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Pediatric measles vaccine expressing a dengue tetravalent antigen elicits neutralizing antibodies against all four dengue viruses

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ABSTRACT

Dengue disease is an increasing global health problem that threatens one-third of the world's population. To control this emerging arbovirus, an efficient preventive vaccine is still needed. Because four serotypes of dengue virus (DV) coexist and antibody-dependent enhanced infection may occur, most strategies developed so far rely on the administration of tetravalent formulations of four live attenuated or chimeric viruses. Here, we evaluated a new strategy based on the expression of a single minimal tetravalent DV antigen by a single replicating viral vector derived from pediatric live-attenuated measles vaccine (MV). We generated a recombinant MV vector expressing a DV construct composed of the four envelope domain III (EDIII) from the four DV serotypes fused with the ectodomain of the membrane protein (ectoM). After two injections in mice susceptible to MV infection, the recombinant vector induced neutralizing antibodies against the four serotypes of dengue virus. When immunized mice were further inoculated with live DV from each serotype, a strong memory neutralizing response was raised against all four serotypes. A combined measles-dengue vaccine might be attractive to immunize infants against both diseases where they co-exist.

1. Introduction

Dengue fever is currently the most significant arboviral disease, and one of the most severe public health problems that threatens 2.5 billion people in tropical and subtropical areas, infects an estimated 50–100 million people and causes about 25,000 death each year [1]. Dengue viruses (DV) are enveloped, positive-stranded RNA viruses. The DV complex consists of four closely related but antigenically distinct DV serotypes, members of the *Flaviviridae* family [2]. Infection by one serotype provides protection against this serotype, but increases the risk of developing severe disease if infection by a different serotype occurs, possibly through an antibody-dependent enhancement (ADE) by cross-reactive DV antibodies [3,4]. Despite decades of efforts, no licensed vaccine against dengue is currently available. A preventive dengue vaccine needs to protect unexposed individuals against all four serotypes of DV. It must be tetravalent, safe for young babies, provide long-lasting protective immunity, and be cost-effective. For these reasons, most of the strategies developed so far rely on tetravalent formulations of four live attenuated vaccines or four chimeric viruses based on the exchange of homologous structural genes between different flaviviruses [5–8]. Both strategies have shown good immunogenicity in clinical trials [9–13] and are progressing to late clinical trials. However, their development is complicated by problems of interference between the serotypes, and difficulty to optimize the formulation [14–17].

To avoid interference problems while still benefiting of the advantageous capacity of the live vaccines, one strategy is to express a single tetravalent DV antigen by a single replicating viral vector. The DV antigen would be of limited size and induce long-lasting protective immunity against DV infection regardless the serotype. The crystal structures of the envelope E protein from TBEV [18], DV1 [19], DV2 [20], DV3 [21] and WNV [22,23] have revealed that it contains three distinct ectodomains EDI, EDII and EDIII. The central EDII contains immunodominant epitopes close to the fusion loop. These epitopes elicit cross-reactive subneutralizing antibodies that can favor the risk of ADE [24–26]. On the opposite, the C-terminal immunoglobulin-like EDIII, a 100 amino-acid sequence stabilized by a disulfide bridge, is involved in receptor binding and contains critical epitopes that elicit both type-specific antibodies (i.e., that cross-neutralize DV but not other Flaviviruses), and serotype-specific antibodies (i.e., that neutralize one DV serotype but not another) [27–33]. For these reasons, the EDIII has emerged as the antigen of choice to develop a tetravalent DV vaccine [34–39].

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With the aim to develop an affordable vaccine that could be used in young children living in tropical areas, we propose a new strategy based on the expression of a minimal tetravalent DV antigen by a vector derived from pediatric live-attenuated measles vaccine (MV). Such a combined measles-dengue vaccine might be attractive to immunize children against measles and dengue in areas where both diseases co-exist. We previously developed a vector derived from the live-attenuated Schwarz strain of measles virus (MV) [40]. MV vaccine is a live-attenuated negative-stranded RNA virus proven to be one of the safest and most effective human vaccines. Produced on a large scale in many countries and distributed at low cost through the extended program on immunization (EPI) of WHO, this vaccine induces life-long immunity to measles after one or two injections [41–43]. We previously showed that MV vector stably expressed different proteins from HIV and flaviviruses and induced strong and long-term specific neutralizing antibodies and cellular immune responses, even in presence of preexisting immunity to MV [44–47]. In collaboration with an industrial vaccine manufacturer, we initiated a clinical development to build GMP and regulatory logistics for a recombinant MV-HIV vector and to evaluate its safety and immunogenicity in humans with preexisting immunity to measles. To adapt this technology to dengue vaccine and as a proof-of-concept, we previously designed and inserted into MV vector a minimal combined dengue antigen composed of the envelope domain III (EDIII) fused to the ectodomain of the membrane protein (ectoM) from DV serotype-1 [48]. Immunization of mice resulted in a long-term production of DV1 serotype-specific neutralizing antibodies that were not cross-reactive with the other DV serotypes. The presence of the pro-apoptotic sequence ectoM was critical to the immunogenicity of EDIII, its adjuvant capacity correlating with its ability to promote the maturation of dendritic cells and the secretion of proinflammatory and antiviral cytokines and chemokines involved in adaptive immunity.

In the present work, we designed tetravalent DV antigens incorporating the EDIII of DV1-4 in combination with the pro-apoptotic ectoM sequence and generated three recombinant MV vectors expressing these antigens. In a mouse model of MV infection, we demonstrated that one of the recombinant vectors is efficient at inducing neutralizing antibodies against DV of the four serotypes.

2. Materials and methods

2.1. 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). Helper 293-3-46 cells (a gift from M.A. Billeter, Zurich University) used for viral rescue were grown in DMEM/10% FCS and supplemented with 1.2 mg of G418 per ml.

2.2. Construction of pTM-MVSchw-TetraA, pTM-MVSchw-TetraB and pTM-MVSchw-TetraC plasmids

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 [40]. The tetrameric antigens have been generated by chemical synthesis by Genecust. The antigens TetraA, TetraB and TetraC have been designed based on the sequences encoding the EDIII from the E protein (aa 295–394) and the ectodomain of the membrane M protein (aa 1–40) from the M protein from strain FGA/89 French Guiana for serotype DV1 [80], Jamaica/N.1409 for DV2 (Genbank accession no. M20558) [81], DV3 H87 (WHO reference strain, Genbank accession no. M93130) and 63632/76 Burma for DV4. When indicated in the construction (see Fig. 1), the original furin-like cleavage site RRDKR was introduced. All the antigens contain in the 5′ end the human calreticulin-derived endoplasmic reticulum targeting signal sequence contained in the pEGFP-RE vector (Clontech). The sequences respect the "rule of six", which stipulates that the number of nucleotides into the MV genome must be a multiple of 6 and contain the *BsiWI* digestion site at the 5′ end, and the *BssHII* digestion site at the 3′ end. The sequences have been optimized for measles virus expression in mammalian cells. The cDNA antigen sequences corresponding to TetraA, TetraB and TetraC were received from Genecust in the pUC57 vector, and 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 [40]. The resulting plasmids were designated as pTM-MVSchw-TetraA, pTM-MVSchw-TetraB and pTM-MVSchw-TetraC.

2.3. Rescue of recombinant MV-TetraA, MV-TetraB and MV-TetraC from the cloned cDNA

Rescue of recombinant Schwarz MV from the plasmids *pTM-MVSchw-TetraA*, *pTM-MVSchw-TetraB* and *pTM-MVSchw-TetraC* was performed as previously described [40] using the helper-cell-based rescue system described by Radecke et al. [82] and modified by Parks et al. [83]. The titers of *MV-TetraA*, *MV-TetraB* and *MV-TetraC* were determined by an endpoint limit dilution assay on Vero cells. The TCID50 was calculated by using the Kärber method.

2.4. Production of recombinant EDIII proteins in Drosophila S2 cells

The EDIII PCR products described above were cloned into pMT/Bip/V5-His A plasmid (Invitrogen) between *BglII* and *NotI* restriction sites. The clones were validated by sequencing. *Drosophila* S2 cells (Invitrogen) were transfected by these plasmids using the Calcium Phosphate Transfection Kit (Invitrogen). Transfected cells were selected by adding 25 μg/ml blasticidin. The EDIII protein production was induced by adding 750 μM $CuSO_4$. Cell culture supernatant was filtered on 0.2 μM filters before concentration on 10,000-MWCo Vivaspin columns (Vivasciences) eluted with PBS. Recombinant proteins were semi-quantified by Western blot using the MAb 9D12 reactive to EDIII from DV [31].

2.5. Production of recombinant EDIII proteins in E. coli

The following DV strains from the collection of Institut Pasteur were used: strain FGA/89 French Guiana for serotype DV1 [84], Jamaica/N.1409 for DV2 [81], PaH881/88 Thailand for DV3 and 63632/76 Burma for DV4. The *Escherichia coli* strain BL21 (DE3) and SB medium have been described [85]. Plasmids pLB11, pLB12, pLB13, pLB14, coding respectively for EDIII from DV serotypes 1, 2, 3, and 4 (residues 296–400) with an hexahistidine tag in C-term, under control of the T7 promoter and pelB signal sequence, were constructed by insertion of RT-PCR products obtained with primers specific for EDIII, in plasmid pET20b+ (Novagen) [70]. The DV-EDIII-H6 recombinant proteins were produced from these plasmids in *E. coli* BL21(DE3). Bacteria were grown at 24 °C in SB medium with ampicillin (200 μg/mL) until $A_{600\,nm}$ = 1.5–2.0 and then induced for 2.5 h with 1 mM IPTG to obtain the expression of the recombinant genes. The purification of DV-EDIII-H6 proteins from bacteria's periplasmic fluid was performed by chromatography on a NiNTA resin (Qiagen, Hilden) and concentration determined by absorbance spectrometry as previously described [69]. The protein fractions were analyzed by SDS-PAGE in reducing conditions. The fractions that were homogeneous at >95%, were pooled, dialyzed against 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, snap frozen in liquid nitrogen, and stored at −80 °C.

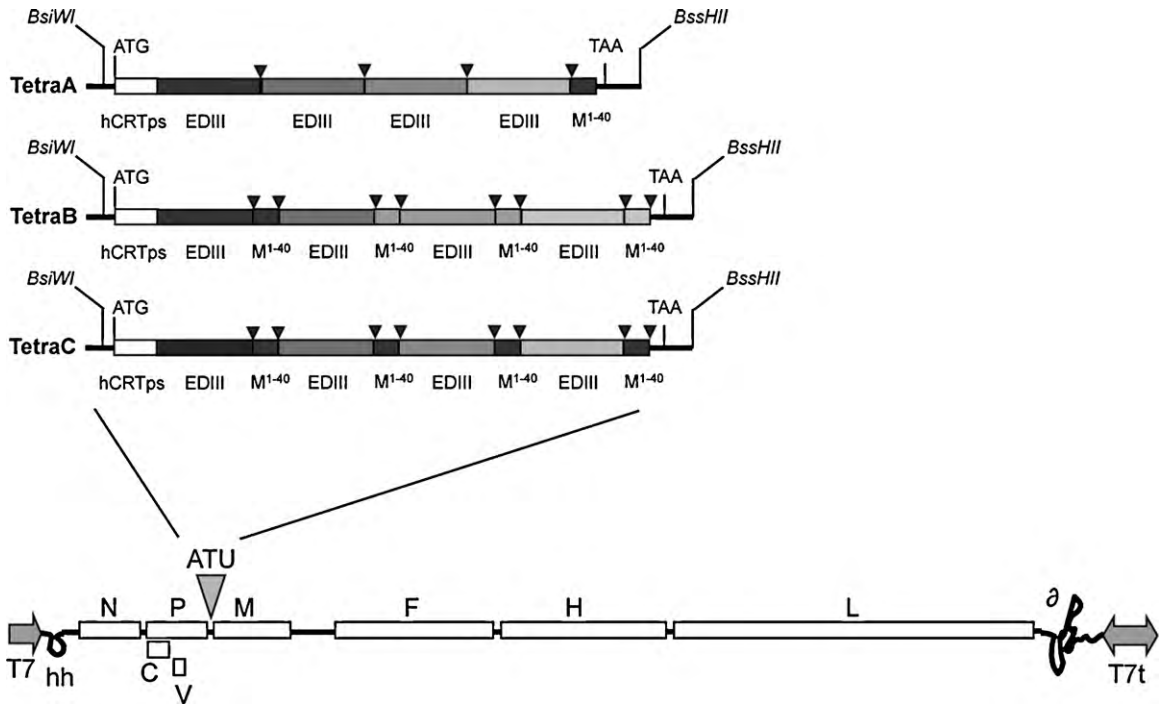

Fig. 1. Schematic representation of DV tetravalent constructs and of recombinant MV vector. The human calreticulin peptide signal sequence (hCRT ps), the DV1, 2, 3 and 4 envelope E domain III (EDIII) and the M ectodomain (ectoM) are indicated. Original furin-like cleavage site RRDKR and amino acid positions are indicated. The TetraA, B and C sequences were cloned into the ATU position of MV vector using BsiWI/BssHII sites. The MV genes are indicated as follows: nucleoprotein (N), phosphoprotein and V/C accessory proteins (PVC), matrix (M), fusion (F), hemagglutinin (H) and polymerase (L). T7 RNA polymerase promoter (T7), T7 RNA polymerase terminator (T7t), hepatitis delta virus ribozyme (h), hammerhead ribozyme (hh).

2.6. Immunofluorescence

Immunofluorescence staining was performed on infected cells, as described elsewhere [86]. Cells were probed with mouse anti-DV1 HyperImmune Ascitic Fluid [87] for the EDIII of DV1, the Mab 3H5 for the EDIII of DV2, the Mab 8A1 for the EDIII of DV3, and mouse anti-DV1 HyperImmune Ascitic Fluid [87] for the EDIII of DV4 (all DV-specific antibodies were used at 1/100 dilution). Cy3-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) was used as secondary antibody (1/100 dilution).

2.7. Western blot assays

Protein lysates from Vero cells infected with recombinant viruses were fractionated by SDS-PAGE gel electrophoresis and transferred to cellulose membranes (Amersham Pharmacia Biotech). DV1, or DV2 EDIII (5 ng) produced in drosophila cells was loaded as a positive control. The blots were probed with the 4E11 mouse Mab for the detection of DV1 EDIII, the Mab 3H5 for the detection of DV2 EDIII, the Mab 8A1 for the detection of DV3 EDIII, and the Mab 1H10 for the DV4 EDIII (all DV-specific antibodies were used at 1/100 dilution). A goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Amersham) was used as a secondary antibody (1/5000 dilution). Peroxidase activity was visualized with an enhanced chemiluminescence detection kit (Pierce).

2.8. Mice experiments

CD46-IFNAR mice susceptible to MV infection were produced as previously described [40]. Mice were housed under specific pathogen-free conditions at the Pasteur Institute animal facility. Six-week-old CD46-IFNAR mice were inoculated intraperitoneally (i.p.) with 10^5 TCID50 of recombinant MV-TetraA, MV-TetraB, MV-TetraC, or MV. To detect the anamnestic response generated by immunization, immunized mice were i.p. inoculated with 10^5 FFU of live strain FGA/89 French Guiana for serotype DV1 [80], Jamaica/N.1409 for DV2 (Genbank accession no. M20558) [81], PaH881/88 Thailand for DV3 and 63632/76 Burma for DV4. All mice experiments were repeated twice or three times and only a representative set of experiments is presented. All experiments were approved and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at Pasteur Institute.

2.9. ELISA for humoral responses

To evaluate the specific antibody responses, mice were bled via the periorbital route at different time after inoculation. Sera were heat inactivated at 56 °C for 30 min and the presence of anti-MV antibodies was detected by ELISA (Trinity Biotech). HRP-conjugated anti-mouse immunoglobulin (Jackson Immuno Research) was used as secondary antibody. Anti-DV antibodies were detected by ELISA using 96-well plates coated with recombinant EDIII proteins from DV1, DV2, DV3, DV4 produced in *E. coli* or synthetic. 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.

2.10. Focus reduction neutralization test

Anti-DV neutralizing antibodies were detected by a focus reduction neutralization test (FRNT) on Vero cells previously described [44] using 50 FFU of Vero-adapted DV1 Hawaï (WHO reference strain, Genbank accession no. AF226687), strain Jamaica/N.1409 for DV2, PaH881/88 Thailand for DV3 and 63632/76 Burma for DV4. The endpoint titer was calculated as the highest serum dilution

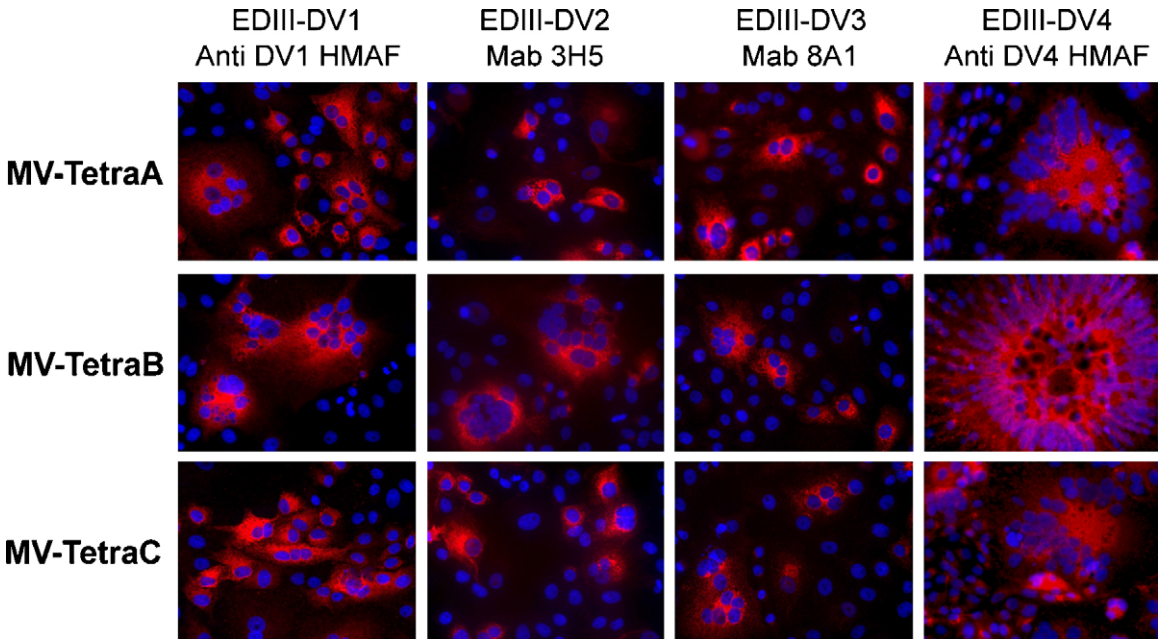

Fig. 2. Immunofluorescence detection of EDIII antigens from DV1, 2, 3, and 4 in Vero cells infected for 24 h with MV-TetraA, B or C. The presence of DV EDIII antigens was detected by immunofluorescence in Vero cells infected for 24 h by the recombinant MV vectors. DV1 EDIII was detected using an anti-DV1 hyper immune-ascitic fluid. DV2 EDIII was detected using the Mab 3H5, DV3 EDIII was detected using the Mab 8A1, and DV4 EDIII was detected using an anti-DV4 hyper immune-ascitic fluid. All DV antibodies were used at 1/100 dilution and secondary antibodies were used at 1/5000 dilution.

tested that reduced the number of FFU by at least 50% (FRNT50) or 90% (FRNT90).

3. Results

3.1. Design of tetravalent DV antigens and their expression by replicating recombinant MV vector

Our previous work demonstrated that DV1 EDIII (100 residues) fused to the 40-long residues of the M protein (ectoM) was remarkably efficient to induce serotype-specific neutralizing antibodies against DV1 [49]. Based on this result, we constructed three tetravalent antigens (TetraA, TetraB, TetraC) designed to express the EDIII from each of the four DV serotypes as individual antigens (Fig. 1). The short segment ($E_{290–300}$) between EDI and EDIII, which is flexible enough to allow the shift of the EDIII during the fusion process [50] was conserved as a linker sequence between each EDIII. To allow the individualization of the four EDIII segments during the processing of the tetravalent antigens, we added to their C-terminal parts the furin-like cleavage site of DV prM protein (RRDKR). TetraA construct was designed to express the four EDIII ordered DV1 to DV4 fused to a single ectoM sequence from DV1 at the C-terminal end. In TetraB and TetraC constructs, four copies of ectoM were added in C-terminal of each EDIII to examine the effect of position, number of copies and serotype origin of the ectoM. In TetraB construct each EDIII was associated with the ectoM of its corresponding serotype, while in TetraC all four ectoM were of serotype 1.

The antigens were generated by chemical synthesis from the sequences $E_{290–394}$ and $M_{1–40}$ from DV1 strain FGA/89, DV2 strain Jam 1409, DV3 Strain H97, and DV4 strain 63632. The sequences were codon-optimized for their expression in mammalian cells. The sequences AAAGGG, AAAAGG, GGGGAA that can lead to an editing by the MV polymerase have been changed to the synonymous sequences. The viral sequences were cloned downstream the cellular calreticulin signal peptide sequence (ssCRT) to address the antigens through the secretion pathway allowing the disulfide bond formation and therefore the correct folding of EDIII [49]. The resulting ss-CRT-TetraA, ss-CRT-TetraB, ss-CRT-TetraC constructs were each inserted as an additional transcription unit (ATU) into MV vector (pTM-MVSchw plasmid), which contains an infectious MV cDNA corresponding to the antigenome of the Schwarz MV vaccine strain (Fig. 1). The recombinant MV-TetraA, MV-TetraB and MV-TetraC were successfully rescued by transfecting the pTM-MVSchw-TetraA, pTM-MVSchw-TetraB and pTM-MVSchw-TetraC plasmids into helper cells and propagation on Vero cells, as previously described [40].

Detection of intracellular DV antigens in recombinant MV-infected Vero cells was first assessed by immunofluorescence analysis using hyperimmune mouse ascitic fluid (HMAF) directed against DV1 or DV4 and monoclonal antibodies (mAbs) specific to EDIII from DV2 (mAb 3H5 [51]) or DV3 (mAb 8A1 [52]) provided by Robert Putnak, Walter Reed Army Institute of Research). HMAF was used for the immunofluorescence detection of DV4 since the monoclonal antibody 8A1 that was used in western blots (see below) did not recognize the epitope under non-denaturing conditions. For DV1 the immunofluorescence assay was performed either with the 4E11 mAb (data not shown) and the anti-DV1 HMAF. As shown in Fig. 2, DV antigens were clearly immunostained indicating that the three recombinant MV vectors were efficient to produce EDIII from the four serotypes of DV. The stability of expression of the correct DV sequences was verified by RT-PCR and sequencing of viral RNAs produced in infected cells after 5 passages of recombinant viruses (data not shown). We analyzed the processing of recombinant DV antigens by monitoring the intracellular and extracellular forms of individual EDIII in Vero cells infected by recombinant MV (Fig. 3). Immunoblotting analysis was performed using mAbs 3H5, 8A1, and 1H10 [51], the latter recognizing specifically the EDIII from DV4. The DV1 EDIII was detected using DV-neutralizing mAb 4E11 [53]. Soluble DV1 EDIII secreted from a stably established *Drosophila* S2 cell line served as a positive control. In cell lysates from MV-TetraA infection, we detected the expected 5 fragments of 13, 25, 37, 49 and 54 kDa, respectively, indicating that the furin cleavage sites are accessible. MV-TetraB and MV-TetraC infections produced a higher number of fragments corresponding to the expected sizes generated by the eight furin cleavage sites (13, 18, 30, 35, 47, 53 65 and

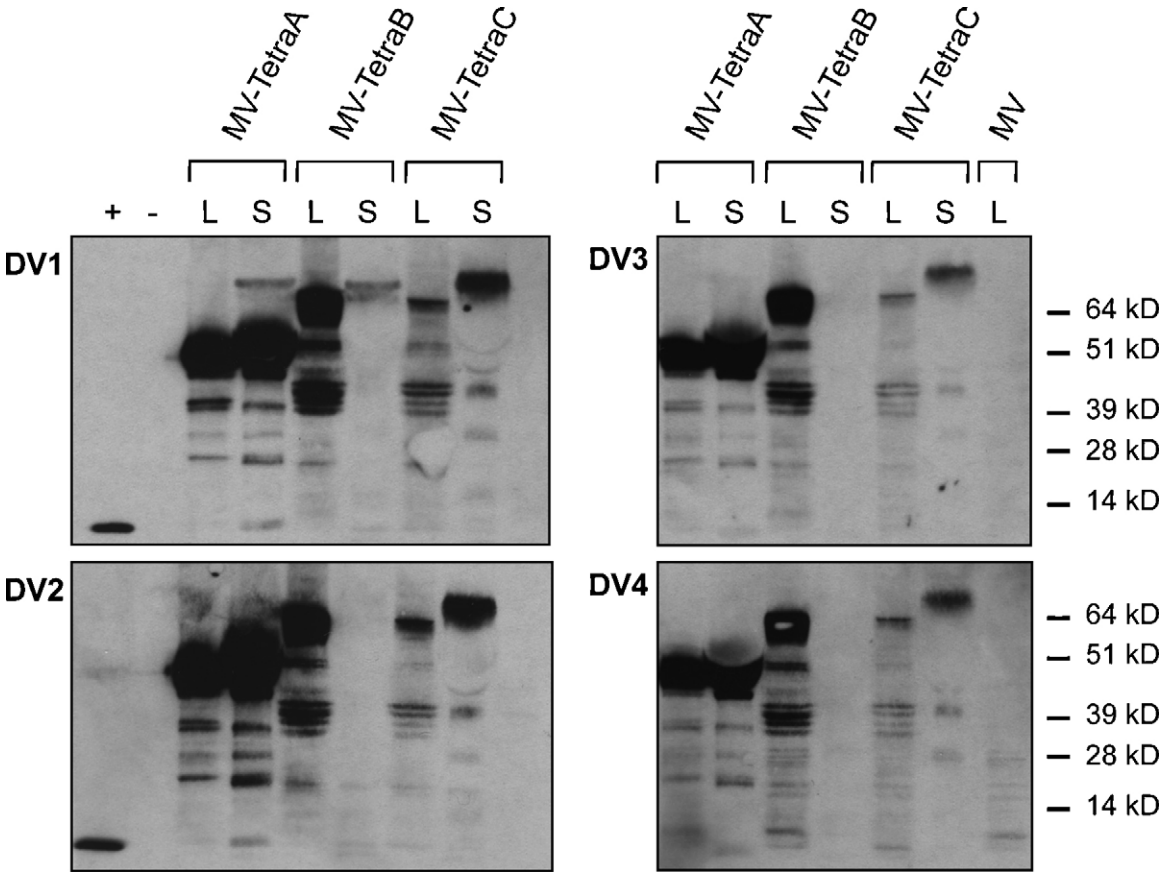

Fig. 3. Expression of EDIII from DV1, 2, 3 and 4. Cell lysates (L) and supernatants (S) of Vero cells infected for 24 h by MV-TetraA, MV-TetraB and MV-TetraC were analyzed by western blot (cell lysates are 20 times more concentrated than supernatants). (+) positive control (5 ng of DV1 or DV2 EDIII produced in drosophila S2 cells). DV1 EDIII was probed with the 4E11 mouse monoclonal anti-DV1 EDIII, DV2 EDIII with the Mab 3H5, DV3 EDIII with the Mab 8A1, and DV4 EDIII with the Mab 1H10. All DV antibodies were used at 1/100 dilution and secondary antibodies were used at 1/5000 dilution.

70 kDa). TetraA and TetraC antigens were also detected in unconcentrated supernatants of infected cells, although the supernatant volume was 100 times larger than the lysate volume, thus indicating an efficient antigen secretion. TetraA antigen was even found in higher concentration in supernatant than in cell lysate and all the cleavage products were secreted. On the contrary, TetraB antigen was not detected in cell supernatants.

We assessed the replication of the recombinant MV-TetraA, MV-TetraB and MV-TetraC viruses on Vero cells, using the same MOI (0.01) as for standard MV stock production (Fig. 4). The growth of MV-TetraA was similar to that of control MV, and its final titer was even slightly higher. In contrast, we found that both MV-TetraB and MV-TetraC were less efficient to replicate in Vero cells and progeny virus production was significantly detected only after two rounds of virus life cycle. The impairment of viral growth was associated to early death of infected cells (data not shown). It is likely that expression of multiple copies of the cytotoxic ectoM sequence potentiates MV-mediated cell death. According to this hypothesis, we reported that accumulation of ectoM by MV vector enhances the apoptotic properties of the recombinant virus [49].

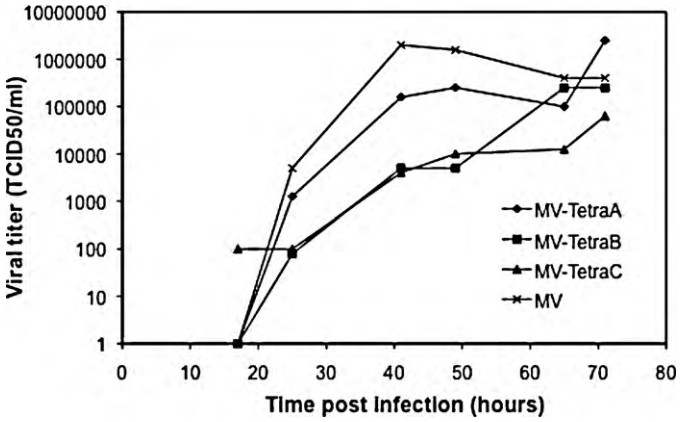

Fig. 4. Growth kinetics of recombinant MV-TetraA, MV-TetraB and MV-TetraC viruses compared with standard MV on Vero cells (MOI 0.01). Cell-associated virus titers are indicated in TCID50.

3.2. MV-TetraA induces neutralizing antibodies against all four DV serotypes in mice

The immunogenicity of the three recombinant MV-TetraA, MV-TetraB and MV-TetraC viruses was investigated in genetically modified CD46-IFNAR mice susceptible to MV infection [54]. The CD46-IFNAR mice express CD46, the human receptor for vaccine MV strains, and lack the INF-α/β receptor. They have been used previously as a model to evaluate the immunogenicity of recombinant MV [40,44–47,55]. Six 12-week-old male CD46-IFNAR mice received two intraperitoneal (i.p.) injections at 4-week interval of 10^5 $TCID_{50}$ of recombinant viruses. A group of three control mice was injected similarly with empty MV vector (10^5 $TCID_{50}$). Specific antibody responses to MV and to the four DV serotypes were determined in pooled sera collected 1 month after the second immunization (Fig. 5). The antibody titers to MV in the four groups of mice were similar to those usually observed in previous experiments [48]. Specific anti-EDIII antibodies to the four DV serotypes

Table 1
Antibody response of CD46-IFNAR mice to immunization with MV-TetraA.

Mice	DV1		DV2		DV3		DV4	
	rEDIII[a]	$PRNT_{50}$[b]	rEDIII[a]	$PRNT_{50}$[b]	rEDIII[a]	$PRNT_{50}$[a]	rEDIII[a]	$PRNT_{50}$[b]
1	5000	20	100	20	300	<10	100	<10
2	5000	10	100	10	100	<10	300	<10
3	10,000	>40	500	20	300	10	300	10
4	50,000	>40	1000	40	>3000	10	1000	10
5	5000	20	300	10	500	10	100	<10
6	50,000	10	<100	10	<100	<10	300	<10
Pool MV	<100	<10	<100	<10	<100	<10	<100	<10

CD46-IFNAR mice were inoculated intraperitoneally (i.p.) twice with 10^5 TCID50 of MV-TetraA, or MV at 1 month of interval (6 mice/group and 3 mice/group, respectively).
[a] Antibody titers against DV1, DV2, DV3 and DV4 were determined by ELISA on individual mouse heat-inactivated serum for MV-TetraA and on pooled-sera for MV control collected 1 month after immunization.
[b] $FRNT_{50}$ represents the highest serum dilution that reduced the number of DV focus-forming units (FFU) on Vero cells by at least 50%.

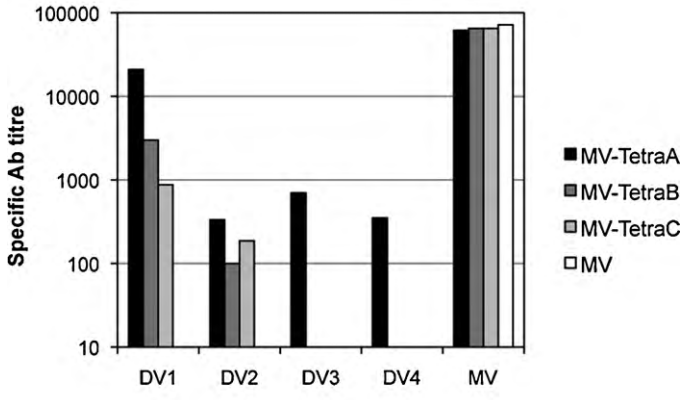

Fig. 5. Antibody response of CD46-IFNAR mice to immunization with MV-TetraA, MV-TetraB and MV-TetraC. CD46-IFNAR mice were inoculated intraperitoneally (i.p.) twice with 10^5 TCID50 of MV, MV-TetraA, MV-TetraB or MV-TetraC at 1 month of interval (6 mice/group). Anti-DV and anti-MV antibody titers were determined by ELISA on pooled heat-inactivated sera collected 1 month after immunization.

were determined using ELISA plates coated with the four recombinant EDIII proteins produced in *E. coli*. Only mice immunized with MV-TetraA vector had antibodies to the four DV serotypes. Mice immunized with MV-TetraB and MV-TetraC vectors had a low titer of antibodies to DV1 and no antibodies against the other serotypes. This result likely reflects the low replicative capacity and the low secretion level of DV antigens by MV-TetraB and MV-TetraC vectors as compared to MV-TetraA (Figs. 3 and 4). For this reason MV-TetraB and MV-TetraC vectors were not further evaluated.

We then determined the neutralizing activity against the four DV serotypes in sera from mice immunized with MV-TetraA using a focus reduction neutralization test (FRNT) on Vero cells (Table 1). Mice immunized with empty MV vector failed to induce specific anti-DV neutralizing antibodies. In contrast, immunization with MV-TetraA raised $FRNT_{50}$ titers against DV1 and DV2 in all immunized animals, against DV3 in 3/6 animals and against DV4 in 2/6 animals tested. This result shows that MV-TetraA was able to induce neutralizing antibodies to the four DV serotypes.

3.3. Memory neutralizing antibodies induced by immunization with MV-TetraA are strongly boosted by intraperitoneal inoculation of live DV1, 2, 3 and 4

The mouse models for DV infection developed to date include infection of severely immuno compromised mice [56–58], non-physiologic routes of infection [59], and mouse-human chimeras [60–63], which all have their limitations. The CD46-IFNAR mice used in this study are not sensitive to DV infection and therefore, do not allow to evaluate the protection conferred by MV-TetraA immunization. We nevertheless assessed the ability of intraperitoneal injection of live DV2 (Jam strain, 10^6 FFU) 2 months after immunization to stimulate anamnestic humoral response against DV1, 2, 3 and 4. The results show that ELISA and neutralizing titers previously induced by immunization with recombinant MV-TetraA were remarkably boosted by live DV2, whereas animals immunized with empty MV vector did not show any boost (Table 2). Both ELISA and FRNT antibody titers show that the tetravalent TetraA antigen has the potential to induce antibodies that can recognize and bind to the four DV serotypes.

To further our effort in assessment of the TetraA construct, we immunized a significant group of 24 CD46-IFNAR mice with two i.p. injections of 10^5 FFU of MV-TetraA and then analyzed the antibody titers in pooled sera collected 1 month after immunization (Table 3). Antibodies to MV were induced in all immunized mice at similar titers. Anti-DV1, -DV2, -DV3 and -DV4 neutralizing activity

Table 2
Antibody response of CD46-IFNAR mice immunized with MV-TetraA and challenged by DV2.

Mice	DV1		DV2		DV3		DV4		MV
	rEDIII[a]	$PRNT_{50}$[b]	rEDIII[a]	$PRNT_{50}$[b]	rEDIII[a]	$PRNT_{50}$[a]	rEDIII[a]	$PRNT_{50}$[b]	
1	200,000	320	5000	2560	2000	320	2000	80	100,000
2	300,000	320	5000	1280	500	1280	1000	80	30,000
3	1,000,000	5120	20,000	2560	1000	160	2000	80	100,000
4	50,000	5120	100,000	5120	20,000	2560	5000	320	30,000
5	300,000	1280	20,000	1280	2000	1280	3000	320	100,000
6	200,000	2560	10,000	2560	500	80	2000	320	100,000
Pool MV	1000	40	1000	640	<100	40	<100	80	100,000

CD46-IFNAR mice were inoculated intraperitoneally (i.p.) twice with 10^5 TCID50 of MV-TetraA, or MV control at 1 month of interval (6 mice/group and 3 mice/group, respectively) and then challenged with DV2 i.p.
[a] Antibody titers against DV1, DV2, DV3, DV4 and MV were determined by ELISA on individual mouse heat-inactivated serum for MV-TetraA and on pooled-sera for MV control collected 1 month after DV inoculation.
[b] $FRNT_{50}$ represents the highest serum dilution that reduced the number of DV focus-forming units (FFU) on Vero cells by at least 50%.

Table 3
Antibody response of CD46-IFNAR mice to immunization with MV-TetraA.

Virus	DV1 $FRNT_{50}$[a]	DV2 $FRNT_{50}$[a]	DV3 $FRNT_{50}$[a]	DV4 $FRNT_{50}$[a]	MV Ab titer[b]
MV-TetraA	20	10	20	20	100,000
MV	<10	<10	<10	<10	100,000

CD46-IFNAR mice were inoculated intraperitoneally (i.p.) twice with 10^5 TCID50 of MV-TetraA or MV at 1 month of interval (24 mice/group and 12 mice/group, respectively).
[a] Neutralizing antibody titers against DV1, DV2, DV3 and DV4 were determined by FRNT50 that represents the highest serum dilution that reduced the number of DV focus-forming units (FFU) on Vero cells by at least 50% on pooled heat-inactivated sera collected 1 month after immunization.
[b] Antibody titers against MV were determined by ELISA.

Table 4
Antibody response of CD46-IFNAR mice immunized with MV-TetraA and challenged by DV1, DV2, DV3 or DV4.

	DV1			DV2			DV3			DV4			MV[a]
	EDIII[a]	$FRNT_{90}$[b]	$FRNT_{50}$[b]	EDIII[a]	$FRNT_{90}$[b]	$FRNT_{50}$[b]	EDIII[a]	$FRNT_{90}$[b]	$FRNT_{50}$[b]	EDIII[a]	$FRNT_{90}$[b]	$FRNT_{50}$[b]	
TetraA/DV1	10,000	160	1280	500	160	5120	500	320	1280	500	20	160	10,000
MV/DV1	500	10	80	10	10	80	10	10	80	10	<10	80	10,000
TetraA/DV2	50,000	40	160	5000	160	5120	1000	80	320	5000	20	80	30,000
MV/DV2	10	<10	80	100	10	320	10	<10	10	10	<10	<10	10,000
TetraA/DV3	30,000	10	80	ND	40	160	2000	160	640	5000	<10	40	30,000
MV/DV3	10	<10	<10	ND	<10	<10	10	<10	320	10	<10	20	10,000
TetraA/DV4	10,000	<10	10	500	80	320	500	40	160	5000	20	160	10,000
MV/DV4	10	<10	<10	10	<10	20	10	<10	20	100	<10	20	10,000

CD46-IFNAR mice were inoculated intraperitoneally (i.p.) twice with 10^5 TCID50 of MV-TetraA, or MV at 1 month of interval (24 mice/group and 12 mice/group, respectively), separated in four groups and then challenged with DV1, DV2, DV3 or DV4 i.p.
[a] Antibody titers against DV1, DV2, DV3, DV4 and MV were determined by ELISA on pooled mouse heat-inactivated sera collected 1 month after DV inoculation.
[b] FRNT90 and FRNT50 represents the highest serum dilution that reduced the number of DV focus-forming units (FFU) on Vero cells by at least 90% or 50%, respectively.

was detected at protective levels in sera of mice immunized with MV-TetraA. $FRNT_{50}$ titers >10 were induced to DV1 in 83% of mice tested, to DV2 in 70%, to DV3 in 58% and to DV4 in 47%. Mice were then separated in four groups of 6 animals and inoculated 2 months after immunization with 10^5 FFU of DV1 (FGA/NA d1d), DV2 (Jam), DV3 (H97) and DV4 (63632) viruses, respectively. The antibody titers were determined in pooled sera collected from each of the four groups of mice 1 month after DV inoculation (Table 4). To determine whether the administration of a DV serotype could elicit an anamnestic response against the other DV serotypes, we analyzed the neutralizing activity of sera from mice inoculated with each DV serotype against the other serotypes. The data clearly show that the administration of any DV serotype stimulated a strong memory neutralizing response against all four serotypes in mice immunized with MV-TetraA as compared to control mice immunized with empty MV vector. The neutralizing titers were increased by one or two log ($FRNT_{50}$ titers up to 5210 and $FRNT_{90}$ titers > 100).

Altogether, these data show that mice immunized with MV-TetraA rapidly develop neutralizing antibodies against the four DV serotypes that are recalled by any DV serotype encountered. Because neutralizing antibodies are correlates of protection against dengue disease, our result suggests that vaccinated individuals might be protected against infection by any serotype, avoiding the risk of ADE after vaccination with this vaccine candidate.

4. Discussion

A preventive dengue vaccine needs to protect unexposed individuals against the four serotypes of DV. It must be tetravalent, safe for young children and provide long-lasting protective immunity. It must be produced at low cost and scaled up at million doses. The strategies developed during the last 25 years have proven that only live attenuated viruses or chimeric viral vectors are able to induce after few injections long term neutralizing antibodies against DV, which are generally accepted as a marker of vaccine efficacy [6,64,65]. Despite the different approaches explored to date, we believe that there still needs to develop a new strategy that would avoid (i) the induction of cross-reactive non-neutralizing antibodies and ADE, (ii) the stability problems of tetravalent formulation, (iii) the DV-associated reactogenicity, (iv) the possible recombination and dissemination by mosquitoes, and that would be absolutely safe for children and rapidly implemented at large scale. To address these challenges, we designed a live recombinant vector derived from pediatric measles vaccine expressing a tetravalent DV antigen designed to induce neutralizing and non-facilitating antibodies against the 4 DV serotypes.

As a proof-of-concept of this new dengue vaccine strategy, we previously evaluated the immunogenic potential of a MV vector expressing a DV1 soluble antigen composed of the EDIII fused with the ectoM [48]. To avoid the induction of cross-reactive non-neutralizing antibodies that may result in antibody-dependent enhancement of secondary DV infections, we designed a DV antigen corresponding to the domain III of the envelope E glycoprotein of DV1 (EDIII). As the EDIII was poorly immunogenic by itself, to reinforce its immunogenicity we added in fusion a second antigen composed of the ectodomain of the membrane M protein of DV1 (ectoM). This small protein (40 amino acids), which is highly conserved among the four DV serotypes, has pro-apoptotic properties [66] that adjuvanted the EDIII immunogenicity through activation of DCs [49]. We demonstrated that the minimal EDIII-ectoM antigen was able to induce long-term specific neutralizing antibodies to DV1, thus making the proof-of-concept of this strategy for dengue vaccine development.

In the present work, taking advantage of the capacity of MV vector to express very stably large amounts of heterologous genetic material [67], we assessed three tetravalent dengue antigenic constructs expressing the EDIII from the four DV serotypes fused either with each of the four ectoM corresponding to each serotype, or only with the ectoM of DV1 (Fig. 1). These tetravalent constructs were inserted into single MV vectors to be expressed as secreted antigens. The corresponding tetravalent recombinant MV vectors were produced by reverse genetics and evaluated for their replicative capacity and the expression of the dengue antigens. The three recombinant MV-DV viruses expressed the DV tetravalent antigens in infected cells. TetraA and TetraC tetravalent antigens were correctly cleaved, indicating a good accessibility of furin cleavage sites in the constructs. Only TetraA was able to induce a high level of

secretion of tetrameric and cleaved antigens from infected cells. The growth kinetic of MV-TetraA vector was similar to that of standard MV Schwarz vaccine, whereas MV-TetraB and MV-TetraC had a reduced replicative capacity, likely due to the expression of four copies of the pro-apoptotic ectoM sequence. Based on its replicative capacity and its capacity to express and secrete high amounts of tetravalent DV antigen, MV-TetraA was chosen as a lead recombinant vector candidate and further evaluated for its immunogenicity in mice. After two injections, MV-TetraA induced neutralizing antibodies against the four serotypes of dengue virus. When mice immunized with MV-TetraA were further inoculated with live DV from each serotype, a strong memory neutralizing response was raised against all four serotypes as compared to control mice immunized with empty MV vector, whatever the serotype injected. The increased anamnestic response observed in vaccinated animals after wild-type DV inoculation demonstrates the presence of memory B and CD4 T cells to the DV antigens. This response resulted in strongly increased levels of neutralizing antibodies. Because DV does not replicate in CD46-IFNAR mice, the antigenic load of incoming virus only stimulated memory. The antibodies induced at low titers by this antigenic boost in control mice vaccinated with empty measles vector are directed against the DV envelope antigens, which are present in viral particles. If wild-type DV replicated, antibodies would be have been induced to other viral antigens that are not present in the vaccine. We looked for anti-NS1 antibodies. They remained absent from all inoculated mice (data not shown).

The induction of cross-neutralizing antibodies is desirable in a tetravalent vaccine formulation, as opposed to deleterious non-neutralizing cross-reactive antibodies. Several monoclonal antibodies that neutralize the four DV serotypes, such as the sub-complex-specific Mab 4E11 and the Mabs 1A1-D2 and 9D12, bind an adjacent epitope centered on the A strand of EDIII at residues K305, K307 and K310 [27,68–70]. Lysine K310 is conserved in all flaviviruses, whereas K305 and K307 are specific of DV4 and DV1, respectively. As frequently observed in other tetravalent strategies [71–73], we detected a lower level of antibodies induced against DV4 and DV3 as compared to DV1 and DV2. This might be due to different surface electrostatic charges between the four EDIII of the tetravalent antigen [74]. However, very low neutralizing titers (FRNT < 10) induced by immunization were able to protect macaques from DV viraemia [72,75].

Concerning the immunogenicity of the EDIII from DV3, Zulueta et al., compared immunogenicity in mice of two fusion proteins expressing the EDIII from two different DV3 strains: the H87 prototype strain that we used for the construction of our tetravalent antigens, and a strain isolated during the 1994 outbreak in Nicaragua [76]. Four amino acids differ in the EDIII of these two strains. It was shown that only the EDIII from the Nicaragua 1994 strain reacted with human anti-sera and induced a higher immune response in mice than the EDIII derived from the H87 prototype strain. These results suggest that not all sequences of the EDIII are equally immunogenic, and may explain the lower response observed against DV3 in our study. To address this issue, a more immunogenic DV3 sequence, such as the Nicaragua 1994 strain, might be inserted in the next generation [52].

Altogether, our data show that mice immunized with MV-TetraA rapidly develop neutralizing antibodies against the four DV serotypes that are recalled by any DV serotype encountered. Because neutralizing antibodies are correlate of protection from dengue disease, this result suggests that vaccinated individuals will be protected against infection by any serotype, avoiding the risk of ADE after vaccination with this vaccine candidate. A major advantage of this strategy is the use of a single vector that avoids the interference observed when the vaccine is composed of a mix of four viral strains. A combined measles-dengue vaccine might be attractive to immunize children against both diseases where they co-exist. Indeed, during the first 2 years of life, children are particularly exposed to dengue severe forms. Hemorrhagic fever cases appear from 8 to 9 months of age, correlating with the vanishing of maternal antibodies, which provide protection until the age of 6–9 months, and the attack rate increases during the second year of life [77–79]. Therefore, a 2-dose measles-dengue combined vaccination should be targeted at 6–9 and 12-month-old infants, corresponding to the age of measles vaccination in areas of high dengue fever endemicity. Such a vaccine might benefit of a large distribution through the WHO program for measles elimination during the next decades, the double measles-dengue valence giving a competitive advantage. We are currently evaluating the protective efficacy of a new improved MV-TetraA recombinant vaccine against DV1-4 infections in macaques, the model of choice for dengue virus infection.

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