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Immunogenicity and protection conferred by an optimized purified inactivated Zika vaccine in mice

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ABSTRACT

After decades of inconsequential infections, and sporadic outbreaks in the Asia-Pacific region between 2007 and 2013, Zika virus caused a widespread epidemic in South America in 2015 that was complicated by severe congenital infections. After the WHO declared a Public Health Emergency of International Concern in February 2016, vaccine development efforts based on different platforms were initiated. Several candidates have since been evaluated in clinical phase I studies. Of these, a Zika purified inactivated vaccine (ZPIV), adjuvanted with aluminum hydroxide, developed by the Walter Reed Army Institute of Research (WRAIR), yielded high seroconversion rates. Sanofi Pasteur further optimized the vaccine in terms of production scale, purification conditions and regulatory compliance, using its experience in flavivirus vaccine development. Here we report that the resulting optimized vaccine (ZPIV-SP) elicited robust seroneutralizing antibody responses and provided complete protection from homologous Zika virus strain challenge in immunocompetent BALB/c mice. ZPIV-SP also showed improved immunogenicity compared with the first-generation vaccine, and improved efficacy in the more permissive interferon receptor-deficient A129 mice. Finally, analysis of the IgG response directed towards non-structural protein 1 (NS1) suggests that viral NS1 was efficiently removed during the optimized purification process of ZPIV-SP. Together, these results suggest that the optimized vaccine is well suited for further evaluation in larger animal models and late-stage clinical studies.

1. Introduction

Zika virus (ZIKV), a flavivirus of the Spondweni virus clade, was first discovered in Africa in 1947 [1]. Following several decades of inconsequential infections on the African continent and in South Asia, the virus emerged in South America in 2015 [2]. It rapidly expanded throughout the South American region to cause widespread disease, particularly in pregnant women where infection led to severe congenital abnormalities or death among fetuses and infants [3]. The combination of the tropism of ZIKV to the cells of the neural lineage and the virus's ability to be transmitted sexually, differentiates it from other members of the flavivirus genus [4,5]. In addition, ZIKV can cause other serious consequences in adults, including Guillain-Barré syndrome and other neurologic conditions [6,7]. However, most infections are asymptomatic or cause only mild disease, including low-grade fever, conjunctivitis and rash, greatly complicating its diagnosis [4].

Despite the recent dramatic decline in ZIKV infections in much of the world, ZIKV remains a public health concern due to its severe impact on infants and the widespread distribution of the vector that transmits the infection, *Aedes aegypti*, placing nearly half of the world's population at risk [8]. In addition, evidence suggests that ZIKV may be transmissible by other mosquito vectors [9], including the widely disseminated species *A. albopictus*, further increasing the risk of a global epidemic. Consequently, many organizations have undertaken the development of Zika vaccine candidates, based on both traditional and more novel vaccine platform technologies [10–11]. Several candidates were shown to elicit protective antibodies in mice and non-human primates, including a ChimeriVax-Zika vaccine developed by Sanofi Pasteur [11–12]. However, only two candidates were assessed in humans, a DNA vaccine [13] and the Zika purified inactivated virus vaccine (ZPIV)

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developed by the Walter Reed Army Institute of Research (WRAIR) [14]. Among those two, only ZPIV yielded robust seroconversion rates in humans.

ZPIV was derived from a strain isolated in Puerto Rico in 2015; [4] it was chemically inactivated, purified and adsorbed to an aluminum hydroxide adjuvant. Preclinical studies demonstrated that ZPIV was immunogenic and completely protective against ZIKV challenge in animal models including mice [15] and macaques [16]. Protection was shown to be durable, lasting for at least one year in monkeys [17]. ZPIV was further evaluated in three phase I studies in healthy adult volunteers and was found to be well-tolerated and induced detectable neutralizing antibody titers in 92% of individuals tested [14].

Sanofi Pasteur sought to optimize ZPIV in terms of production scale and regulatory compliance, by establishing a seed lot system, using a more stringent process of purification and controlled inactivation in an entirely animal-free process. A secondary objective was to produce a vaccine free of NS1 to allow the discrimination between vaccination and natural infection using a NS1-specific serological assay. Here we report on the immunogenicity and protective efficacy of the resulting optimized ZPIV (ZPIV-SP) in two murine models, the immunocompetent BALB/c mice and the immunodeficient type I interferon receptor (IFNAR)-knockout (KO) A129 mice. BALB/c mice were previously used to test ZPIV immunogenicity and efficacy, however, they were moderately permissive to ZIKV replication [13] and did not replicate the disease phenotype seen in humans, limiting the efficacy assessment. We thus also used the more permissive A129 mice which have been shown to be highly susceptible to ZIKV infection with widespread dissemination of viral RNA to multiple target organs [16–18] and which, therefore, represent a more stringent model to test Zika vaccine efficacy.

In both murine models, we demonstrate that ZPIV-SP induces high neutralizing antibody responses and provides complete protection. Moreover, compared with ZPIV, ZPIV-SP shows improved immunogenicity. Finally, the enhanced antibody response correlated with improved efficacy in the highly permissive A129 mice model compared with the first generation vaccine.

2. Material and methods

2.1. Virus and cells

ZIKV virus PRVABC59 isolate from Puerto Rico (ZIKV-PR) [4] was received from the U.S. Centers for Disease Control and Prevention (Fort Collins, USA) and propagated in Sanofi Pasteur Vero cells.

2.2. ZPIV and ZPIV-SP vaccines

The first-generation ZPIV produced from the ZIKV-PR strain was kindly provided by WRAIR [14]. The optimized vaccine (ZPIV-SP) was prepared at Sanofi Pasteur (Marcy l'Etoile, France) using the WRAIR process as a starting point with the following improvements. Briefly, after an initial amplification of ZIKV-PR in Vero cells, viral RNA was extracted and transfected into Sanofi Pasteur's serum-free (SPSF) Vero cells. Recovered virus was amplified, plaque-purified twice and further amplified to generate a pre-Master Seed Lot from which a Master and a Working Seed Lot were derived. The drug product was then produced in a 180 L bioreactor using SPSF Vero cells. The virus was clarified, purified by ultracentrifugation with a modified cut-off and chromatography, and then inactivated by formalin treatment. The parameters of the purification and inactivation steps were optimized compared with the conditions used by WRAIR. The drug product was adjusted for ZIKV Envelope (E) antigenic content by ELISA to 400 Antigenic Unit (AU)/mL (corresponding to 10 μg/mL of proteins of ZPIV) and freeze-dried. The freeze-dried drug product was then reconstituted with 1 mg/mL aluminium hydroxide (Brenntag Biosector, Frederikssund, Denmark) for a final vaccine formulation of 200 AU in 500 μL.

2.3. ZIKV quantification ELISA

In order to formulate ZPIV-SP, the vaccine ZIKV E antigen content was quantified using a sandwich ELISA based on two conformational human neutralizing monoclonal antibodies (mAb) isolated from ZIKV infected patients and specific to ZIKV E and to E-dimer epitopes respectively (Richard S. et al., Manuscript in preparation).

2.4. Murine models

To determine the relative potency of ZPIV-SP, we used two murine models of immunogenicity and protection previously described [15,18,20,21].

The first model utilized BALB/c mice to evaluate the ability of the vaccine to induce an appropriate immune response in immunocompetent animals; it also served as a direct comparison to the previously published data of [15].

For the immunogenicity study, immunocompetent BALB/c ByJ female mice (8–11 weeks old, Charles River Laboratories, Saint Germain-sur-l'Arbresle, France) were vaccinated on Days 0 (D0) and 21 (D21), by the intramuscular (i.m.) route, with either aluminium hydroxyde in diluent (sham vaccine; n = 5) or escalating doses of ZPIV or ZPIV-SP (n = 5 each) ranging from 0.37 to 40 AU. Blood samples were collected 3 weeks after the last vaccine dose for immunogenicity evaluation.

For the protection study, BALB/c mice received 40 AU of ZPIV or ZPIV-SP, or sham vaccine (Alum diluent) (n = 15 each). Blood was collected on D21 for immunogenicity evaluation and all animals were challenged on Day 28 (D28) with 10 plaque-forming units (PFU) of ZIKV-PR by the intravenous (i.v.) route. On Days 2 (D2), 3 (D3) and 4 (D4) post-infection (p.i.), 5/15 animals from each group were bled for viremia analysis.

The second model utilized IFNAR-KO A129 mice to further evaluate vaccine efficacy. Eight- to 10-week-old A129 male mice (B&K UNIVERSAL, London, United Kingdom) were vaccinated on D0 and D21 by the i.m. route with 40 AU of either ZPIV or ZPIV-SP, or sham vaccine (n = 12 each). Plasma was collected on Day 42 (D42) using dipotassium EDTA (EDTA-K2) anticoagulant (Sigma-Aldrich, St. Louis, MO, USA) for immunogenicity evaluation. Four weeks after the last vaccine dose, 10/12 mice from each group were challenged with 10^3 PFU of ZIKV-PR by the subcutaneous (s.c.) route; 2/12 were used as non-infected controls. Plasma collected on D4 and Day 6 (D6) p.i. as well as selected organs (i.e. spleen, testes and brain) collected on D6 p.i. after sacrifice, were analyzed for viral RNA detection by qRT-PCR.

All animal experiments were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care accredited animal facilities, in compliance with European Directive 2010/63 and French national regulations. The protocols were approved by the Sanofi Pasteur Ethical Committee for Animal Experimentation and all efforts were made to reduce the use of animals and to minimize pain and distress in application of the 3Rs principles. All blood sampling were performed under isofluorane anesthesia.

2.5. Viral RNA extraction

Viral RNA was extracted from plasma, half of each spleen, one cerebral lobe and both testes. For plasma, RNA was isolated with

the NucleoSpin 96 virus kit (Macherey Nagel, Duren, Germany). Organs were placed in RNAlater (Invitrogen, Carlsbad, CA, USA) and stored at −70 °C. Between 30 and 100 mg of each organ were homogenized in GentleMacs M tubes (Miltenyi Biotec, Paris, France) containing RLT Plus lysis buffer (Qiagen, Hilden, Germany). Homogenate fractions were placed on a genomic DNA (gDNA) eliminator plate (Qiagen) and mixed with 70% ethanol. RNA was purified on an RNeasy plate (Qiagen) and eluted in nuclease-free water. The quantity and quality of RNA were analyzed using DNA 5 K / RNA / Charge Variant LabChip (PerkinElmer, Waltham, MA, USA) on LabChip GXII (PerkinElmer).

2.6. Viral genomic titration

Viral RNA was quantified using a ZIKV-specific quantitative reverse transcription-PCR (qRT-PCR) assay targeting the NS5 gene, using the following primers: 5′GGAAGAAGTTCCGTTTTG (forward) and 5′GCCAATCAGTTCATCTTG (reverse) and the 5′Fam-CCACCACTTCAACA-MGB NFQ probe, with a one-step Quantitect Probe RT-PCR kit (Qiagen). RNA titers were expressed either in $\log_{10}$ copies/mL (plasma) or in $\log_{10}$ copies/mg (organs).

For plasma, the limit of quantification (LOQ) of the qRT-PCR was 3.2 $\log_{10}$ Geq/mL and the limit of detection (LOD) 3.0 $\log_{10}$ Geq/mL. An arbitrary value of half of the LOD was assigned to samples below the LOD, and of 3.0 $\log_{10}$ Geq/mL for values between the LOD and LOQ.

2.7. E-specific and NS1-specific ELISA IgG

Ninety-six-well plates were coated with recombinant E protein (Meridian Life Science Inc., Memphis, USA) or recombinant non-structural protein 1 (NS1) protein (Native Antigen Company, Oxford, UK) in carbonate buffer, pH 9.6. Following blocking with PBS-Tween20-milk for 60 min at 37 °C, 2-fold diluted serum samples were added and incubated for 90 min at 37 °C. Washing steps were performed between incubation steps with PBS-Tween. Anti-mouse IgG horseradish peroxidase-conjugate (Southern Biotech, Birmingham, US) was added and incubated for another 90 min at 37 °C before color development with tetramethylbenzidine substrate (Tebu-Bio Laboratories, Le-Perray-en-Yvelines, France). Optical density was measured at 450–650 nm with an automatic plate reader. For E-specific IgGs titers were calculated using an anti-ZIKV mouse reference serum regression curve; for NS1-specific IgGs, titers were calculated as the reciprocal dilution of the serum giving an optical density of 1 using the tendency function. The titer of the reference was previously calculated by the average of several determinations of the reciprocal dilution giving an optical density of 1.0. All titers were expressed in $\log_{10}$ ELISA Units (EU). Based on common laboratory practices, the LOD was defined at 1.3 $\log_{10}$ EU, and titers below LOD were assigned a value of 1.0 $\log_{10}$ EU.

2.8. MN_{50} neutralization assay

Neutralizing antibodies were measured by a high-throughput microneutralization (MN_{50}) assay based on the dengue SN_{50} assay previously described [22] with the following modifications. Briefly, heat-inactivated plasma or serum samples were serially 4-fold diluted, mixed with ZIKV-PR at 2.7 $\log_{10}$ cell culture infectious dose 50%/mL and incubated for 1 h at 37 °C. The mix was then added to Vero cell monolayers in 96-well plates. Following 4–5 days of incubation, cells were fixed with 85% acetone at −20 °C, dried, blocked with PBS-Tween20-skim milk, and incubated with biotinylated pan-flavivirus 4G2 mAb (HB112, Biotem, Apprieu, France). After washing, streptavidin-alkaline phosphatase was added to the cells. Infected cells were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium in Levamisole substrate. Positive wells were defined when at least one colored infectious focus was detected, and negative wells when none were observed (i.e. neutralized). For each dilution, the total number of negative wells was recorded and the reciprocal dilution corresponding to 50% of viral neutralization was calculated according to the least square method and expressed as the neutralization $\log_{10}$ MN_{50} titer. The LOD is 1.0 $\log_{10}$ MN_{50} titer, corresponding to the reciprocal of the first dilution tested and titers below LOD were assigned a value of 0.7 $\log_{10}$ MN_{50} titer.

2.9. Statistical analyses

Statistical analyses were performed on antibody responses measured after the last vaccine dose. Prior to analysis, MN_{50} titers and E-specific IgG ELISA titers were log10-transformed. Groups of animals with >50% of non-responders were excluded from the analysis. Dose-effect analyses were modelled using a linear model including the two products (ZPIV and ZPIV-SP), log10-transformed doses (continuous factor) and the interaction between these two factors. The slopes of the linear regression of ZPIV and ZPIV-SP were compared for E-specific IgG titers on D42. As non-significant interaction was observed, the two dose effects are parallel. The two products were then compared globally (regardless of the dose). The fold difference between the slopes of the two products was estimated and compared to zero using a Student's *t*-test into the model. In the protection studies, ZPIV and ZPIV-SP were compared using either one-way analysis of variances or a non-parametric Kruskall Wallis test depending on the data distribution.

To investigate the link between protection and MN_{50} titer levels a logistic regression model was used.

All analyses were performed using the SAS® v9.4 software with an alpha level of 5% and an interaction of 10%.

3. Results

3.1. ZPIV-SP elicits enhanced neutralizing antibodies and E-specific IgG responses in immunocompetent mice

BALB/c mice received increasing doses of ZPIV or ZPIV-SP, or sham vaccine on D0 and D21. Production of seroneutralizing antibodies and E-specific IgG were evaluated 3 weeks after the last vaccine dose. Across all doses tested, ZPIV-SP consistently produced greater neutralizing antibody titers than those elicited by ZPIV (Fig. 1A). ZPIV-SP was highly potent in mice that received two doses $\geq$10 AU, with mean MN_{50} titers reaching 1.8 log10 for the 40 AU dose (Fig. 1A). E-specific IgG responses were consistent with those measured by seroneutralization: vaccination with ZPIV-SP induced a significant mean 2.2-fold higher response than that measured after vaccination with ZPIV (p-value = 0.01). Following two injections, regardless of the vaccine injected, all mice immunized with a dose $\geq$ 10 AU displayed E-specific IgG responses. The mean responses ranged from 1.0 to 3.2 $\log_{10}$ EU and 1.1 to 3.5 $\log_{10}$ EU for ZPIV and ZPIV-SP, respectively (Fig. 1B).

3.2. ZPIV-SP elicits complete protection against wild-type ZIKV-PR in immunocompetent mice

We next evaluated the ability of a single dose of either ZPIV or ZPIV-SP to protect BALB/c mice from viremia induced by infection with wild-type ZIKV-PR. As expected, a single dose (40 AU) of either of the two vaccines produced very low levels of E-specific IgG and neutralizing antibodies, with ZPIV-SP eliciting a higher percentage of responders (Fig. 2A). However, these modest antibody levels were sufficient to completely protect BALB/c mice from a viral challenge with 10 PFU of the homologous ZIKV-PR: no

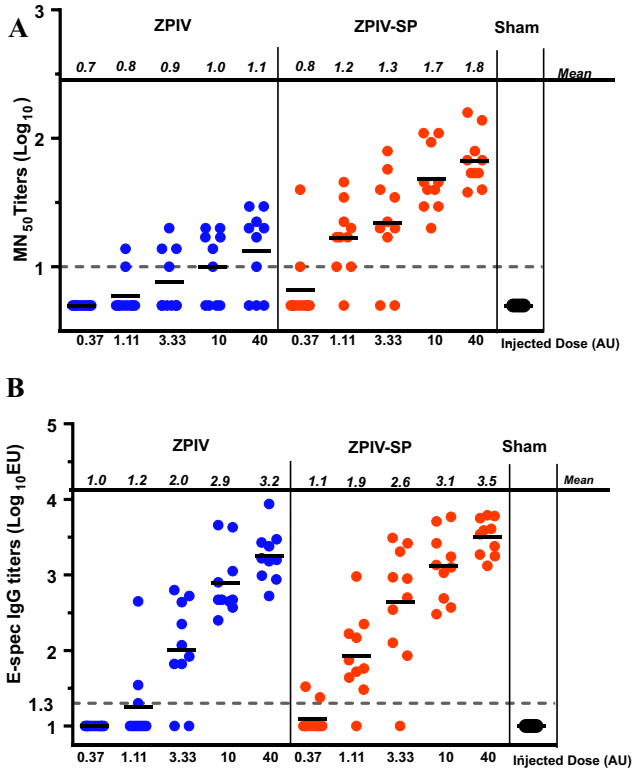

Fig. 1. Immunogenicity of ZPIV vaccines in BALB/c mice. BALB/c mice were immunized on Day 0 and 21 with increasing doses of either Walter Reed Army Institute of Research Zika vaccine (ZPIV) or optimized ZPIV (ZPIV-SP) (n = 10 each) or with sham vaccine (Alum diluent (sham) (n = 5) by the intramuscular route in the quadriceps (100 μL). Sera were collected from all animals on Day 42 for humoral response measurements. (**A**) ZIKV-specific microneutralizing (MN_{50}) titers. (**B**) E-specific IgG antibody titers. Dots represent individual animals and bars represent the mean. The dotted lines represent the limit of detection..

detectable virus was found in blood on D2, D3 or D4 p.i. (Fig. 2B). This was in contrast to results obtained in sham-immunized animals which showed viremia from D2 p.i. in some animals and in all animals by D4 p.i..

3.3. ZPIV-SP elicits protective immune responses in IFNAR-KO mice

In IFNAR-KO A129 mice, both ZPIV and ZPIV-SP vaccines elicited E-specific IgG in all animals. Importantly, all mice immunized with ZPIV-SP developed detectable neutralizing titers, and the mean titer was significantly higher than with ZPIV (2.1 vs 1.4 $\log_{10}$ MN_{50}, p-value = 0.0035) (Fig. 3A). For comparison, ZIKV-PR infection in IFNAR-KO A129 mice elicited MN_{50} mean titers of 2.8 $\log_{10}$ MN_{50} (data not shown). ZPIV-SP also induced more potent E-specific IgG responses than ZPIV on D42 (p = 0.0006) (Fig. 3B).

Upon challenge with ZIKV-PR sham-immunized IFNAR-KO A129 mice demonstrated a marked viremia on D6 p.i., with 100% of animals displaying high levels of viral RNA (5.2–7.2 $\log_{10}$ copies/mL) in plasma (Fig. 3C). Similarly, the majority of sham-immunized animals showed detectable levels of viral RNA in all three target organs evaluated (up to 4.2 $\log_{10}$ copies/mg) (Fig. 3 D–F). In contrast, viral RNA was not detected at any time point in plasma or organs from mice immunized with ZPIV-SP (Fig. 3C–F). ZPIV displayed an intermediary profile: 2/10 ZPIV-immunized mice did not yield detectable RNA either in plasma or organs, while the remainder demonstrated a later onset of viremia at D6 p.i. (up to 6.6 $\log_{10}$ copies/mL), though at levels lower than those measured in the sham-immunized group. Moreover, some of these viremic animals had detectable RNA in spleen (5/10), testes (3/10), and brain (1/10), at levels comparable to those of sham-immunized mice (Fig. 3D–F).

Interestingly, using a logistic model, we observed a significant link between protection and MN_{50} titers (p-value = 0.01), although no absolute protective titer could be identified (Fig. 4).

3.4. ZPIV-SP does not elicit NS1-specific IgG response in mice

All ZPIV-immunized BALB/c mice mounted a high NS1-specific IgG response after two vaccine doses (mean ELISA titer of 5.0 log10 EU), while no detectable IgG responses were measured after immunization with ZPIV-SP (Fig. 5).

4. Discussion

The recent Zika outbreak was declared by WHO to be a public health emergency, stimulating an urgent call for development of an efficient vaccine. Chemical inactivation of viruses has led to successful vaccines for other viruses including one flavivirus, the Japanese encephalitis virus, and is viewed as an attractive approach due to the inherent safety profiles and rapid development timelines of these vaccines. Several organizations have developed ZIKV vaccines using this technology [23] and a number of these vaccine candidates have been described to be highly protective in animal models [15,16,24]. The most advanced of these vaccines, ZPIV, developed by WRAIR, was further shown to induce serum neutralizing antibodies in three phase I trials following two i.m. injections [14]. However, the serum neutralizing titers obtained in two of these studies showed a decrease between 2 and 4 weeks after the second injection and approached the predicted protection threshold of 100, determined previously in non-human primates (NHPs) [16]. The NHP study also showed a decline in neutralizing titers between 2 and 4 months following the two immunizations [16]. These results suggest that a second boost might be required, or that this inactivated vaccine may need to be optimized to improve its immunogenicity and ensure sustained protection in humans. Addition of a second boost is currently being evaluated in a phase I trial (K. Modjarrad, personal communication). In order to further develop ZPIV, Sanofi Pasteur produced a well characterized, highly purified vaccine ZPIV-SP. After two immunizations, ZPIV-SP provided superior immunogenicity when compared with the first generation vaccine in both immunocompetent (BALB/c) and immunodeficient (IFNAR-KO A129) mice, with no induction of NS1-specific responses, and demonstrated complete protection in the highly permissive A129 mice.

The efficacy results in BALB/c mice observed in this study are consistent with those reported by Larocca et al. [15]. supporting further that a single dose of 1 μg ZPIV (or 40 AU) is able to induce complete protection against viremia. In both studies, protection was observed despite low to undetectable neutralizing antibody responses. Whether other immune parameters, such as cell-mediated immunity, played a role in vaccine efficacy in this model needs to be further investigated. However, immunocompetent rodents such as BALB/c mice are naturally resistant to ZIKV infection, displaying only a very transient viral replication upon an invasive i.v. viral challenge. The model is therefore poorly reflective of the course of infection in humans, thereby restricting the protective efficacy assessment of vaccine candidates.

Immunodeficient mice such as IFNAR-KO or IFNAR-depleted were shown to be highly permissive to ZIKV infection and displayed a similar invasion profile to that observed in humans including replication in testes and brain [18,21,25]. Following ZIKV infection, IFNAR-KO A129 mice showed signs of illness, viremia, and weight loss but no mortality [19]. They thus represent a non-lethal challenge model that matches the human ZIKV

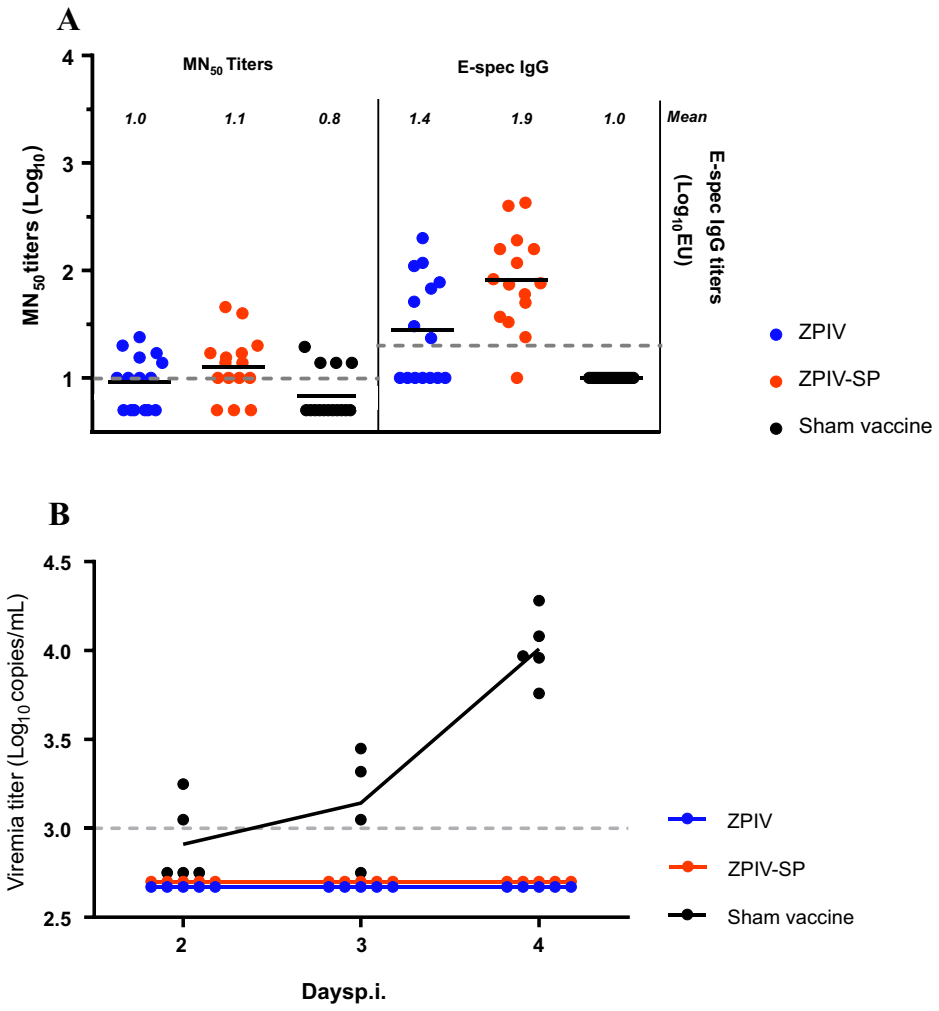

Fig. 2. Protective efficacy of ZPIV vaccines in BALB/c mice. BALB/c mice were immunized once with 40 AU of Walter Reed Army Institute of Research Zika vaccine (ZPIV) or optimized ZPIV (ZPIV-SP) or with (sham vaccine (Alum diluent) by the intramuscular route in the quadriceps (100 μL) (n = 15 each) and sera were collected from all animals on Day 21. Mice were challenged on Day 28 with Zika virus PRVABC59 isolate from Puerto Rico (10 PFU) by the intravenous route (200 μL) and viral RNA was measured by quantitative reverse transcription-PCR (qRT-PCR) on Days 2, 3 and 4 post-infection (p.i.) (**A**) E-specific IgG antibody titer and ZIKV-specific microneutralizing (MN_{50}) titers. Dots represent individual animals and black bars represent mean. Dotted lines represent the limit of detection. (**B**) Viremia measured by qRT-PCR. Dots represent individual animals and solid lines represent the mean viremia. Dotted lines represent the limit of detection.

pathology in adults, and fulfill the 3R animal welfare recommendations. In preliminary infection studies in A129 mice, we have observed robust viral RNA titers in plasma and target organs with a peak at D6 p.i. (data not shown). Using this model, and in contrast to what was observed in the BALB/c mice challenge model, we demonstrated here that, after two vaccinations, ZPIV-SP induced complete protection, while ZPIV conferred only partial protection. Moreover, data analysis using a logistic model showed that the protection was linked to the level of induced neutralizing antibodies. The observed protection from viral dissemination to target organs following immunization with ZPIV-SP is encouraging as it suggests that the vaccine could protect not only from viremia but also from virus propagation to neural tissue, which is critical during prenatal development, and to testes, which are an important reservoir for sexual transmission [26]. Whether this vaccine can also protect against transplacental transmission has not been directly studied.

Serum neutralizing antibody levels have been proposed to be a correlate of protection against Zika infection and similar correlates have been established for other flaviviruses, including Japanese encephalitis, West Nile and yellow fever viruses [27]. More specifically, in a rhesus monkey model of ZIKV infection, neutralizing antibody responses were shown to correlate with efficacy against viremia with a protective threshold observed at around 2.0 $\log_{10}$ MN_{50} titers [16]. Here, in the protection A129 mouse model, while neither correlation nor protective threshold could be demonstrated, we showed a link between the level of neutralizing antibody responses and protection. Moreover, for most mice (18/20), a MN_{50} titer of 1.5 $\log_{10}$ or above could predict protection; interestingly, all the ZPIV-SP vaccinated mice (10/10) displayed MN_{50} titers above 1.5 $\log_{10}$ and were protected. Additionally, it is worth noting that ZPIV-SP contains the E domain III and E quaternary epitopes as demonstrated by ZIKV quantification ELISA (Richard S. et al., Manuscript in preparation), which are targeted by potent neutralizing antibodies [28,29].

The anti-NS1 IgG response induced by the optimized vaccine was investigated to assess its potential use as a specific biomarker of ZIKV infection. The absence of NS1-specific IgG response observed after ZPIV-SP immunization in mice suggests that, if confirmed in humans, including non ZIKV flavivirus-primed individuals, ZIKV NS1 serology could be used as a surrogate of ZIKV natural infection during clinical development of ZPIV-SP.

In addition, this observation suggests that ZPIV-SP has a lower NS1 content compared to ZPIV, which has been confirmed by

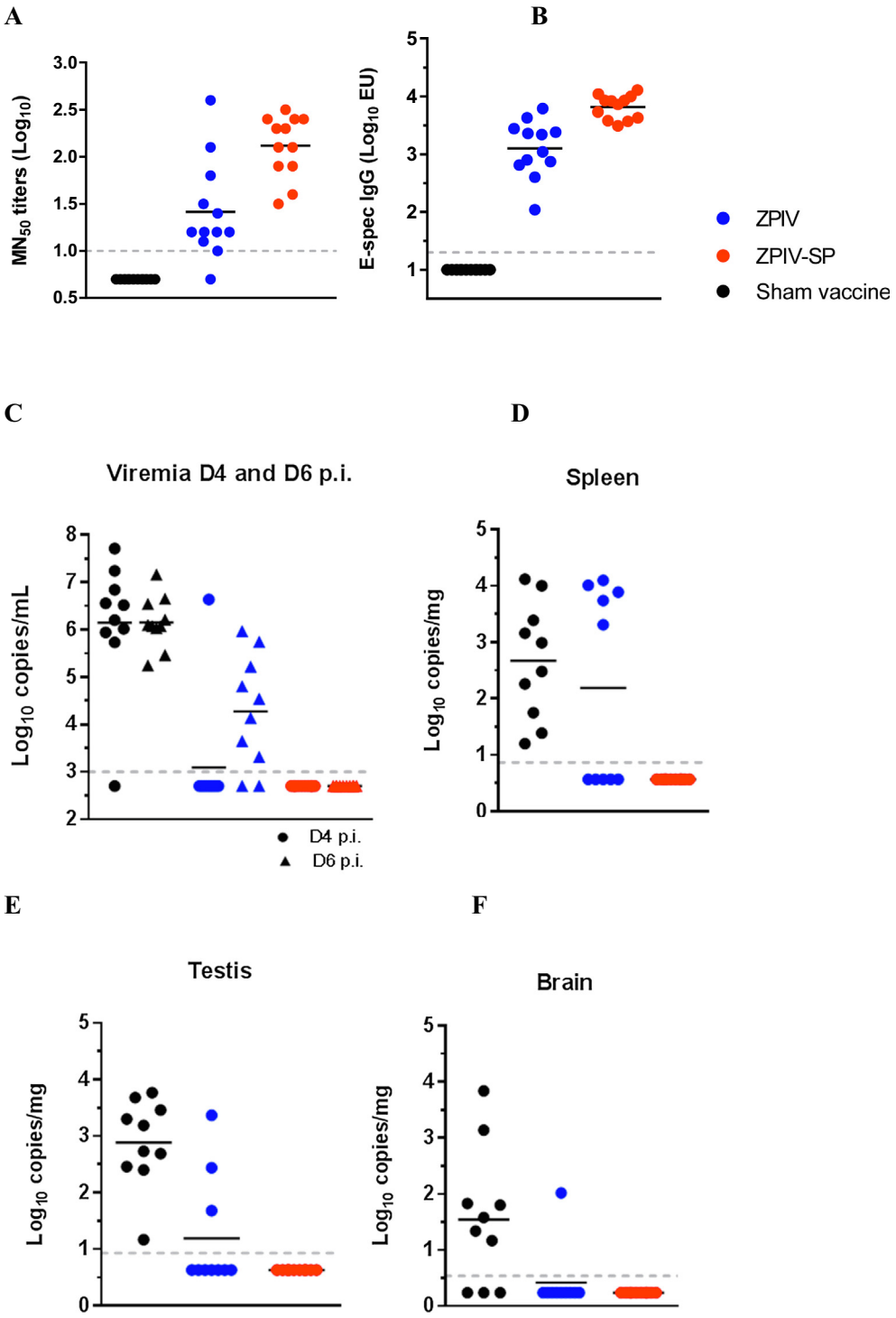

Fig. 3. Protective efficacy of ZPIV vaccines in A129 mice. A129 mice were immunized on Day 0 and Day 21 with 40 AU of either Walter Reed Army Institute of Research Zika vaccine (ZPIV) or optimized ZPIV (ZPIV-SP) or with sham vaccine (Alum diluent) by the intramuscular route (n = 12/group). Plasma was collected from all animals on Day 42. Then, mice were challenged on Day 49 with ZIKV-PRPR (10^3 PFU) by the subcutaneous route (n = 10). **(A)** ZIKV-specific microneutralizing titers (MN_{50}). **(B)** E-specific IgG antibody titers. Black bars represent the means. Viremia was measured by qRT-PCR on D4 and D6 post-infection (p.i.) in plasma **(C)** and on D6 p.i. in spleen, testes and brain **(D, E, F respectively)** (n = 10/group). For each single mouse, both testes were analyzed individually; among the two, the highest viral RNA titer per animal is depicted. Points represent individual animals and solid lines represent the mean viremia. Dotted lines represent the limit of detection.

quantitative ELISA (data not shown). This difference in NS1 content could be the result of optimization of the purification process allowing a more efficient removal of NS1. Interestingly, in terms of immunogenicity and efficacy, the induction of the NS1-specific response in our study did not correlate with a higher efficacy of the vaccine, despite a protective role being previously described for ZIKV NS1 in mice using a live recombinant vector [30] and for dengue and yellow fever NS1 using purified proteins [31,32] or passive transfer of antibodies [33].

The reasons for the improved immunogenicity and efficacy of the optimized vaccine observed here in murine models can only be speculated at this stage, as the main biochemical characteristics

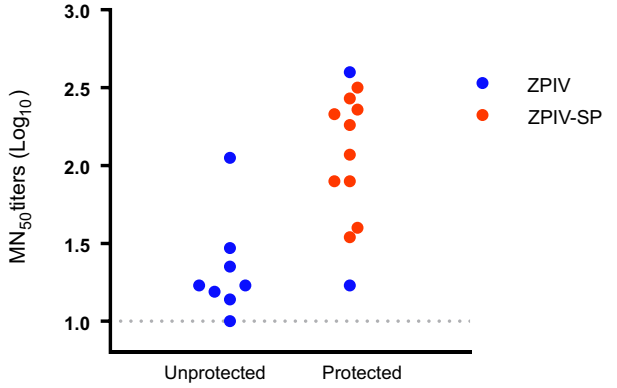

Fig. 4. Link between the neutralizing titers on Day 42 and protection. Comparison of microneutralizing MN_{50} titers in vaccinated A129 mice before challenge (Day 42) in protected (no viremia detected in plasma and organs) as compared to infected animals (viremia in the plasma and potentially some in organs). Mice were vaccinated with Walter Reed Army Institute of Research Zika vaccine (ZPIV) or optimized ZPIV (ZPIV-SP). Points represent individual MN_{50} titers. Dotted lines represent the limit of detection.

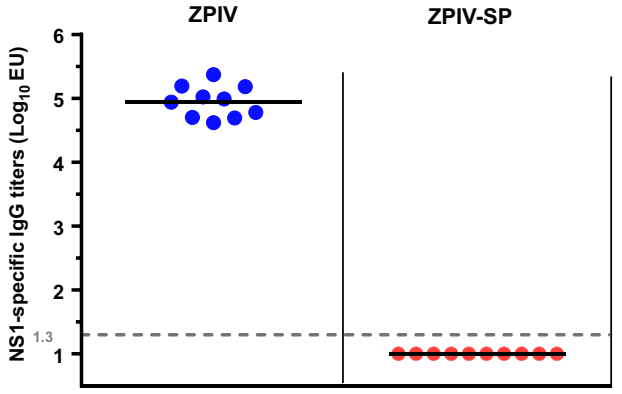

Fig. 5. NS1-specific antibody responses elicited by ZPIV vaccines in BALB/c mice. BALB/c mice were immunized on D0 and D21 with 40 AU of either Walter Reed Army Institute of Research Zika vaccine (ZPIV) or optimized ZPIV (ZPIV-SP) (n = 10 each) by the intramuscular route in the quadriceps (100 μL). NS1-specific IgG titers were measured by ELISA on Day 42. Dots represent individual animals and bars represent the mean. Dotted lines represent the quantification limit of detection.

such as E antigen content, purity, and particle size were assessed on both vaccines and found to be similar. The role of NS1 on the neutralizing antibody response is unknown, but some immunodominance of NS1 cannot be excluded, although no impact on the E-specific-IgG responses was observed. However, besides the more efficient removal of NS1, the improved purification process may also allow a higher integrity of viral particles potentially leading to a better epitope presentation, as well as to a higher content of encapsidated viral RNA able to trigger innate signaling through recognition by Toll-Like Receptors. The superior efficacy of the ZPIV-SP vaccine in mice needs to be further evaluated in NHPs before its evaluation in humans.

4.1. Conclusions

Because the durability of vaccine responses is critically important to ensure the lack of materno-fetal transmission during the entire reproductive life of a woman, whether the improved immunogenicity and efficacy observed in mice with our optimized vaccine will confer improved durability of immunity will need to be demonstrated in long-term studies in larger animal models and in humans. Together, these results suggest that the optimized vaccine is well suited for further evaluation in late-stage clinical studies.

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Conflicts of interest

All authors are employees of Sanofi Pasteur.

Author contribution

JH led the vaccine program and collaboration with WRAIR. SC and ER coordinated the production and characterization of the vaccine. VL, MCB, CB and FB conceived and designed the experiments, CB executed the experiments, CB, MCB, VL and JH analyzed and interpreted the experiments. JH, VL, MCB, CB and FB contributed to the writing of this manuscript. All authors approved the final version of the paper.

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