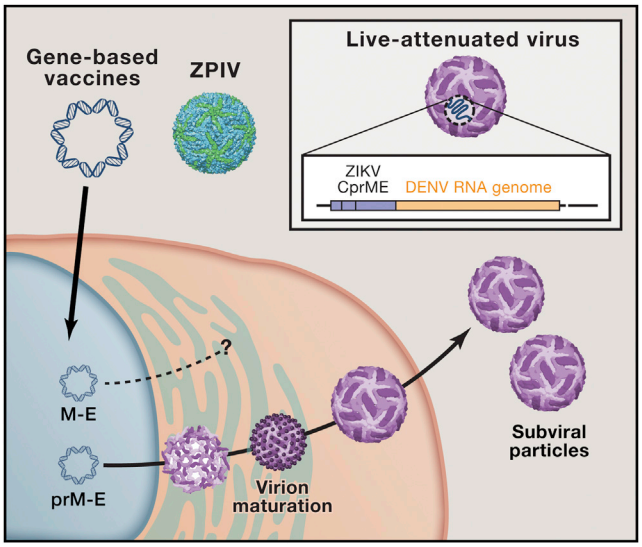

Figure 1. Multiple Platforms for a Protective ZIKV Vaccine

The emergence and rapid spread of ZIKV has created an urgent need for a safe and effective ZIKV vaccine. Multiple vaccine platforms, guided by considerable experience with other flaviviruses, are being developed and tested in clinical studies. Gene-based vaccine approaches, including nucleic acid (DNA or mRNA) or other viral vector expression platforms, provide a rapid means to introduce viral antigens of any sequence into the candidate vaccine. ZIKV vaccines encoding the structural proteins M-E and prM-E have been developed. Expression of prM-E in vitro results in the production of subviral particles that traffic through the secretory pathway and undergo virion maturation in an analogous process to infectious virions, resulting in a particulate antigen. The manner in which E is presented following transfection of M-E, and whether this is secreted from cells, remains unknown. ZIKV purified inactivated virions (ZPIV) have been developed and show excellent immunogenicity in preclinical studies. Finally, live-attenuated virus approaches developed for DENV vaccines can be adapted for ZIKV, including chimeric viruses encoding ZIKV structural proteins in a DENV or YFV backbone or ZIKV attenuated by genetic modifications, are also being developed. This cost-effective approach, if successful, has the potential to be combined with other live-attenuated flavivirus vaccines.

adventitious fusion as newly synthesized virions traffic through the secretory pathway (Lorenz et al., 2002). Whether E expressed in this context exists as a dimer and is incorporated into virus-like particles awaits further biochemical study and may present an opportunity to study relationships between the structure of distinct E protein immunogens and the resulting antibody repertoires elicited by vaccination (Figure 1).

Purified inactivated virus (PIV) vaccines are licensed for vaccination against JEV and TBEV (Halstead and Thomas, 2011; Jarmer et al., 2014). A similar inactivated vaccine (ZPIV) is being developed by the Walter Reed Army Institute of Research (WRAIR) using a Puerto Rican isolate (strain: PRVABC59) propagated in mammalian cells, purified, and inactivated by formalin (Larocca et al., 2016). The administration of one dose of ZPIV and adjuvant was sufficient for preventing viremia in semi-permissive BALB/c mice after ZIKV challenge when delivered by an intramuscular, but not subcutaneous, route. Challenge studies performed using Rhesus macaques revealed uniform protection from infection when animals were challenged 4 weeks after receiving two subcutaneous doses of ZPIV given at 4-week intervals (Abbink et al., 2016). Neutralization activity achieved in NHPs using this regimen was quite high (a reciprocal EC_{50} titer ~5,000 by a MN assay). Phase I studies of the ZPIV candidate are anticipated to start in the fall of 2016.

Live-attenuated vaccine (LAV) candidates also are being developed based on strategies proven safe and effective for DENV (Kirkpatrick et al., 2016). While multiple strategies for attenuation are possible, the creation of chimeric flaviviruses encoding heterologous prM and E genes has been used extensively (Durbin and Whitehead, 2010). The properties of these LAVs require careful study to characterize and confirm the level of attenuation and stability prior to large-scale clinical evaluation. Effective LAVs should be single dose and can be economically produced in large quantities, reducing the financial burden required for widespread distribution in low-resource areas where vaccines may be needed most. In addition, ZIKV and DENV LAVs may be formulated together to provide protection against multiple pathogens. Because of the unusual capacity of ZIKV to be transmitted sexually and to cross the placenta, LAVs for ZIKV will be most effective as a childhood vaccine delivered prior to sexual maturity. While the safety profile of many chimeric flaviviruses has been excellent (Kirkpatrick et al., 2016; Sirivichayakul et al., 2016), use of the currently available DENV LAV is limited to those greater than 9 years of age (Guy and Jackson, 2016; Hadinegoro et al., 2015). Clinical studies to establish the safety of ZIKV LAVs in an adolescent population will be required. Phase I studies to demonstrate safety and immunogenicity of a candidate developed by the Laboratory of Infectious Diseases at the National Institute of Allergy and Infectious Diseases (NIAID) are anticipated in 2017.

Concluding Remarks

The urgent need for interventions to blunt the impact of ZIKV on global health has stimulated rapid progress in our understanding of ZIKV biology, pathogenesis, and immunity. Antibodies that bind E proteins on the virus particle contribute substantially to protection against infection, may be elicited by candidate vaccines, and hold promise as therapeutics. Many important questions remain, including the characteristics of a vaccine-elicited immune response capable of preventing infection and vertical transmission. Will sterilizing immunity be required, or will a reduction in viremia be sufficient to protect the fetus from disease? While ADE with heterologous immune sera was expected from prior flavivirus studies, does this process play a role in shaping the immune response following ZIKV infection or in modulating disease? Can more specific diagnostics be developed using insight into ZIKV TS and CR responses? While these and numerous other questions require epidemiological studies and more detailed study of antibody response, the remarkable pace of progress in the months since the scope and complexity of the ZIKV crisis became clear supports cautious optimism that solutions to prevent ZIKV infection and transmission will be found soon.

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