RECOMBINANT MEASLES VIRUS EXPRESSING CHIKUNGUNYA VIRUS POLYPEPTIDES AND THEIR APPLICATIONS

FIELD OF THE INVENTION

The invention is directed to recombinant Measles virus expressing Chikungunya virus polypeptides, and concerns in particular virus like particles (VLP) that contain envelope and capsid proteins of a Chikungunya virus at their surface. These particles are recombinant infectious particles able to replicate in a host after an administration. The invention provides means, in particular nucleic acids, vectors, cells and rescue systems to produce these recombinant infectious particles. The invention also relates to the use of these recombinant infectious particles, in particular under the form of a composition, more particularly in a vaccine formulation, for the treatment or prevention of an infection by Chikungunya virus.

BACKGROUND OF THE INVENTION

Chikungunya virus (CHIKV) is a positive-strand RNA virus of the genus *Alphavirus* within the family of *Togaviridae,* first isolated in Tanzania in 1952.

Infection by this virus causes human disease that is characterized by symptoms similar to dengue fever, with an acute febrile phase during two to five days, followed by a prolonged arthralgic disease that affects the joints of the extremities. CHIKV is endemic in Africa, India and South-East Asia and is transmitted by Aedes mosquitoes through an urban or sylvatic transmission cycle. In 2006, an outbreak of CHIKV fever occurred in numerous islands of the Indian Ocean (the Comoros, Mauritius, Seychelles, Madagascar, Reunion island...), before jumping to India where an estimated 1.4 million cases have been reported. More recently, imported infections have been described in Europe, and around 200 endemic cases have been reported in Italy (Jose, J. *et al.*, A structural and functional perspective of alphavirus replication and assembly. Future Microbiol, 2009. 4(7): p. 837-56). Clinically, this CHIKV epidemic was accompanied by more severe symptoms than previous outbreaks, with reports of severe polyarthralgia and myalgia, complications and deaths.

The CHIKV genome is an 11.8 kb, single-stranded RNA molecule of positive polarity. This virus is closely related to Semliki Forest virus (SFV), Sindbis virus (SINV), and other Old-World alphaviruses, and more distantly related to New-World alphaviruses like Venezuelan equine encephalitis virus (Griffin, D.E., Alphaviruses, in Fields Virology, 5th ed., D.M. Knipe, Editor 2007, Wolters Kluwer, Lippincott Williams & Wilkins. p. 1023-1067). The genomic RNA is capped, and directly translated into a full-length non-structural polyprotein (nsP) called P1234, which is encoded by the 5′ two-thirds of the genome (Jose, J., J.E. Snyder, and R.J. Kuhn, A structural and functional perspective of alphavirus replication and assembly. Future Microbiol, 2009. 4(7): p. 837-56 ; Kuhn, R.J., Togaviridae: the viruses and their replication, in Fields Virology, 5th ed., D.M. Knipe, Editor 2007, Wolters Kluwer, Lippincott Williams & Wilkins. p. 1001-1022). This precursor cleaves itself to produce P123 and nsP4 that carries the RNA-dependent RNA polymerase activity. These proteins, together with cellular co-factors, assemble into a replication complex that produces antisense genomic RNA molecules. Subsequent cleavage of P123 into nsP1 and P23 gives rise to a polymerase complex making both sense and antisense genomic RNA. Further processing of P23 into nsP2 and nsP3 gives rise to a polymerase complex making only positive-sense genomic RNA molecules. In addition to replicating the viral genome, this viral protein complex transcribes a 26S subgenomic RNA from the 3′ extremity of the viral genome. This messenger RNA is translated into a polyprotein precursor, which is cleaved by a combination of viral and cellular enzymes to produce a capsid protein (C), two major envelope proteins (E1 and E2), and two smaller accessory peptides, E3 and 6k. Once assembled, CHIKV virions are spherical particles of 65–70 nm in diameter, essentially composed of genomic RNA molecules associated with capsid proteins, and enveloped in a host-derived lipid membrane decorated by E1-E2 heterodimers organized in an icosahedral lattice (Voss, J.E., et al., Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. Nature, 2010. 468(7324): p. 709-12).

The disease severity, as much as the evolution and spread of the virus into new geographic areas are a serious health public matter that needs to be fixed. In order to solve this problem, vaccines with live attenuated virus, with chimeric alphavirus, with recombinant DNA or with virus-like particles have been developed.

A formalin inactivated vaccine was shown to be immunogenic in non-human primates and humans, but the high amount of antigen required for mass immunization that needs to be prepared under BSL-3 conditions is a limitation for the development of this strategy (Tiwari, M., et al., Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. (Vaccine, 2009. 27(18): p. 2513-22). The live attenuated TSI-GSD-218 CHIKV vaccine developed by the US-Army was immunogenic but caused side effects in Phase II clinical trials associated with reversion to virulence raising safety issues. Therefore, although the results obtained with vaccines based on live attenuated virus show that an efficient immunization can be achieved by this way, those vaccines are still questionable as there could be a risk of possibly side effects (Edelman R *et al.*, Am J Trop Med Hyg. 2000 Jun;62(6):681-685). Chimeric alphavirus vaccine strategies encoding the E1, E2 and capsid proteins from CHIKV are immunogenic in mice (Wang, E., et al., Chimeric alphavirus vaccine candidates for Chikungunya. Vaccine, 2008. 26(39): p. 5030-9), but the ability of alphavirus to easily recombine raizes safety issues against the development of such strategies (Weaver, S.C., et al., Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. J Virol, 1997. 71(1): p. 613-23).

Another strategy, which has been explored is to design recombinant DNA construct for use as a vaccine. DNA based CHIKV vaccines encoding the E1, E2 and capsid protein have been shown to be immunogenic in mice and non-human primates (Muthumani, K., et al., Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. Vaccine, 2008. 26(40): p. 5128-34 ; Mallilankaraman, K., et al., A DNA vaccine against chikungunya virus is protective in mice and induces neutralizing antibodies in mice and nonhuman primates. PLoS Negl Trop Dis, 2011. 5(1): p. e928), but the DNA strategies do not induce the strong neutralizing immune response required for CHIKV clearance in humans. The disadvantages of DNA vaccines are that high quantities of DNA are required to induce an immune response and multiple booster vaccinations must be performed. The need for multiple boosts and high quantities of DNA injected into the nuclei of many cells raises concern regarding the fact that DNA vaccines can integrate into the host DNA and cause insertional mutagenesis. Therefore a recent study reports using DNA vaccines combined with live attenuated virus (WO2011/082388). Although this technique

allows reducing drawbacks of live attenuated virus and DNA vaccines, there is still a need in providing a vaccine with reduced side effects.

In order to avoid drawbacks of live attenuated virus and DNA vaccines, other types of vaccines have been developed such as vaccines based on virus-like particles (VLPs) which are obtained by expressing structural proteins of the Chikungunya virus. These structural proteins are able to self-assemble in virus-like particles. On that basis, vaccines comprising polynucleotides encoding all Chikungunya virus structural proteins have been worked out (Akahata, W., et al., A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. Nat Med, 2010. 16(3): p. 334-8). However, VLPs produced *in vitro* are expensive to manufacture and require three administrations for a complete immunity, therefore these vaccines are not cheaply affordable. The CHIKV VLPs strategy disclosed in Akata *et al.* required several immunizations with an adjuvant to induce protection. For this reason, there is still a need for the design of improved vaccines that would enable the CHIKV VLPs to be generated *in vivo* in infected cells, in particular in infected cells of a host, and thus to provide an efficient and long-lasting immunity, especially which induces life-long immunity after only a single or two administration steps.

DESCRIPTION OF THE INVENTION

To this end, the inventors achieved the production of vaccines based on recombinant infectious replicative measles virus recombined with polynucleotides encoding Chikungunya virus antigens, which are recovered when the recombinant virus replicates in particular in the host after administration. The invention thus relates to a live CHIKV vaccine active ingredient based on the widely used Schwarz measles pediatric vaccine. In a preferred embodiment, this recombinant live MV-CHIKV vaccine yields CHIK virus-like particles by replicating in infected cells.

Measles virus is a non-segmented single-stranded, negative-sense enveloped RNA virus of the genus *Morbilivirus* 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 and 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 Measles viruses to generate recombinant Measles virus particles stably expressing structural antigens of Chikungunya virus, in particular as VLPs.

The invention thus relates to a nucleic acid construct which comprises a polynucleotide encoding at least one Chikungunya virus (CHIKV) structural protein said polynucleotide being operably linked, in particular cloned into a cDNA molecule which encodes the nucleotide sequence of the full-length, infectious antigenomic (+) RNA strand of a measles virus (MV).

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.

In a particular embodiment of the invention, the construct is prepared by cloning a polynucleotide encoding one structural protein or a plurality of structural proteins of CHIKV in the cDNA encoding the full-length antigenomic (+) RNA of the measles virus. 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 the at least one protein of the CHIKV, or each of these polynucleotides, is cloned into an ATU (Additional Transcription Unit) inserted in the cDNA of the measles virus. 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-dependant expression of a transgene, such as a promoter of the gene preceding, in MV cDNA, the insert represented by the polynucleotide encoding the CHIKV protein(s) inserted into a multiple cloning sites cassette.

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 the at least one structural protein of the CHIKV) that it contains.

The polynucleotide encoding at least one structural protein of CHIKV may thus be inserted in any intergenic region of the cDNA molecule of the measles virus in particular in an ATU. Particular constructs of the invention are those illustrated in the examples.

In a particular embodiment, when several distinct polynucleotides are present in the DNA construct, each of these polynucleotides encoding at least one structural protein of the CHIKV may be inserted in different sites of MV cDNA, possibly in distinct ATU of the cDNA of the measles virus.

In a preferred embodiment of the invention, the polynucleotide encoding at least one of the structural proteins of CHIKV is inserted in the intergenic region of the P and M genes of the measles virus cDNA molecule, in particular in an ATU.

The expression “encoding” used in the present application 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 of the invention 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 CHIKV 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 CHIKV 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 CHIKV infection or against CHIKV 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 CHIKV, 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, the cDNA encoding the nucleotide sequence of the full-length, infectious antigenomic (+) RNA strand of MV in nucleic acid construct complies with the rule of six (6) of the measles virus genome.

The organization of the genome of measles viruses 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 measles virus, 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. In this regard the rule of six applies individually to the cDNA encoding the nucleotide sequence of the full-length infectious antigenomic (+) RNA strand of the measles virus possibly but not necessarily to the polynucleotide cloned into said cDNA and encoding one or more protein(s) of the CHIKV.

According to a particular aspect of the invention, the nucleic acid construct comprises the following gene transcription units encompassing from 5' to 3':

(a) a polynucleotide encoding the N protein of a MV,
(b) a polynucleotide encoding the P protein of a MV,
(c) the polynucleotide encoding at least one CHIKV structural protein,
(d) a polynucleotide encoding the M protein of a MV,
(e) a polynucleotide encoding the F protein of a MV,
(f) a polynucleotide encoding the H protein of a MV, and
(g) a polynucleotide encoding the L protein of a MV,

said polynucleotides and nucleic acid construct being operably linked and under the 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 Measles virus. 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 cDNA molecule encoding the full-length, infectious antigenomic (+)RNA strand of a measles virus is characteristic of or is obtained from an attenuated strain of MV.

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 Measles virus 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 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 MV 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.

According to 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.

According to 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 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 measles virus 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 measles virus. 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 CNCM 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 nucleic acid construct of the invention is suitable and intended for the preparation of recombinant infectious replicative measles - Chikungunya virus (MV-CHIKV) 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-CHIKV virus and yield of CHIKV structural protein(s), in particular CHIKV VLPs. The pTM-MVSchw plasmid is suitable to prepare the transfer vector, by insertion of the CHIKV polynucleotide(s) necessary for the expression of CHIKV structural protein(s), in particular CHIKV VLPs.

The invention thus relates to a transfer vector, which is used for the preparation of recombinant MV-CHIKV particles when rescued from helper cells. The transfer vector of the invention is advantageously a plasmid, in particular a plasmid obtained from a Bluescript plasmid.

The invention also concerns the use of the transfer vector to transform cells suitable for rescue of viral MV-CHIKV particles, in particular to transfect or to transduce such cells respectively with plasmids or with viral vectors harbouring the nucleic acid construct of the invention, said cells being selected for their capacity to express required measles virus 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, replicative MV-CHIKV particles.

The invention also relates to the cells or cell lines thus transformed by the transfer vector of the invention and by further polynucleotides providing helper functions and proteins.

Polynucleotides are thus present in said cells, which encode proteins that include in particular the N, P and L proteins of a measles virus (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-CHIKV 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, the cDNA clone of a measles virus is from the same measles virus strain as the N protein and/or the P protein and/or the L protein. In another embodiment, the cDNA clone of a measles virus is from a different strain of virus than the N protein and/or the P protein and/or the L protein.

The invention thus relates to a process for the preparation of recombinant infectious measles virus 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-CHIKV virus expressing at least one structural protein of CHIKV.

According to a particular embodiment this process comprises:

1) transfecting helper cells with a nucleic acid construct according to the invention 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-CHIKV virus expressing at least one structural protein of CHIKV.

According to another particular embodiment of the invention the method for the production of recombinant infectious MV-CHIKV virus comprises :

1) recombining a cell or a culture of cells stably producing a RNA polymerase, the nucleoprotein (N) of a measles virus and the polymerase cofactor phosphoprotein (P) of a measles virus, with a nucleic acid construct of the invention and with a vector comprising a nucleic acid encoding a RNA polymerase large protein (L) of a measles virus, and

2) recovering the infectious, MV-CHIKV virus from said recombinant cell or culture of recombinant cells.

According to a particular embodiment of said process, recombinant MV are produced, which express CHIKV structural protein, in particular CHIKV VLPs wherein the particles express a combination of antigens, e.g. CE3E26KE1 antigens of CHIK virus. As an illustration, a process to rescue recombinant MV expressing CHIKV structural proteins, in particular CHIKV VLPs comprises the steps of :

1) cotransfecting helper cells, in particular HEK293 helper cells, that stably express T7 RNA polymerase, and measles N and P proteins with (i) a transfer vector, in particular a plasmid, comprising cDNA encoding the full-length antigenomic (+)RNA of a measles virus recombined with at least one polynucleotide encoding CHIKV structural proteins, for example encoding the CHIKV-CE3E26KE1 antigens and with (ii) a vector, especially a plasmid, encoding the MV L polymerase cDNA;
2) cultivating said cotransfected helper cells in conditions enabling the production of MV-CHIKV recombinant virus;
3) propagating the thus produced recombinant virus by co-cultivating said helper cells of step 2) with cells enabling said propagation such as Vero cells;
4) recovering replicating MV-CHIKV recombinant virus and CHIKV structural proteins, in particular CHIKV Virus Like Particles, in particular CHIKV-CE3E26KE1 VLPs.

Such a process, together with the constructs and conditions used are illustrated in Figure 1B.

As used herein, "*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 measles virus, 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 MV-CHIKV virus of the invention may involve a transfer of cells transformed as described herein. "*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-CHIKV virus *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-CHIKV virus 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-CHIKV virus. 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 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-CHIKV virus appropriate for use as immunization compositions.

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

As defined herein the nucleic acid construct of the invention and the MV-CHIKV virus of the invention encode or express at least one CHIKV structural protein.

By "Chikungunya virus structural protein" is meant a “protein” as defined herein the sequence of which is identical to a counterpart in a strain of CHIKV, including a polypeptide which is a native mature or precursor of a structural protein of CHIKV or is a fragment thereof or a mutant thereof as defined herein 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 Chikungunya virus capsid or envelope protein. Amino acid sequence identity can be determined by alignment by one of skill in the art using manual alignments or using the numerous alignment programs available (for example, BLASTP – http://blast.ncbi.nlm.nih.gov/). Fragments or mutants of CHIKV structural proteins of the invention may be defined with respect to the particular amino acid sequences illustrated herein.

According to the invention, the polynucleotide encoding at least one of the structural proteins of CHIKV encodes one or a plurality of proteins selected in the group of structural glycoproteins, structural polypeptides and capsid protein.

In a particular embodiment, the glycoproteins encompass the envelope glycoproteins E1, E2 and E3 envelope glycoproteins. The polypeptides encompass the 6K polypeptide and the capsid protein. In the following paragraphs the terms protein or glycoprotein will be used interchangeably to designate the E1, E2, or E3 glycoproteins or their combinations.

According to a particular embodiment of the invention, the polynucleotide encoding the CHIKV structural proteins is selected in the group of:

- A polynucleotide encoding one of the E1, E2, E3, 6K or C proteins;
- A fusion polynucleotide encoding several proteins selected among the E1, E2, E3, 6K and C proteins;
- A polynuclotide encoding the E3-E2-6K-E1 polyprotein.
- A polynucleotide which encodes the C-E3-E2-6K-E1 polyprotein and in particular the open reading frame (ORF) derived from a CHIKV genome or a corresponding cDNA which encodes said polyprotein;
- Any of these polynucleotides which has been modified in order to encode a mutated form of one or more of these proteins, in particular a mutated form of the E2 protein.

A particular polynucleotide in this respect encodes a soluble form of the E2 protein (sE2) or encodes the ectodomain of the E2 protein or its soluble form (sE2Δstem). In a particular embodiment, the polynucleotide encodes one of the following polypeptides: E3-sE2-6K-E1, E3-sE2Δstem-6K-E1, C-E3-sE2-6K-E1, and C-E3-sE2Δstem-6K-E1.

As used herein, the term “ectodomain” means the domain of glycoprotein E2 which extends outside the viral particle and is responsible for attachment to and entry into cells during infection by CHIKV viral particles.

According to a particular embodiment, the polynucleotide encodes a single epitope of a CHIKV structural protein or encodes a polyepitope resulting from expression of

repeated epitopes (having identical or similar sequences) or multiple distinct epitopes of one or many CHIKV structural proteins.

By way of illustration a polyepitope is formed by fusion of repeated polynucleotides encoding the E2 EP3 single epitope located at N-terminus of the E2 glycoprotein proximal to a furin E2/E3-cleavage site. The amino acid sequence of the E2 EP3 epitope is disclosed in Kam Y. W et al. (EMBO Mol Med 4, 330-343) and as SEQ ID NO: 33.

According to a particular embodiment of the invention, several polynucleotides wherein each polynucleotide encodes at least one structural protein of CHIKV are combined or fused to form a polynucleotide encoding several structural proteins of the CHIKV. These polynucleotides may distinguish from each other by the fact that they code for proteins of various strains of the CHIKV. As a result, the polynucleotide encodes a polyepitope as described herein, such as a polyepitope of single E2 EP3 epitope.

The polynucleotide(s) encoding at least one structural protein(s) of CHIKV is (are) cloned in the cDNA molecule encoding the full-length infectious antigenomic (+)RNA strand of a measles virus, to give rise to the nucleic acid construct of the invention, possibly into different sites.

According to one aspect of the invention, a polynucleotide encoding at least one Chikungunya virus (CHIKV) structural protein is derived from the genome of isolated and purified wild strain(s) of CHIKV. Wild strains of CHIKV can be for example the Ross strain (GenBank: AF490259.3), or the S27 strain (GenBank: AF339485.1), both isolated from patients during the 1952 Tanzania outbreak or, a strain isolated during the Senegal 1983 outbreak and named Ae. furcifer (GenBank: AY726732.1).

According to another aspect of the present invention, a polynucleotide encoding at least one CHIKV structural protein derives from the following purified and isolated wild strains of CHIKV: 05.61, 05.115, 05.209, 06.21, 06.27 and 06.49, which have been described in WO2007/105111. The nearly complete genome sequences of these CHIKV isolates representing distinct geographic origins, time points and clinical

forms of the Indian Ocean outbreak of Chikungunya virus have been sequenced and disclosed in WO2007/105111. 11,601 nucleotides were determined, corresponding to positions 52 (5'NTR) to 11,667 (3'NTR, end of third Repeat Sequence Element) in the nucleotide sequence of the 1952 Tanzanian isolate S27 taken as a reference (total length 11 ,826 nt).

The genome sequences of the isolates 05.61, 05.115, 05.209, 06.21, 06.27 and 06.49 presented in WO2007/105111 and from which the polynucleotides according to the present invention may be derived in a particular embodiment of the invention are organized as follows. Coding sequences consist of two large open reading frames (ORF) of 7,422 nt and 3,744 nt encoding the nonstructural polyprotein (2,474 amino-acids) and the structural polyprotein (1,248 amino-acids), respectively. The non structural polyprotein is the precursor of proteins nsP1 (535 aa), nsP2 (798 aa), nsP3 (530 aa) and nsP4 (611 aa), and the structural polyprotein is the precursor of proteins C (261 aa), p62 (487 aa, precursor to E3 - 64 aa - and E2 - 423 aa), 6K (61 aa), and E1 (439 aa). Cleavage sites characteristic of the alphavirus family in the non-structural and structural polyproteins are conserved. Glycosylation sites in E3, E2 and E1 are also conserved. The disclosure of the genome sequences is incorporated herewith by reference.

According to one embodiment, a polynucleotide encoding the CHIKV structural protein(s) is derived from the genome of the following group of wild strains of CHIKV designated: 06.115, 06.21, 06.27 and 06.49, previously described.

The term “derive” appearing in the definition of the polynucluotides merely specifies that the sequence of said polynucleotide may be identical to the corresponding sequence in a CHIKV strain or may vary to the extent that it encodes structural protein(s) of CHIKV that meet(s) the definition of the “protein” according to the present invention. Accordingly, the term does not restrict the production mode of the polynucleotide.

The polynucleotide(s) and nucleic acid construct of the invention may rather be prepared in accordance with any known method in the art and in particular may be

cloned, obtained by polymerization especially using PCR methods or may be synthesized.

The nucleic acid constructs of the invention are further defined as including one of the following polynucleotides encoding at least one CHIKV structural proteins.

According to a particular embodiment, a polynucleotide which encodes one or several structural protein(s) of CHIKV encodes a soluble form of the glycoprotein E2. As an example such a polynucleotide comprises a coding domain which has the sequence of SEQ ID NO: 2, 4, 6, 8.

In a particular embodiment, a polynucleotide which encodes one or several structural protein(s) of CHIKV encodes the E2 ectodomain. As an example, such a polynucleotide comprises a coding domain which has the sequence of SEQ ID NO: 10, 12, 14.

In another embodiment of the invention, the polynucleotide encoding a soluble form of the glycoprotein E2 encodes one polypeptide having an amino acid sequence selected in the group of SEQ ID NO: 3, 5, 7, 9.

In another embodiment of the invention, the polynucleotide encoding the ectodomain of the glycoprotein E2 encodes one polypeptide having an amino-acid sequence selected in the group of SEQ ID NO: 11, 13, 15.

According to a particular embodiment of the invention, the polynucleotide encoding structural protein of CHIKV encompasses one of the above defined nucleotide sequences encoding the soluble form of the E2 glycoprotein or the ectodomain of said protein and comprises further, a polynucleotide encoding one of the E3, E1, 6K or C protein, or a polynucleotide encoding any combination thereof.

Accordingly particular polynucleotides of the invention encoding structural polyprotein E2-6K-E1 of France/2010 strains isolated in Fréjus are selected in the following group: polynucleotides comprising a coding domain which has the sequence of SEQ ID NO: 16 (GenBank: CCA61130.1) and 20 (GenBank: CCA61131.1).

Preferred polynucleotide of the invention encodes structural proteins C-E3-E2-6K-E1 from a strain of CHIKV. In a particular embodiment, the polynucleotide encodes polyprotein C-E3-E2-6K-E1 of one of the strains named S27 or 06.49 and have respectively the sequences of SEQ ID NO: 20 and 27.

In another embodiment of the invention, the polynucleotide which is used encodes the E2 EP3 epitope or a polyepitope formed with a repetition of this epitope or comprising such repetition. This polynucleotide has in particular the nucleotide sequence disclosed as SEQ ID No: 32.

According to a preferred embodiment, the invention also concerns modifications and optimization of the polynucleotide to allow an efficient expression of the Chikungunya virus proteins at the surface of chimeric infectious particles of MV-CHIKV in the 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, in particular may be modified for the Maccaca codon usage or for the human codon usage. This optimization allows increasing the efficiency of chimeric infectious particles production in cells without impacting the expressed protein(s).

In particular, the optimization of the polynucleotide encoding the CHIKV protein(s) 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 the polynucleotides according to this particular embodiment of the invention, the following editing-like sequences from Measles virus can be mutated: AAAGGG, AAAAGG, GGGAAA, GGGGAA, as well as their complementary sequence: TTCCCC, TTTCCC, CCTTTT, CCCCTT. For example, AAAGGG can be mutated in AAAGGC, AAAAGG can be mutated in AGAAGG or in TAAAGG or in GAAAGG, and GGGAAA in GCGAAA.

An embodiment of a modified and optimized polynucleotide according to the present invention is as defined in SEQ ID NO:29. This polynucleotide encodes the soluble form of the envelope protein E2 without the stem region.

An embodiment of a modified and optimized polynucleotide encoding all the structural proteins C-E3-E2- 6K-E1 is as defined in SEQ ID NO: 31.

These optimized polynucleotides according to this particular embodiment of the invention as defined in SEQ ID NO: 29 and 31, present mutations in BsiWI and BssHII sites inside the sequences but not at the end of these sequences in order to maintain the sites for cloning purposes.

Therefore, according to this particular embodiment the invention provides nucleic acid constructs comprising polynucleotides which increase the efficiency of chimeric MV-CHIKV infectious particles production.

Other optimized polynucleotides comprising the sequences coding for structural proteins of the CHIKV and also suitable for use in the nucleic acid constructs of the invention are optimized fusion polynucleotides encoding one of the following combinations of structural proteins: E3-E2-6K-E1, E3-sE2-6K-E1, E3-sE2Δstem-6K-E1, C-E3-E2-6K-E1, C-E3-sE2-6K-E1, and C-E3-sE2Δstem-6K-E1.

The invention also relates to nucleic acid construct wherein the polynucleotide encoding at least one structural protein of CHIKV encodes one of the following polypeptides.

Examples of amino acid sequences of CHIK virus structural proteins according to preferred embodiments of the invention and related to a soluble form of the glycoprotein E2 from the following strains designated 05.115, 06.21, 06.27 and 06.49, are as defined in SEQ ID NO: 3, 5, 7, 9.

Examples of amino acid sequences of CHIK virus structural proteins according to preferred embodiments of the invention and related to the ectodomain of the glycoprotein E2 from the following strains designated 05.115, 06.21, 06.27 and 06.49, are as defined in SEQ ID NO: 11, 13, 15.

In a particular embodiment, the invention relates to fusion proteins constituted by the structural proteins E2-6K-E1 of a France/2010 strain isolated in Fréjus. These proteins result respectively from the expression of the polynucleotides which have the sequence of SEQ ID NO: 16 (GenBank: CCA61130.1) and 18 (GenBank: CCA61131.1). Their sequences are defined respectively in SEQ ID NO: 17 and 19.

In a particularly preferred embodiment, the invention concerns fusion proteins constituted by all CHIKV structural proteins. These proteins results from the expression of the polynucleotides encoding all the structural proteins C-E3-E2- 6K-E1 and are defined in SEQ ID NO: 21, 22, 23, 24, 25, 26 and 28.

The structural proteins C-E3-E2-6K-E1 obtained are able to auto-assemble into CHIKV virus-like-particles (VLPs), in the CHIKV-MV 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. Virus Like Particles in accordance with the invention do not carry genetic information encoding the proteins of the Virus Like Particles, in general, virus-like particles lack a viral genome and, therefore, are noninfectious and non replicative. In

accordance with the present invention, Virus Like Particles can be produced in large quantities and are expressed together with CHIKV-MV recombinant particles.

In a particular embodiment, the invention relates to a soluble form of the glycoprotein E2 without the stem region from the strain of CHIKV designated 06.49. This glycoprotein results from the expression of the modified and optimized polynucleotide as defined in SEQ ID NO:29. Its sequence is defined in SEQ ID NO: 30.

According to another aspect, the invention relates to recombinant CHIKV-Measles virus particles expressing the Chikungunya virus structural protein(s) as defined herein, in particular by reference to their nucleic acid and polypeptide sequences. The recombinant CHIKV-MV virus advantageously expresses the CHIKV structural proteins as VLPs.

The invention also concerns the association, in a composition, of virus-like particles of CHIKV structural proteins, in particular VLPs of C-E3-E2-6K-E1, with CHIKV-MV infectious replicative virus particles.

According to a preferred embodiment of the invention, the recombinant measles virus 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 measles virus strain adapted for vaccination, enable the production of recombinant Measles-Chikungunya infectious and replicative virus and the production of CHIKV-VLPs for use in immunogenic compositions, preferably protective or even vaccine compositions.

Advantageously, the genome of the recombinant Measles-Chikungunya infectious virus 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-CHIKV can also be achieved *in vivo* in

the host, in particular the human host to which recombinant MV-CHIKV is administered.

The invention also concerns a composition or an assembly of active ingredients which comprises the recombinant Measles-Chikungunya replicative virus in association with VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins. These compositions or assemblies induce an immune response, in particular a protective immune response, against Chikungunya virus, and in particular elicit antibodies production directed against Chikungunya virus structural proteins and/or elicit a cellular immune response against CHIKV infection. These compositions 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.

The invention relates in particular to a composition for administration to children.

The invention is also directed to an immunogenic composition, in particular a vaccine composition and in particular to a vaccine composition for administration to children. Said composition or vaccine is used for protection against CHIKV infection in a prophylactic treatment. Such a vaccine composition has advantageously active principles (active ingredients) which comprise recombinant Measles– Chikungunya infectious replicative viral particles rescued from the vector which has been defined herein associated with VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins.

In the context of the invention, the terms “associated” or “in association” refer to the presence, in a unique composition, of both MV-CHIKV recombinant viral particles and CHIKV structural proteins, in particular as VLPs, usually as physically separate entities.

The invention also concerns the recombinant MV-CHIKV infectious replicating virus particles in association with CHIKV-Virus structural proteins, in particular CHIKV-

Virus Like Particles expressing CHIKV structural proteins, in particular CHIKV-CE3E26KE1 VLPs or the composition according to the invention, for use in the treatment or the prevention of an infection by Chikungunya virus in a subject, in particular in a human.

The invention also concerns MV-CHIKV infectious, replicative virus and associated CHIKV-Virus structural proteins, in particular associated VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins for use in an administration scheme and according to a dosage regime that elicit an immune response, advantageously a protective immune response, against CHIKV virus 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 MV-CHIKV infectious, replicative virus and associated CHIKV structural proteins, in particular associated VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins.

Alternatively it may require multiple doses administration in a prime-boost regimen. Priming and boosting may be achieved with identical active ingredients consisting of MV-CHIKV infectious, replicative virus and associated CHIKV structural proteins, in particular associated VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins.

Alternatively priming and boosting administration may be achieved with different active ingredients, involving MV-CHIKV infectious, replicative virus and associated CHIKV structural proteins, in particular associated VLPs of CHIKV structural proteins, such as VLPs of CE3E26KE1 proteins in at least one of the administration steps and other active immunogens of CHIKV, such as CHIKV proteins or VLPs expressing C-E3-E2-6K-E1 polyprotein, in other administration steps.

The invention also concerns an assembly of different active ingredients including as one of these ingredients MV-CHIKV infectious, replicative virus and associated CHIKV structural proteins, in particular associated VLPs of CHIKV structural proteins,

such as VLPs of CE3E26KE1 proteins. The assembly of active ingredients is advantageously for use in immunization of a host, in particular a human host.

The inventors have shown that administration of MV-CHIKV infectious, replicative virus and associated VLPs of CHIKV structural proteins elicit an immune response and especially elicit antibodies that are cross-reactive for various CHIKV strains, at least in the ECSA genotype. 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 CHIKV, can elicit an immune response against a group of strains of CHIKV, in particular a group of strains of the ECSA genotype, in particular a group of strains encompassing CHIKV strain India, CHIKV strain Congo, CHIKV strain Thailand and CHIKV strain La Réunion.

Considering available knowledge on doses of vaccines suitable for other pathogens (such as HBV or HPV) which involve the administration of Virus Like Particles (VLP) and also for known human MV vaccines, the inventors have determined that the recovery of CHIKV-VLPs with the recombinant MV-CHIKV virus enables proposing administration of effective low doses of the active ingredients. Indeed, considering that the recombinant MV-CHIKV virus enables production of around 10^4 CHIKV-VLPs per recombinant MV-CHIKV replicated particle, and considering that the currently known doses for human MV vaccines are in the range of 10^3 to 10^4 pfu, a suitable dose of recombinant MV-CHIKV virus 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-CHIKV 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 composition or the vaccine defined herein may also be used for protection against an infection by the measles virus.

DESCRIPTION OF THE DRAWINGS

Figure 1 Schematic representation of ORFs for structural proteins of Chikungunya virus and for the backbone of MV genome - MV-CHIKV constructs including antigens of said CHIK virus for expression by Measles virus are proposed.

Figure 1B. Rescue of recombinant MV expressing CHIKV VLP

Figure 2 Immunofluorescence detection of E2 antigen in Vero cells infected by recombinant MV-CHIKV for 24h at MOI 0.1
E2 was detected using the anti-E2 Mab 3E4 used at 1/100 dilution and secondary antibody were used at 1/5000 dilution.

Figure 3: Expression of E2 and capsid proteins by MV-CHIKV vectors.
Cell lysates (cells) and supernatants (SN) of Vero cells infected for 24 h by MV-sE2Δstem, and MV-CE3E26KE1 analyzed by western blot. E2 was probed with the 3E4 Mab, and C protein was detected using an anti-capsid Mab (from P. Desprès) used at 1/100 dilution and secondary antibodies were used at 1/5000 dilution.

Figure 4. Electron microscopy analysis of CHIKV VLPs secreted in the supernatant of Vero cells infected by MV- CE3E26KE1 recombinant virus at MOI 0.1. Scale bar 200 nm (left) and 100 nm (right). Red arrows indicate the specific arrangement of spikes on particles surface and the icosahedral symmetry of the capsid protein inside the particles.

Figure 5. Sequence of the truncated sE2 expressed by MV-sE2 recombinant virus (156 aa, 19 kDa).

Figure 6: Growth kinetics of recombinant MV-sE2Δstem, and MV-CE3E26KE1 viruses compared with standard MV on Vero cells (MOI 0.01). Cell-associated virus titers are indicated in TCID50.

Figure 7: Immunization and challenge schedule of example 2.

Figure 8: Survival curve of mice lethally challenged with 100 PFU of CHIKV-06-49 after two immunizations with MV-CE3E26KE1 recombinant virus.

Figure 9: Immunization and challenge schedule of example 3.

Figure 10: Survival curve of mice lethally challenged with 100 PFU of CHIKV-06-49 after a single immunization with MV-CE3E26KE1 recombinant virus.

Figure 11: Immunization and challenge schedule of example 4.

Figure 12: Survival curve of mice lethally challenged with 100 PFU of CHIKV-06-49 after immunization with different doses of MV-CE3E26KE1 recombinant virus.

Figure 13: Passive transfer of immune sera and challenge schedule of example 5.

Figure 14: Survival curve of mice lethally challenged with 100 PFU of CHIKV-06-49 after passive transfer of MV-CE3E26KE1 immune sera.

Figure 15: Cell-mediated immune responses elicited in splenocytes of CD46-IFNAR mice immunized by a single injection of 106 TCID50 of MV-CHIKV.

Figure 16: Immunization and challenge schedule of example 6.

Figure 17: Survival curve of MV pre-immune mice lethally challenged with 100 PFU of CHIKV-06-49 after immunization with MV-CE3E26KE1.

Figure 18: PRNT assays performed against CHIK prior to first immunization on day 90 (prior to boost) and day 111 (21 days after boost).

EXAMPLES

Construction and characterization of recombinant Measles virus vectors expressing Chikungunya virus proteins.

The inventors designed three Chikungunya Virus antigens based on peptide sequences from native proteins of the strain 06-49 of Chikungunya virus. The native proteins enabling preparation of these peptide sequences were the five structural proteins which consist of capsid (C) envelope and accessory proteins E1, E2, E3 and 6K.

The first construct was directed to the expression of the soluble form of the envelope protein E2 (sE2), the second construct to the expression of the sE2 without the stem region (sE2Δstem), and the third construct was directed to the expression of all viral structural proteins (C-E3-E2-6K-E1) (Fig. 1). The experiment protocols have been described herein with respect to this latter construct.

Cell culture. Vero (African green monkey kidney) cells were maintained in DMEM GlutaMAX™ (Gibco-BRL) supplemented with 5% heat-inactivated fetal calf serum (FCS, Invitrogen, Frederick, MD). HEK-293-T7-MV helper cells (WO2008/078198) used for recombinant measles virus rescue were grown in DMEM supplemented with 10% FCS.

Construction of pTM-MVSchw-CE3E26KE1. The plasmid pTM-MVSchw, which contains an infectious MV cDNA corresponding to the anti-genome of the Schwarz MV vaccine strain, has been described elsewhere (Combredet, C., *et al.*, A molecularly cloned Schwarz strain of measles virus vaccine induces strong immune responses in macaques and transgenic mice. J Virol, 2003. 77(21): p. 11546-54). The cDNA encoding for the structural CE3E26KE1 CHIKV antigens was generated by chemical synthesis (GenScript, USA). It contains the sequence for viral structural proteins C-E3-E2-6K-E1 from CHIKV strain 06-49 (WO2007/105111). The complete sequence respects the “rule of six”, which stipulates that the number of nucleotides into the MV genome must be a multiple of 6, and contains *BsiWI* restriction site at the 5’ end, and *BssHII* at the 3’ end. The sequence was optimized for measles virus expression in mammalian cells. This cDNA was inserted into *BsiWI/BssHII*-digested pTM-MVSchw-ATU2, which contains an additional transcription unit (ATU) between the phosphoprotein (P) and the matrix (M) genes of the Schwarz MV genome (Combredet, C., *et al.*, A molecularly cloned Schwarz strain of measles virus vaccine induces strong immune responses in macaques and transgenic mice. J Virol, 2003.

77(21): p. 11546-54). The resulting plasmid was designated as pTM-MVSchw-CE3E26KE1.

Rescue of recombinant MV-CE3E26KE1. Rescue of recombinant Schwarz MV-CHIKV from the plasmid pTM-MVSchw-CE3E26KE1 was performed as previously described using a rescue system previously described (Radecke, F., *et al.*, Rescue of measles viruses from cloned DNA. Embo J, 1995. 14(23): p. 5773-84 ; WO2008/078198). Viral titers were determined by endpoint limit dilution assay on Vero cells and TCID50 was calculated by using the Kärber method.

Immunofluorescence. Immunofluorescence staining was performed on infected cells, as described elsewhere (Lucas, M., *et al.*, Infection of mouse neurons by West Nile virus is modulated by the interferon-inducible 2'-5' oligoadenylate synthetase 1b protein. Immun. Cell Biol., 2003. 81: p. 230-236). Cells were probed with mouse anti-E2 (3E4) and anti-capsid antibodies. Cy3-conjugated goat anti mouse IgG antibody Cy3 conjugated (Jackson Immunoresearch laboratories), was used as secondary antibody.

Western blot assays. Protein lysates from Vero cells infected with recombinant virus were fractionated by SDS-PAGE gel electrophoresis and transferred to cellulose membranes (Amersham Pharmacia Biotech). The blots were probed with mouse Mab 3E4 anti-E2 and anti-capsid. A goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Amersham) was used as a secondary antibody. Peroxidase activity was visualized with an enhanced chemiluminescence detection kit (Pierce).

Analysis of VLP production by electron microscopy. Vero cells (3 x T–150 flasks) were infected with MV–CHIKV recombinant virus at MOI 1. Supernatants collected after 36 h of infection were clarified by centrifugation at 3000 rpm for 30 min, layered on 20% sucrose cushion in PBS and centrifuged at 41,000 rpm for 2 h in a SW41 rotor. Pellets were resuspended in PBS with 1% BSA and analysed by electron microscopy. Negative staining was made by 2% uranyl acetate on copper grids coated with carbon and glow discharged just before use. The samples were observed at 80kV with a Jeol JEM1200 (Tokyo, Japan) transmission electron

microscope. Images were recorded using an Eloise Keenview camera and the Analysis Pro-software version 3,1 (Eloise SARL, Roissy, France).

Mice experiments. CD46-IFNAR mice susceptible to MV infection were produced as previously described (Combredet, C., *et al.*, A molecularly cloned Schwarz strain of measles virus vaccine induces strong immune responses in macaques and transgenic mice. J Virol, 2003. 77(21): p. 11546-54). Mice were housed under specific pathogen-free conditions at the Pasteur Institute animal facility. For immunization, six-week-old CD46-IFNAR mice were inoculated intraperitoneally (i.p.) with 10^5 TCID50 of recombinant MV-CE3E26KE1 or MV. For protection assays, immunized mice were i.p inoculated with 100 pfu of CHIKV 06-49 strain and mortality was followed for 2 weeks. All experiments were approved and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at Pasteur Institute. For passive transfer study, CD46-IFNAR mice were inoculated intraperitoneally with 20µl of pooled sera from 6 mice immunized with 10^5 TCID50 of MV-CE3E26KE1. Control mice received either 20 µl of pooled sera from mice immunized with 10^5 TCID50 of empty MVSchw or 20 µl of anti-CHIKV HMAF. The sera were diluted in a total volume of 100 µl in PBS before passive transfer at 24h and 16h before challenge with 100 pfu of CHIKV 06-49 strain, and then 12h post-challenge to mimic antibody persistence in infected animals. Mice mortality was analyzed for 2 weeks to determine protection.

Analysis of humoral immune responses. To evaluate the specific antibody responses, mice were bled via the periorbital route at different time after immunization. Sera were heat inactivated at 56°C for 30 min and anti-MV antibodies were detected by ELISA (ENZYGNOST-Siemens). HRP-conjugated anti-mouse immunoglobulin (Jackson Immuno Research) was used as secondary antibody. Anti-CHIKV antibodies were detected with a specific ELISA. Briefly, 96-wells plates were coated with a recombinant CHIKV-E2 protein produced in E. Coli. HRP-conjugated anti-mouse immunoglobulin was used as secondary antibody. The endpoint titers of pooled sera were calculated as the reciprocal of the last dilution giving twice the absorbance of sera from MV inoculated mice that served as negative controls. Anti-CHIKV neutralizing antibodies were measured by using a plaque reduction neutralization test (PRNT). Vero cells were seeded into 12-well plates for 24 h.

Serum samples were serially diluted in DMEM Glutamax/2% FCS. Dilutions of 100µl were incubated for 2 h at 37°C, under gentle agitation, with an equal volume of CHIKV containing 100 pfu of 06-49 strain. Remaining infectivity was then assayed on Vero cell monolayers overlaid with DMEM GlutaMAX™/2% FCS containing 0.8% final (wt/vol) carboxy methylcellulose. After 3 days of incubation, cells were fixed and stained with crystal violet for plaque count determination. The endpoint neutralization titer was calculated as the highest serum dilution tested that reduced the number of plaques by at least 50% (PRNT50).

Analysis of cell mediated immune response. Six-week-old CD46+/-IFNα/βR-/- mice were inoculated intraperitoneally with 10^6 TCID50 of MV-CHIKV recombinant virus. Control mice were immunized with 10^6 TCID50 of empty MV vector. Mice were euthanized at 7 days post-infection and spleens were collected. Splenocytes from immunized mice were incubated in RPMI, 10% FCS, and 10 IU of recombinant human interleukin-2 (rh-IL-2; Boehringer Mannheim). Their capacity to secrete IFN-γ upon stimulation was tested by enzyme-linked immunospot (ELISPOT) assay. Cells were stimulated 18h by concanavalin A (5 µg/ml; Sigma) as a positive control, RPMI–IL-2 (10 U/ml) as a negative control, CHIKV (MOI 1), or MV (MOI 1). Multiscreen-HA 96-well plates were coated overnight at 4°C with 5 µg of anti-mouse IFN-γ/ml (R4-6A2; Pharmingen) in PBS, washed, and then incubated with 100 µl of RPMI and 10% FCS for 1 h at 37°C. The medium was replaced by 100 µl of cell suspension ($5x10^5$ splenocytes per well in triplicate) and 100 µl of stimulating agent. After 2 h at 37°C, heated-FCS (10%) was added, and the plates were incubated for 18h at 37°C. After washing, biotinylated anti-mouse IFN-γ antibody (XMG1.2; Pharmingen) was added and the plates were incubated for 2 hours at room temperature. Streptravidin-alkaline phosphatase conjugate (Roche) was used as secondary step. Spots were developed with BCIP/ NBT (Promega) and counted (ELISpot Reader; Bio-Sys).

Expression of CHIKV virus-like particles by recombinant MV vector. CHIKV VLPs have been shown to elicit protective immunity against CHIKV infection (Akahata, W., *et al.*, A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. Nat Med, 2010. 16(3): p. 334-8). To benefit of this capacity, the inventors designed a recombinant MV vector able to

induce the secretion of CHIKV VLPs. To this aim, a cDNA encoding the C-E3-E2-6K-E1 structural proteins required for CHIKV VLPs production was chemically synthetized (Genscript) and optimized for measles virus expression in mammalian cells, then introduced into an additional transcription unit (ATU) of the Schwarz MV vaccine infectious cDNA (Figure 1). The recombinant MV-CHIKV virus was obtained by transfecting this plasmid into HEK-293 helper cells and propagation on Vero cells. Virus stocks were grown on Vero cells and titer was determined.

High amounts of CHIKV VLPs were secreted in the culture medium of infected cells, as demonstrated by immunofluorescence, western blot and electron microscopy.

This strategy provides a live recombinant MV vaccine virus secreting CHIKV VLPs at each round of replication. It was not expected that the assembly of native alphavirus particles would take place and that these VLPs would not hamper the simultaneous replication of a paramyxovirus. This is demonstrated here for the first time. Because MV vaccine is industrially produced as a crude viral extract, the batches of recombinant MV-CHIKV contain both live MV virus and non-replicating CHIKV VLPs. This strategy allows benefiting of the advantageous immunogenic property of multimeric antigens displayed on VLPs with no need of fastidious and expensive purification and concentration process. Moreover, no adjuvant is needed as the VLPs benefit of the advantageous immunogenic characteristics of live vaccines, such as balanced Th1 response and long-term memory.

The expression of CHIKV E2 and capsid antigens was demonstrated in infected Vero cells by immunofluorescence using a specific antibody (Mab 3E4) directed against the E2 protein of CHIKV (Figure 2). To look for the presence of secreted VLPs, the culture medium of infected cells was clarified by low-speed centrifugation, then layered onto a 20% sucrose cushion and concentrated by centrifugation at 41,000 rpm for 2 h in a SW41 rotor. Pellets were dissolved in PBS with 1% BSA. Proteins extracted from cell lysates and from concentrated culture media were fractionated by SDS-PAGE gel electrophoresis and transferred to cellulose membranes. The blots were probed with the 3E4 mouse Mab produced by hybridoma deposited at the CNCM (Collection Nationale de Cultures de Microorganismes, Paris, France) on September 6, 2007 under number I-3824, in the name of Institut Pasteur for the detection of E2 and an anti-capsid Mab (Figure 3).

The E2 protein was found both in cell lysates and in concentrated supernatant of infected cells at the correct size (46KDa), indicating the capacity of MV-CHIKV virus to induce the secretion of high-density particles containing the E2 protein. The capsid protein was also found in high-density particles, confirming the formation of CHIKV-VLPs. The presence of both C and E2 proteins in concentrated supernatant of infected cells suggests the formation of CHIKV VLPs. To observe their physical presence, the inventors analyzed by electron microscopy the pellets concentrated from supernatant of MV-CHIKV infected cells. The images revealed the presence of high amount of particles of size and morphology similar to those described after wild type CHIKV infection (Pletnev, S.V., *et al.*, Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. Cell, 2001. 105(1): p. 127-36 ; Zhang, W., *et al.*, Placement of the structural proteins in Sindbis virus. J Virol, 2002. 76(22): p. 11645-58) (Figure 4). The observed particles present an external diameter of 65 nm and a core diameter of 40 nm. The surface organization indicates the presence of spikes on the surface of the VLPs, similarly arranged than for other alphaviruses (Pletnev, S.V., *et al.*, Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. Cell, 2001. 105(1): p. 127-36 ; Zhang, W., *et al.*, Placement of the structural proteins in Sindbis virus. J Virol, 2002. 76(22): p. 11645-58). This observation confirms that infection of Vero cells by recombinant MV-CHIKV virus enables the secretion of high amounts of CHIK VLPs that self assemble.

The E2 protein was also expressed and secreted at the correct size (46KDa) by cells infected with Measles virus – sE2Δstem recombinant viruses, and Measles virus - CE3E26KE1 recombinant viruses.

Unfortunately, the analysis of MV-sE2 infected cells showed repeatedly the expression of a truncated form of the E2 protein. The inventors sequenced the E2 mRNA produced by MV-sE2 virus after RT-PCR amplification of infected cells. The analysis demonstrated the presence of a mutation generating a STOP codon, responsible for the truncation (Fig. 5).

The inventors then compared the replication rate of Measles Virus-sE2, Measles Virus-sE2Δstem, and Measles Virus-CE3E26KE1 recombinant viruses on Vero cells to standard Measles virus stock production, using a low MOI (0.01) (Fig. 6). The growth of MV-sE2 was similar to that of control MV. The growth of Measles virus-sE2Δstem and Measles virus-CE3E26KE1 recombinant viruses was slightly delayed, but their final titers were in the same range as that of empty Measles virus.

Immunogenicity of MV-sE2 and MV-CE3E26KE1 and protection in CD46-IFNAR mice.

CD46-IFNAR mice susceptible to MV infection were used to assess the immunogenicity of the recombinant MV-CHIKV viruses and their protective efficacy. These mice express the human CD46 gene with human-like tissue specificity and lack the type-I interferon receptors. Mice were housed under specific pathogen-free conditions at the Pasteur Institute animal facility and all experiments were approved and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at Pasteur Institute. Six-week-old CD46-IFNAR mice were inoculated intraperitoneally (i.p.) with doses ranging from 10^3 to 10^5 TCID50 of MV–CHIKV recombinant viruses and boosted 1 month later with the same dose of recombinant viruses. Control mice were immunized with the same dose of empty MVSchw vector. For antibody determination, blood samples were collected via the periorbital route 1 month after the first inoculation, then at 2 or 4 weeks after boosting.

Previous studies have shown that IFNAR mice are susceptible to lethal Chikungunya virus infection, showing pathological manifestations of infection and providing a model to evaluate immune mechanisms of protection (Couderc et al; 2008).

CD46-IFNAR mice susceptible to Measles virus infection were used to assess the immunogenicity of the recombinant Measles-Chikungunya viruses and their protective efficacy. These mice express the human CD46 gene with human-like tissue specificity and lack the type-I interferon receptors. Mice were housed under specific pathogen-free conditions at the Pasteur Institute animal facility and all experiments were approved and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at Pasteur Institute.

<u>Experiment 1</u> Analysis of the immunogenicity and the protective efficacy of Measles virus-CE3E26KE1 recombinant viruses in CD46-IFNAR mice.

Six-week-old CD46-IFNAR mice susceptible to Measles virus infection were intraperitoneally inoculated with 2.10^4 $TCID_{50}$ of Measles virus - Chikungunya virus recombinant viruses and boosted 1 month later with the same dose of recombinant viruses. Control mice were immunized with the same dose of empty Measles virus Schwarz vector (MV Schw). For antibody determination, blood samples were collected via the periorbital route 1 month after the first inoculation, then at 2 weeks after boosting. Mice were then challenged by i.p. injection of 100 pfu of Chikungunya virus 06-49 strain for evaluating protection (Immunization and challenge schedule is given in figure 7).

To evaluate the specific antibody responses, mice were bled at different time-points after inoculation. Sera were heat inactivated at 56°C for 30 min and anti - Chikungunya virus antibodies were detected by ELISA. 96-well plates were coated with recombinant Chikungunya virus-E2 protein produced in *E. coli*. HRP-conjugated anti-mouse immunoglobulin was used as secondary antibody and mouse antibodies anti-Chikungunya virus was used as a positive control. Anti-Chikungunya virus neutralizing antibodies were detected by a plaque reduction neutralization test (PRNT) (Warter L et al. JIM 2011 (D4 enclosed) and Russell PK et al. JIM 1967) on Vero cells using 50 PFU of Chikungunya virus-06-49 (produced on Vero cells). The endpoint titer was calculated as the highest serum dilution tested that reduced the number of PFU by at least 50% (PRNT50) or 90% (PRNT90).

A single injection of Measles virus-CE3E26KE1 recombinant viruses induced high antibody titers, which were strongly boosted by a second injection (table1 – figure 8). After two immunizations, high neutralizing titers were induced (PRNT50 = 450-4050 and PRNT90 = 50-450). All animals immunized with 104 or 105 TCID50 were protected from CHIKV lethal challenge with 100 PFU of CHIKV-06-49, whereas immunization with the lower dose (103 TCID50) protected 83% of the animals.

Table 1. Antibody response of CD46-IFNAR mice to immunization with MV-sE2 and MV-CE3E26KE1 (determined in pooled mice sera)

	Elisa 1 dose	Elisa 2 dose	PRNT50	PRNT90
MV	<100	<100	<50	<50
MV-CHIK.sE2	450	4000	<50	<50
MV-CHIK.CE3E26KE1	4000	>12000	1350	150
Anti-CHIKV HMAF	ND	ND	4050	450

After two immunizations, high neutralizing titers were induced (PRNT50 = 1350 and PRNT90 = 150) that protected mice from a lethal challenge with 100 PFU of Chikungunya virus-06-49.

<u>Experiment 2</u> Analysis of the immunogenicity and the protective efficacy of a single dose of Measles virus-CE3E26KE1 recombinant virus in CD46-IFNAR mice.

Six-week-old CD46-IFNAR mice were i.p. inoculated with 10^5 $TCID_{50}$ of Measles virus-CE3E26KE1 recombinant viruses. Control mice were immunized with the same dose of empty Measles virus Schw vector. Blood samples were collected via the periorbital route 2 weeks after immunization for antibody determination, and then mice were challenged by i.p. injection of 100 pfu of Chikungunya virus 06-49 (Immunization and challenge schedule is given in figure 9).

The Measles virus-CE3E26KE1 recombinant viruses induced high antibody titers after a single injection (table 2 – figure 10), and neutralizing titers that were sufficient to confer protection against a lethal challenge with 100 PFU of Chikungunya virus-06-49 in IFNAR mice.

Table 2. Antibody response elicited in CD46-IFNAR mice after a single immunization with MV-CE3E26KE1 virus

	MV Elisa titer	CHIKV Elisa titer	CHIKV PRNT50	CHIKV PRNT90
MV	10 000	<100	<50	<50
MV-CHIKV	10 000	4 050	150	50
Anti-CHIKV HMAF	ND	ND	12150	450

Experiment 3 Determination of the protective dose of Measles virus-CE3E26KE1 recombinant virus in CD46-IFNAR mice.

Six-week-old CD46-IFNAR mice were inoculated intraperitoneally (i.p.) with doses ranging from 10^3 to 10^5 TCID50 of MV-CHIKV recombinant virus and boosted one month later with the same dose. Control mice were immunized with the same dose of empty MVSchw vector. One month after the last immunization, mice were challenged by i.p. injection of 100 pfu of CHIKV 06-49 (Immunization and challenge schedule is given in figure 11). For antibody determination, blood samples were collected via the periorbital route 1 month after the first inoculation, then 1 month after boosting, just before challenge. Specific Elisa's were performed to detect anti-MV and anti-CHIKV binding antibodies. Anti-CHIKV neutralizing antibodies titers were determined by a plaque reduction neutralization test (PRNT) on Vero cells.

The results are given in table 3 and figure 12.

Table 3. Antibody response after immunization with different doses of MV-CE3E26KE1

	1st immunisation				2nd immunisation			
	MV Elisa titer	CHIKV Elisa titer	CHIKV PRNT50	CHIKV PRNT90	MV Elisa titer	CHIKV Elisa titer	CHIKV PRNT50	CHIKV PRNT90
MV 10^5	10 000	<100	<50	<50	300 000	<100	<50	<50
MV-CHIKV 10^3	1 000	1 350	50	<50	3 000	2 700	450	50
MV-CHIKV 10^4	3 000	4 050	150	50	30 000	12 150	1 350	150
MV-CHIKV-10^5	3 000	12 150	450	150	300 000	48 600	4 050	450

Both anti-MV and anti-CHIKV antibody titers increased when the dose of recombinant MV increased. A single immunization with MV-CE3E26KE1 virus induced high antibody titers, which were boosted by the second injection. After two immunizations, high neutralizing titers were induced (PRNT50 = 450-4050 and PRNT90 = 50-450). All animals immunized with 10^4 or 10^5 $TCID_{50}$ were protected from CHIKV lethal challenge with 100 PFU of CHIKV-06-49, whereas immunization with the lower dose (10^3 TCID50) protected 83% of the animals (Figure 12).

Experiment 4 Evaluation of the protection conferred by passive transfer of sera from mice immunized with recombinant MV-CE3E26KE1 virus.

Six-week-old CD46-IFNAR mice were i.p. inoculated with 20 µl of pooled sera from mice immunized with 10^5 TCID50 of recombinant MV-CE3E26KE1. Control mice received either 20 µl of pooled sera from mice immunized with 10^5 TCID50 of empty Measles virus Schwarz or 20 µl of anti-Chikungunya virus HMAF. The sera were diluted in a total volume of 100 µl PBS. The sera were transferred at 24h and 16h before challenge with 100 pfu of Chikungunya virus 06-49, and then 12h post-challenge to mimic antibody persistence in infected animals. Mice mortality was analyzed for 2 weeks to determine protection (Immunization and challenge schedule is given in figure 13).

Passive transfer of immune sera of mice immunized with MV-CE3E26KE1 virus protected 83% of recipient mice from lethal Chikungunya virus challenge, while mice that received anti-Chikungunya virus HMAF were fully protected. In contrast, mice that received immune sera from mice immunized with empty Measles viruses all died. These results indicate that humoral immune responses induced by Measles virus-CE3E26KE1 recombinant viruses confer protection against Chikungunya virus infection in CD46-IFNAR mice (figure 14).

Experiment 5 Induction of specific cell-mediated immune responses.

To determine whether immunization with MV-CHIKV elicited cell-mediated immune responses, we measured by ELISPOT assay the capacity of splenocytes from immunized mice to secrete IFN-γ upon specific ex-vivo stimulation. Splenocytes were collected 7 days after a single immunization and both MV-specific and CHIKV-specific responses were evaluated. CHIKV and MV were used at an MOI of 1 for splenocytes stimulation. A significant number of CHIKV-specific cells (up to 300/106 splenocytes, mean 150/106) were detected (Figure 15), which represents one third of MV-specific response in similar stimulation condition (up to 600/106 splenocytes, mean 500/106). All mice immunized with MV-CHKV, except one out of eight, had a significant CMI response to CHIKV. In contrast, control mice immunized with empty

MVSchw had a similar MV-specific response but no CHIKV-specific response. These results show that a single inoculation of MV-CHIKV induced high levels of CHIKV- and MV-specific cellular immune response in the spleen of immunized mice.

Experiment 6 Analysis of Measles virus pre-immunity impact on the immunogenicity and protective efficacy of recombinant MV-CE3E26KE1 virus in CD46-IFNAR mice.

Six-week-old CD46-IFNAR mice were i.p. inoculated with 5.10^3 $TCID_{50}$ of empty Measles virus Schwarz (Group1 Figure 16) to mimic pre-immunity. Three months later, these mice were injected twice with 10^5 $TCID_{50}$ of Measles virus-CE3E26KE1 recombinant viruses at one month of interval. Control mice were immunized with 10^5 $TCID_{50}$ of Measles virus-CE3E26KE1 recombinant viruses (Group 2) or 10^5 $TCID_{50}$ of empty Measles virus Schw (Group 3). For antibody determination, blood samples were collected via the periorbital route as indicated in Figure 16, and mice were then challenged by i.p. injection of 100 pfu of Chikungunya virus 06-49 strain for protection assays.

This experiment demonstrates that CD46-IFNAR mice previously immunized with 5.10^3 TCID50 of empty Measles viruses are able to mount a protective Chikungunya virus immune response after immunization with Measles virus-CE3E26KE1 recombinant viruses. The ELISA and PRNT titers (Table 4 – figure 17) remain high and in the same range of that induced in naive mice (ELISA titer unchanged and PRNT titers reduced by one-fold dilution). 100% of the vaccinated animals were protected from Chikungunya virus lethal challenge in both pre-immune and naive groups of animals immunized with Measles virus-CE3E26KE1 recombinant viruses.

Table 4. Antibody response of CD46-IFNAR mice to MV-CE3E26KE1 in the presence of pre-immunity to MV vector

Immunizations	1st	2nd				3rd			
	MV Elisa	MV Elisa	CHIKV Elisa	CHIKV PRNT50	CHIKV PRNT90	MV Elisa	CHIKV Elisa	CHIKV PRNT50	CHIKV PRNT90
MV + MV-CHIKV	3 000	30 000	12 150	150	50	30 000	150 000	12 150	150
MV-CHIKV	ND	10 000	12 150	450	150	100 000	150 000	12 150	450
MV	ND	10 000	<50	<50	<50	100 000	<50	<50	<50

EXPERIENCE-7: Cross-reactivity of Abs elicited by vaccination

To determine whether immunization with MV-CHIKV elicited cross-reactive antibody response to different CHIKV primary isolates, sera obtained from animals of experiment 4 were tested for their ability to neutralize different CHIKV primary isolates. Four strains belonging to the ECSA genotype were chosen.

- CHIKV strain India, clinical isolate n° 3710 (NRC for Arbovirus, France), isolated in 2011. Passage 1 on Vero cells (December 2011), virus titer 6.3 log PFU/ml
- CHIKV strain Congo, clinical isolate n° 525 (NRC for Arbovirus, France), isolated in 2011. Passage 1 on Vero cells (June 2011), virus titer 6.5 log PFU/ml
- CHIKV strain Thailand, clinical isolate n° 1499 (NRC for Arbovirus, France), isolated in 2009. Passage on C6/36 cells (December, 2009), virus titer 6.3 log PFU/ml
- CHIKV strain La Réunion, clinical isolate 2006.49 (NRC for Arbovirus, France), isolated in 2006. Passage 3 on Vero cells (April 4th, 2011), virus titer 7.3 log PFU/ml

Anti-CHIKV neutralizing antibodies were detected by use of a plaque reduction neutralization test (PRNT). Vero cells were seeded into 12µwell plates for 24 h. Serum samples were serially diluted in DMEM Glutamax/2% FCS. Dilutions 100µl were incubated for 2 h at 37°C, under gentle agitation, with an equal volume of CHIKV containing 100 pfu of 06-49 strain. Remaining infectivity was then assayed on Vero cell monolayers overlaid with DMEM GlutaMAX/2% FCS containing 0.8% final (wt/vol) carboxy methylcellulose. After 3 days of incubation, cells were fixed and stained with crystal violet for plaque count determination. The endpoint neutralization titer was calculated as the highest serum dilution tested that reduced the number of plaques by at least 50% ($PRNT_{50}$).

Table 5. Cross-reactivity of neutralizing Ab from mice immunized with MV-CHIKV

	PRNT50 post 1st		PRNT50 post 2nd		PRNT50 post challenge	
	MV-CHIKV	MV	MV-CHIKV	MV	MV-CHIKV	MV
06-49	50	< 50	1350	< 50	450	ND
Inde	150	< 50	1350	< 50	13250	ND
Congo	< 50	< 50	1350	< 50	1350	ND
Thai	150	< 50	4050	< 50	12150	ND

The result shows that immunization of mice with MV-CHIKV induces cross-reactive antibodies that are able to neutralize several primary isolates of CHIKV from different countries. Interestingly, the challenge of animals with the 06-49 La Reunion virus results in even broadening the response and boosts the neutralization of Indian and Thai isolates to very strong levels. Only ECSA genotype was tested because of its availability at Institut Pasteur.

EXPERIENCE-8: Immunogenicity of MV-CHIKV in cynomolgus macaques

The immunogenicity of recombinant measles virus vaccine against Chikungunya was tested in non-human primates. Two groups of four cynomolgus macaques (*macaca fascicularis*) previously selected to be seronegative for flaviviruses and measles virus were vaccinated subcutaneously with 10^4 or 10^5 $TCID_{50}$ MV-CHIKV on day 0 then boosted on day 90 with the same dose. Serum and plasma were collected and stored at -20°C for later analysis. Neutralizing antibodies to Chikungunya virus were detected by using a PRNT assay.

Table 6.

Group	Animal	Code	Vaccine	Dose	Reciprocal CHIK-PRNT			
					Day 0	Day 21	Day 90	Day 111
D	D1	12/9	MV-CHIK	10^4	<10	574	<10	14
	D2	12/4	MV-CHIK	10^4	<10	142	73	97
	D3	12/5	MV-CHIK	10^4	<10	16	17	121
	D4	12/10	MV-CHIK	10^4	<10	70	83	118
E	E1	12/16	MV-CHIK	10^5	<10	247	101	382
	E2	12/23	MV-CHIK	10^5	<10	103	178	355
	E3	12/26	MV-CHIK	10^5	<10	6173	151	652
	E4	12/22	MV-CHIK	10^5	<10	97	386	228

Results presented in Table 6 show that all monkeys developed high titers of antibodies that neutralized CHIKV. The highest dose was more efficient than the lower dose. In most animals, boosting was efficient, as shown on figure 18 (day 90, the day of boost versus day 111, 21 days after boost). These results demonstrate the immunogenicity of MV-CHIKV vaccine candidate in non-human primates.

GENERAL CONCLUSION

The inventors have generated a recombinant MV-CHIK virus expressing stably the complete structural proteins CE3E26KE1 of CHIKV strain 06.49. Vero cells infected by this recombinant virus expressed high levels of CHIKV proteins and secreted high density VLPs. The recombinant virus was slightly delayed in growth kinetics but yielded similar titers that empty MV vector. Evaluated in CD46-IFNAR mice susceptible to MV infection, this vaccine candidate induced high levels of neutralizing antibodies to CHIKV depending on the dose and number of administrations (PRNT50

= 450-4050; PRNT90 = 150-450). All immunized mice were repeatedly protected, even after a single administration, demonstrating the strong immune capacity of this vaccine candidate. The passive transfer of immune sera in naïve animals conferred protection from lethal challenge, even in these highly susceptible mice. Lastly, the inventors demonstrated that the presence of pre-existing immunity to MV vector in CD46-IFNAR mice did not prevent the induction of protective immunity after immunization with MV- CE3E26KE1 vaccine candidate. In view of the results, the recombinant vector thus obtained deserves to be evaluated in a reliable non-human primate model of infection.

CLAIMS

1. A nucleic acid construct which comprises a polynucleotide encoding the C-E3-E2-6K-E1 structural proteins of a Chikungunya virus (CHIKV), said polynucleotide being operably linked, in particular cloned into a cDNA molecule which encodes the nucleotide sequence of the full-length, infectious antigenomic (+) RNA strand of a measles virus (MV).

2. The nucleic acid construct according to claim 1, characterized in that said nucleic acid construct complies with the rule of 6 of measles genome.

3. The nucleic acid construct according to claim 1 or 2, which comprises gene transcription units encompassing from 5' to 3':

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

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

(c) the polynucleotide encoding the C-E3-E2-6K-E1 structural proteins of CHIKV,

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

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

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

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

said polynucleotides being operably linked in the nucleic acid construct and under the 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, characterized in that said Measles virus is an attenuated virus strain, said strain being in particular selected in the group 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, characterized in that said polynucleotide encoding the C-E3-E2-6K-E1 structural proteins of CHIKV has been optimized for the Macacca codon usage or has been optimized for the human codon usage.

6. The nucleic acid construct according to any one of claims 1 to 5, characterized in that *measles editing-like sequences* have been deleted from said polynucleotide encoding the C-E3-E2-6K-E1 structural proteins of CHIKV.

7. The nucleic acid construct according to any one of claims 1 to 6, characterized in that said Chikungunya virus is from the strain designated 06-49 strain.

8. The nucleic acid construct according to any one of claims 1 to 6, characterized in that the ectodomain of envelope protein E2 has the sequence of SEQ ID NO: 11, 13, 15.

9. The nucleic acid construct according to any one of claims 1 to 6, characterized in that said C-E3-E2-6K-E1 structural proteins are as defined in SEQ ID NO: 21, 22, 23, 24, 25, 26 and 28.

10. The nucleic acid construct according to any one of claims 1 to 6, characterized in that said nucleic acid construct is as defined in SEQ ID NO: 20, 27 and 31.

11. A transfer vector plasmid which comprises a nucleic acid construct of any one of claims 1 to 10.

12. The transfer vector plasmid according to claim 11, characterized in that said vector is pMV-CHIKV.

13. Cells characterized in that said cells are transformed with a nucleic acid construct according to any one of claims 1 to 10 or a transfer vector plasmid according to claim 11 or 12, in particular eukaryotic cells, such as avian cells, in particular CEF cells, mammalian cells or yeast cells.

14. Recombinant infectious replicating MV-CHIK virus particles, characterized in that said virus particles are the product of expression of a nucleic acid construct according to any one of claims 1 to 10 or a transfer vector plasmid according to claim 11 or 12, in a host cell producing, in particular a host cell stably producing, at least an

RNA polymerase recognized by said host cell, a nucleoprotein (N) of a MV, a phosphoprotein (P) of a MV and optionally a MV RNA-polymerase large protein (L).

15. The recombinant infectious replicating MV-CHIK virus particles according to claim 14, characterized in that said virus particles are the product of expression of a nucleic acid construct as defined in SEQ ID NO: 27 or 31.

16. A composition or an assembly of active ingredients comprising the recombinant infectious replicating MV-CHIK virus particles according to claim 14 or 15, in association with CHIKV-C-E3-E2-6K-E1 VLPs and a pharmaceutically acceptable vehicle.

17. Use of the composition or the assembly of active ingredients according to claim 16 for the manufacture of a vaccine in the elicitation of a protective immune response against CHIK virus by the elicitation of antibodies directed against said CHIKV protein(s) and/or of a cellular immune response, in a host, in particular a human host in need thereof.

18. Use of the recombinant infectious replicating MV-CHIK virus particles according to claim 14 or 15 in association with CHIKV-C-E3-E2-6K-E1 VLPs, or the composition or the assembly of active ingredients according to claim 16, in the manufacture of a medicament for the prevention of an infection by Chikungunya virus in a subject, in particular in a human.

19. A process to rescue recombinant measles virus (MV) expressing the C-E3-E2-6K-E1 structural proteins of a Chikungunya virus (CHIKV), in particular CHIKV-C-E3-E2-6K-E1 Virus Like Particles (VLPs) comprising the steps of:

1) cotransfecting helper cells, in particular HEK293 helper cells, that stably express T7 RNA polymerase, and measles N and P proteins with (i) a transfer vector plasmid according to claim 11 or 12 and with (ii) a vector, especially a plasmid, encoding the MV L polymerase;

2) cultivating said cotransfected helper cells in conditions enabling the production of MV-CHIKV recombinant virus;

3) propagating the thus produced recombinant virus by co-cultivating said helper

cells of step 2) with cells enabling said propagation such as Vero cells;
4) recovering replicating MV-CHIKV recombinant virus and the C-E3-E2-6K-E1 structural proteins of CHIKV, in particular CHIKV-C-E3-E2-6K-E1 VLPs.

20. The process according to claim 19, wherein the transfer vector plasmid comprises a nucleic acid construct as defined in SEQ ID NO: 27 or 31.