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(54) Title: RECOMBINANT MEASLES VIRUS EXPRESSING ZIKA VIRUS PROTEINS AND THEIR APPLICATIONS

(57) Abstract: The present invention relates to recombinant measles virus expressing Zika virus proteins and their applications, in particular in inducing preventive protection against Zika virus. The present invention is directed to recombinant measles virus (MV) expressing at least (i) the precursor of membrane (prM) protein of a Zika virus (ZIKV), and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKVor a truncated version thereof, and concerns recombinant infectious particles of said MV-ZIKV able to replicate in a host after an administration, and also Virus Like Particles (VLPs) that contain these ZIKV proteins at their surface. The present invention provides means, in particular nucleic acids, vectors, cells and rescue systems to produce these recombinant infectious particles and VLPs. The present invention also relates to the use of these recombinant infectious particles and/or VLPs, in particular under the form of a composition, more particularly in a vaccine formulation, for the prevention of an infection by ZIKV or for the preventive protection against clinical outcomes of ZIKV infection.

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RECOMBINANT MEASLES VIRUS EXPRESSING ZIKA VIRUS PROTEINS AND THEIR APPLICATIONS

The present invention relates to recombinant measles virus expressing Zika virus proteins and their applications, in particular in inducing preventive protection against Zika virus. The present invention is directed to recombinant measles virus (MV) expressing at least (i) the precursor of membrane (prM) protein of a Zika virus (ZIKV), and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof, and concerns recombinant infectious particles of said MV-ZIKV able to replicate in a host after an administration, and also Virus Like Particles (VLPs) that contain these ZIKV proteins at their surface. The present invention provides means, in particular nucleic acids, vectors, cells and rescue systems to produce these recombinant infectious particles and VLPs. The present invention also relates to the use of these recombinant infectious particles and/or VLPs, in particular under the form of a composition, more particularly in a vaccine formulation, for the prevention of an infection by ZIKV or for the preventive protection against clinical outcomes of ZIKV infection.

ZIKV is an emerging mosquito-borne flavivirus. Although it was initially isolated in 1947, to date there are no specific treatments or any vaccines available against ZIKV disease, making it a truly neglected and emerging disease. The recent rapid spread of ZIKV in previously unaffected regions such as South Pacific islands and Latin America has provided strong epidemiological evidence that infection with this virus might be associated with neurological complications in adults and with an increase in severe congenital brain malformations of new-borns. Consequently, the World Health Organization (WHO) has declared the recent outbreak of the ZIKV a public health emergency.

ZIKV was initially isolated from a rhesus monkey in the Zika forest in Uganda in 1947 (*Gubler DJ, et al., eds. Fields Virology, 5th edn. Philadelphia, PA: Lippincott Williams & Wilkins Publishers, 2007: 1155–227; Dick GWA, et*

al. Trans R Soc Trop Med Hyg 1952; 46: 509–20). The first human infection was reported in Nigeria in 1954 (*Macnamara FN. Trans R Soc Trop Med Hyg 1954; 48: 139–45*). Like dengue and chikungunya viruses, ZIKV adapted from an ancestral transmission cycle involving non-human primates and a broad spectrum of forest mosquito species as vectors to an urban cycle involving humans as reservoirs and the widely distributed Aedes mosquitoes as vectors (*Musso D, et al. Lancet 2015; 386: 243–44*). Since the 1950s, ZIKV had only been reported as circulating sporadically in Africa and Southeast Asia. In 2007, ZIKV was isolated for the first time in the Pacific, on the Micronesian island of Yap (*Duffy MR, et al. N Engl J Med 2009; 360: 2536–43*). Between October 2013 and April 2014, French Polynesia experienced the largest Zika outbreak ever reported at that time (*Cao-Lormeau VM, et al. Emerg Infect Dis 2013; 20: 1085–86*). More than 32,000 patients were suspected of ZIKV infection. Between 2014 and 2015, ZIKV spread to other Pacific islands, notably the Cook Islands and Easter Island (Chile). In March 2015, Brazil reported the autochthonous transmission of ZIKV (*Zanluca C, et al. Mem Inst Oswaldo Cruz 2015; 110: 569–72*) and declared an unprecedented outbreak 6 months later (*Dyer O. BMJ 2015; 351: h6983*) with preliminary estimates of 440,000 to 1.3 million cases of infection through December 2015 (*European Centre for Disease Prevention and Control, December 10, 2015*). As of March 2016, ZIKV infection has been reported from 43 countries and territories worldwide.

The current Zika epidemic is the largest epidemic ever recorded for this virus (*Abushouk et al. An updated review of Zika virus, J. Clin. Virol. 2016, 84, 53-58*). Although infection with ZIKV was usually associated with mild disease, its emergence in the Americas has coincided with a steep increase in patients developing Guillain-Barré syndrome. Moreover, infection with ZIKV has been linked to the birth of babies with neurological complications, in particular congenital microcephaly (*WHO. Guillain-Barré syndrome – El Salvador. Jan 21, 2016; ECDC. Rapid risk assessment. Zika virus epidemic in the Americas: potential association with microcephaly and Guillain-Barré syndrome. Dec 10, 2015; Soares de Araújo J, et al. Microcephaly in northeast Brazil: a review of 16 208 births between 2012 and 2015*), and it was shown that when pregnant

women are exposed to ZIKV during the first trimester of pregnancy, the risk of microcephaly for the newborn is increased 50 times from 2/10 000 to 1/100 (*Cauchemez S, et al. Association between Zika virus and microcephaly in French Polynesia, 2013–15: a retrospective study. The Lancet 2016*). In February 2016, the WHO declared the suspected link between ZIKV and neurological disorders and neonatal malformations a Public Health Emergency of International Concern.

In this context, in March 2016, experts gathered at WHO agreed that the development of a preventive vaccine is a major priority to respond to Zika epidemics in the future. Pragmatic strategies were asked to fast track the development of a safe and effective vaccine. Due to the established link between ZIKV infection and the appearance of congenital microcephaly in babies born to infected mothers, one could argue that a Zika vaccine has to be suitable for use in pregnant women. However, no licensed vaccine is currently recommended for use during pregnancy. Moreover, with the demonstrated association of Zika infection with Guillain-Barré syndrome, the observation of possible sexual transmission, and the appearance of developmental defects probably appearing very early in pregnancy, it is very likely that the vaccine should be addressed to the general population. In any case a Zika vaccine will have to demonstrate an excellent safety profile, particularly concerning the risk of neurotropism.

To allow fast track development of a Zika vaccine, the inventors used one of the safest and most efficacious vaccines available, the live-attenuated measles vaccine, as a delivery vector for ZIKV protective antigens to ensure the timely availability of a preventive vaccine whenever a new epidemic occurs. This delivery platform technology has demonstrated proof of principle in humans and a preclinical track record of rapid adaptability and effectiveness for a variety of pathogens. Moreover, the manufacturing process for these measles vector-based vaccines has been optimized to give higher yields and purity than the standard manufacturing measles vaccine process. It uses standard equipment and thus lends itself to further scale up as well as technology transfer to low and middle-income countries.

Measles vaccination has been used for more than 40 years in over 1 billion children and is approximately 93% efficacious after one administration and 97% after 2 administrations. Attenuated measles vaccine strains have been shown to be genetically stable. Reversion to pathogenicity or integration into the host cell genome is virtually impossible and has never been observed. Taking advantage of these characteristics, the inventors previously cloned the attenuated measles Schwarz vaccine virus and developed a method to genetically manipulate this negative strand RNA virus into a versatile chimeric or recombinant vector (*Combredet, C. et al., 2003, J Virol, 77(21): 11546-11554*).

A prophylactic vaccine against ZIKV, as for any other target, has to be safe and efficacious. In addition, the special epidemiology of a rapidly emerging virus, affecting both industrialized and developing countries, and the threat of infections during pregnancy causing serious birth defects, calls for a number of additional features for an ideal ZIKV vaccine.

ZIKV infection during pregnancy is strongly suspected to cause birth defects. Although live vaccines are generally contraindicated during pregnancy, measles infections have not been connected to birth defects (*Rasmussen SA, et al. Obstet Gynecol. 2015 Jul;126 (1):163-70*), and accidental application of the MMR vaccine during pregnancy was not connected to congenital birth defects (*Swamy GK, et al. Obstet Gynecol. 2015 Jan;125(1):212-26*). In contrast to measles-based vaccine according to the invention, a live-attenuated Zika vaccine approach would raise very significant safety concerns if accidentally applied during pregnancy. It has to be seriously questioned, if a vaccine against Zika intended for use during pregnancy could be developed and licensed in any acceptable time frame to stop the current epidemic. Instead, a vaccine for adolescents with minimal safety concerns for accidental use during pregnancy seems the most practical and realistic intervention to eliminate Zika-induced disease. A measles-based vaccine would exactly meet that target profile.

The measles-based approach of the invention can meet all of the relevant criteria of a future ZIKV vaccine at least equally well or better than

alternative approaches. In particular a non-adjuvanted measles-based ZIKV vaccine for children, adolescents and travelers represents one of the most likely candidates to be developed in a short time frame, has an excellent safety and efficacy profile, and has production and cost characteristics that are compatible with its use also in countries of limited economic strength.

To this end, a sequential development path was defined by the inventors. The first stage was the construction and characterisation of recombinant MV expressing at least ZIKV prM-E or E proteins as soluble secreted antigens. The characterisation included demonstration of Zika antigen expression, established growth characteristics in a production cell line, and analysis of genetic stability. Preclinical immunogenicity and protective efficacy of selected recombinant MV-Zika vaccine was evaluated in CD46-IFNAR mice susceptible to MV infection. The currently best candidate selected was evaluated for immunogenicity and protective efficacy in non-human primate model of ZIKV infection.

The inventors achieved the production of vaccines based on recombinant infectious replicative MV recombined with polynucleotides encoding at least ZIKV prM-E or E antigens, which are recovered when the recombinant virus replicates in particular in the host after administration. The invention thus relates to a live ZIKV vaccine active ingredient based on the widely used measles, in particular measles from the Schwarz strain, pediatric vaccine. In a preferred embodiment, this recombinant live MV-ZIKV vaccine yields ZIKV VLPs by replicating in infected cells.

MV is a non-segmented single-stranded, negative-sense enveloped RNA virus of the genus *Morbillivirus* within the family of *Paramyxoviridae*. This virus has been isolated in 1954 (*Enders, J. F., and T. C. Peebles. 1954. Propagation in tissue cultures of cytopathogenic agents from patients with measles. Proc. Soc. Exp. Biol. Med. 86:277-286*), and live-attenuated vaccines have been derived from this virus since then to provide vaccine strains, in particular from the Schwarz strain. Measles vaccines have been administered to hundreds of millions of children over the last 30 years and have proved its efficiency and safety. It is produced on a large scale in many countries and is

distributed at low cost. For all these reasons, the inventors used attenuated MVs to generate recombinant MV particles stably expressing prM-E or E antigens of ZIKV, and possibly capable of expressing also VLPs.

The invention thus relates to a nucleic acid construct which comprises:

(1) a polynucleotide encoding at least (i) the precursor of membrane (prM) protein of a Zika virus (ZIKV), and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof; and

(2) a cDNA molecule encoding a full-length, infectious antigenomic (+) RNA strand of a measles virus (MV);

wherein the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof is operatively linked, in particular cloned into the cDNA molecule.

A nucleic acid construct according to the invention is in particular a purified DNA molecule, obtained or obtainable by recombination of various polynucleotides of different origins, operably linked together.

The expression "*operably linked*" refers to the functional link existing between the different polynucleotides of the nucleic acid construct of the invention such that said different polynucleotides and nucleic acid construct are efficiently transcribed and if appropriate translated, in particular in cells or cell lines, especially in cells or cell lines used as part of a rescue system for the production of chimeric infectious MV particles of the invention or in host cells, especially in human cells.

In a particular embodiment of the invention, the construct is prepared by cloning a polynucleotide encoding at least (i) the prM protein of a ZIKV, and the E protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof, in the cDNA encoding a full-length, infectious antigenomic (+) RNA strand of a MV. Alternatively, a nucleic acid construct of the invention may be prepared using steps of synthesis of nucleic acid fragments or polymerization from a template, including by PCR.

In a particular embodiment of the invention, the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, is cloned into an ATU (Additional Transcription Unit) inserted in the cDNA of the MV. ATU sequences are known from the skilled person and comprise, for use in steps of cloning into cDNA of MV, cis-acting sequences necessary for MV-dependent expression of a transgene, such as a promoter of the gene preceding, in MV cDNA, the insert represented by the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, and a multiple cloning sites cassette for insertion of said polynucleotide.

When used to carry out the invention, the ATU is advantageously located in the N-terminal sequence of the cDNA molecule encoding the full-length (+)RNA strand of the antigenome of the MV and is especially located between the P and M genes of this virus or between the H and L genes. It has been observed that the transcription of the viral RNA of MV follows a gradient from the 5' to the 3' end. This explains that, when inserted in the 5' end of the coding sequence of the cDNA, the ATU will enable a more efficient expression of the heterologous DNA sequence (*e.g.* the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof) that it contains.

The polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, may thus be inserted in any intergenic region of the cDNA molecule of the MV in particular in an ATU. Particular constructs of the invention are those illustrated in the examples.

In a preferred embodiment of the invention, the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated

version thereof, is inserted in the intergenic region of the P and M genes of the MV cDNA molecule, in particular in an ATU.

As used herein, the expression "*encoding*" defines the ability of the nucleic acid molecules to be transcribed and where appropriate translated for product expression into selected cells or cell lines. Accordingly, the nucleic acid construct may comprise regulatory elements controlling the transcription of the coding sequences, in particular promoters and termination sequences for the transcription and possibly enhancer and other cis-acting elements. These regulatory elements may be heterologous with respect to the ZIKV polynucleotide sequences.

The term "*protein*" is used interchangeably with the terms "*antigen*" or "*polypeptide*" and defines a molecule resulting from a concatenation of amino acid residues. In particular, the proteins disclosed in the application originate from the ZIKV and are structural proteins that may be identical to native proteins or alternatively that may be derived thereof by mutation, including by substitution (in particular by conservative amino acid residues) or by addition of amino acid residues or by secondary modification after translation or by deletion of portions of the native proteins(s) resulting in fragments having a shortened size with respect to the native protein of reference. Fragments are encompassed within the present invention to the extent that they bear epitopes of the native protein suitable for the elicitation of an immune response in a host in particular in a human host, preferably a response that enables the protection against ZIKV infection or against ZIKV associated disease. Epitopes are in particular of the type of B epitopes involved in the elicitation of a humoral immune response through the activation of the production of antibodies in a host to whom the protein has been administered or in whom it is expressed following administration of the infectious replicative particles of the invention. Epitopes may alternatively be of the type of T epitopes involved in elicitation of Cell Mediated Immune response (CMI response). Fragments may have a size representing more than 50% of the amino-acid sequence size of the native protein of ZIKV, preferably at least 90% or 95%. Alternatively, fragments may be short polypeptides with at least 10 amino acid residues, which harbor

epitope(s) of the native protein. Fragments in this respect also include polyepitopes as defined herein.

In a particular embodiment of the invention, said nucleic acid construct complies with the rule of six (6) of the MV genome, *i.e.* the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, taken together with the cDNA molecule encoding the full-length, infectious antigenomic (+) RNA strand of the MV consist of a number of nucleotides that is a multiple of six.

The organization of the genome of MVs and their replication and transcription process have been fully identified in the prior art and are especially disclosed in Horikami S.M. and Moyer S.A. (*Curr. Top. Microbiol. Immunol. (1995) 191, 35-50*) or in Combredet C. *et al* (*Journal of Virology, Nov 2003, p11546-11554*) for the Schwarz vaccination strain of the virus or for broadly considered negative-sense RNA viruses, in Neumann G. et al (Journal of General Virology (2002) 83, 2635-2662).

The "*rule of six*" is expressed in the fact that the total number of nucleotides present in a nucleic acid representing the MV(+) strand RNA genome or in nucleic acid constructs comprising same is a multiple of six. The "rule of six" has been acknowledged in the state of the art as a requirement regarding the total number of nucleotides in the genome of the MV, which enables efficient or optimized replication of the MV genomic RNA. In the embodiments of the present invention defining a nucleic acid construct that meets the rule of six, said rule applies to the nucleic acid construct specifying the cDNA encoding the full-length MV (+) strand RNA genome and all inserted sequences, when taken individually or collectively. In this regard the rule of six applies to the cDNA encoding the full-length infectious antigenomic (+) RNA strand of the MV possibly and to the polynucleotide cloned into said cDNA and encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof.

In a particular embodiment of the invention, the nucleic acid construct comprises the following polynucleotides from 5' to 3':

(a) a polynucleotide encoding the N protein of the MV;

(b) a polynucleotide encoding the P protein of the MV;

(c) the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof;

(d) a polynucleotide encoding the M protein of the MV;

(e) a polynucleotide encoding the F protein of the MV;

(f) a polynucleotide encoding the H protein of the MV; and

(g) a polynucleotide encoding the L protein of the MV;

wherein said polynucleotides are operably linked in the nucleic acid construct and under a control of viral replication and transcription regulatory sequences such as MV leader and trailer sequences.

The expressions "*N protein*", "*P protein*", "*M protein*", "F protein" , "*H protein*" and "*L protein*" refer respectively to the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin protein (H) and the RNA polymerase large protein (L) of a MV. These components have been identified in the prior art and are especially disclosed in Fields, Virology (*Knipe & Howley, 2001*).

In a preferred embodiment of the invention, the measles virus is an attenuated virus strain.

An "*attenuated strain*" of measles virus is defined as a strain that is avirulent or less virulent than the parent strain in the same host, while maintaining immunogenicity and possibly adjuvanticity when administered in a host *i.e.*, preserving immunodominant T and B cell epitopes and possibly the adjuvanticity such as the induction of T cell costimulatory proteins or the cytokine IL-12.

An attenuated strain of a MV accordingly refers to a strain which has been serially passaged on selected cells and, possibly, adapted to other cells to produce seed strains suitable for the preparation of vaccine strains, harboring a stable genome which would not allow reversion to pathogenicity

nor integration in host chromosomes. As a particular "attenuated strain", an approved strain for a vaccine is an attenuated strain suitable for the invention when it meets the criteria defined by the FDA (US Food and Drug Administration) *i.e.*, it meets safety, efficacy, quality and reproducibility criteria, after rigorous reviews of laboratory and clinical data (www.fda.gov/cber/vaccine/vacappr.htm).

Particular attenuated strains that can be used to implement the present invention and especially to derive the MV cDNA of the nucleic acid construct are the Schwarz strain, the Zagreb strain, the AIK-C strain and the Moraten strain. All these strains have been described in the prior art and access to them is provided in particular as commercial vaccines.

In a particular embodiment of the invention, the cDNA molecule is placed under the control of heterologous expression control sequences. The insertion of such a control for the expression of the cDNA, is favorable when the expression of this cDNA is sought in cell types which do not enable full transcription of the cDNA with its native control sequences.

In a particular embodiment of the invention, the heterologous expression control sequence comprises the T7 promoter and T7 terminator sequences. These sequences are respectively located 5' and 3' of the coding sequence for the full length antigenomic (+)RNA strand of MV and from the adjacent sequences around this coding sequence.

In a particular embodiment of the invention, the cDNA molecule, which is defined hereabove is modified i.e., comprises additional nucleotide sequences or motifs.

In a preferred embodiment, the cDNA molecule of the invention further comprises, at its 5'-end, adjacent to the first nucleotide of the nucleotide sequence encoding the full-length antigenomic (+)RNA strand of the MV approved vaccine strain, a GGG motif followed by a hammerhead ribozyme sequence and which comprises, at its 3'-end, adjacent to the last nucleotide of said nucleotide sequence encoding the full length anti-genomic (+)RNA strand, the sequence of a ribozyme. The Hepatitis delta virus ribozyme (δ) is appropriate to carry out the invention.

The GGG motif placed at the 5' end, adjacent to the first nucleotide of the above coding sequence improves the efficiency of the transcription of said cDNA coding sequence. As a requirement for the proper assembly of measles virus particles is the fact that the cDNA encoding the antigenomic (+)RNA of the nucleic acid construct of the invention complies with the rule of six, when the GGG motif is added, a ribozyme is also added at the 5' end of the coding sequence of the cDNA, 3' from the GGG motif, in order to enable cleavage of the transcript at the first coding nucleotide of the full-length antigenomic (+)RNA strand of MV.

In a particular embodiment of the invention, in order to prepare the nucleic acid construct of the invention, the preparation of a cDNA molecule encoding the full-length antigenomic (+) RNA of a MV disclosed in the prior art is achieved by known methods. Said cDNA provides especially the genome vector when it is inserted in a vector such as a plasmid.

A particular cDNA molecule suitable for the preparation of the nucleic acid construct of the invention is the one obtained using the Schwarz strain of MV. Accordingly, the cDNA used within the present invention may be obtained as disclosed in WO2004/000876 or may be obtained from plasmid pTM-MVSchw deposited by Institut Pasteur at the Collection Nationale de Culture de Microorganismes (CNCM), 28 rue du Dr Roux, 75724 Paris Cedex 15, France, under No I-2889 on June 12, 2002, the sequence of which is disclosed in WO2004/000876 incorporated herein by reference. The plasmid pTM-MVSchw has been obtained from a Bluescript plasmid and comprises the polynucleotide coding for the full-length measles virus (+) RNA strand of the Schwarz strain placed under the control of the promoter of the T7 RNA polymerase. It has 18967 nucleotides and a sequence represented as SEQ ID NO: 1. cDNA molecules (also designated cDNA of the measles virus or MV cDNA for convenience) from other MV strains may be similarly obtained starting from the nucleic acid purified from viral particles of attenuated MV such as those described herein.

The cDNA used within the present invention may also be obtained from plasmid pTM2-MVSchw-gfp deposited by Institut Pasteur at the Collection

Nationale de Culture de Microorganismes (CNCM), 28 rue du Dr Roux, 75724 Paris Cedex 15, France, under No I-2890 on June 12, 2002. It has 19795 nucleotides and a sequence represented as SEQ ID NO: 2. This plasmid contains the sequence encoding the eGFP marker that may be deleted.

The nucleic acid construct of the invention is suitable and intended for the preparation of recombinant infectious replicative measles – Zika virus (MV-ZIKV) and accordingly said nucleic acid construct is intended for insertion in a transfer genome vector that as a result comprises the cDNA molecule of the measles virus, especially of the Schwarz strain, for the production of said MV-ZIKV and yield of at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, in particular ZIKV VLPs. The pTM-MVSchw plasmid or the pTM2-MVSchw plasmid is suitable to prepare the transfer vector, by insertion of the ZIKV polynucleotide(s) necessary for the expression of at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof. The recombinant infectious replicating MV-ZIKV particles may be recovered from rescue helper cells or in production cells and may optionally be recovered with VLP expressing the ZIKV antigens disclosed in accordance with the invention.

The invention thus relates to a transfer vector, which is used for the preparation of recombinant MV-ZIKV particles when rescued from helper cells. Advantageously, the transfer vector of the invention is a transfer vector plasmid suitable for transfection of said helper cells or of production cells, comprising the nucleic acid construct of the invention, in particular is a plasmid obtained from a Bluescript plasmid, such as pMV-ZIKV.

In a particular embodiment of the invention, the transfer vector plasmid has the sequence of SEQ ID NO: 165, SEQ ID NO: 166 or SEQ ID NO: 167, preferably has the sequence of SEQ ID NO: 165.

The invention also concerns the use of said transfer vector to transform cells suitable for rescue of viral MV-ZIKV particles, in particular to transfect or to transduce such cells respectively with plasmids or with viral vectors

harboring the nucleic acid construct of the invention, said cells being selected for their capacity to express required MV proteins for appropriate replication, transcription and encapsidation of the recombinant genome of the virus corresponding to the nucleic acid construct of the invention in recombinant infectious replicating MV-ZIKV particles.

In a preferred embodiment, the invention relates to transformed cells comprising inserted in their genome the nucleic acid construct according to the invention or comprising the transfer vector plasmid according to the invention, wherein said cells are in particular eukaryotic cells, such as avian cells, in particular CEF cells, mammalian cells such as HEK293 cells or yeast cells.

Polynucleotides are thus present in said cells, which encode proteins that include in particular the N, P and L proteins of a MV (*i.e.*, native MV proteins or functional variants thereof capable of forming ribonucleoprotein (RNP) complexes), preferably as stably expressed proteins at least for the N and P proteins functional in the transcription and replication of the recombinant viral MV-ZIKV particles. The N and P proteins may be expressed in the cells from a plasmid comprising their coding sequences or may be expressed from a DNA molecule inserted in the genome of the cell. The L protein may be expressed from a different plasmid. It may be expressed transitory. The helper cell is also capable of expressing a RNA polymerase suitable to enable the synthesis of the recombinant RNA derived from the nucleic acid construct of the invention, possibly as a stably expressed RNA polymerase. The RNA polymerase may be the T7 phage polymerase or its nuclear form (nlsT7).

In an embodiment of the invention, the cDNA clone of MV is from the same MV strain as the N protein and/or the P protein and/or the L protein. In another embodiment of the invention, the cDNA clone of a MV is from a different strain of virus than the N protein and/or the P protein and/or the L protein.

The invention also relates to a process for the preparation of recombinant infectious measles virus (MV) particles comprising:

1) transferring, in particular transfecting, the nucleic acid construct of the invention or the transfer vector containing such nucleic acid construct in a

helper cell line which also expresses proteins necessary for transcription, replication and encapsidation of the antigenomic (+)RNA sequence of MV from its cDNA and under conditions enabling viral particles assembly; and

2) recovering the recombinant infectious MV-ZIKV particles expressing at least (i) the prM protein of a ZIKV, and the E protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof.

In a particular embodiment of the invention, this process comprises:

1) transfecting helper cells with a nucleic acid construct according to the invention and with a transfer vector, wherein said helper cells are capable of expressing helper functions to express an RNA polymerase, and to express the N, P and L proteins of a MV virus ;
2) co-cultivating said transfected helper cells of step 1) with passaged cells suitable for the passage of the MV attenuated strain from which the cDNA originates ;
3) recovering the recombinant infectious MV-ZIKV particles expressing at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof.

In another particular embodiment of the invention, the method for the production of recombinant infectious MV-ZIKV particles comprises :

1) recombining a cell or a culture of cells stably producing a RNA polymerase, the N protein of a MV and the P protein of a MV, with a nucleic acid construct of the invention and with a vector comprising a nucleic acid encoding the L protein of a MV, and

2) recovering the recombinant infectious MV-ZIKV particles from said recombinant cell or culture of recombinant cells.

In a particular embodiment of said process, recombinant MV are produced, which express at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, in particular ZIKV VLPs expressing the same ZIKV protein(s).

Preferably, the invention relates to a process to rescue recombinant infectious measles virus-Zika virus (MV-ZIKV) particles expressing at least (i) the precursor of membrane (prM) protein of a ZIKV, and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof, and ZIKV VLPs expressing the same ZIKV protein(s), comprising:

1) co-transfecting helper cells, in particular HEK293 helper cells, that stably express T7 RNA polymerase, and measles N and P proteins with (i) the transfer vector plasmid according to the invention and with (ii) a vector, especially a plasmid, encoding the MV L polymerase;

2) cultivating said co-transfected helper cells in conditions enabling the production of recombinant MV-ZIKV particles;

3) propagating the thus produced recombinant MV-ZIKV particles by co-cultivating said helper cells of step 2) with cells enabling said propagation such as Vero cells;

4) recovering replicating infectious replicating MV-ZIKV particles expressing at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, and ZIKV VLPs expressing the same ZIKV protein(s).

According to a particular embodiment of said process, the transfer vector plasmid has the sequence of SEQ ID NO: 165, SEQ ID NO: 166 or SEQ ID NO: 167, preferably has the sequence of SEQ ID NO: 165.

As used herein, the term "*recombining*" means introducing at least one polynucleotide into a cell, for example under the form of a vector, said polynucleotide integrating (entirely or partially) or not integrating into the cell genome (such as defined above).

According to a particular embodiment, recombination can be obtained with a first polynucleotide, which is the nucleic acid construct of the invention. Recombination can, also or alternatively, encompasses introducing a polynucleotide, which is a vector encoding a RNA polymerase large protein (L) of a MV, whose definition, nature and stability of expression has been described herein.

In accordance with the invention, the cell or cell lines or a culture of cells stably producing a RNA polymerase, a nucleoprotein (N) of a measles virus and a polymerase cofactor phosphoprotein (P) of a measles virus is a cell or cell line as defined in the present specification or a culture of cells as defined in the present specification, *i.e.*, are also recombinant cells to the extent that they have been modified by the introduction of one or more polynucleotides as defined above. In a particular embodiment of the invention, the cell or cell line or culture of cells, stably producing the RNA polymerase, the N and P proteins, does not produce the L protein of a measles virus or does not stably produce the L protein of a measles virus, *e.g.*, enabling its transitory expression or production.

The production of recombinant infectious replicating MV-ZIKV particles of the invention may involve a transfer of cells transformed as described herein. The term "*transfer*" as used herein refers to the plating of the recombinant cells onto a different type of cells, and particularly onto monolayers of a different type of cells. These latter cells are competent to sustain both the replication and the production of infectious MV-ZIKV particles, *i.e.*, respectively the formation of infectious viruses inside the cell and possibly the release of these infectious viruses outside of the cells. This transfer results in the co-culture of the recombinant cells of the invention with competent cells as defined in the previous sentence. The above transfer may be an additional, *i.e.*, optional, step when the recombinant cells are not efficient virus-producing culture, *i.e.*, when infectious MV-ZIKV particles cannot be efficiently recovered from these recombinant cells. This step is introduced after further recombination of the recombinant cells of the invention with nucleic acid construct of the invention, and optionally a vector comprising a nucleic acid encoding a RNA polymerase large protein (L) of a measles virus.

In a particular embodiment of the invention, a transfer step is required since the recombinant cells, usually chosen for their capacity to be easily recombined are not efficient enough in the sustaining and production of recombinant infectious MV-ZIKV particles. In said embodiment, the cell or cell

line or culture of cells of step 1) of the above-defined methods is a recombinant cell or cell line or culture of recombinant cells according to the invention.

Cells suitable for the preparation of the recombinant cells of the invention are prokaryotic or eukaryotic cells, particularly animal or plant cells, and more particularly mammalian cells such as human cells or non-human mammalian cells or avian cells or yeast cells. In a particular embodiment, cells, before recombination of its genome, are isolated from either a primary culture or a cell line. Cells of the invention may be dividing or non-dividing cells.

According to a preferred embodiment, helper cells are derived from human embryonic kidney cell line 293, which cell line 293 is deposited with the ATCC under No. CRL-1573. Particular cell line 293 is the cell line disclosed in the international application WO2008/078198 and referred to in the following examples.

According to another aspect of this process, the cells suitable for passage are CEF cells. CEF cells can be prepared from fertilized chicken eggs as obtained from EARL Morizeau, 8 rue Moulin, 28190 Dangers, France, or from any other producer of fertilized chicken eggs.

The process which is disclosed according to the present invention is used advantageously for the production of infectious replicative MV-ZIKV particles and optionally VLPs expressing ZIKV antigens appropriate for use as immunization compositions.

The invention thus relates to an immunogenic composition whose active principle comprises infectious replicative MV-ZIKV particles rescued from the nucleic acid construct of the invention and in particular obtained by the process disclosed.

The nucleic acid construct of the invention and the MV-CHIKV of the invention encode or express at least (i) the prM protein of a ZIKV, and the E protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof.

By "protein of a ZIKV" is meant a “protein” as defined herein, the sequence of which is identical to a counterpart in a strain of ZIKV, including a polypeptide which is a native mature or precursor of a protein of ZIKV or is a

fragment thereof or a mutant thereof as defined herein. In the present invention, a "*protein of a ZIKV*" is in particular an antigen (prM or E or their derivatives as disclosed herein) designed using a consensus sequence for the ZIKV. In particular, said antigen is designed using the consensus amino acid sequence of Zika viruses as observed circulating from 2015 and onward, notably to include the S139N change that generated a novel potential N glycosylation site in prM that was absent from the African lineage, and the V763M in E. Thus the inventors included this S139N mutation that was present in all Asian lineage sequences, but did not include single mutations in particular isolates. The inventors observed that the amino acid sequence of the Asian strain BeH818995 (GenBank: KU365777) corresponded to the consensus amino acid sequence of Zika viruses as observed circulating from 2015 and onward.

In particular a fragment or a mutant having at least 50%, at least 80%, in particular advantageously at least 90% or preferably at least 95% amino acid sequence identity to a naturally occurring ZIKV capsid or envelope protein. Amino acid sequence identity can be determined by alignment by one skilled in the art using manual alignments or using the numerous alignment programs available. Fragments or mutants of ZIKV proteins of the invention may be defined with respect to the particular amino acid sequences illustrated herein.

According to a preferred embodiment, the invention also concerns modifications and optimization of the polynucleotide to allow an efficient expression of the at least (i) prM of ZIKV, and E protein of ZIKV or truncated version thereof, or (ii) E protein of ZIKV or truncated version thereof, at the surface of chimeric infectious particles of MV-ZIKV in the host, in particular the human host.

According to this embodiment, optimization of the polynucleotide sequence can be operated avoiding cis-active domains of nucleic acid molecules: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; ARE, INS, CRS sequence elements; repeat

sequences and RNA secondary structures ; cryptic splice donor and acceptor sites, branch points.

The optimized polynucleotides may also be codon optimized for expression in a specific cell type. This optimization allows increasing the efficiency of chimeric infectious particles production in cells without impacting the expressed protein(s).

In a particular embodiment of the invention, the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, has been optimized for a Macaca codon usage or has been optimized for a human codon usage.

The optimization of the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof may be performed by modification of the wobble position in codons without impacting the identity of the amino acid residue translated from said codon with respect to the original one.

Optimization is also performed to avoid editing-like sequences from Measles virus. The editing of transcript of Measles virus is a process which occurs in particular in the transcript encoded by the *P* gene of Measles virus. This editing, by the insertion of extra G residues at a specific site within the *P* transcript, gives rise to a new protein truncated compared to the P protein. Addition of only a single G residue results in the expression of the V protein, which contains a unique carboxyl terminus (*Cattaneo R et al., Cell. 1989 Mar 10;56(5):759-64*).

In a particular embodiment of the invention, *measles editing-like sequences* have been deleted from said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof. The following *measles editing-like sequences* can be mutated: AAAGGG, AAAAGG, GGGAAA, GGGGAA, TTAAA, AAAA, as well as their complementary sequence: TTCCCC, TTTCCC, CCTTTT, CCCCTT, TTTAA,

TTTT. For example, AAAGGG can be mutated in AAAGGC, AAAAGG can be mutated in AGAAGG or in TAAAGG or in GAAAGG, and GGGAAA in GCGAAA.

In a particular embodiment of the invention, the native and codon-optimized nucleotide sequences of the polynucleotide encoding particular peptides/proteins/antigen as well as the amino acid sequences of these peptides/proteins/antigen of the invention are the sequences disclosed as SEQ ID Nos: 3-164 and mentioned in Table 1 below. These sequences are also represented in **Figures 3A-3D**.

In a particular embodiment of the invention, the transfer vector plasmid pTM2-MVSchw_A1_Zikasp_ZikaprME has the optimized sequence of SEQ ID NO: 165, the transfer vector plasmid pTM2-MVSchw_insert 4 has the native sequence of SEQ ID NO: 166 and the transfer vector plasmid pTM2-MVSchw_insert 5 has the native sequence of SEQ ID NO: 167, as mentioned in Table 1 below.

In another particular embodiment of the invention, the native nucleotide sequences of the polynucleotide encoding insert 4 or insert 5 as well as the amino acid sequences of said insert 4 or insert 5 of the invention are the sequences disclosed as SEQ ID Nos: 168-171 and mentioned in Table 1 below. Insert 4 (SEQ ID NO: 169) is similar to Zikasp_Zika_prMEd404 (SEQ ID NO: 54) but with a shorter sp in 5'. Insert 5 (SEQ ID NO: 171) is similar to Zikasp'_ZikaEd445 (SEQ ID NO: 75) with 2 minor differences in 5'.

Table 1. Native and codon-optimized nucleotide sequences of the polynucleotide encoding particular peptides/proteins as well as amino acid sequences of these peptides/proteins used in the invention.

Name of the compound, *i.e.* peptide/protein/antigen (abbreviation)	SEQ ID NO of the native nucleotide sequence of the polynucleotide encoding the compound	SEQ ID NO of the codon-optimized nucleotide sequence of the polynucleotide encoding the compound	SEQ ID NO of the amino acid sequence of the compound
Signal peptide from the capsid of ZIKV (**sp**)	3	4	5
Signal peptide from the membrane protein of ZIKV (**sp'**)	6	7	8
Signal peptide from the capsid of JEV (**JEVsp**)	9	10	11
Signal peptide from the fusion protein of MV (**MVsp**)	12	13	14
Modified signal peptide from the fusion protein of MV (**MVsp'**)	15	16	17
Precursor of membrane (**prM**) protein of ZIKV	18	19	20
Full-length **E** protein of ZIKV	21	22	23
E protein of ZIKV truncated at amino acid position 456 (**Ed456**)	24	25	26
E protein of ZIKV truncated at amino acid position 445 (**Ed445**)	27	28	29
E protein of ZIKV truncated at amino acid position 404 (**Ed404**)	30	31	32
E stem region of ZIKV	33	34	35
Intermediate domain between E stem and E anchor regions of ZIKV	36	37	38
E anchor region of ZIKV	39	40	41
Transmembrane (TM) and intracytoplasmic tail of MV F protein	42	43	44
Zikasp_ZikaprME protein (A1)	45	46	47
Zikasp_Zika_prMEd456 protein (A2)	48	49	50
Zikasp_Zika_prMEd445 protein (A3)	51	52	53
Zikasp_Zika_prMEd404 protein (A4)	54	55	56
Zikasp_ZikaE protein (A5)	57	58	59
Zikasp_ZikaEd456 protein (A6)	60	61	62
Zikasp_ZikaEd445 protein (A7)	63	64	65
Zikasp_ZikaEd404 protein (A8)	66	67	68
Zikasp'_ZikaE protein (A9)	69	70	71
Zikasp'_ZikaEd456 protein (A10)	72	73	74
Zikasp'_ZikaEd445 protein (A11)	75	76	77
Zikasp'_ZikaEd404 protein (A12)	78	79	80
JEVsp_ZikaprME protein (B1)	81	82	83
JEVsp_Zika_prMEd456 protein (B2)	84	85	86

JEVsp_Zika_prMEd445 protein (B3)	87	88	89
JEVsp_Zika_prMEd404 protein (B4)	90	91	92
JEVsp_ZikaE protein (B5)	93	94	95
JEVsp_ZikaEd456 protein (B6)	96	97	98
JEVsp_ZikaEd445 protein (B7)	99	100	101
JEVsp_ZikaEd404 protein (B8)	102	103	104
MVsp_ZikaprME (C1)	105	106	107
MVsp_Zika_prMEd456 (C2)	108	109	110
MVsp_Zika_prMEd445 (C3)	111	112	113
MVsp_Zika_prMEd404 (C4)	114	115	116
MVsp_ZikaE (C5)	117	118	119
MVsp_ZikaEd456 (C6)	120	121	122
MVsp_ZikaEd445 (C7)	123	124	125
MVsp_ZikaEd404 (C8)	126	127	128
MVsp_ZikaprME_MVTMintracyto (C9)	129	130	131
MVsp_Zika_MVTMintracytoE (C10)	132	133	134
MVsp'_ZikaprME (D1)	135	136	137
MVsp'_Zika_prMEd456 (D2)	138	139	140
MVsp'_Zika_prMEd445 (D3)	141	142	143
MVsp'_Zika_prMEd404 (D4)	144	145	146
MVsp'_ZikaE (D5)	147	148	149
MVsp'_ZikaEd456 (D6)	150	151	152
MVsp'_ZikaEd445 (D7)	153	154	155
MVsp'_ZikaEd404 (D8)	156	157	158
MVsp'_ZikaprME_MVTMintracyto (D9)	159	160	161
MVsp'_Zika_MVTMintracytoE (D10)	162	163	164

Name of the transfer vector plasmid	SEQ ID NO
pTM2-MVSchw_A1_Zikasp_ZikaprME (optimized sequence)	165
pTM2-MVSchw_insert 4 (native sequence)	166
pTM2-MVSchw_insert 5 (native sequence)	167

Name of the compound, *i.e.* peptide/protein/antigen (abbreviation)	SEQ ID NO of the native nucleotide sequence of the polynucleotide encoding the compound	SEQ ID NO of the amino acid sequence of the compound
Insert 4	168	169
Insert 5	170	171

In a particular embodiment of the invention, said ZIKV is from the African lineage, in particular from the African strain ArB1362 (GenBank: KF383115) or African isolate IbH_30656 (GenBank: HQ234500), or from the Asian lineage, in particular from the Asian strain BeH818995 (GenBank: KU365777), preferably is from ZIKV strains that circulated in the Pacific and Americas since 2013.

In another particular embodiment of the invention, said ZIKV corresponds to various lineages of ZIK viruses including strains that circulated in the Pacific and Americas since 2013.

In a preferred embodiment of the invention, the prM protein of the ZIKV has an amino acid sequence which is a consensus amino acid sequence representative of the prM sequences of a selection of various strains of ZIKV including from the Asian lineage, in particular is from the ZIKV strain BeH818995. The E protein of the ZIKV or the truncated version thereof has an amino acid sequence which is a consensus amino acid sequence representative of the E sequences of a selection of various strains of ZIKV including from the Asian lineage, in particular is from the ZIKV strain BeH818995.

In a particular embodiment of the invention, said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV (sp) or the signal peptide from the capsid of a JEV (JEVsp) or the signal peptide from the fusion protein of MV (MVsp) or the modified signal peptide from the fusion protein of MV (MVsp') and the signal peptide from the membrane protein of the ZIKV (sp'), or

said polynucleotide encoding at least (ii) the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV (sp) or the signal peptide from the membrane protein of the ZIKV (sp') or the signal peptide from the capsid of a JEV (JEVsp) or the signal peptide from the fusion protein of MV (MVsp) or the modified signal peptide from the fusion protein of MV (MVsp').

In a preferred embodiment of the invention, said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV and the signal peptide from the membrane protein of the ZIKV, or

said polynucleotide encoding at least (ii) the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV or the signal peptide from the membrane protein of the ZIKV.

In a particular embodiment of the invention, the polynucleotide encoding the E protein encodes either the full-length E protein or its soluble form lacking the two C-terminal transmembrane domains of the full-length E protein.

In a preferred embodiment of the invention, the polynucleotide encoding the truncated version of the E protein is selected from the group consisting of (i) the polynucleotide encoding the E protein truncated at amino acid position 456 of the full-length E protein of the ZIKV, *i.e.* the E protein lacking the anchor region and the intermediate domain between the stem and anchor regions, (ii) the polynucleotide encoding the E protein truncated at position 445 of the full-length E protein of the ZIKV, *i.e.* the E protein lacking the anchor region, the intermediate domain between the stem and anchor regions and a fragment of the second helix that composed the stem region, and (iii) the polynucleotide encoding the E protein truncated at position 404 of the full-length E protein of the ZIKV, *i.e.* the E protein lacking the stem region, the intermediate domain between the stem and anchor regions, and the anchor region.

In a preferred embodiment of the invention, the polynucleotide encodes the prM protein of the ZIKV whose sequence is SEQ ID NO: 20, and the polynucleotide encodes the E protein of the ZIKV or the truncated version thereof whose sequence is selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 29 and SEQ ID NO: 32.

In a preferred embodiment of the invention, the polynucleotide encoding the prM protein of the ZIKV has the sequence of SEQ ID NO: 19, and the polynucleotide encoding the E protein of the ZIKV or a truncated version

thereof has a sequence selected from the group consisting of SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 and SEQ ID NO: 31.

In a particular embodiment of the invention, said nucleic acid construct comprises a sequence selected from the group consisting of SEQ ID NOs: 45-164 and 168-171.

In a preferred embodiment of the invention, said nucleic acid construct comprises a sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 70, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 168 and SEQ ID NO: 170, preferably has the sequence of SEQ ID NO: 46, SEQ ID NO: 55 or SEQ ID NO: 76, more preferably has the sequence of SEQ ID NO: 46.

In a preferred embodiment of the invention, the nucleic acid construct comprises the sequence from nucleotide at position 83 to nucleotide at position 18404 in the sequence of SEQ ID NO: 165, or the sequence from nucleotide at position 83 to nucleotide at position 18074 in the sequence of SEQ ID NO: 166, or the sequence from nucleotide at position 83 to nucleotide at position 17702 in the sequence of SEQ ID NO: 167.

The invention also concerns recombinant infectious replicating measles virus-Zika virus (MV-ZIKV) particles, which comprise as their genome a nucleic acid construct according to the invention.

In a particular embodiment of the invention, said recombinant infectious replicating MV-ZIKV particles are rescued from a helper cell line expressing an RNA polymerase recognized by said cell line, for example a T7 RNA polymerase, a nucleoprotein (N) of a MV, a phosphoprotein (P) of a MV, and optionally an RNA polymerase large protein (L) of a MV, and which is further transfected with the transfer vector plasmid according to the invention.

Said recombinant infectious replicating MV-ZIKV particles are thus produced by a method comprising expressing the nucleic acid construct according to the invention in a host cell comprising an RNA polymerase recognized by said host cell, for example a T7 RNA polymerase, a nucleoprotein (N) of a MV, a phosphoprotein (P) of a MV, and optionally an RNA polymerase large protein (L) of a MV.

According to a particular embodiment of the invention, said particles comprise in their genome a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 70, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 168 and SEQ ID NO: 170, preferably has the sequence of SEQ ID NO: 46, SEQ ID NO: 55 or SEQ ID NO: 76, more preferably has the sequence of SEQ ID NO: 46.

The obtained at least (i) prM protein of the ZIKV, and E protein of the ZIKV or truncated version thereof, or (ii) E protein of the ZIKV or truncated version thereof, are also able to auto-assemble into ZIK virus-like-particles (VLPs), with the MV-ZIKV particles.

As used herein, the term "*virus-like particle*" (VLP) refers to a structure that in at least one attribute resembles a virus but which has not been demonstrated to be infectious as such. VLPs in accordance with the invention do not carry genetic information encoding the proteins of the VLPs, in general, VLPs lack a viral genome and, therefore, are non-infectious and non-replicative. In accordance with the present invention, VLPs can be produced in large quantities and are expressed together with recombinant infectious MV-ZIKV particles. Said VLPs are VLPs of ZIKV.

According to another aspect, the invention relates to recombinant infectious MV-ZIKV particles expressing at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, in particular by reference to their nucleic acid and polypeptide sequences. The recombinant infectious MV-ZIKV advantageously expresses at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, as VLPs.

The invention also relates to a composition or an assembly of active ingredients comprising the recombinant infectious replicating MV-ZIKV particles according to the invention, and a pharmaceutically acceptable vehicle.

The invention also concerns the association, in a composition, of VLPs comprising at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, with recombinant infectious replicating MV-ZIKV-MV particles.

According to a preferred embodiment of the invention, the recombinant MV vector is designed in such a way and the production process involves cells such that the virus particles produced in helper cells transfected or transformed with said vector, originated from a MV strain adapted for vaccination, enable the production of recombinant infectious replicating MV and the production of ZIKV-VLPs for use in immunogenic compositions, preferably protective or even vaccine compositions.

Advantageously, the genome of the recombinant infectious MV-ZIKV particles of the invention is replication competent. By "replication competent', it is meant a nucleic acid, which when transduced into a helper cell line expressing the N, P and L proteins of a MV, is able to be transcribed and expressed in order to produce new viral particles.

Replication of the recombinant virus of the invention obtained using MV cDNA for the preparation of the recombinant genome of MV-ZIKV can also be achieved *in vivo* in the host, in particular the human host to which recombinant MV-ZIKV is administered.

The invention also concerns a composition or an assembly of active ingredients comprising the recombinant infectious replicating MV-ZIKV particles according to the invention, in association with ZIKV-VLPs expressing the same ZIKV protein(s) as said MV-ZIKV particles.

According to a preferred embodiment of the invention, said composition or assembly of active ingredients is used in the elicitation of an immune response, in particular a protective immune response, against ZIKV by the elicitation of antibodies directed against said ZIKV protein(s), and/or of a cellular immune response, in a host, in particular a human host in need thereof.

Said composition or assembly of active ingredients accordingly may comprise a suitable vehicle for administration *e.g.* a pharmaceutically

acceptable vehicle to a host, especially a human host and may further comprise but not necessarily adjuvant to enhance immune response in a host. The inventors have indeed shown that the administration of the active ingredients of the invention may elicit an immune response without the need for adjuvantation.

According to a particular embodiment of the invention, said composition or assembly of active ingredients comprises a pharmaceutically acceptable vehicle.

The invention relates in particular to a composition, in particular an immunogenic composition, preferably a vaccine composition for administration to children, adolescents or travelers.

In a particular embodiment, said composition or vaccine is used for preventive protection against African and Asian strains of ZIKV.

Said composition or vaccine is used for protection against ZIKV infection or against clinical outcomes of infection by ZIKV (protection against ZIKV disease) in a prophylactic treatment. Such a vaccine composition has advantageously active principles (active ingredients) which comprise recombinant infectious replicating MV-ZIKV particles rescued from the vector as defined herein optionally associated with VLPs comprising the same ZIKV proteins.

In the context of the invention, the terms “associated” or “*in association*” refer to the presence, in a unique composition, of both recombinant infectious replicating MV-ZIKV particles and the above-mentioned ZIKV proteins, in particular as VLPs, usually as physically separate entities.

The invention also concerns the recombinant infectious replicating MV-ZIKV particles according to the invention in association with the above-mentioned ZIKV proteins, in particular in association with ZIKV-VLPs expressing the same ZIKV proteins, or the composition or the assembly of active ingredients according to the invention, for use in the prevention of an infection by ZIKV in a subject, or in the prevention of clinical outcomes of infection by ZIKV in a subject, in particular in a human.

The invention also concerns the recombinant infectious replicating MV-ZIKV particles according to the invention in association with the above-mentioned ZIKV proteins, in particular in association with ZIKV-VLPs expressing the same ZIKV proteins, for use in an administration scheme and according to a dosage regime that elicit an immune response, advantageously a protective immune response, against ZIKV infection or induced disease, in particular in a human host.

The administration scheme and dosage regime may require a unique administration of a selected dose of the recombinant infectious replicating MV-ZIKV particles according to the invention in association with the above-mentioned ZIKV proteins, in particular in association with ZIKV-VLPs expressing the same ZIKV proteins.

Alternatively it may require multiple administration doses in a prime-boost regimen. Priming and boosting may be achieved with identical active ingredients consisting of said recombinant infectious replicating MV-ZIKV particles in association with the above-mentioned ZIKV proteins, in particular in association with ZIKV-VLPs expressing the same ZIKV proteins.

Alternatively priming and boosting administration may be achieved with different active ingredients, involving said recombinant infectious replicating MV-ZIKV particles in association with the above-mentioned ZIKV proteins, in particular in association with ZIKV-VLPs expressing the same ZIKV proteins, in at least one of the administration steps and other active immunogens of ZIKV, such as the above-mentioned ZIKV proteins or ZIKV-VLPs expressing the same ZIKV proteins, in other administration steps.

Administration of recombinant infectious replicating MV-ZIKV particles according to the invention in association with ZIKV-VLPs expressing the same ZIKV proteins elicits an immune response and especially elicits antibodies that are cross-reactive for various ZIKV strains. Accordingly, it has been shown that administration of the active ingredients according to the invention, when prepared with the coding sequences of a particular strain of ZIKV, can elicit an immune response against a group of strains of ZIKV.

Considering available knowledge on doses of vaccines suitable for other pathogens (such as HBV or HPV) which involve the administration of VLPs and also for known human MV vaccines, the inventors have determined that the recovery of ZIKV-VLPs with the recombinant MV-ZIKV enables proposing administration of effective low doses of the active ingredients. Indeed, considering that the recombinant MV-ZIKV enables production of around 10^4 ZIKV-VLPs per recombinant infectious replicating MV-ZIKV particle, and considering that the currently known doses for human MV vaccines are in the range of 10^3 to 10^5 TCID50, a suitable dose of recombinant MV-ZIKV to be administered may be in the range of 0.1 to 10ng, in particular 0.2 to 6ng, and possibly as low as 0.2 to 2ng. For comparison doses of VLPs administered in the case of HBV or HPV vaccines are in the range of 10µg which means that a dose of recombinant MV-ZIKV vaccine could comprise around 2 000 or up to 5 000 to 10 000 times less VLPs.

According to a particular embodiment of the invention, the immunogenic or vaccine composition defined herein may also be used for protection against an infection by the measles virus.

Other features and advantages of the invention will be apparent from the examples which follow and will also be illustrated in the figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of Zika virus genome.

Figure 2. Phylogenetic trees of the main human pathogenic flaviviruses based on the amino acid sequences of the E protein (left) and of the polymerase NS5 protein (right). JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; POWV, Powassan virus; SLEV, Saint Louis encephalitis virus; TBEV, tick-borne encephalitis virus; YFV, yellow fever virus; WNV, West Nile virus.

Figure 3. Schematic representation of Zika virus antigens. Protein domains are drawn to scale. Zika, Zika virus; JEV, Japanese encephalitis virus; MV, Measles virus. A. 12 variants of the Zika antigen, where the native signal peptide from the capsid (sp) or from the membrane protein (sp') of Zika virus is used. B. 8 variants of the chimeric JEV-Zika antigen, where a signal peptide of the capside of JEV is used. C. 10 variants of the MV-Zika antigen, where the signal peptide of the fusion protein of MV (MVsp) is used. D. 10 variants of the MV Zika antigen, where a modified signal peptide of the fusion protein of MV (MVsp') is used.

Figure 4. **Schematic representation of MV Vector.** MV genes are indicated: N (nucleoprotein), PVC (phoshoprotein and V/C proteins), M (matrix), F (fusion), H (hemagluttinin), L (polymerase), T7 (T7 RNA polymerase promoter), hh (hammerhead ribozyme), T7t (T7 RNA polymerase terminator), ∂ (hepatitis delta virus ribozyme), red arrows (additional transcription units).

Figure 5. **Single immunization in mice.** A) Zika antibody response measured in mice sera by ELISA at one month after a single immunization. MV-prMEd404 (native sequence, insert 4); MV-ssEd445 (native sequence, insert 5). B) Survival of immunized mice after challenge by Zika virus. C) Zika virus viremia in serums of immunized mice (determined by RT-qPCR) at different days after challenge. D) IFN-gamma Elispot detected in splenocytes of mice one week after immunization with MV-Zika or control MVSchw viruses. Elispots are detected against MV (Schwarz), Zika virus (Zika) and Concanavalin A as a control.

Figure 6. **Prime-boost immunization in mice.** A) Zika antibody response measured in mice sera by ELISA at day 30, 45 and 55 after two immunizations. B) Detection of Zika virus neutralizing antibodies in the sera of mice immunized with two injections of MV-prMEd404 (native sequence, insert 4), MV-ssEd445 (native sequence, insert 5). C) Survival of immunized mice after challenge with

low dose of Zika virus. D) Zika virus viremia in serums of immunized mice (determined by RT-qPCR) at different days after challenge.

Figure 7. Recombinant MV expressing the full-length prME Zika antigen (construct A1) produces Zika VLPs. Vero cells were infected with three different clones of rMV-Zika_A1 (1, 2, 3) for 48 hours. Cell lysates and medium were collected. Supernatant medium was clarified by low-speed centrifugation (1500 rpm) then concentrated by ultracentrifugation on a 20% sucrose cushion for 3 hours (36000 rpm). All material was analyzed by western blot to detect the Zika E protein (50 kD) with 4G2 panflavi monoclonal antibody. (A) Cell lysates, (B) Concentrated medium, (C) Non-concentrated medium and positive and negative controls. Positive control is a lysate of Vero cells transfected for 48 hours with pcDNA5 plasmid expressing the Zika A1 antigen. The positive E protein recovered in panel B after ultracentrifugation demonstrates that high density VLPs were produced in the supernatant of infected Vero cells.

Figure 8. Zika virus antigen expression assay. HEK293T cells were transfected with each codon-optimized construct, and cell lysates and medium were collected after 48h. Supernatant medium was clarified by low-speed centrifugation (1500 rpm), and then a fraction was concentrated by ultracentrifugation on a 20% sucrose cushion for 3 hours (36000 rpm). All material was analyzed by western blot to detect the Zika virus E protein (~50 kD) with the 4G2 pan-flavivirus antibody. (L) Cell lysates, (S) non-concentrated medium, and (U) ultracentrifugated medium.

Figure 9. Expression of Zika virus antigen A1 from measles vector and growth curve of recombinant MV-Zika-A1. (A) Immunofluorescence analysis showed large syncytia in Vero cells infected for 24 hours with MV-Zika-A1 (the Zika virus E protein was detected with the 4G2 pan-flavivirus antibody). (B) Replication of recombinant MV-Zika-A1 virus on Vero cells at 32°C after infection with a multiplicity of infection of 0.01 (titers were determined by limiting dilution and the Karber method).

Figure 10. Antibody response to ZIKV in immunized CD46-IFNAR$^{-/-}$ mice. The antibody titers against ZIKV EDIII were determined using indirect ELISA in mice sera collected after prime and boost with MV-ZIKV-A1, MV-prMEd404 (native sequence, insert 4), MV-ssEd445 (native sequence, insert 5), MV-ZIKV-A12 or control empty MV-Schwarz. Readings from wells coated with mock antigens were subtracted from wells with ZIKV-EDIII and the ZIKV specific IgG titers were calculated as the reciprocal of the highest dilution of an individual serum giving an absorbance of 0.5. A strong antibody response to ZIKV was induced in immunized mice with slightly higher values for A1 (highly reproducible) and A12 (more variability).

Figure 11. ZIKV neutralizing antibody titers in immunized CD46-IFNAR$^{-/-}$ mice. Neutralizing antibody titers against ZIKV were determined by using plaque reduction neutralizing tests ($PRNT_{50}$) in mice sera collected after last boost with MV-ZIKV-A1, MV-prMEd404 (native sequence, insert 4), MV-ssEd445 (native sequence, insert 5), MV-ZIKV-A12 or control empty MV-Schwarz and before challenge. The strongest neutralizing titers were observed with the MV-ZIKV-A1 construct.

Figure 12. Protection of immunized CD46-IFNAR$^{-/-}$ mice from ZIKV non-lethal challenge. Mice immunized twice with MV-ZIKV-A1, MV-ZIKV-A12 or control empty MV-Schwarz were challenged with 10^3 ffu of ZIKV (Asian-South American lineage, isolated in December 2015) one month after the last immunization. Viral loads were determined by RT-qPCR. LOD indicates the limit of detection of the RT-qPCR. Mice immunized with construct MV-ZIKV-A1 were all protected from viremia while mice immunized with MV-ZIKV-A12 or empty MV Schwarz control were infected.

Figure 13. Protection of immunized CD46-IFNAR$^{-/-}$ mice from ZIKV lethal challenge. Mice immunized twice with MV-ZIKV-A1, or control empty MV-Schwarz were challenged with 10^3 ffu of ZIKV (Mouse adapted strain of the African lineage) one month after the last immunization. Animals were

monitored for morbidity and mortality for 15 days. All animals immunized with MV-ZIKV-A1 survived without presenting signs of disease, while all control mice died by day 8.

EXAMPLES

Generation of vaccine candidates

Previous experiences with different flaviviruses (dengue, West Nile, Japanese encephalitis, tick-borne encephalitis) widely demonstrated that the flaviviral surface envelope (E) proteins are able to elicit protective neutralizing antibodies that allow reducing virus infectivity. The ZIKV genome consists of a single-stranded positive sense RNA molecule of ~10800 kb of length with 2 flanking non-coding regions (5' and 3' NCR) and a single long open reading frame encoding a polyprotein that is cleaved into three structural proteins (capsid (C), precursor of membrane (prM), envelope (E)) and seven non-structural proteins (NS) (**Figure 1**). The E protein (53 kDa) is the major virion surface protein involved in various aspects of the viral cycle, mediating binding to target cells and membrane fusion.

The inventors therefore chose to express the Zika virus E protein. Several forms of E protein were selected in order to express either soluble secreted proteins or anchored proteins onto the surface of VLPs. The following Zika virus antigens were cloned and expressed from a mammalian expression plasmid in human cells: prM-E and different forms of E with or without the stem or anchor region. These proteins contain either the original signal peptide sequence of Zika virus E or a heterologous signal peptide sequence from JEV or MV fusion protein. These proteins contain the signalase cleavage site located between the prM and the E sequences (**Figures 3A, 3B, 3C, 3D**).

Antigens selection and design

The Zika antigens were selected based on previous works concurring into suggesting that envelope antigens of flaviviruses may be able to elicit neutralizing antibodies and T cell responses. Selecting a suitable antigen should however take into consideration the evolution of the virus over time and

the variety of existing virus strains. To this end, the inventors reconstructed the phylogeny of representative members of the flavivirus family, including Zika virus, using only the amino acid region of the flavivirus polyprotein corresponding to the envelope (E) gene. Unlike phylogenetic analyses based on the full genome, or the polymerase (NS5) of flaviviruses, where the closest relative of Zika virus are neurotropic viruses such as Saint-Louis Encephalitis virus, the inventors noticed that Zika E appeared closer to DENV E (**Figure 2**) (*Barba-Spaeth, et al. Nature 2016, 536, 48-53)*. The inventors then proceeded to identify the different domains of Zika membrane (M), its precursor (prM) and E proteins through structural homology modelling based on available data on DENV (*Ekins et al. Illustrating and homology modeling the proteins of the Zika virus, F1000Research 2016, 5:275*). The inventors also identified the signal peptides at the end of the Capsid (C) gene, just upstream of prM, using again homology modelling with dengue virus as a reference, as well as publicly available algorithms to predict signal peptide sequences (http://sigpep.services.came.sbg.ac.at/signalblast.html; http://www.cbs.dtu.dk/services/SignalP/; http://www.predisi.de/). The inventors chose to include the signal peptide sequence to induce the export and secretion of the candidate antigen, either the full-length prM-E, or the E only, outside the cells. For the E antigen, the inventors also predicted the signal peptide at the end of M, just upstream of E, and designed versions of the antigen using this native signal (**Figure 3A**). In addition, the inventors also designed chimeric antigens where the native signal peptide of Zika virus was replaced with the signal peptide present at the end of JEV C (**Figure 3B**), or the signal peptide present at the N-terminal of the fusion protein (F) of MV (**Figure 3C**), hypothesizing that these sequences would provide enhanced export of the candidate antigens. The inventors designed an additional version of the chimeric antigen including the signal peptide of F from MV, where two amino acids corresponding to the junction between the end of the signal peptide of F and the beginning of F itself were removed (**Figure 3D**).

Secondly, the inventors also designed shorter variations of the antigens by removing C terminal fragments of the E protein corresponding to the

predicted stem and/or anchor domains, including the intermediate region between the stem and anchor (as predicted by comparison to DENV). The aim of these modifications that reduced antigens size was to generate antigens that were able to form VLPs. For a third variant, the inventors removed the anchor, the intermediate domain between the anchor and the stem, as well as a fragment of the second helix that composed the stem, this time in homology modelling with WNV (variant Ed445).

Finally, the inventors designed chimeric prM-E and E antigens using the signal peptide from MV F protein, and replacing Zika E anchor by the transmembrane (TM) and intracytoplasmic tail of MV F protein (**Figures 3C and 3D**).

For the selection sequence of the antigen itself, the inventors analyzed all publicly available sequences of Zika virus (both Asian and African lineages), as well as unpublished sequences generated by the inventors, from the epidemic in South America and Pacific. Based on the epidemiological data reporting an association of congenital syndromes and neurological afflictions in adults with only the Asian lineage, the inventors designed an antigen using the consensus amino acid sequence of Zika viruses as observed circulating from 2015 and onward, notably to include the S139N change that generated a novel potential N glycosylation site in prM that was absent from the African lineage, and the V763M in E.

The sequences were codon-optimized for Homo sapiens expression and adapted to measles vector cloning and to the “rule of six” (total number of nucleotides divisible by 6). Regions very rich (> 80%) or very poor (< 30%) in GC were avoided to increase RNA stability, a high CAI value (0.97) was obtained to increase translation efficacy, the following CIS active sequences were avoided: internal TATA-boxes, chi-sites, ribosomal entry sites, AT- or GC-rich sequence stretches, ARE, INS, CRS elements, repeat sequences, RNA secondary structures, cryptic splice donor and acceptor sites, branch points. The following measles virus editing sequences were avoided where possible: AAAGGG, AAAAGG, GGGAAA, GGGGAA, TTAAA, AAAA, and also their complementary sequences on the same strand: TTCCCC, TTTCCC,

CCTTTT, CCCTT, TTTAA, TTTT. The enzyme restriction sites BssHII, BsiWI were avoided internally and inserted at both ends for cloning purpose.

Antigen expression in mammalian cells

The optimized antigen sequences were cloned into pcDNA5 mammalian expression plasmid and transfected into HEK293 cells. The size and level of expression of each antigen were characterized after western blotting using appropriate antibodies for detection.

Antigen expression in measles vector

The optimized Zika antigen sequences were inserted into the MV vector in different additional transcription units, according to the desired level of expression. After sequencing of the measles vector plasmids expressing the different Zika antigens, the replicating recombinant vectors were generated by reverse genetics using a cell-based system previously developed (*Combredet, C. et al., 2003, J Virol, 77(21): 11546-11554*), and the rescued viruses were amplified and titrated on Vero cells. The recombinant viruses were grown on Vero cells to document the expression of Zika proteins detected both in supernatants and in cells by using Western Blot and indirect immunofluorescence staining with appropriate antibodies. The presence of Zika virus VLPs (in prM/E expressing vectors) was identified after ultracentrifugation of culture medium and western blot (**Figure 7**). The correct processing of antigens in infected cells was checked by Western Blot. The vectors with the best expression capacity of Zika antigens were isolated by serial dilution and single plaque cloning before amplification on Vero cells.

Growth capacity of recombinant vaccine virus

The growth capacity of selected vaccine viruses was compared with standard MV Schwarz. Growth curve analysis was performed in Vero cell culture by using different multiplicity of infection then titration.

Stability of recombinant vaccine virus

The best vaccine vectors selected were tested for their genetic stability by serial passaging over 10 cell culture passages on Vero cell culture followed by western blot for antigen expression and full sequencing analysis.

Preclinical evaluation of first MV-Zika recombinant in mice

Single immunization

The two recombinant vectors MV-prMEd404 (native sequence, insert 4) and MV-ssEd445 (native sequence, insert 5) were evaluated in CD46/IFNAR mice susceptible to measles infection. Mice were immunized with one or two intraperitoneal injections with defined infectious units of vaccine virus and functional antibodies and cell-mediated immune responses were analysed using both standard and specifically developed assays. Binding antibodies to Zika virus were determined with ELISA and neutralizing antibodies with specific plaque reduction neutralization test (PRNT). The T cell responses were analysed by Elispot assay using Zika virus-specific peptides for *ex vivo* stimulation of splenic cells. The vaccine vectors were then tested for protective efficacy: immunized mice were challenged with a lethal dose of Zika virus. A dose-response challenge was previously established in CD46/IFNAR mice showing that doses between 10^2 and 10^6 focus forming unit (ffu) of Zika virus African strain HD78788 (adapted to mouse) efficiently kill these mice.

In a first experiment 6 mice per group were immunized with a single intraperitoneal injection of 10^6 TCID50 of MV-prMEd404 (native sequence, insert 4), MV-ssEd445 (native sequence, insert 5) or empty MVSchw as a control. Blood was taken before immunization and at day 30 after immunization, and Zika virus ELISA titers were determined (**Figure 5A**).

The immunized mice were then challenged at day 30 by intraperitoneal injection of 10^6 ffu of Zika virus African strain HD78788 (mouse adapted). Morbidity and mortality were controlled during 12 days (**Figure 5B**) and Zika virus viremia was determined in serum by qRT-PCR (**Figure 5C**).

To determine T-cell response to the vaccine, another group of CD46/IFNAR mice were immunized by MV-prMEd404 (insert 4) or empty

MVSchw and spleens were collected at 8 days after immunization. Elispot assay was performed on freshly extracted splenocytes using MVSchw or Zika virus to re-stimulate T-cells or concanavalin A as a control (**Figure 5D**).

Prime-boost immunization

In a second set of experiments, groups of CD46/IFNAR mice were immunized with two successive intraperitoneal injections of 10^6 TCID50 of MV-prMEd404 (native sequence, insert 4), MV-ssEd445 (native sequence, insert 5) or empty MVSchw as a control. Blood was taken before immunization and at day 30, 45 and 55 after immunizations and Zika virus ELISA titers were determined (**Figure 6A**). Neutralizing antibodies were determined in sera collected at day 50 using a specific neutralization test of Zika virus (**Figure 6B**). The immunized mice were then challenged at day 60 by intraperitoneal injection of 10^6 ffu of Zika virus African strain HD78788 (mouse adapted). Morbidity and mortality were controlled during 12 days (**Figure 6C**) and Zika virus viremia was determined in serum by qRT-PCR at days 2, 4 and 6 post infection (**Figure 6D**).

Preclinical evaluation in Non-Human Primates (NHP)

Validation of the ZIKV strain used in the NHP challenge study

Because little is known about the physiopathology of ZIKV in cynomolgus macaque (*Macaca fascicularis*), two animals were inoculated in a preliminary assay with three doses of Zika wild-type virus (10^4, 10^5 and 10^6 pfu) to assess the viral stock and associated clinics in macaques. These two animals were submitted to the same follow-up than vaccinated and challenged animals but for a 6-month period. The following points were addressed: Virology (qRT-PCR; clinics (Rash, Fever); Blood cell count (Lymphocytes, Monocytes, Granulocytes, platelettes); Biochemistry (ASAT, ALAT, CRP); Non-specific (innate and inflammatory) and specific immune response: Cytokines/chemokines by luminex, NK, B and T cell profile (14 colors flow cytometry), Antibodies (neutralizing, binding) on serial sera samples, T cells functional response and memory cells (ELISpot, ICS). Shedding of the virus in

biological fluid (saliva, tears, genital fluids) was assessed by qRT-PCR and/or isolation methods at various time-points.

Vaccine immunogenicity study in NHP

Macaques were immunized with one or two subcutaneous injections at 3 months interval of defined infectious units of vaccine virus. Humoral and cell-mediated immune responses were determined at different times post immunization. Macaques were then challenged with infectious doses of ZIKV. Infectious viremia and clinical signs were determined. For this task, twenty-one adult cynomolgus macaques were selected to be negative for anti-flaviviruses and anti-measles antibodies; Two groups of 7 animals were vaccinated with a single dose or a prime boost regiment with the best MV-ZIKV recombinant virus (MV-prMEd404 native) selected. Immunity (Humoral and cell associated) was explored and virology was followed up to 1 month post vaccination. Clinics and biological parameters are assessed in parallel to a third group of 7 animals vaccinated with the control empty MVSchw strain following the prime boost schedule. Antibody neutralization titer was determined.

Vaccine Efficacy study in NHP

Immunized NHP were challenged with ZIKV two months after immunization. ZIKV viremia level (qRT-PCR) was analyzed in blood, saliva and tears. Inflammation and immune response was assessed in plasma (neutralizing Ab, cytokines).

Expression assays

The expression assays performed for all constructs generated (**Figure 8**) showed a strong expression for several of them. Signal was detected in the ultracentrifugated fraction, which was compatible with the generation of virus-like particles, in varying amounts for some candidate antigens, notably A1 and A12. These two antigens were thus further cloned into the measles vector and demonstrated high-level expression as shown by immunofluorescence

(**Figure 9A**). The recombinant MV-ZIKV-A1 vector replicated similarly to standard MV Schwarz virus, although with a lower final titer (**Figure 9B**).

Tested for their immunogenicity in CD46/IFNAR mice, MV-ZIKV-A1 and MV-ZIKV-A12 vectors elicited strong immune responses following a prime and boost regimen with 1-month interval, comparable to MV-prMEd404 and MV-ssEd445 vectors, as detected by ELISA (**Figure 10**). However, different amounts of neutralizing antibodies were induced (**Figure 11**). Only the candidate MV-ZIKV-A1 induced a strong neutralizing response (2 log stronger). This correlated with the complete protection conferred to mice by immunization with MV-ZIKV-A1 (**Figure 12**) against viremia, as well as protection from a lethal challenge (**Figure 13**).

In conclusion, this study demonstrated that the A1 full-length Zika antigen expressed in MV vector was able to provide sterile protection from infectious and lethal challenge of immunized animals, correlating with strong neutralizing antibody induction.

CLAIMS

1. A nucleic acid construct which comprises:

(1) a polynucleotide encoding at least (i) the precursor of membrane (prM) protein of a Zika virus (ZIKV), and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof; and

(2) a cDNA molecule encoding a full-length, infectious antigenomic (+) RNA strand of a measles virus (MV);

wherein the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof is operatively linked, in particular cloned into the cDNA molecule.

2. The nucleic acid construct according to claim 1, characterized in that the polynucleotide of (1) and the cDNA molecule of (2) together consist of a number of nucleotides that is a multiple of six.

3. The nucleic acid construct according to claim 1 or 2, comprising the following polynucleotides from 5' to 3':

(a) a polynucleotide encoding the N protein of the MV;

(b) a polynucleotide encoding the P protein of the MV;

(c) the polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof;

(d) a polynucleotide encoding the M protein of the MV;

(e) a polynucleotide encoding the F protein of the MV;

(f) a polynucleotide encoding the H protein of the MV; and

(g) a polynucleotide encoding the L protein of the MV;

wherein said polynucleotides are operably linked in the nucleic acid construct and under a control of viral replication and transcription regulatory sequences such as MV leader and trailer sequences.

4. The nucleic acid construct according to any one of claims 1 to 3, wherein said measles virus is an attenuated virus strain selected from the group consisting of the Schwarz strain, the Zagreb strain, the AIK-C strain and the Moraten strain.

5. The nucleic acid construct according to any one of claims 1 to 4, wherein said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, has been optimized for a Macaca codon usage or has been optimized for a human codon usage.

6. The nucleic acid construct according to any one of claims 1 to 5, wherein *measles editing-like sequences* have been deleted from said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof.

7. The nucleic acid construct according to any one of claims 1 to 6, wherein said ZIKV is from the African lineage, in particular from the African strain ArB1362 (GenBank: KF383115) or African isolate IbH_30656 (GenBank: HQ234500), or from the Asian strain, in particular from the Asian strain BeH818995 (GenBank: KU365777).

8. The nucleic acid construct according to any one of claims 1 to 7, wherein said polynucleotide encoding at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV and the signal peptide from the membrane protein of the ZIKV, or
wherein said polynucleotide encoding at least (ii) the E protein of the ZIKV or the truncated version thereof, further encodes (iii) the signal peptide from the capsid of the ZIKV or the signal peptide from the membrane protein of the ZIKV.

9. The nucleic acid construct according to any one of claims 1 to 8, wherein the polynucleotide encoding the E protein encodes either the full-length E protein or its soluble form lacking the two C-terminal transmembrane domains of the full-length E protein.

10. The nucleic acid construct according to any one of claims 1 to 8, wherein the polynucleotide encoding the truncated version of the E protein is selected from the group consisting of (i) the polynucleotide encoding the E protein truncated at amino acid position 456 of the full-length E protein of the ZIKV, (ii) the polynucleotide encoding the E protein truncated at amino acid position 445 of the full-length E protein of the ZIKV and (iii) the polynucleotide encoding the E protein truncated at amino acid position 404 of the full-length E protein of the ZIKV.

11. The nucleic acid construct according to any one of claims 1 to 10, wherein the polynucleotide encodes the prM protein of the ZIKV whose sequence is SEQ ID NO: 20, and the polynucleotide encodes the E protein of the ZIKV or the truncated version thereof whose sequence is selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 26, SEQ ID NO: 29 and SEQ ID NO: 32.

12. The nucleic acid construct according to any one of claims 1 to 11, wherein the polynucleotide encoding the prM protein of the ZIKV has the sequence of SEQ ID NO: 19, and the polynucleotide encoding the E protein of the ZIKV or the truncated version thereof has a sequence selected from the group consisting of SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 28 and SEQ ID NO: 31.

13. The nucleic acid construct according to any one of claims 1 to 12, wherein said nucleic acid construct comprises a sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 70, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 168 and SEQ ID NO:

170, preferably has the sequence of SEQ ID NO: 46, SEQ ID NO: 55 or SEQ ID NO: 76, more preferably has the sequence of SEQ ID NO: 46.

14. The nucleic acid construct according to any one of claims 1 to 13, which comprises the sequence from nucleotide at position 83 to nucleotide at position 18404 in the sequence of SEQ ID NO: 165, or the sequence from nucleotide at position 83 to nucleotide at position 18074 in the sequence of SEQ ID NO: 166, or the sequence from nucleotide at position 83 to nucleotide at position 17702 in the sequence of SEQ ID NO: 167.

15. A transfer vector plasmid, comprising the nucleic acid construct according to any one of claims 1 to 14.

16. The transfer vector plasmid according to claim 15, whose sequence is SEQ ID NO: 165, SEQ ID NO: 166 or SEQ ID NO: 167, preferably is SEQ ID NO: 165.

17. Transformed cells comprising inserted in their genome the nucleic acid construct according to any one of claims 1 to 14 or comprising the transfer vector plasmid according to claim 15 or 16, wherein said cells are in particular eukaryotic cells, such as avian cells, in particular CEF cells, mammalian cells or yeast cells.

18. Recombinant infectious replicating measles virus-Zika virus (MV-ZIKV) particles, which comprise as their genome a nucleic acid construct according to any one of claims 1 to 14.

19. Recombinant infectious replicating MV-ZIKV particles according to claim 18, which are rescued from a helper cell line expressing an RNA polymerase recognized by said cell line, for example a T7 RNA polymerase, a nucleoprotein (N) of a MV, a phosphoprotein (P) of a MV, and optionally an

RNA polymerase large protein (L) of a MV, and which is further transfected with the transfer vector plasmid according to claim 15 or 16.

20. The recombinant infectious replicating MV-ZIKV particles according to claim 18 or 19, wherein said particles comprise in their genome a polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO: 46, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 70, SEQ ID NO: 76, SEQ ID NO: 79, SEQ ID NO: 168 and SEQ ID NO: 170, preferably has the sequence of SEQ ID NO: 46, SEQ ID NO: 55 or SEQ ID NO: 76, more preferably has the sequence of SEQ ID NO: 46.

21. A composition or an assembly of active ingredients comprising the recombinant infectious replicating MV-ZIKV particles according to any one of claims 18 to 20, in association with ZIKV-Virus Like Particles (VLPs) expressing the same ZIKV protein(s) as said MV-ZIKV particles, and a pharmaceutically acceptable vehicle.

22. The composition or the assembly of active ingredients according to claim 21 for use in the elicitation of a protective immune response against ZIKV by the elicitation of antibodies directed against said ZIKV protein(s), and/or of a cellular immune response, in a host, in particular a human host in need thereof.

23. The recombinant infectious replicating MV-ZIKV particles according to any one of claims 18 to 20 in association with ZIKV-VLPs expressing the same ZIKV protein(s), or the composition or the assembly of active ingredients according to claim 21, for use in the prevention of an infection by ZIKV in a subject or in the prevention of clinical outcomes of infection by ZIKV in a subject, in particular in a human.

24. A process to rescue recombinant infectious measles virus-Zika virus (MV-ZIKV) particles expressing at least (i) the precursor of membrane (prM)

protein of a ZIKV, and the envelope (E) protein of a ZIKV or a truncated version thereof, or (ii) the E protein of a ZIKV or a truncated version thereof, and ZIKV Virus Like Particles (VLPs) expressing the same ZIKV protein(s), comprising:
1) co-transfecting helper cells, in particular HEK293 helper cells, that stably express T7 RNA polymerase, and measles N and P proteins with (i) the transfer vector plasmid according to claim 15 or 16 and with (ii) a vector, especially a plasmid, encoding the MV L polymerase;
2) cultivating said co-transfected helper cells in conditions enabling the production of recombinant MV-ZIKV particles;
3) propagating the thus produced recombinant MV-ZIKV particles by co-cultivating said helper cells of step 2) with cells enabling said propagation such as Vero cells;
4) recovering recombinant infectious replicating MV-ZIKV particles expressing at least (i) the prM protein of the ZIKV, and the E protein of the ZIKV or the truncated version thereof, or (ii) the E protein of the ZIKV or the truncated version thereof, and ZIKV VLPs expressing the same ZIKV protein(s).

25. The process according to claim 24, wherein the transfer vector plasmid has the sequence of SEQ ID NO: 165, SEQ ID NO: 166 or SEQ ID NO: 167, preferably has the sequence of SEQ ID NO: 165.

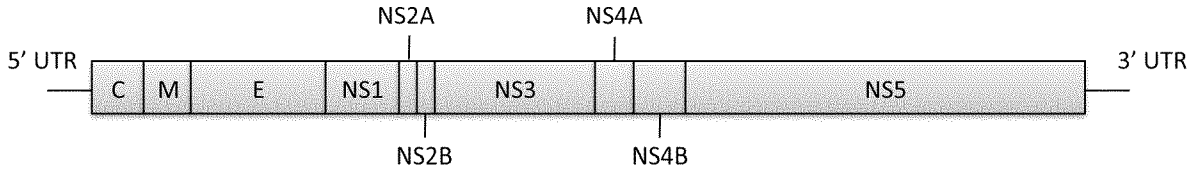


Figure 1

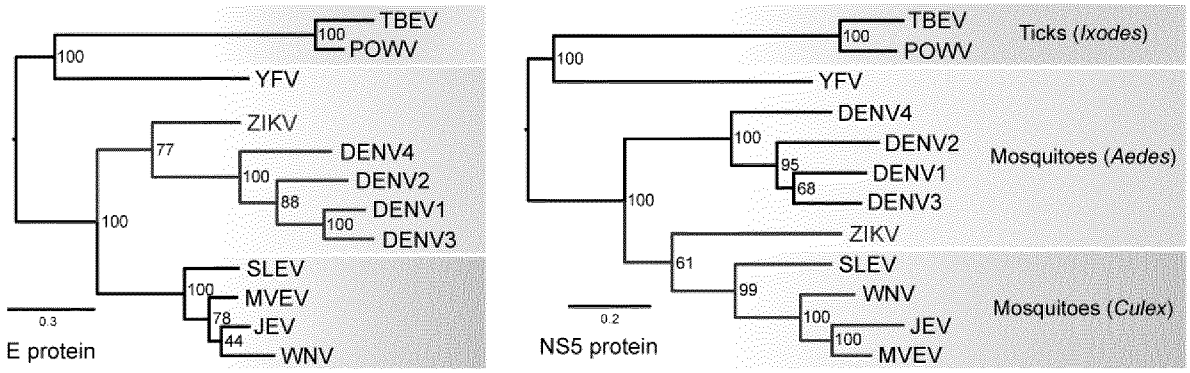


Figure 2

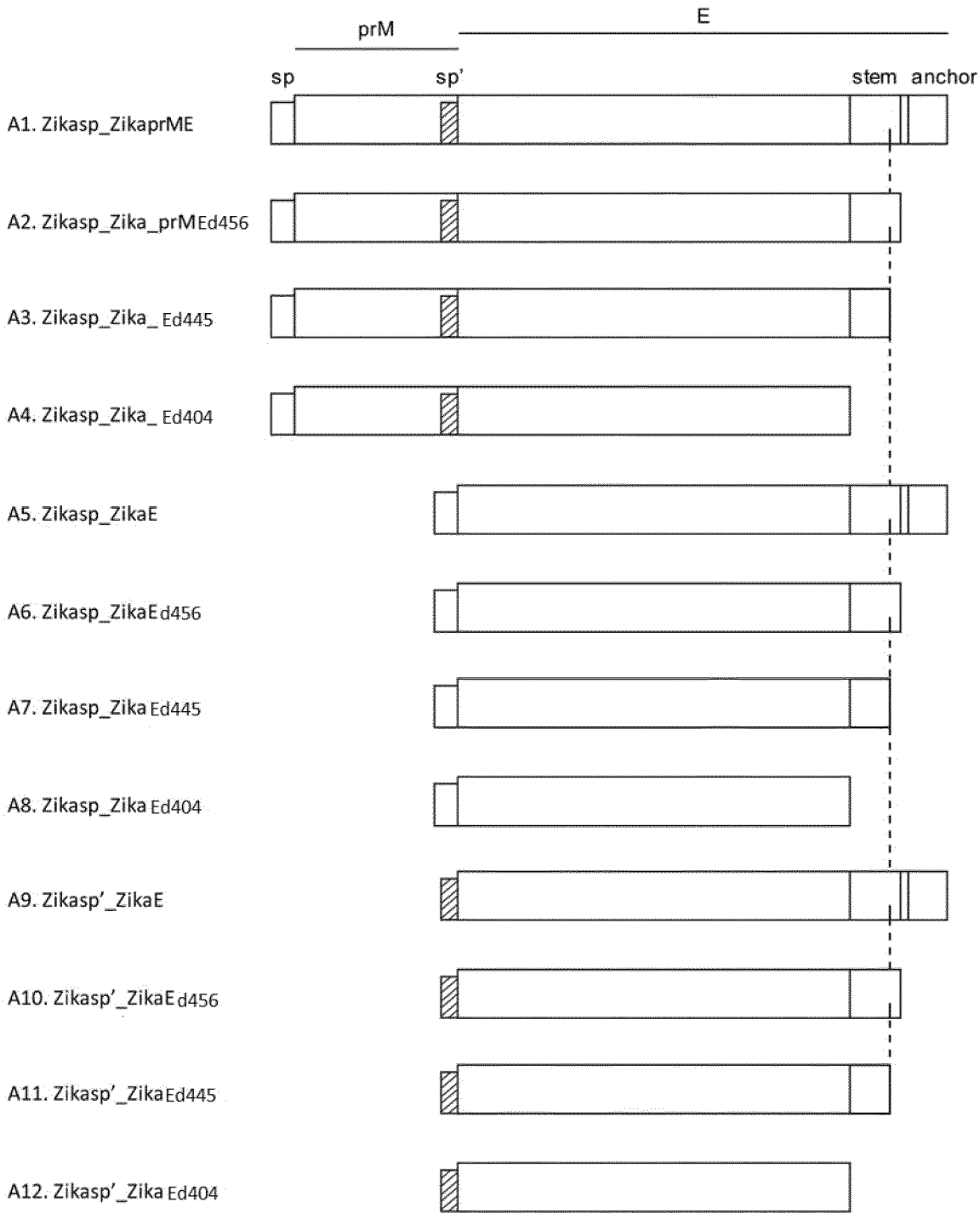

prM
E
sp
sp'
stem
anchor
A1. Zikasp_ZikaprME
A2. Zikasp_Zika_prMEd456
A3. Zikasp_Zika_ Ed445
A4. Zikasp_Zika_ Ed404
A5. Zikasp_ZikaE
A6. Zikasp_ZikaEd456
A7. Zikasp_Zika Ed445
A8. Zikasp_Zika Ed404
A9. Zikasp'_ZikaE
A10. Zikasp'_ZikaEd456
A11. Zikasp'_ZikaEd445
A12. Zikasp'_Zika Ed404

Figure 3A

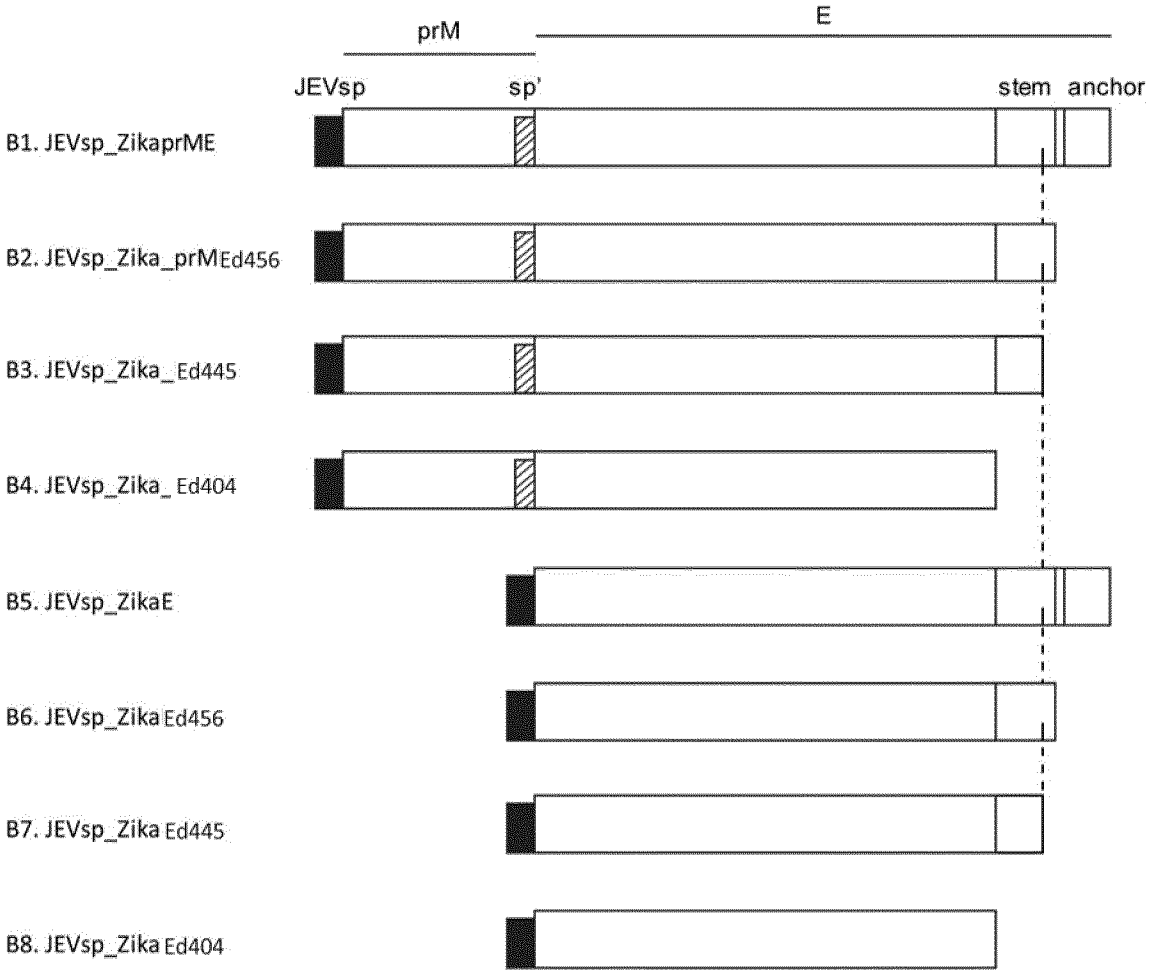

prM
E
JEVsp
sp'
stem
anchor
B1. JEVsp_ZikaprME
B2. JEVsp_Zika_prMEd456
B3. JEVsp_Zika_Ed445
B4. JEVsp_Zika_Ed404
B5. JEVsp_ZikaE
B6. JEVsp_Zika Ed456
B7. JEVsp_Zika Ed445
B8. JEVsp_Zika Ed404

Figure 3B

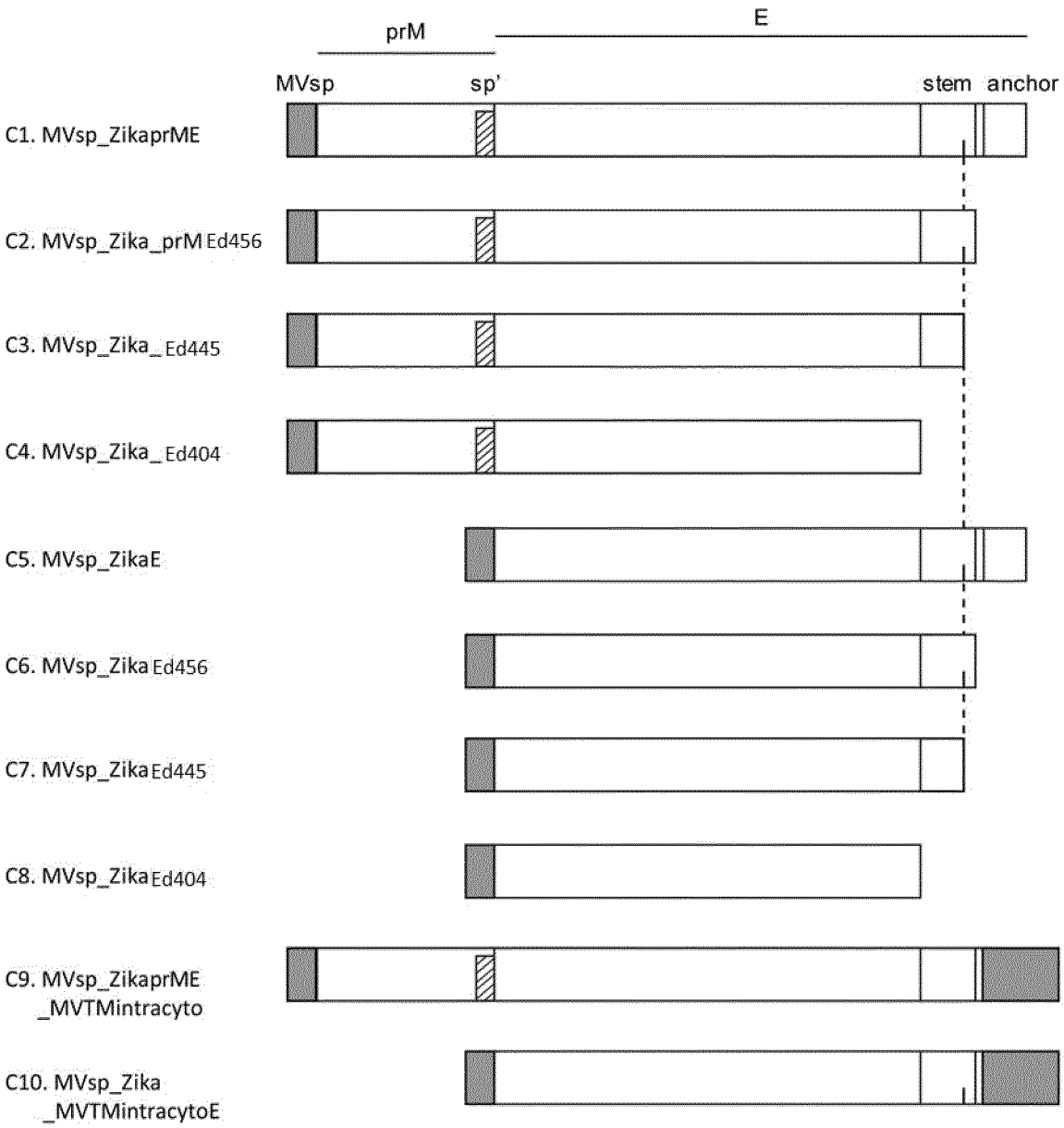

prM
E
MVsp
sp'
stem
anchor
C1. MVsp_ZikaprME
C2. MVsp_Zika_prM Ed456
C3. MVsp_Zika_ Ed445
C4. MVsp_Zika_ Ed404
C5. MVsp_ZikaE
C6. MVsp_Zika Ed456
C7. MVsp_Zika Ed445
C8. MVsp_Zika Ed404
C9. MVsp_ZikaprME
_MVTMintracyto
C10. MVsp_Zika
_MVTMintracytoE

Figure 3C

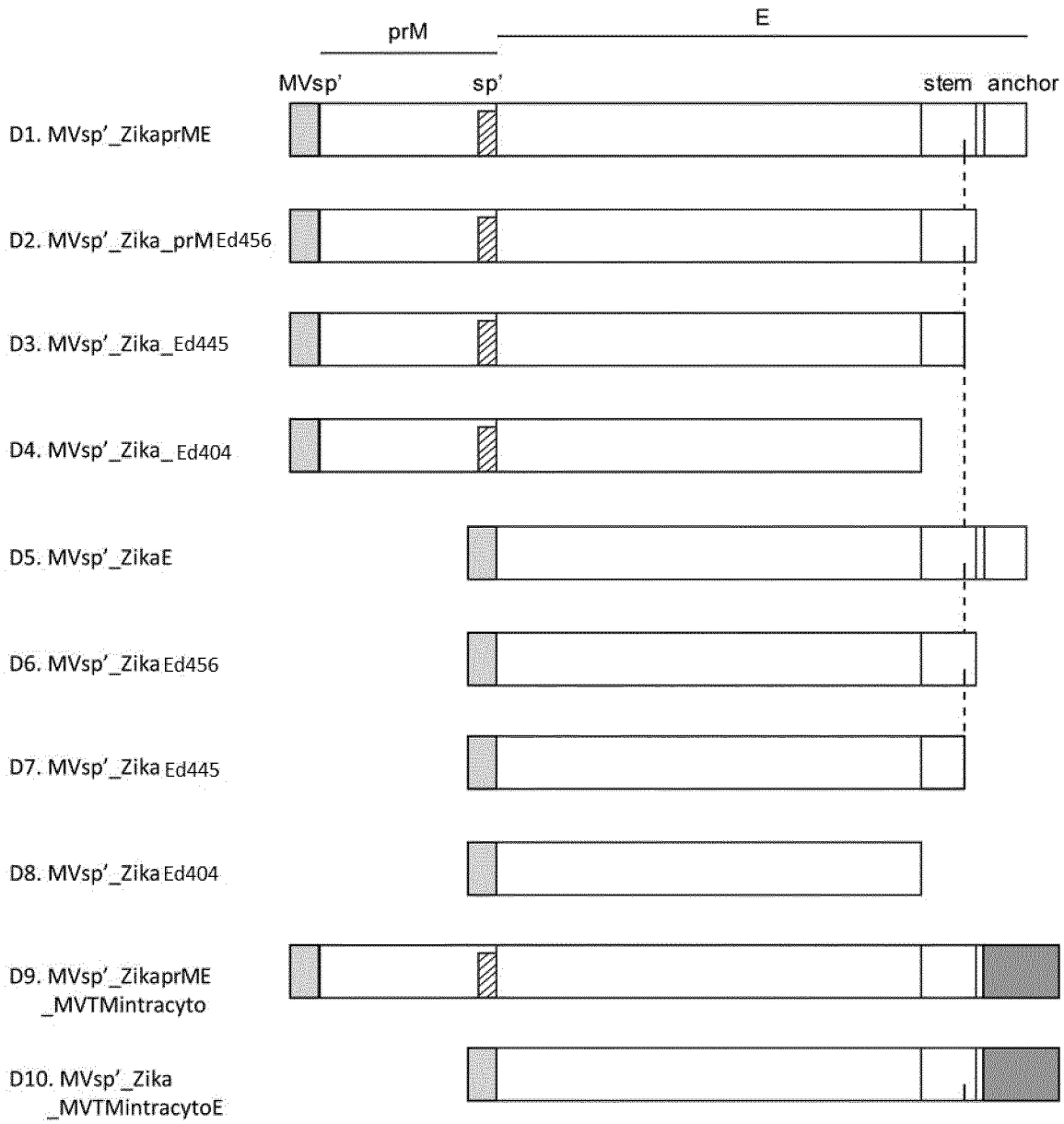

prM
E
MVsp'
sp'
stem
anchor
D1. MVsp'_ZikaprME
D2. MVsp'_Zika_prM Ed456
D3. MVsp'_Zika_Ed445
D4. MVsp'_Zika_ Ed404
D5. MVsp'_ZikaE
D6. MVsp'_Zika Ed456
D7. MVsp'_Zika Ed445
D8. MVsp'_Zika Ed404
D9. MVsp'_ZikaprME _MVTMintracyto
D10. MVsp'_Zika _MVTMintracytoE

Figure 3D

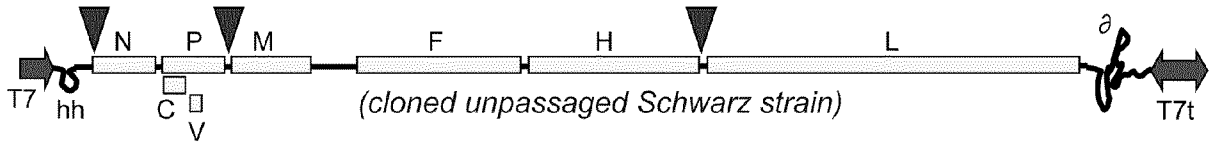
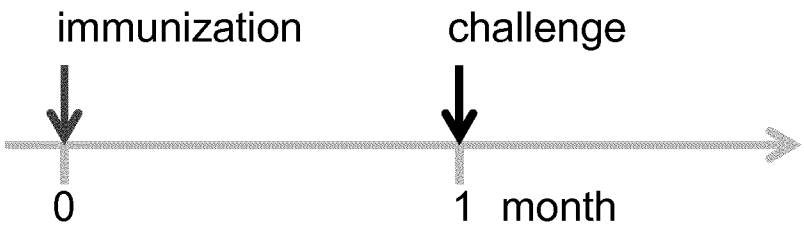


Figure 4



6 CD46-IFNAR mice per group

Immunization: MV vectors 10^6 $TCID_{50}$/mouse

Challenge: ZIKV African strain 10^6 pfu/mouse

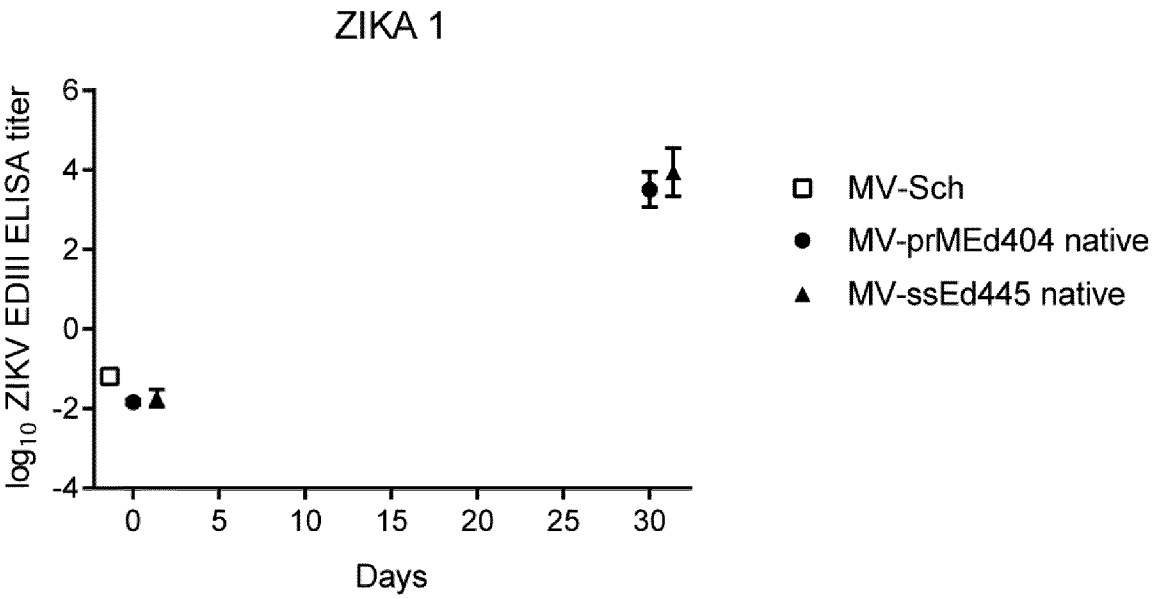


Figure 5A

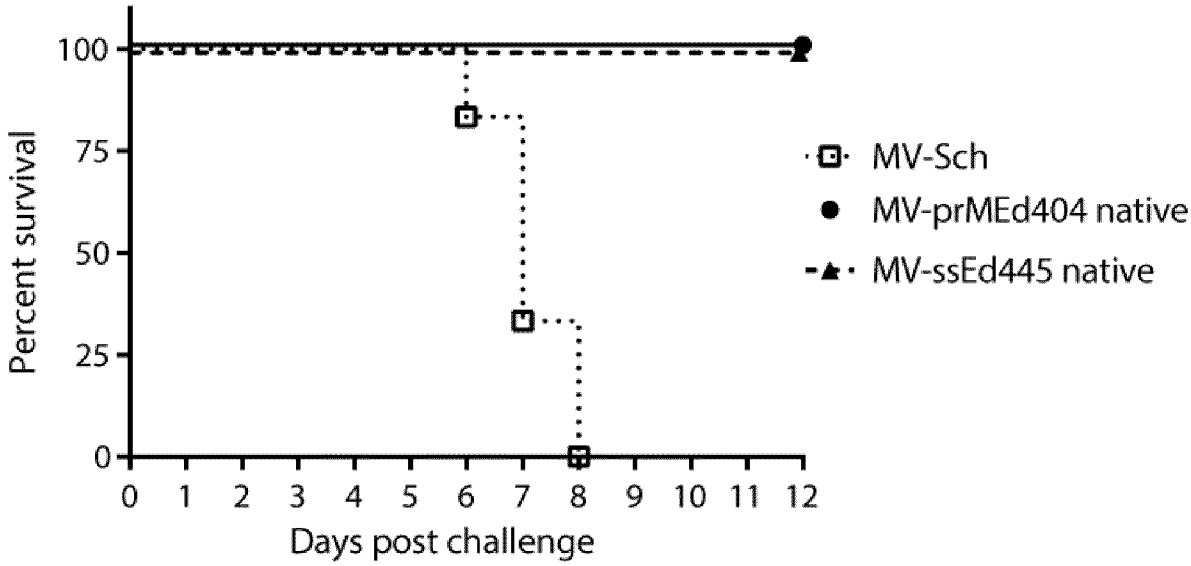


Figure 5B

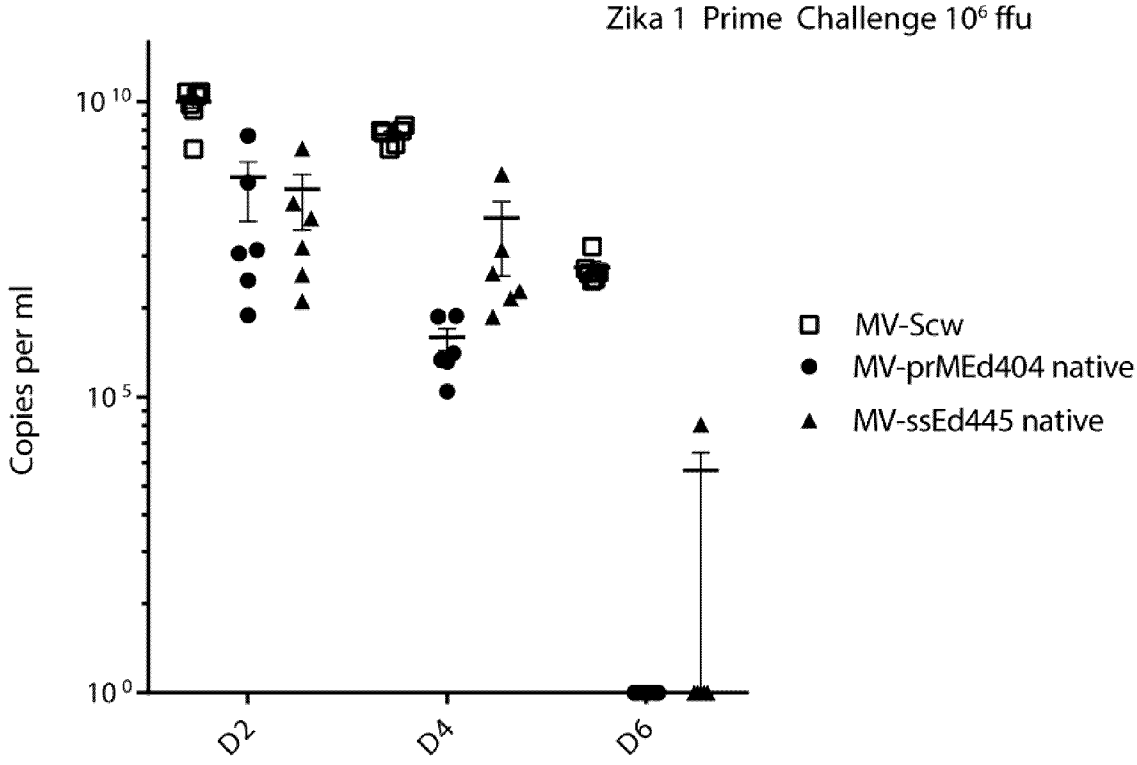


Figure 5C

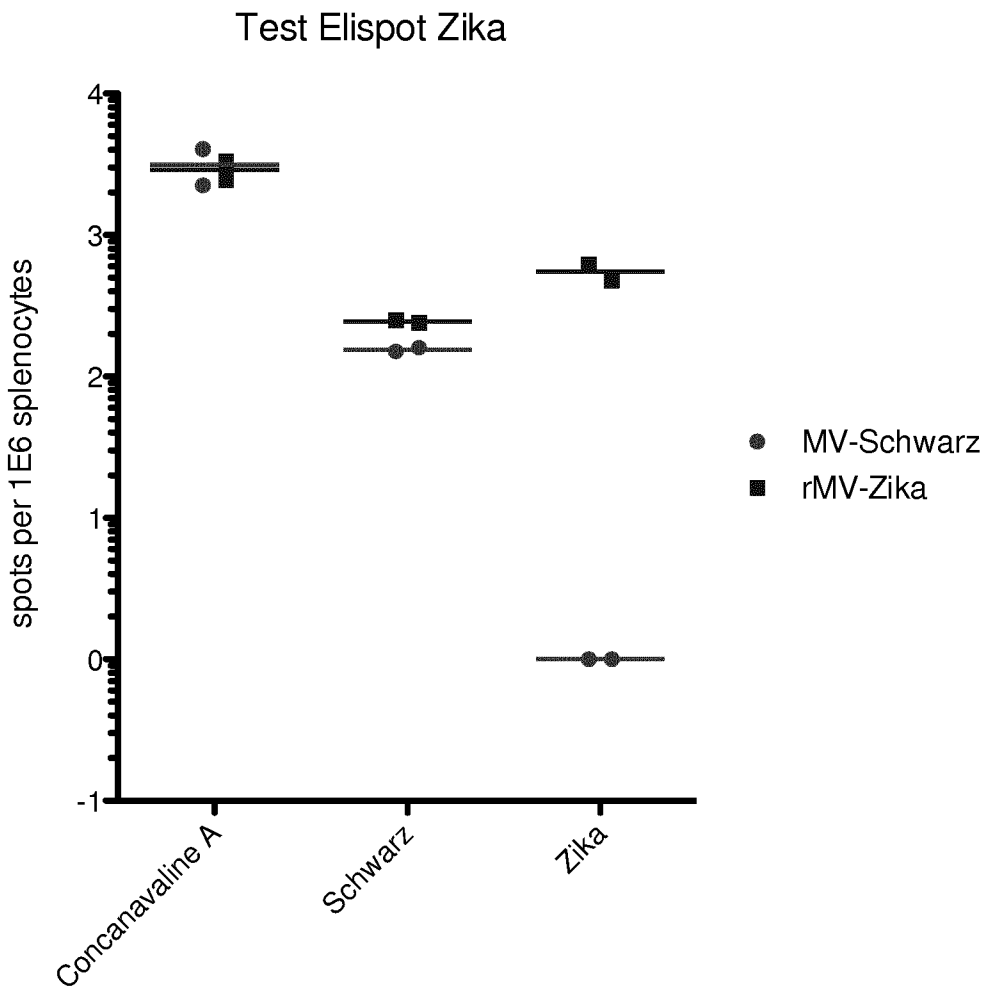
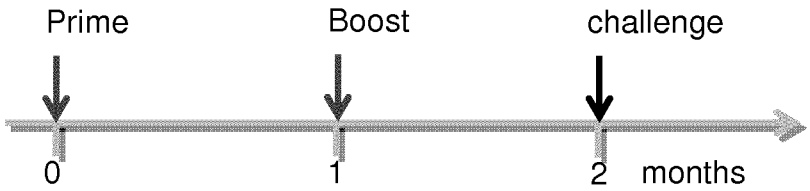

Test Elispot Zika
spots per 1E6 splenocytes
4
3
2
1
0
-1
Concanavaline A
Schwarz
Zika
MV-Schwarz
rMV-Zika

Figure 5D



6 CD46-IFNAR mice per group
Immunization: MV vectors 10^6 $TCID_{50}$/mouse
Challenge: ZIKV African strain 10^3 pfu/mouse

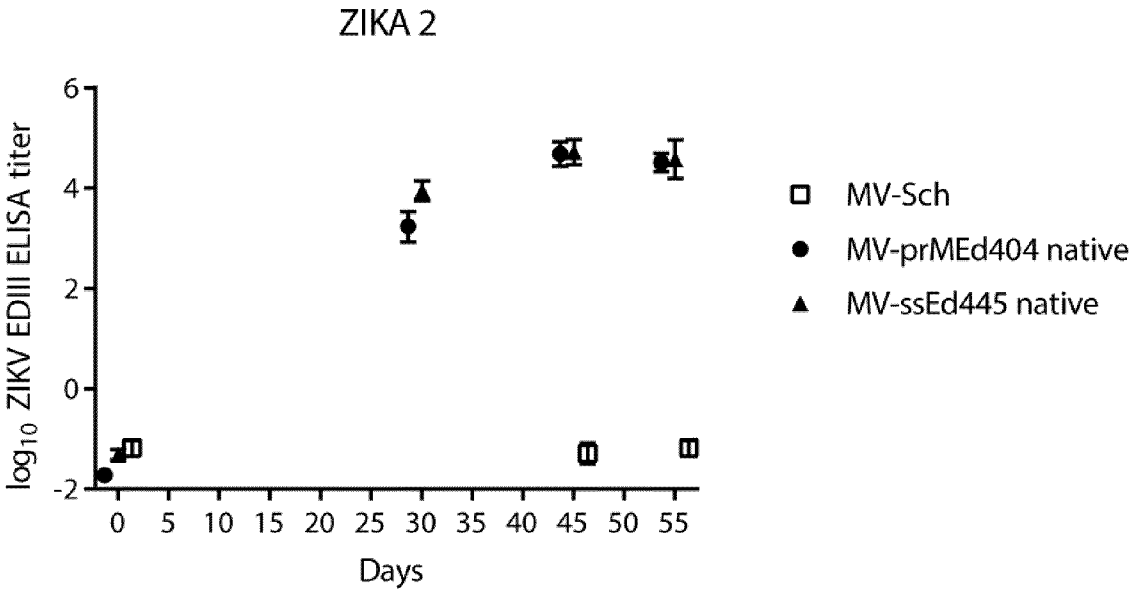
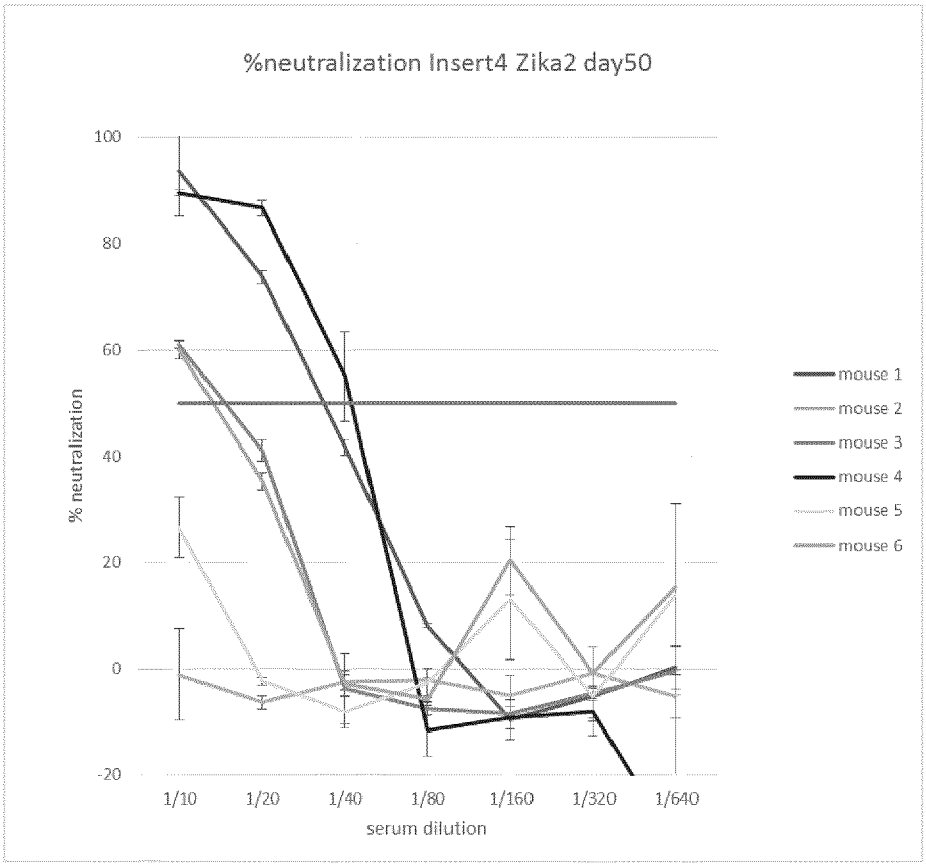


Figure 6A


%neutralization Insert4 Zika2 day50
100
80
60
40
20
0
-20
% neutralization
1/10
1/20
1/40
1/80
1/160
1/320
1/640
serum dilution
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
mouse 6

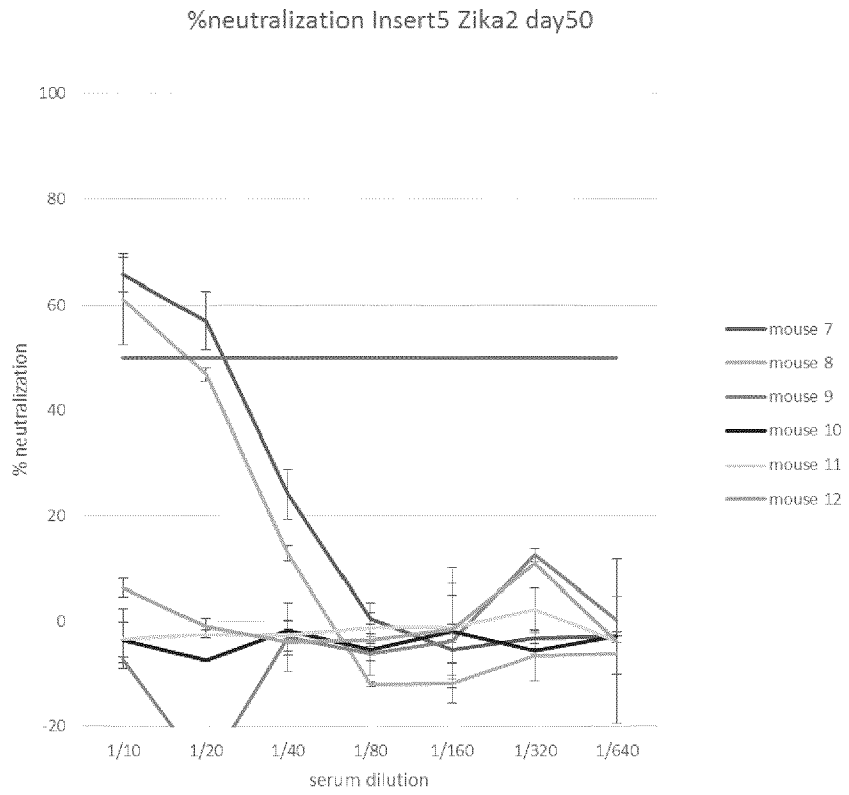

%neutralization Insert5 Zika2 day50
100
80
60
40
20
0
-20
% neutralization
1/10
1/20
1/40
1/80
1/160
1/320
1/640
serum dilution
mouse 7
mouse 8
mouse 9
mouse 10
mouse 11
mouse 12

Figure 6B

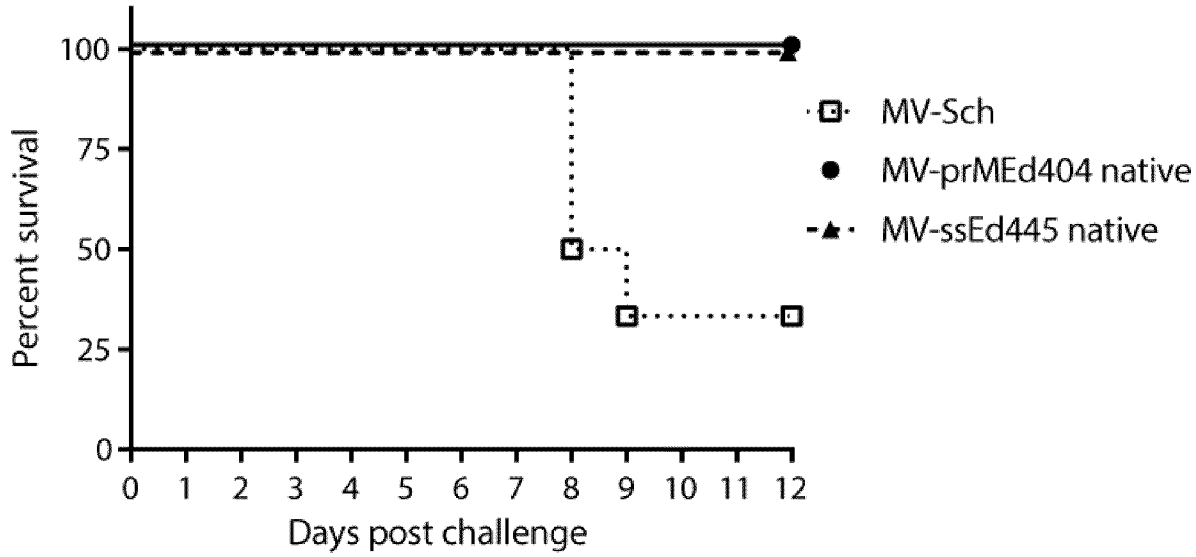


Figure 6C

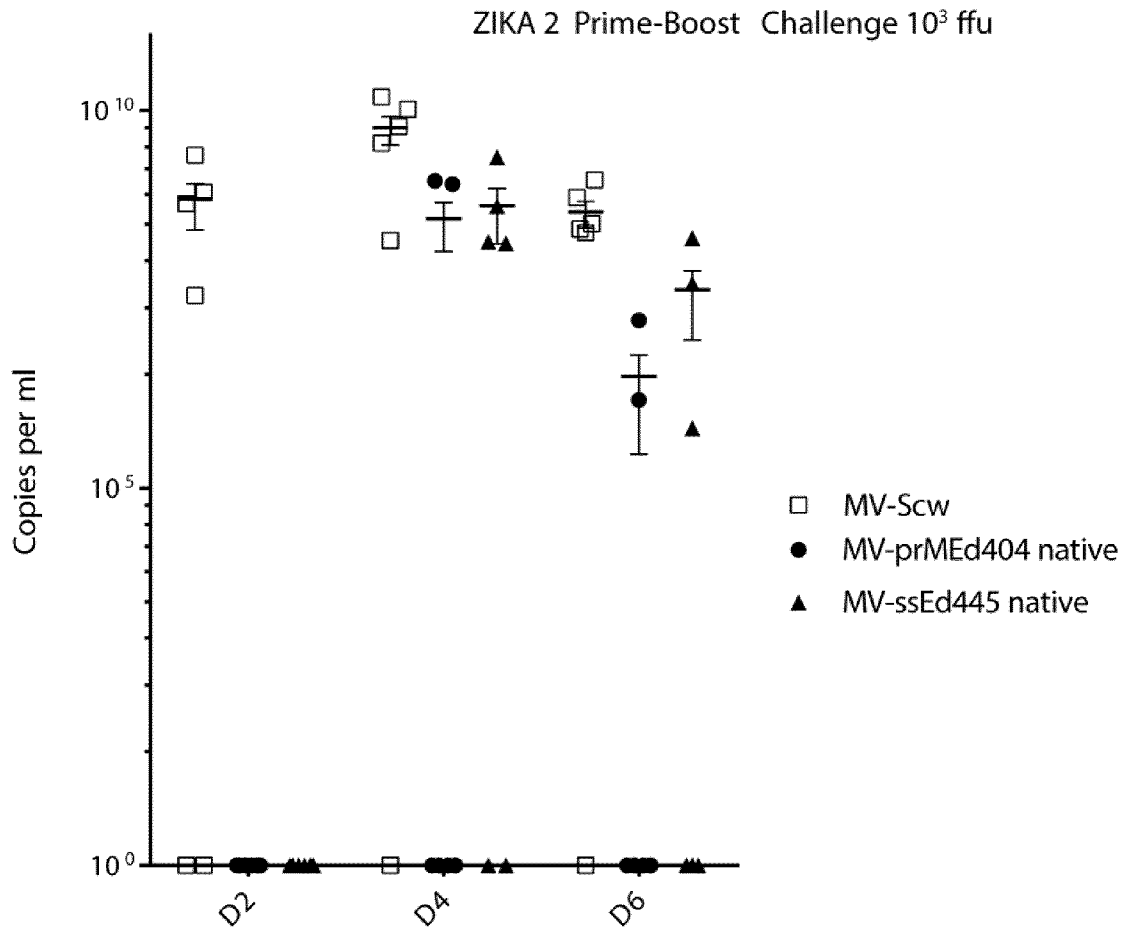


Figure 6D

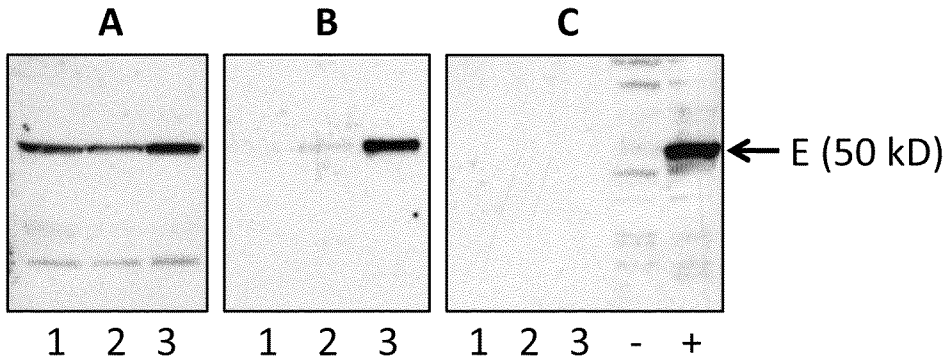


Figure 7

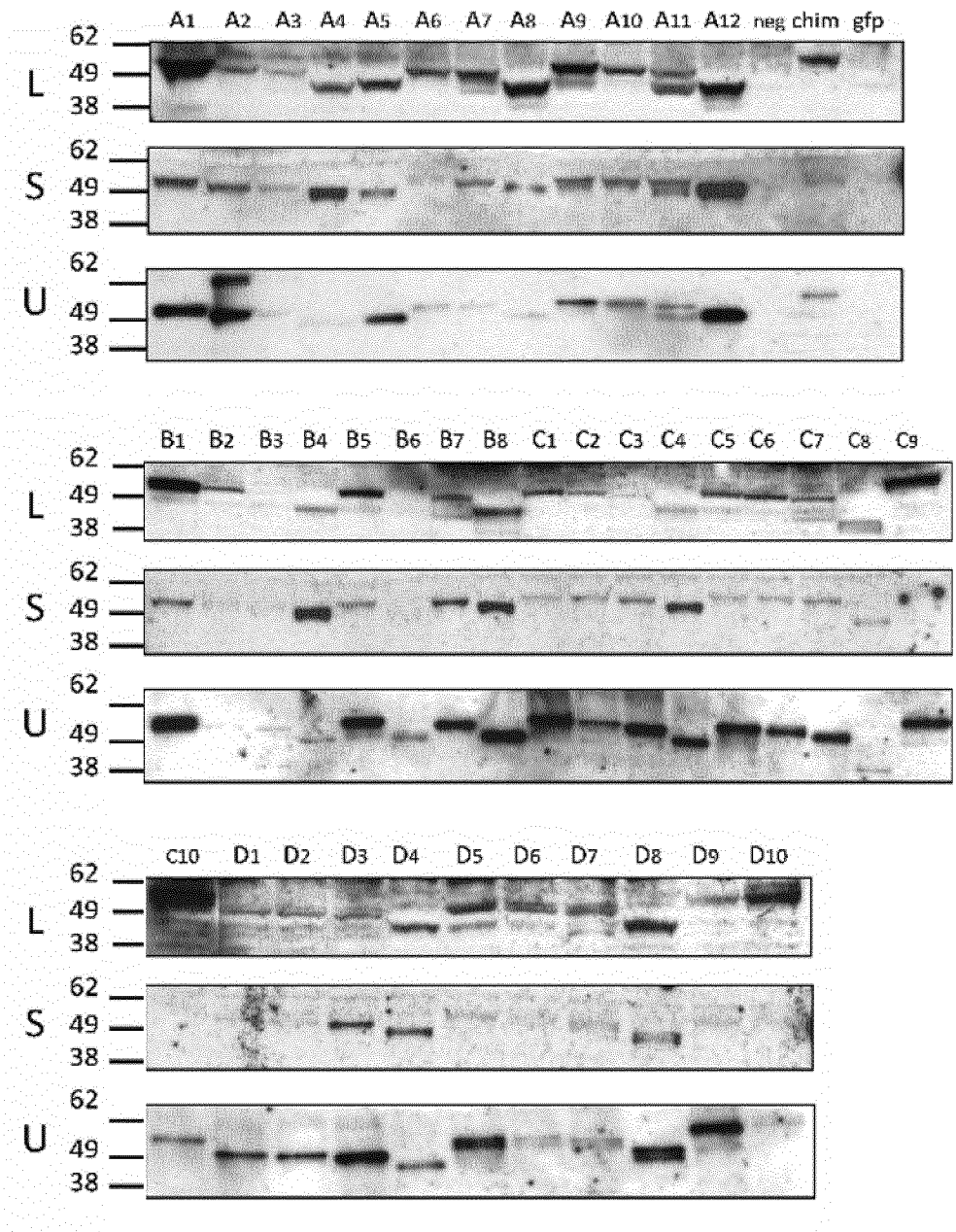

A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 neg chim gfp
L
S
U
62
49
38
B1 B2 B3 B4 B5 B6 B7 B8 C1 C2 C3 C4 C5 C6 C7 C8 C9
C10 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10

Figure 8

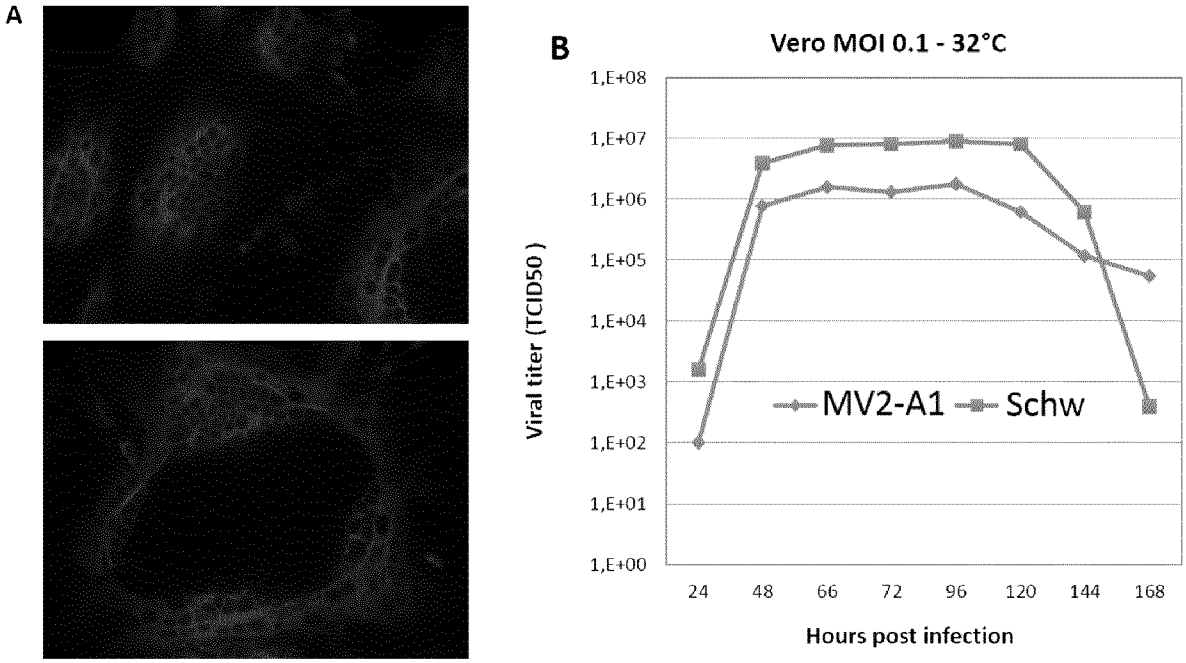


Figure 9

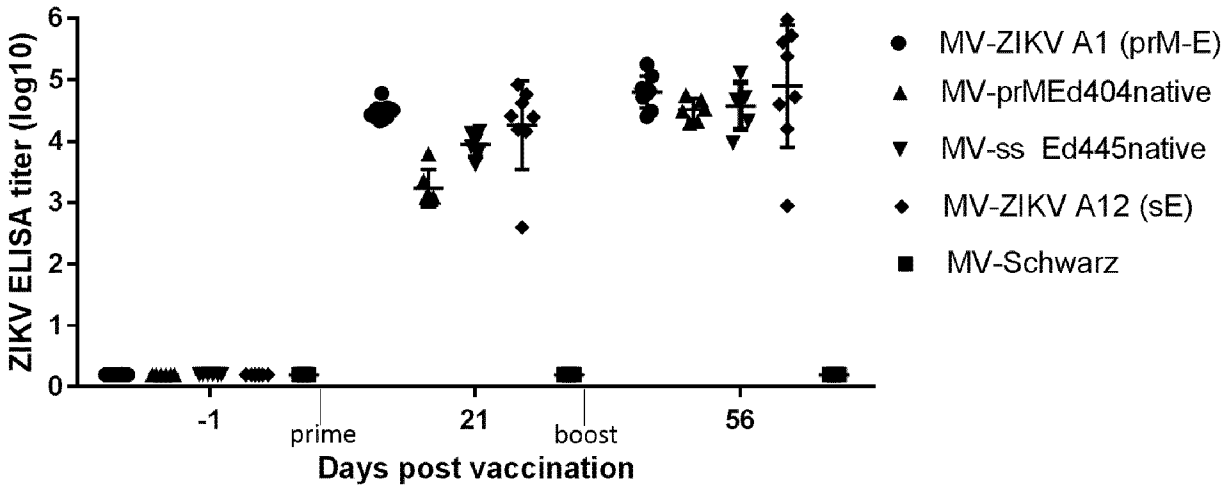


Figure 10

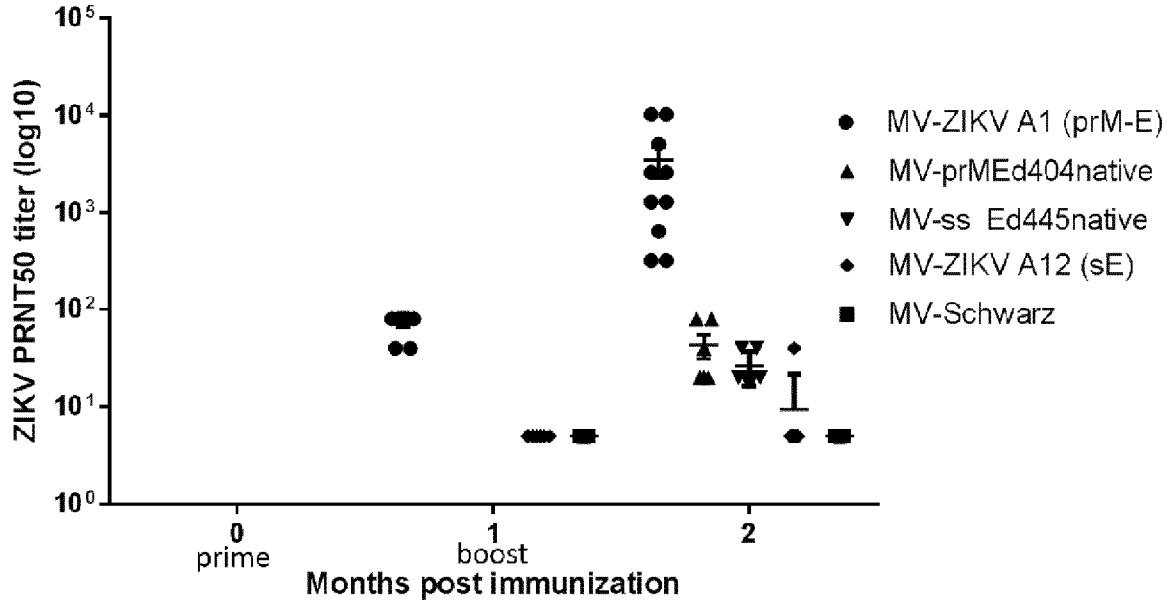


Figure 11

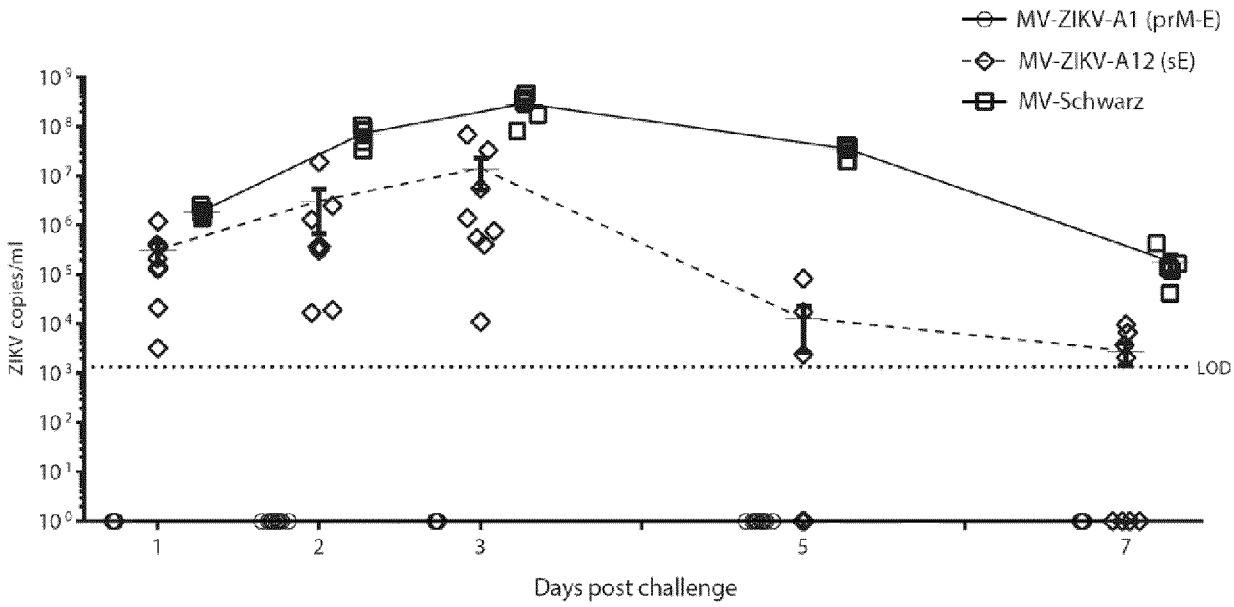


Figure 12

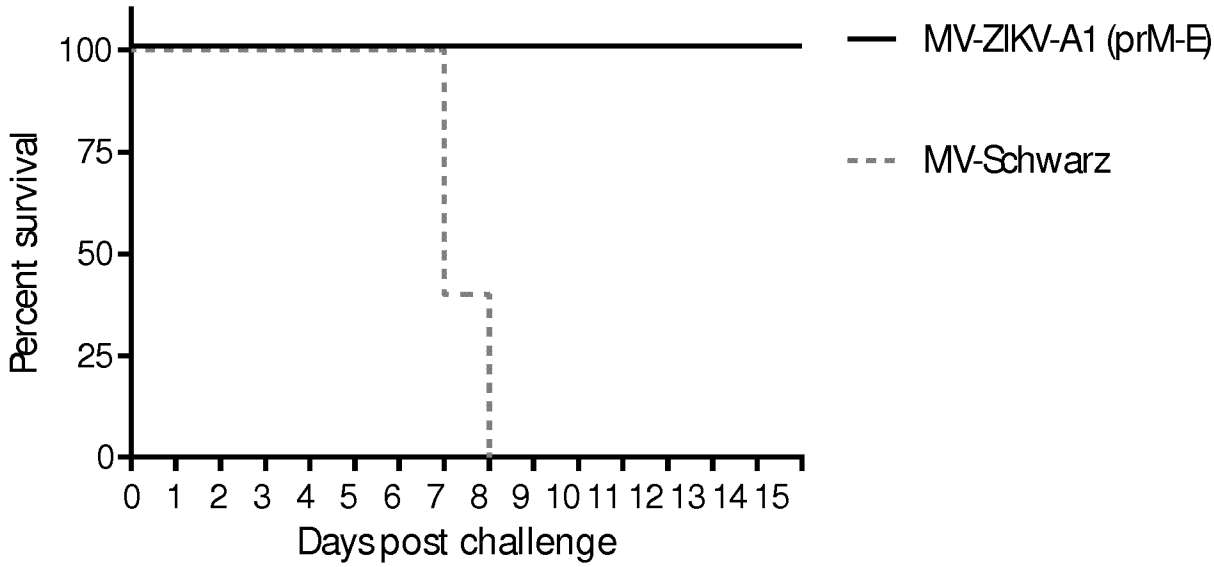

100
75
50
25
0
Percent survival
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post challenge
MV-ZIKV-A1 (prM-E)
MV-Schwarz

Figure 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/064943

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 A61K39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Anna Durbin ET AL: "Role of a ZIKV CHIM in vaccine evaluation", , 2 June 2017 (2017-06-02), XP055419451, Retrieved from the Internet: URL:https://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0ahUKEwiruOOVpo7XAhWDbFAKHYyVChoQFggmMAA&url=http://www.who.int/entity/blueprint/what/norms-standards/1_Durbin_CHIM.pdf?ua=1&usg=AOvVaw3sraJTiT8oBPlV6yWhMhVq [retrieved on 2017-10-26]	1
Y	page 22; table all	2-25

-/--

X Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2018	02/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fellows, Edward

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Usp/institut Pasteur/fiocruz: "Workshop - Beyond Zika - A tripartite Initiative", , 29 August 2016 (2016-08-29), XP055419564, Retrieved from the Internet: URL:http://www.usp.br/aucani/zika/archive/Presentations_Afternoon_August_30.pdf [retrieved on 2017-10-26] page 5 - page 6	2-25
Y	PENELOPE KORAKA ET AL: "Bioinformatics in New Generation Flavivirus Vaccines", JOURNAL OF BIOMEDICINE AND BIOTECHNOLOGY, vol. 9, no. 5, 1 January 2010 (2010-01-01) , page 17PP, XP055314273, US ISSN: 1110-7243, DOI: 10.1155/2010/864029 figure all; example all; table all	2-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/064943

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: