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- (71) Applicant: INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY - ICGEB [IT/IT]; Padriciano 99, I-34149 Trieste (IT).
- (72) Inventors: BURRONE, Oscar; c/o Padriciano 99, I-34149 Trieste (IT). BESTAGNO, Marco; c/o Padriciano 99, I-34149 Trieste (IT). POGGIANELLA, Monica; c/o Padriciano 99, I-34149 Trieste (IT).
- (74) Agents: SPADARO, Marco et al.; Cantaluppi & Partners S.r.l., Viale Della Tecnica 205, I-00144 Roma (IT).
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(54) Title: ANTI-DENGUE VIRUS GENETIC VACCINE BASED ON THE ENVELOPE PROTEIN ECTODOMAINS

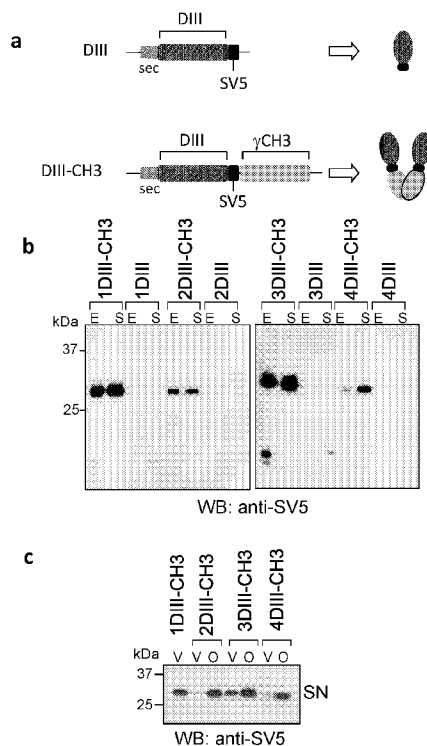


Figure 1

(57) Abstract: The present invention refers to a genetic vaccine for dengue virus of all serotypes or for any virus belonging to the Flavivirus genus. Said vaccine is based on the envelope protein E ectodomains, more in particular on the DIII domain of dengue virus or of other Flavivirus. A genetic construct encoding a fusion protein wherein said domain of envelope protein E is fused to a carboxyterminal domain from the constant region of an immunoglobulin is disclosed in the present invention. The use of said construct allows for an efficient secretion of the fusion protein from the cells and thus for a high antibody response.



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ANTI-DENGUE VIRUS GENETIC VACCINE BASED ON THE ENVELOPE PROTEIN ECTODOMAINS

DESCRIPTION

The present invention refers to the field of genetic engineering and virology.

- 5 In particular, it refers to a genetic vaccine for the dengue virus or for any virus belonging to the Flavivirus genus, more in particular to a vaccine based on the envelope protein E ectodomains, more in particular based on the DIII domain of dengue virus or of other Flavivirus.

BACKGROUND OF THE INVENTION

- 10 Dengue virus is a positive-sense RNA virus belonging to the Flavivirus genus of the family Flaviviridae. Dengue virus is widely distributed throughout the tropical and semitropical regions of the world and is transmitted to humans by mosquito vectors. It is estimated to cause in each year more than 50 million cases of dengue fever (DF), 500,000 cases of dengue hemorrhagic fever (DHF) and 25,000 deaths, with 2.5 billion people at risk
15 (Guzman, M.G. et al., *Nature Rev. Microbiol.*, 8, S7-S16, 2010). At the moment there is as yet no dengue vaccine approved for use; a chimeric dengue-yellow fever live vaccine has undergone a phase III clinical trial (Guy, B. et al., *Vaccine*, 29, 7229–7241, 2011), but failed in providing complete protection against all four dengue serotypes at the same time (Sabchareon A., et al., *Lancet*, 380, 1559-1567, 2012). Another DNA-based vaccine has
20 been subjected to a phase I trial (Beckett C.G., et al., *Vaccine*, 29, 960-968, 2011)

- Dengue genome encodes three structural proteins, C (capsid), pr-M (membrane) and E (envelope) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, that include an RNA-directed RNA polymerase, and a protease involved in processing of the long viral polyprotein, produced as a consequence of continuous
25 translation of the RNA viral genome (Chambers, T. J. et al., *Ann. Rev. Microbiol.* 44, 649-688, 1990; Bollati, M. et al., *Antiviral Res.* 87, 125-148, 2010).

Protein C encapsidates the RNA genome, while proteins M (obtained after cleavage of the pr-M precursor) and E are associated to the membrane envelope and participate in virus entry into the cell.

- 30 Protein E is the main component of the viral envelope. It is a membrane-associated glycoprotein containing two trans-membrane domains and an ectodomain composed of three sub-domains (I, II, III) and a stem region. Domain II (E-DII) is responsible for E protein homodimerization; domain III (E-DIII) can fold independently into an

immunoglobulin like module and is involved in receptor binding. Most anti-dengue neutralizing antibodies are directed against E-DIII that is therefore considered as a promising target for vaccination (Guzman M.G. et al., *Expert Rev. Vaccines*, 9, 137-147, 2010).

- 5 There are four serotypes known of dengue virus ("DENV 1" through "DENV 4"), which share approximately 60%-74% amino acid residue identity with one another in the E protein and induce cross-reacting antibodies (Heinz, F.X. & Stiasny, K., *Vaccine*, 30, 4301-4306, 2012). However, neutralizing antibodies to the structural proteins of one serotype of dengue provide only limited protection against other serotypes, and in addition
10 appear to predispose to the more severe forms of dengue (DHF) upon re-infection by another serotype (Guzman, M.G. et al., *Front. in Dengue Virus Res.*, Hanley, K.A. & Weaver, S.C. Eds., *Caister Academic Press*, Norfolk, U.K., 79-101, 2010). In fact, recognition of viral particles by cross-reacting, but not neutralizing, antibodies may lead to increased Fc receptor-mediated uptake of incompletely neutralized virus by monocytes,
15 macrophages, and dendritic cells, which have been shown to be primary major targets of dengue virus infections in humans, thus causing the phenomenon known as antibody-dependent enhancement of infection (ADE) (Heinz, F.X. & Stiasny, K., *Vaccine*, 30, 4301-4306, 2012; Halstead, S.B. et al., *Lancet Infect. Dis.*, 10, 712-722, 2010). This is critical in dengue vaccine development, since an immunogen that does not elicit fully neutralizing
20 antibodies to all four serotypes may contribute to disease, rather than prevent infection.

Experimental anti-dengue vaccines in animal models have exploited genetic immunization with plasmids or viruses expressing various recombinant forms of the DIII domain of dengue envelope protein E (E-DIII) (Mota, J. et al., *Vaccine*, 23, 3469-3476, 2005; Khanam, S., et al., *Vaccine*, 24, 6513-6525, 2006; Ramanathan, M.P. et al., *Vaccine*, 27,
25 6444-6453, 2009; Azevedo, A.S. et al., *PLoSOne* 6(7): e20528, 2011). However, these constructs elicit only a weak immune response with low neutralizing titres of the sera of immunized animals.

Dengue tetravalent vaccines based on envelope domain III have already been disclosed, for example in the following patent literature.

- 30 WO2008152652 and WO2007034507 disclose recombinant envelope domain III based proteins wherein the domains DIII of the four serotypes of dengue virus are linked by penta-glycine linkers. In WO2008152652 the construct can further comprise a secretory peptide from *S. cerevisiae*.

WO2010057159 and US2010291144 disclose DNA vaccines comprising constructs with a DIII consensus sequence, which can further include a leader sequence deriving from IgG or IgE.

US6455509 discloses an eukaryotic plasmid expression vector which includes at least part (92%) of the envelope gene and optionally the preM gene of Dengue virus.

In EP2484376 DIII can be fused to the sequence that codifies the signal peptide of the human tissue plasminogen activator (t-PA).

The fusion of DIII with a heterologous domain is not disclosed nor foreseen in none of the above documents.

US2009074781 discloses a dengue vaccine active against multiple virus serotypes, comprising a peptide designed on the basis of the DIII domains of 4 serotypes. The construct does not comprise further components.

US2013071419 discloses a vaccine comprising a sequence codifying for DIII fused with a sequence codifying for the region p28 of the complement protein c3d. A tetravalent composition may be administered.

In WO2012045063 a tetravalent vaccine for dengue comprising a polypeptide for the DIII domain is disclosed; the fusion protein can further include an adjuvant polypeptide.

An efficient and specific DNA vaccine for dengue virus is still needed. In particular, a vaccine active against all four serotypes of dengue virus is strongly needed. More in particular, a vaccine based on the envelope protein domains, especially based on the DIII domain, with enhanced immunogenicity and induction of high titres of neutralising antibodies against all the four serotypes is still under search.

Some attempts have been made in the prior art to improve immunogenicity of the E protein or one of its domains, for example by fusion with heterologous proteins.

Staropoli I, Clément JM, Frenkiel MP, Hofnung M, Deubel V. "Dengue virus envelope glycoprotein can be secreted from insect cells as a fusion with the maltose-binding protein.", J Virol Methods. 1996 Feb;56(2):179-89, discloses a fusion protein wherein the gene encoding the envelope glycoprotein E of the dengue type 2 virus deleted of its C-terminal 102 amino acids (D2Edelta102) was fused to the maltose-binding protein (MalE).

The obtained protein is produced in insect cells and secreted in the extracellular compartment.

Purdy DE, Chang GJ, "Secretion of noninfectious dengue virus-like particles and identification of amino acids in the stem region involved in intracellular retention of envelope protein.", *Virology*, 2005 Mar 15;333(2):239-50, discloses chimeric plasmids expressing flavivirus premembrane/membrane (prM/M) and envelope (E) proteins in the form of virus-like particles. In particular, constructs wherein the carboxy-terminal region of Dengue E protein gene is replaced with the corresponding sequence of Japanese encephalitis virus (JEV) are reported.

WO2011115583 refers to a platform for retrovirus-like particle (VLP)-display of vaccine antigens. Among others, it discloses CD16-RigE, a chimeric human membrane glycoprotein consisting of the CD 16 ectodomain fused to the transmembrane domain and cytoplasmic tail of the gamma chain of the high affinity receptor of IgE, wherein the CD16 ectodomain can be replaced by the envelope glycoprotein domain III (E:DIII) of serotype I of Dengue virus (DEN1). The resulting fusion protein DIII-DEN1-RigE was found to traffic to the plasma membrane of human and insect cells and to be exposed at the cell surface.

Murrell S, Wu SC, Butler M, "Review of dengue virus and the development of a vaccine." *Biotechnol Adv.* 2011 Mar-Apr;29(2):239-47 (Epub 2010 Dec 10), discloses different approaches to develop candidate vaccines for dengue viral infections. Some DNA vaccines and recombinant protein vaccines are reported. In particular, several strategies used to improve the immunogenicity of the DENV domain III antigen are reported, as fusion to the Staphylococcal A protein, the Maltose Binding Protein of *Escherichia coli*, and the P64K protein from *Neisseria meningitidis*. Also, it is reported that the main disadvantage of DNA vaccine is the low immunogenicity elicited in hosts.

The problem of providing a vaccine wherein Dengue protein E or one of its domains could efficiently exercise its immunogenic activity has thus been addressed in the prior art in many different ways. However, a DNA vaccine able to elicit an efficient immunization when administered to a subject is still under search and strongly needed.

More in particular, the highly inefficient secretion of the DIII domain when expressed alone in mammalian cells leads to a poor anti-DIII antibody response, following genetic immunization. Thus, a system for enhancing secretion of DIII and consequently improving antibody response is still needed.

The inventors of the present invention have found that when any domain of envelope protein E of all four serotypes of dengue virus, in particular any of the ectodomains DI, DII and DIII, is fused with at least one carboxyterminal domain of an immunoglobulin constant region, preferably a dimerizing domain, for example the CH3 domain of immunoglobulin G

(γ CH3) or the CH4 domain of immunoglobulin E (ϵ CH4), the obtained fusion proteins are endowed with a high secretion efficiency.

This allows for an enhanced bioavailability and thus eventually a higher immunogenicity of said fusion proteins, with respect to the prior art. The new genetic constructs of the present invention encoding the above described fusion proteins can thus be used to efficiently immunise mammals, thanks to the efficient expression of the antigen and subsequent stimulation of the immune response. Importantly, following administration to a subject in need of immunization of said constructs, high titres of neutralising antibodies are generated against all four dengue virus serotypes: a tetravalent vaccine is thus provided with the present invention.

Also, when the envelope protein domain included in said genetic construct is DIII, it can be the DIII of any virus belonging to the Flavivirus genus.

SUMMARY OF THE INVENTION

It is an object of the present invention, a genetic construct comprising a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding any domain of envelope protein E of dengue virus and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.

In a preferred embodiment, said domain of envelope protein E is anyone of the ectodomains DI, DII and DIII. In a more preferred embodiment it is DIII.

When the envelope protein domain included in said genetic construct is DIII, it can be the DIII of any virus belonging to the Flavivirus genus.

Thus in an alternative embodiment of the invention, the genetic construct comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding the domain DIII of envelope protein E of a virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.

Said immunoglobulin can be of human, murine or other animal origin.

In a preferred embodiment, said carboxyterminal domain is selected from the group consisting of γ CH3, α CH3, ϵ CH4, μ CH4, C κ and C λ domains from any immunoglobulin subclass.

Optionally, the construct of the invention can further comprise a nucleotide sequence encoding a tag, said sequence being preferably included between the sequence encoding the domain of protein E and the sequence encoding one or more carboxyterminal domains.

- 5 It is a preferred object of the present invention a genetic construct comprising a polynucleotide sequence selected from the group consisting of: SEQ ID NO: 1, 2, 3 and 4.

aagcttgtcgacc**ATGGGCTGGAGCCTGATCCTCCTGTTCTCGTCGCTGTGGCTACAGGTGTGCA**
CTCTCAGGGCGCGCATGCCTCATATGTAATGTGCACAGGGTCATTCAAGTTAGAGAAGGAAGT
GGCTGAGACCCAGCATGGAAGTGTCTAGTGCAGGTTAAATACGAAGGAACAGATGCACCAT
10 GCAAGATCCCCTTCTCGTCCCAAGATGAGAAGGGAGTAACCCAGAATGGGAGATTGATAACA
GCCAACCCCATAGTCACTGACAAAGAAAAACCAGTCAACATTGAAGCGGAGCCACCTTTTGGT
GAGAGCTACATTGTGGTAGGAGCAGGTGAAAAGGCCTTGAACTAAGCTGGTTCAAGAAGGG
AAGCAGTATAGGGAAAATGTTTGAAGCAACTGCCCGTGGAGCACGAAGGATGGCCATCCTGG
GAGGTACCGGAGCTAGCGGCAAACCAATCCCAAACCCACTGCTGGGCCTGGATGGATCCGG
15 GGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAG
AACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTG
GGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGAC
GGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGT
CTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCT
20 GTCTCCGGGTAAATGAgaattc (SEQ ID NO. 1)

Sec

E-DIII

SV5

hCH3

25 aagcttgtcgacc**ATGGGCTGGAGCCTGATCCTCCTGTTCTCGTCGCTGTGGCTACAGGTgTGCA**
CTCTCAGGGCGCGCACGCCTCATACAGCATGTGCACTGGAAAGTTCAAGGTGGTCAAGGAAA
TGCGAGAGACTCAGCACGGGACTATTGTCATTAGGGTCCAGTACGAGGGCGACGGGTCTCCA
TGCAAGATTCCCTTCGAGATCATGGATCTGGAAAAACGGCACGTGCTGGGCAGACTGATTAC
CGTGAACCCTATCGTCACAGAGAAGGACAGCCCAGTGAATATTGAGGCCGAACCCCTTTCCG
30 GGGATTCTATATCATTATCGGAGTCGAACCCGGCCAGCTGAACTGAACTGGTTTAAGAAAG
GAAGCAGTATTGGGCAGATGATTGAGACCACTATGAGAGGGGCTAAGCGAATGGCTATCCTG
GGCGGTACCGGAGCTAGCGGCAAACCAATCCCAAACCCACTGCTGGGCCTGGATGGATCCG
GGGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAA
GAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGT
35 GGGAGAGCAATGGGCAGCCGGAGAACAACACTACAAGACCACGCCTCCCGTGCTGGACTCCGA
CGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAAC

GTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC
CTGTCTCCGGGTAAATGAgaattc (SEQ ID NO. 2)

Sec

E-DIII

5 SV5

hCH3

aagcttgtcgacc**ATGGGCTGGAGCCTGATCCTCCTGTTCTCGTCGCTGTGGCTACAGGTgTGCA**
CTCTCAGGGCGCGCACGCCTCCTACGCAATGTGCCTGAATACTTTTGTCTGAAGAAAGAGG
TGTCTGAAACCCAGCATGGAACCTATCCTGATCAAGGTCGAGTACAAGGGGGAAGACGCTCCA
10 TGCAAAATTCCCTTCAGCACCGAGGATGGACAGGGGAAGGCACACAACGGACGGCTGATTAC
CGCTAATCCTGTGGTCAAAAAGAAAGAGGAACCCGTGAACATCGAGGCAGAACCCCTTTTG
GCGAATCCAATATCGTCATTGGAATCGGCGACAAGGCCCTGAAAATCAACTGGTATAGAAAAG
GGAGTTCAATCGGAAAAATGTTTCGAGGCAACCGCAAGAGGAGCAAGACGCATGGCAATTCTG
GGAGGTACCGGAGCTAGCGGCAAACCAATCCCAAACCCACTGCTGGGCCTGGATGGATCCG
15 GGGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAA
GAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGT
GGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGA
CGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAAC
GTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC
20 CTGTCTCCGGGTAAATGAgaattc (SEQ ID NO. 3)

Sec

E-DIII

SV5

hCH3

25 aagcttgtcgacc**ATGGGCTGGAGCCTGATCCTCCTGTTCTCGTCGCTGTGGCTACAGGTgTGCA**
CTCTCAGGGCGCGCATGCAAGCTACACTATGTGCAGCGGGAAATTCAGCATCGACAAGGAAA
TGGCAGAAACCCAGCATGGAACAACCTGTGGTCAAAGTCAAGTACGAGGGAGCAGGAGCTCCA
TGCAAGGTGCCCATCGAGATTGAGACGTGAACAAGGAAAAAGTGGTCGGCAGAATCATTAG
CTCCACTCCTCTGGCTGAAAACACCAATAGTGTGACAAACATTGAGCTGGAACCCCTTTTCGG
30 GGATAGTTACATCGTGATTGGCGTCGGCAATTCAGCACTGACCCTGCACTGGTTCCGGAAGG
GATCTAGTATCGGCAAAATGTTTGAGAGCACATATAGGGGCGCCAAGCGCATGGCTATTCTG
GGCGGTACCGGGGCTAGCGGAAAACCAATCCCCAACCCCTCTGCTGGGCCTGGACGGATCCG
GGGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAA
GAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGT
35 GGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGA

CGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAAC
GTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC
CTGTCTCCGGGTAAATGAgattc (SEQ ID NO. 4)

Sec

5 E-DIII

SV5

hCH3

wherein Sec is the secretory domain, E-DIII is the E-DIII domain, SV5 is the exemplary SV5 tag and hCH3 is the hCH3 IgG domain.

10 In a preferred embodiment, said construct does not contain any kind of tag.

The protein codified by any genetic construct according to the invention is also within the scope of the present invention. Said protein is also herein referred to as "fusion protein" or "chimeric protein".

In particular, a protein comprising an aminoacidic sequence selected from the group
 15 consisting of SEQ ID NO: 5, 6, 7 and 8, respectively coded by gene sequences SEQ ID NO. 1, 2, 3 and 4, is a preferred object of the present invention.

MGWSLILLFLVAVATGVHSQGAHASVVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFS
SQDEKGVLTQNGRLITANPIVTDKEKPVNIEAEPFPGESYIVVGAGEKALKLSWFKKGSSIGKMF
ARGARRMAILGGTGASGKPIPNPLLGLDGS
 20 QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
KLSLSLSPGK (SEQ ID NO. 5)

Sec

E-DIII

SV5

25 hCH3

MGWSLILLFLVAVATGVHSQGAHASYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEI
MDLEKRHVLGRLITVNPIVTEKDSPVNIEAEPFPGDSYIIIGVEPGQLKLNWFKKGSSIGQMIETTMR
GAKRMAILGGTGASGKPIPNPLLGLDGS
 30 QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
LSLSLSPGK (SEQ ID NO. 6)

Sec

E-DIII

SV5

hCH3

MGWSLILLFLVAVATGVHSQGAHASYAMCLNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFST
EDGQGKAHNRLITANPVVTKKEEPVNIEAEPFGESENVIGIGDKALKINWYRKGSSIGKMFATA
RGARRMAILGGTGASGKPIPNLLGLDGSQQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 5 DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK
SLSLSPGK (SEQ ID NO. 7)

Sec

E-DIII

SV5

hCH3

MGWSLILLFLVAVATGVHSQGAHASYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIE
IRDVNKEKVVGRIISSTPLAENTNSVTNIELEPPFGDSYIVIGVGNLALTLHWFRKGSSIGKMFESTY
RGAKRMAILGGTGASGKPIPNLLGLDGSQQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 15 DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQK
SLSLSPGK (SEQ ID NO. 8)

Sec

E-DIII

SV5

hCH3

wherein Sec is the secretory domain, E-DIII is the E-DIII domain, SV5 is the exemplary SV5 tag and hCH3 is the hCH3 IgG domain.

In a preferred embodiment, said construct does not contain any kind of tag.

Vectors and means of delivery of the genetic constructs of the invention are within the scope of the present invention.

Antibodies raised against any protein codified by a genetic construct according to the invention are further objects of the present invention.

The genetic constructs of the invention, the proteins codified by said genetic constructs and the antibodies induced by said constructs can be used as medicaments.

In particular, the genetic constructs of the invention and the proteins codified by said genetic constructs can be used as a medicament for the prevention of dengue infections, in particular of all the four serotypes.

In another embodiment of the invention, they can be used as a medicament for the prevention of infections of any virus belonging to the Flavivirus genus, provided that the

envelope protein of said virus is subdivided in three subdomains and that the genetic construct comprises a sequence encoding the DIII domain of said virus.

A vaccine comprising at least one genetic construct of the invention or at least one protein codified by said genetic construct, provided that said genetic construct or protein induces neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants is also an object of the present invention.

DESCRIPTION OF THE INVENTION

Definitions

Within the meaning of the present invention, "DIII" is the DIII domain of the envelope protein E of dengue virus or of any virus belonging to the Flavivirus genus whose envelope protein is subdivided in three subdomains. "DIII", "E-DIII" and "domain III" are used in the present invention as synonyms.

A "fusion protein" is a protein created through the joining of two or more genes which originally coded for separate proteins. It is also herein referred to as "chimeric protein".

A "genetic construct" is a functional unit necessary for the expression of a gene. It is characterized by a nucleotide sequence and it can be a DNA or RNA sequence.

A "secretion leader peptide" is a short peptide sequence, usually present at the N-terminus of a protein, necessary for the secretion of the linked protein or polypeptide.

Figures

Figure 1. **a**: Schematic representation of the SV5-tagged recombinant E-DIII domain alone or combined with the human γ CH3 domain; sec: secretion signal. **b**: Western blotting analysis of cell culture supernatants (S) or cellular extracts (E) from HEK-293 cells transfected with vectors expressing E-DIII of the indicated serotypes, fused or non-fused with the CH3 domain. **c**: Western blotting analysis of cell culture supernatants from cells transfected with vectors encoding E-DIII- γ CH3 chimeras of the indicated serotypes, where the DIII sequence is either the original viral sequence (V) or the codon-optimized one (O). E-DIII containing proteins were detected with anti-SV5 antibodies.

Figure 2. **a**: Scheme of the ELISA test to evaluate the immunologic response of mice immunized with E-DIII- γ CH3. Streptavidin-coated plates were used to capture biotinylated chimeras E-DIII- ϵ CH4-BAP and mouse sera were assayed at different dilutions to evaluate their anti-DIII titres; sec: secretion signal; BAP: biotin acceptor peptide. In **b** are shown the responses of mice immunized with E-DIII- γ CH3 of the indicated serotypes, at

1:2000 dilutions, against the corresponding E-DIII- ϵ CH4, compared with the activity of a preimmune serum (PI), at the same dilution against the same antigens.

Figure 3. **a**: Scheme of the E-DIII- ϵ CH4-GPI chimeras expressed on cell surface that are used in cytofluorimetry experiments; sec: secretion signal; GPI: glycosyl-phosphatidyl-inositol anchor sequence. **b**: FACS analysis of Sp2/0 cells expressing on their surface E-DIII- ϵ CH4-GPI proteins of the indicated serotypes. Each cell line was assayed with sera from mice immunized against the different serotypes, as well as with preimmune serum (PI). It is evident that sera from mice immunized against each serotype cross-react efficiently with DIIs of the other serotypes.

Figure 4. **a**: Scheme of the complete Env protein ectodomain fused to the transmembrane domain of human MHC-I α allele A2 (TM); sec: secretion signal. **b**: FACS analysis of transiently transfected HEK293 cells expressing on their surface serotype 3 E-DIII- ϵ CH4-GPI (ϵ DIII-GPI), or serotype 3 Env ectodomain (m3E), or mock transfected (mock); cells were assayed with anti-serotype 3 DII sera (anti-3DII) or preimmune sera (PI).

Figure 5. Plaque Reduction Neutralization Test (PRNT). Vero cells were infected with dengue virus serotype 2 (DENV-2) and treated with sera from mice immunized with E-DIII from the corresponding serotype. The result of a typical assay for serotype 2 is represented in **a**, where plaque reduction on cells treated with anti-E-DIII serum (right) is visible by comparison with cells treated with preimmune serum (center); on the left are non infected cells. In **b** is indicated the level of neutralization in comparison with the preimmune serum of eight individual sera from anti-serotype 2 E-DIII mice.

Detailed description of the invention

It is an object of the present invention a genetic construct comprising a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding any domain of envelope protein E of dengue virus and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.

The carboxyterminal domain from the constant regions of an immunoglobulin is also herein referred to as "carboxyterminal domain".

The secretion leader peptide comprised in said construct can be one of the secretion leader peptides known in the art. A suitable peptide has the following aminoacidic sequence: MGWSLILLFLVAVATGVHS (SEQ ID NO: 9), encoded by the following

nucleotide sequence: atgggctggagcctgatcctcctgttcctcgctgctgtggctacaggtgtgcactct (SEQ ID NO. 10).

When the domain of protein E is DIII, it is preferably the DIII of one of the following dengue virus strains: DENV1 strain Nauru Island, DENV2 strain NGC, DENV3 strain 3H87 and DENV4 strain Dominica.

In a preferred embodiment of the invention, the nucleotide sequence encoding any domain of envelope protein E of dengue virus is a nucleotide sequence encoding for domain III.

In a more preferred embodiment, said nucleotide sequence is selected from the group consisting of

DENV1-DIII

CAGGGCGCGCATGCCTCATATGTAATGTGCACAGGGTCATTCAAGTTAGAGAAGGAA
GTGGCTGAGACCCAGCATGGAAGTGTCTAGTGCAGGTAAATACGAAGGAACAGAT
GCACCATGCAAGATCCCCTTCTCGTCCCAAGATGAGAAGGGAGTAACCCAGAATGG
GAGATTGATAACAGCCAACCCCATAGTCACTGACAAAGAAAAACCAAGTCAACATTGA
AGCGGAGCCACCTTTTGGTGAGAGCTACATTGTGGTAGGAGCAGGTGAAAAGGCCT
TGAAACTAAGCTGGTTCAAGAAGGGAAGCAGTATAGGGAAAATGTTTGAAGCAACTG
CCCGTGGAGCACGAAGGATGGCCATCCTGGGA (SEQ ID NO: 11),

DENV2-DIII

CAGGGCGCGCACGCCTCATAACAGCATGTGCACTGGAAAGTTCAAGGTGGTCAAGGA
AATCGCAGAGACTCAGCACGGGACTATTGTCATTAGGGTCCAGTACGAGGGCGACG
GGTCTCCATGCAAGATTCCCTTCGAGATCATGGATCTGGAAAAACGGCACGTGCTGG
GCAGACTGATTACCGTGAACCTATCGTCAAGAGAAAGGACAGCCAGTGAATATTG
AGGCCGAACCCCTTTTCGGGGATTCTATATCATTATCGGAGTCGAACCCGGCCAG
CTGAAACTGAACTGGTTTAAGAAAGGAAGCAGTATTGGGCAGATGATTGAGACCACT
ATGAGAGGGGCTAAGCGAATGGCTATCCTGGGC (SEQ ID NO: 12),

DENV3-DIII

CAGGGCGCGCACGCCTCCTACGCAATGTGCCTGAATACTTTTGTCTGAAGAAAGAG
GTGTCTGAAACCCAGCATGGAAGTATCCTGATCAAGGTGAGTACAAGGGGGAAGA
CGCTCCATGCAAAATTCCCTTCAGCACCGAGGATGGACAGGGGAAGGCACACAACG
GACGGCTGATTACCGCTAATCCTGTGGTCAAAAGAAAGAGGAACCCGTGAACATCG
AGGCAGAACCCCTTTTGGCGAATCCAATATCGTCATTGGAATCGGCGACAAGGCCC

TGAAAATCAACTGGTATAGAAAAGGGAGTTCAATCGGAAAAATGTTTCGAGGCAACCG
CAAGAGGAGCAAGACGCATGGCAATTCTGGGA (SEQ ID NO: 13),

and

DENV4-DIII

5 CAGGGCGCGCATGCAAGCTACACTATGTGCAGCGGGAAATTCAGCATCGACAAGGA
AATGGCAGAAACCCAGCATGGAACAACCTGTGGTCAAAGTCAAGTACGAGGGAGCAG
GAGCTCCATGCAAGGTGCCCATCGAGATTCGAGACGTGAACAAGGAAAAAGTGGTC
GGCAGAATCATTAGCTCCACTCCTCTGGCTGAAAACACCAATAGTGTGACAAACATT
GAGCTGGAACCCCCTTTTCGGGGATAGTTACATCGTGATTGGCGTCGGCAATTCAGCA
10 CTGACCCTGCACTGGTTCCGGAAGGGATCTAGTATCGGCAAAATGTTTGAGAGCACA
TATAGGGGCGCCAAGCGCATGGCTATTCTGGGC (SEQ ID NO: 14).

The carboxyterminal domain is from the constant region of an immunoglobulin selected from the group consisting of human, murine or other animal origin.

In a preferred embodiment, said carboxyterminal domain is selected from the group
15 consisting of γ CH3, α CH3, ε CH4, μ CH4, C κ and C λ domains from any immunoglobulin subclass, said immunoglobulin being of human, murine or other animal origin, preferably of human origin.

All the nucleotide and amminoacidic sequences of said domains are known in the art, therefore said sequences are not reported in the present description.

20 For example, the human γ CH3 domain has the following amminoacidic sequence:
QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS
DGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO.
15).

A tag can be inserted between the sequence of the domain from protein E and the
25 sequence of the one or more carboxyterminal domains, for example between the DIII and the CH3 domain, to facilitate the detection of the obtained recombinant proteins. Suitable tags are, for example, SV5, FLAG, c-myc, HA, His6, HSV, T7, which are all tags known in the art. A preferred tag is the SV5, which has the amminoacidic sequence GKPIPNPLLGLD (SEQ ID NO: 16).

30 In a preferred embodiment of the invention, the genetic construct comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding the domain III of envelope protein E of dengue virus and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.

In a more preferred embodiment, the genetic construct comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding the domain III of envelope protein E of dengue virus and a nucleotide sequence encoding the γ CH3 domain from immunoglobulin G (construct E-DIII- γ CH3).

- 5 In another preferred embodiment, the genetic construct comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding the domain III of envelope protein E of dengue virus and a nucleotide sequence encoding the ϵ CH4 domain from immunoglobulin E (construct E-DIII- ϵ CH4).

10 In an alternative embodiment, the genetic construct comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding the domain III of envelope protein E of any virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.

- 15 The DNA fragments encoding the viral DIII of the invention are preferably codon-optimized in order to improve their expression in mammalian cells. Codon-optimization is achieved by the methods known in the art for chemical synthesis of defined DNA sequences (Jestin, J.L. & Kempf, A., *J. Mol. Evol.*, 69, 452-457, 2009, Wang, P.G. et al., *PLoSOne* 4(12): e8325, 2009).

- 20 Synthesis and assembly of the genetic constructs of the invention can be carried out according to methods known in the art. (Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning. A laboratory manual*. New York, Cold Spring Harbor Laboratory Press). The suitable methods and protocols to be used for the construction of a specific construct according to the present invention can be chosen from the skilled in
25 the art according to the common knowledge in the technical field.

In a preferred embodiment, the genetic construct comprises a polynucleotide sequence selected from the group consisting of: SEQ ID NO: 1, 2, 3 and 4.

- Expression vectors comprising a construct according to the present invention are within the scope of the present invention. Suitable vectors are commercially available. More
30 specifically, the genetic constructs of the present invention can be inserted in any expression vector able to sustain expression in mammalian cells.

Exemplary vectors are pcDNA₃ (Life Technologies) and pVAX (Life Technologies).

The skilled person knows how to insert the construct of the invention in the desired expression vector using suitable restriction sites; for example the genetic construct can be included in the HindIII/EcoRI restriction sites of an expression vector.

In an embodiment of the invention, the genetic construct of the invention is introduced into a host cell. The host cell can be a prokaryotic or a eukaryotic cell. Preferably, it is a mammalian cell. When the host cell is a prokaryotic cell, a suitable prokaryotic expression vector should be used. The construct can be introduced into a host cell by transfection with any procedure suitable for entry into a cell, for example biological or physical methods, so that the molecules of the present invention are expressed by the host cell.

A host cell transfected with an expression vector comprising the genetic construct of the invention is also within the scope of the present invention.

When transfecting a host cell with the construct of the invention, the corresponding fusion protein is expressed. A properly folded antigen is thus obtained and secreted.

Induction of antibody responses following genetic immunization requires that the antigen is well expressed and secreted from the cells of the host, in order to produce significant availability of antigen for effective stimulation of B cells.

Secretion of envelope protein domains, in particular DIII domains, from mammalian cells with the engineered domain containing only an aminoterminal secretion peptide is highly inefficient and therefore unable to induce antibody responses against the engineered domain.

The construct of the invention solves the problem of inefficiency of secretion, providing a fusion protein comprising any domain of envelope protein E that is efficiently secreted from mammalian cells, mainly thanks to the presence of one or more carboxyterminal domains from the constant regions of an immunoglobulin.

A particularly efficient secretion has been obtained by a fusion protein comprising DIII fused with the IgG CH3 domain.

The construct of the present invention thus encodes for an engineered recombinant protein consisting (from N to C termini) of three essential elements:

- i) a secretion leader peptide to allow secretion from mammalian cells (sec);
- ii) the antigenic codon-optimised sequence of any E protein domain, and
- iii) one or more carboxyterminal domains from the constant regions of an immunoglobulin.

This latter characteristic is the more important one, since the inventors of the present invention have found that the carboxyterminal domain from the constant regions of an immunoglobulin renders the domain from protein E secretable.

The proteins codified by the genetic constructs of the invention are within the scope of the present invention.

In a preferred embodiment, said protein comprises the amino acid sequence of domain III of envelope protein E of dengue virus. In a more preferred embodiment, said amino acid sequence is selected from the group consisting of

DENV1-DIII

10 QGAHASYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSSQDEKGVQTQNGR
LITANPIVTDKEKPVNIEAEPFPGESYIVVGAGEKALKLSWFKKGSSIGKMFPEATARGARR
MAILG (SEQ ID NO: 17),

DENV2-DIII

15 QGAHASYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLIT
VNPIVTEKDSPVNIEAEPFPGDSYIIIGVEPGQLKLNWFKKGSSIGQMIETTMRGAKRMAIL
G (SEQ ID NO: 18),

DENV3-DIII

20 QGAHASYAMCLNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGLRI
TANPVVTKKEEPVNIEAEPFPGESNIVIGIGDKALKINWYRKGSSIGKMFPEATARGARRMA
ILG (SEQ ID NO: 19)

and

DENV4-DIII

25 QGAHASYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRII
SSTPLAENTNSVTNIELEPPFPGDSYIVIGVGNSALTLHWFRKGSSIGKMFESTYRGAKRM
AILG (SEQ ID NO: 20).

In a more preferred embodiment, the protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO. 5, 6, 7 and 8. Said protein is codified, for example, by the polynucleotides of SEQ ID NO. 1, 2, 3 and 4.

30 Said proteins can be obtained by a genetic construct according to the invention by methods known in the art.

Antibodies raised against the E protein domain encoded by any genetic construct according to the invention are a further object of the present invention. Said antibodies

can be obtained following immunization starting from the constructs of the invention or from the codified fusion proteins according to methods known in the art.

Said antibodies can be used, for example, for Passive Humoral Immunotherapy for preventing or treating dengue virus or other Flavivirus infections.

- 5 The delivery to a subject of a genetic construct according to the invention, comprising the sequence of any domain of protein E, induces high antibody titres against said domain.

In particular, the delivery to a subject of a genetic construct comprising the DIII sequence from dengue virus induces for all virus serotypes high anti-DIII antibody titres.

- 10 These antibodies recognise DIII when expressed alone as well as when expressed as part of the full length ectodomain of protein E in all four serotypes.

Sera of the subjects vaccinated with said genetic construct have high titres of virus infectivity neutralising activity.

More importantly, the constructs according to the invention are efficient in inducing neutralizing responses against any of the four dengue virus serotypes.

- 15 In particular, vaccination of a subject with four constructs of SEQ ID NO: 1, 2, 3 and 4 (tetravalent vaccine) induces neutralising antibodies to all four serotypes with comparable activity.

The genetic construct according to the invention can be used as a medicament.

Also, the protein codified by said construct can be used as a medicament.

- 20 In particular, said construct or protein can be used as a medicament for the prevention of dengue infections of all the four serotypes.

- In another embodiment, it can be used as a medicament for the prevention of infections of any virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains and that said genetic construct comprises a
25 nucleotide sequence encoding the domain DIII of said virus.

The construct can be administered alone or in combination with other therapeutic agents to enhance antigenicity, for example adjuvants.

- A vaccine against any of the four dengue virus serotypes comprising at least one genetic construct according to the invention or at least one protein codified by said genetic
30 construct, provided that said genetic construct or protein induces neutralizing antibodies,

and pharmaceutically acceptable carriers, excipients and optionally adjuvants is also within the scope of the present invention.

A vaccine against any of the four dengue virus serotypes comprising at least one genetic construct according to the invention or at least one protein codified by said genetic
5 construct, provided that said genetic construct or protein induces cross-neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants is also within the scope of the present invention.

In a preferred embodiment, said vaccine comprises at least four constructs, each one comprising a nucleotide sequence encoding domain III of a different dengue serotype.

10 A vaccine against any virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, comprising at least one genetic construct according to the invention or at least one protein codified by said genetic construct, wherein the nucleotide sequence encoding any domain of envelope protein E encodes the DIII domain, provided that said genetic construct or protein induces
15 neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants, is a further object of the present invention.

A vaccine against any virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, comprising at least one genetic construct according to the invention or at least one protein codified by said genetic
20 construct, wherein the nucleotide sequence encoding any domain of envelope protein E encodes the DIII domain, provided that said genetic construct or protein induces cross-neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants, is a further object of the present invention. Said vaccine can be used for preventing infections from any virus belonging to the Flavivirus genus, provided that
25 the envelope protein of said virus is subdivided in three subdomains. Examples of said virus are Dengue hemorrhagic fever virus, West Nile virus, Tick-borne encephalitis virus, Japanese encephalitis virus, yellow fever virus, Kyasanur Forest Disease virus, Omsk hemorrhagic fever virus, St. Louis encephalitis virus, Powassan virus, Murray Valley encephalitis virus.

30 In a preferred embodiment, said genetic vaccine comprises a construct comprising a nucleotide sequence encoding domain DIII and a nucleotide sequence encoding the CH3 domain of IgG.

In a preferred embodiment, the vaccine comprises at least one construct comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO. 1, 2, 3 and 4.

In a more preferred embodiment, the vaccine comprises four constructs, one comprising the polynucleotide sequence of SEQ ID NO. 1, one comprising the polynucleotide
5 sequence of SEQ ID NO. 2, one comprising the polynucleotide sequence of SEQ ID NO. 3 and one comprising the polynucleotide sequence of SEQ ID NO. 4. This embodiment of the vaccine is also herein referred to as tetravalent formulation.

Within the meaning of the present invention, neutralizing antibodies means fully neutralizing antibodies to all four dengue virus serotypes.

10 The genetic construct in the vaccine can be in the form of naked DNA or RNA.

Different ways of delivery of the construct can be used, including viral vectors (for example, adeno-associated virus (AAV)).

The preparation of DNA vaccines is within the framework of general knowledge. Reference can be made to Saltzman W. M., Shen H., Brandsma J.L. eds. (2006) DNA
15 Vaccines. Methods and Protocols. 2nd Edition. Totowa, NJ, Humana Press.

The vaccine can be in the form of a preparation for intradermal, intramuscular, intraperitoneal, parenteral, intravenous, subcutaneous, intranasal or oral administration but other forms are equally suitable.

Administration is generally achieved by injection.

20 The vaccine doses can be determined properly by a physician considering the type of dosage form, method of administration, the age of the patient, weight, etc. This activity falls within the normal activities of a person skilled in the art.

A vaccine according to the invention can be used for immunizing an animal subject. Therefore, the vaccine of the invention for veterinary use is also an object of the present
25 invention.

In this connection, an exemplary list of virus infections that can be treated with the present invention are: Japanese encephalitis virus, Kyasanur Forest disease virus, Louping ill virus, Murray Valley encephalitis virus, Omsk hemorrhagic fever virus, St. Louis encephalitis virus, Tick-borne encephalitis virus, Wesselsbron virus, West Nile virus,
30 Yellow fever virus.

A method for immunizing a subject against dengue virus infection of any of the four serotypes comprising administration of the genetic construct of the invention to a subject is also provided.

A method for immunizing a subject against Flavivirus infection comprising administration of the genetic construct of the invention, wherein said genetic construct comprises a nucleotide sequence encoding DIII, to a subject is also provided.

Said subject is an animal, preferably a mammalian, more preferably it is a human.

The present invention will be now further illustrated by the following non-limiting examples.

EXAMPLES

Materials and Methods

Recombinant E-DIII constructs

The sequences coding for dengue virus (DENV) envelope ectodomain and E-DIII domain were obtained from DENV1 strain Nauru Island (GenBank accession number U88535.1), DENV2 strain New Guinea C (NGC, GenBank accession number AF038403), DENV3 strain 3H87 (GenBank accession number M93130) and DENV4 strain Dominica (GenBank accession number AF326573.1).

E-DIII sequences of dengue virus serotype 1, 2, 3 and 4 were produced as synthetic genes by GenScript (Piscataway, NJ, USA). E-DIII sequences of DENV serotype 2, 3 and 4 were further codon optimized to improve expression in mammalian cells.

E-DIII coding fragments from each serotype were inserted in pCDNA₃ vectors (Life Technologies) already containing sequences encoding a secretion signal from mouse immunoglobulins (sec) and the SV5 tag, or sec, SV5 and the CH3 domain of human IgG (γ CH3), to yield the pCDNA₃-sec-E-DIII-SV5 and pCDNA₃-sec-E-DIII-SV5- γ CH3, respectively, that were used for mice vaccination.

To test the presence of anti-E-DIII antibodies in sera of vaccinated mice, the E-DIII domain of each serotype was engineered by adding at the C-terminus the CH4 domain from human IgE (ϵ CH4) fused either with GPI anchor signal (to produce a membrane E-DIII- ϵ CH4-GPI protein) or with BAP (Biotin Acceptor Peptide) sequence (to obtain a secreted biotinylated E-DIII- ϵ CH4 molecule). To build vectors coding for the E-DIII- ϵ CH4-GPI molecules, HindIII-KpnI fragments from the four pCDNA₃-sec-E-DIII-SV5- γ CH3, containing the sec-E-DIII coding sequences, were inserted in the vector pCDNA₃- ϵ mSIP/ Δ EMPD-GPI (Poggianella M. et al., *J. Immunol.*, 177, 3597-3605, 2006), to yield

the plasmids pCDNA₃-sec-E-DIII-εCH4-GPI. To produce vectors expressing secreted biotinylated E-DIII-εCH4 molecules, HindIII-Bsu36I fragments from the four plasmids pCDNA₃-sec-E-DIII-εCH4-GPI were excised and inserted in the corresponding sites of the previously described bigenic vector pCDNA₃-sec-SV5-CysεCH3CH4 BAP-BirA (Predonzani A. et al., *BMC Biotechnology* 8:41, 2008), to yield the vectors pCDNA₃-E-DIII-εCH4-BAP-BirA, expressing at the same time the E-DIII-εCH4-BAP molecules and the biotin ligase BirA, which effectively biotinylates BAP-tagged proteins.

To test if anti-E-DIII antibodies bind the envelope ectodomain expressed on cellular membranes, the ectodomain was engineered with the exogenous trans-membrane domain of human MHC-Iα allele A2. To this purpose the complete envelope ectodomains (composed of domains DI, DII and DIII) of the four serotypes were synthesized and inserted into pcDNA₃ vectors (pcDNA₃-Env). The sequence coding for the MHC-Iα trans-membrane domain (TMD) was amplified from the template pcDNA-BAP-MHC-Iα (Petrus G. et al., *PLoS ONE*, 6(8): e23712, 2011) with the oligonucleotides 5'-CCG GAA TTC GGA GCC GTC TTC CCA GCC-3' (SEQ ID NO. 21) and 5'-ATA GGG CCC TTA ACC TTC ATT TTT AAA GAT- 3' (SEQ ID NO: 22). The amplified fragment was ligated into EcoRI and Apal sites of the four pCDNA₃-Env vectors, obtaining the final constructs pCDNA₃-Env-TMD.

Cell-lines and dengue virus strains

HEK293 and HEK293T/17 cells from American Type Culture Collection (Rockville, MD, numbers CRL-1573 and CRL-11268, respectively) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated foetal calf serum (FCS) (Life Technologies), 50 µg/ml gentamycin (Life Technologies) and 2 mM L-glutamine (Sigma). To select HEK293 stable transfected clones, 0.4 mg/ml G418 (Geneticin, Life Technologies) was added.

BHK-21 cells were grown in DMEM medium supplemented with 10% heat inactivated FCS, 50 µg/ml gentamycin and 2 mM L-glutamine.

Vero cells were maintained in DMEM medium supplemented with 10% heat inactivated FCS, 50 µg/ml gentamycin, 2 mM L-glutamine and 4.5 g/l glucose.

Sp2/0 cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat inactivated FCS, 50 µg/ml gentamycin, 2 mM L-glutamine, 1 mM sodium pyruvate; Sp2/0 stable transfected clones were grown in selective medium containing 0.4 mg/ml Geneticin.

DENV1 strain Hawaii A, DENV2 strain NGC, DENV3 strain TC3 and DENV4 strain TC25 were used for plaque neutralization reduction test. All DENV strains were propagated in BHK21 cells, cultivated in DMEM supplemented with 2% heat inactivated FCS, 50 µg/ml gentamycin, 2 mM L-glutamine and maintained at 36°C and 5% CO₂.

5 **Detection of recombinant dengue molecules expression in HEK 293 and Sp2/0 transfected cells**

Transient transfections of HEK293T/17 cells and stable transfections of HEK293 cells were performed essentially as described by Sambrook *et al.*, using circular or linearized plasmids respectively.

- 10 HEK 293T/17 cells were seeded in 6-well plates at 5×10^5 cells/well density and transfected using standard calcium phosphate method with between 2.5 and 5 µg of plasmids. 24 h after transfection the culture medium was replaced with a serum-free medium or with a serum free medium supplemented with 100 µM biotin (Sigma), to produce the biotinylated protein *in vivo*, according to the method described (Predonzani A.
15 *et al.*, *BMC Biotechnology* 8:41, 2008), and 48 h post-transfection cellular extracts and supernatants from transiently transfected cells were collected. To remove free biotin, after collection, the supernatants containing biotinylated molecules were extensively dialyzed against PBS. Cellular extracts were prepared at 4°C in 100 µl of TNN lysis buffer (100 mM Tris-HCl, pH 8, 250 mM NaCl, 0.5% NP-40), supplemented with Protease Inhibitor
20 Cocktail (PIC, Sigma) according to manufacturer's instructions. The expression and secretion of the recombinant dengue molecules were confirmed by western blot.

- To investigate mice immune responses elicited by DNA vaccines using an ELISA assay, recombinant biotinylated E-DIII molecules, fused to human εCH4, or recombinant biotinylated envelope ectodomain were expressed in stable transfected HEK293 cells. 10⁷
25 HEK 293 cells were transfected with 15 µg of DNA using calcium phosphate technique. Stable clones were selected by culture in medium containing 0.4 mg/ml G418 and secretion of biotinylated proteins was confirmed by western blot. Supernatants from stable transfected cells were collected after 72 h of culture in serum-free medium supplemented with biotin and dialyzed against PBS.

- 30 To analyze mice antibody responses elicited by DNA vaccines using a cytofluorimetric assay, recombinant E-DIII molecules, fused to human εCH4, were expressed through a GPI anchor in stable transfected Sp2/0 cells. Transfections were performed by electroporation as previously described (Bestagno M. *et al.*, *Biochemistry*, 40, 10686-

10692, 2001). Clones were analyzed by FITC-labelled goat anti-hu IgE (KPL, Gaithersburg, MD, USA) in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with CellQuest software.

Samples of the cell lysates and supernatants were separated in 10% SDS-PAGE gels in
5 reducing conditions and then transferred onto polyvinylidene difluoride membrane (Millipore). The membranes were blocked for 1 h with 5% Milk in PBST (PBS supplemented with 0.1% Tween-20), probed 1h at RT with anti-SV5 monoclonal antibody (Life Technologies) and after washing with PBST they were incubated for 1h at RT with HRP-linked goat anti-mouse IgG antibody (Jackson ImmunoResearch, Newmarket, UK);
10 alternatively, after blocking membranes were incubated with HRP-labelled goat anti-human IgE (KPL). Signals were visualized by ECL (Pierce, Rockford, IL, USA).

Balb/c mice vaccinations

Female Balb/c mice, 5-6 weeks old, were purchased from Harlan (Milan, Italy). They were housed at the Animal House of ICGB (Trieste, Italy). All animal procedures were
15 performed in accordance with institutional guidelines and in compliance with national and international laws and policies.

Four groups of 10 female Balb/c mice were vaccinated with each monovalent dengue DNA vaccine using Gene Gun (Bio-Rad, Hercules, CA, USA) delivery as previously described (Cesco-Gaspere M. et al., *Scand. J. Immunol.*, 68, 58-66, 2008). A group of
20 non-immunized mice was also used as negative control and a group of mice was vaccinated with all four serotypes. Animals were immunized three times at fifteen days intervals with 1 µg DNA for monovalent vaccines or 2 µg DNA (0.5 µg each) for tetravalent vaccines and blood was collected at 45th day and 60th day. Animals were bled by sub-mandibular puncture. Serum samples were collected and stored at -20°C until use.

Antibody assays (anti-E-DIII analysis)

Mice sera were analyzed for the presence of anti-E-DIII antibodies by ELISA as well as by flow-cytometry.

In ELISA, the specificity and the cross-reactivity of E-DIII immunized mouse sera for each dengue virus serotype was confirmed using *in vivo* biotinylated E-DIII as coating antigen.

30 MaxiSorp Nunc Immuno-Plates (Nunc, Roskilde, Denmark) were coated with 100 µl/well of avidin diluted at 5 µg/ml in 50mM Na₂CO₃/NaHCO₃ buffer pH 9.5 and incubated overnight at 4°C. After three washing with PBST buffer (0.05% Tween 20 in PBS pH 7.4) and blocking with 100 µl/well of 1% BSA in PBS (PBS-BSA) for 1 h 30 min at RT, the

dialyzed biotinylated-E-DIII antigen (100 µl/well), diluted in PBS, was incubated at 4°C overnight. The plates were then washed three times with PBST buffer and sera from immunized mice were added at dilutions ranging from 1:1000 to 1:10,000 in PBS-BSA. After incubating for 2h at RT, the plates were washed three times and 100 µl/well of HRP-conjugated goat anti-mouse IgG γ chain specific antibody (Jackson ImmunoResearch) (1:20,000 in PBS-BSA) was added and incubated for 1 h. Bound conjugate was detected with TMB substrate (Sigma) and plates incubated at room temperature for 5 min. The reaction was stopped by addition of 1N H₂SO₄ and optical density was measured at 450 nm (OD₄₅₀).

- 5 conjugated goat anti-mouse IgG γ chain specific antibody (Jackson ImmunoResearch) (1:20,000 in PBS-BSA) was added and incubated for 1 h. Bound conjugate was detected with TMB substrate (Sigma) and plates incubated at room temperature for 5 min. The reaction was stopped by addition of 1N H₂SO₄ and optical density was measured at 450 nm (OD₄₅₀).
- 10 The anti-dengue IgG titres were determined as the reciprocal of the highest serum dilution at which OD₄₅₀ was ten times higher than that of the non-immune serum.

Specific and cross-reactive immune responses against the E-DIII were analyzed on stable Sp2/0 E-DIII transfectants by cytofluorimetry on a FACSCalibur flow cytometer using anti-E-DIII sera (1:1000), followed by Alexa₄₈₈-conjugated goat anti-mouse IgG (1:1000) (Jackson ImmunoResearch).

15

Plaque reduction neutralization test (PRNT)

PRNT was carried out on Vero cells in 24 multi-well plates, following procedures described in "Guidelines for plaque reduction neutralization testing of human antibodies to dengue virus" (World Health Organization, Geneva, Switzerland, 2007).

- 20 The day before neutralization test, cells were seeded at a density of 160,000 cells/well.

De-complemented mouse sera samples (30 min at 56°C) were serially diluted (from 1:12.5 to 1:400) in DMEM serum free medium followed by addition of an equal volume of dengue virus containing 50 PFU/reaction. The neutralization reaction was performed in a final volume of 60 µl and it was incubated 1 h 30 min at 36°C.

- 25 After washing with DMEM serum free media, Vero cells were infected in duplicate with 25 µl of neutralization mixture for 1h at 36°C and after this time, the viral inoculum was discarded and cells were overlaid with 1 ml of DMEM supplemented with 2% FCS and 3% carboxymethylcellulose (Sigma). Plates were incubated at 36°C for 8-10 days, depending on the serotype. After this period, cells were washed twice with PBS solution, fixed for 1 h
- 30 with paraformaldehyde (Sigma) 3.7% and stained with 1% crystal violet for 20 min. Plaques were counted and percentage of plaque reduction was calculated as [(average number of plaques in pre-immune control wells – average number of plaques in test well)/ average number of plaque in pre-immune control well] x 100. Neutralizing antibody titres

were expressed as maximum serum dilution yielding a 50% plaque number reduction (PRNT₅₀)

Results

Example 1 - Secretion of the DIII constructs

- 5 Induction of antibody responses following genetic immunization requires that the antigen is well expressed and secreted from the mammalian cells of the host, for effective stimulation of B cells.

We therefore produced different genetic constructs with E-DIII sequences from the four dengue serotypes (from aa 298 to 416, or 296 to 414 for serotype 3, of the viral envelope
10 protein). Each E-DIII was also provided, at its N-terminus, with a sequence encoding a secretion leader peptide to allow translocation into the ER and the secretory pathway and at the C-terminus with the dimerising γ CH3 domain from the IgG Heavy-chain (Figure 1a). To facilitate detection, the 11 amino acids long SV5-tag was also included.

Expression of the recombinant E-DIIIs, with or without the dimerising γ CH3 domain, was
15 first tested by transfections into HEK293 cells. As shown in Fig. 1b, in the absence of the γ CH3 domain, DIIIs from the four serotypes were not secreted. In contrast, when fused to the γ CH3 domain, active secretion was obtained in all cases. We therefore adopted the DIII-CH3 format as the antigenic design to be used in plasmid-DNA immunisations.

Since it was observed a large heterogeneity in the expression levels among the four
20 serotypes, DIII sequences from serotypes 2, 3 and 4 were further codon-optimized to maximize expression efficiency in mammalian cells (Wang, P.G. et al., PLoSOne 4(12): e8325, 2009). Large differences in DIII-CH3 secretion efficiencies were actually observed between the codon-optimized and the non-optimized versions of the E-DIII sequence: the codon-optimized versions showed in fact high and comparable secretion levels for all
25 serotypes (Figure 1c).

Example 2 - Gene-gun immunisations induce DIII specific antibody responses

Groups of 10 animals for each serotype were immunised by gene-gun delivery of the DIII-CH3 plasmid DNA constructs, with a total of three shots at 15 days intervals. Mice sera were then tested for anti-DIII antibodies by ELISA, using *in vivo* mono-biotinylated
30 recombinant DIIIs proteins (fused to the human ϵ CH4 domain, to precisely evaluate anti-DIII response) secreted from mammalian cells, that were captured on Streptavidin-coated plates (schematically shown in Figure 2a). As shown in figure 2b, strong antibody titres

were elicited for each serotype. Sera were tested in plates containing comparable amounts of recombinant DIII proteins. For all 4 serotypes antibody titres (expressed as the dilution producing a 10 fold higher value over the negative serum) were of around 1:4000 (Table 1). In the same table is also shown that sera from mice immunized with plasmid expressing E-DIII without CH3 have much lower antibody titres.

	DIII	DIII-γCH3
Serotype 1	>1:200	>1:4000
Serotype 2	>1:100	>1:5000
Serotype 3	>1:200	>1:4000
Serotype 4	>1:50	>1:3000

Table 1. Titres of sera from mice immunized against E-DIII (first column) or E-DIII- γ CH3 (second column) of each serotype, tested in ELISA on the corresponding E-DIII- ϵ CH4. Titre is calculated as the dilution giving an OD₄₅₀ 10 times higher than the preimmune serum at the same dilution.

Sera were then tested by cytofluorimetry on stable Sp2/0 transfectant clones displaying DIII domains expressed on cell surface membrane through a GPI anchor (Figure 3a). As expected, these antibodies reacted equally well to membrane displayed DIII and were able to cross-react with DIII domains from the other serotypes (Figure 3b), indicating that genetic immunization can raise cross-reacting antibodies.

More important, the induced antibodies were able to recognise the different DIIs also when expressed as part of the full-length E-ectodomain (containing also E domains DI and DII, Figure 4a). For all four serotypes the reactivity against the E-ectodomain was comparable to the one against DIII alone, as exemplified for serotype 3 in Figure 4b. These results indicated that most of the antibody response was directed against DIII epitopes well exposed in the complete envelope ectodomain. In addition, these antibodies proved to be directed mostly against conformational epitopes, since sera positive in ELISA and FACS on native DIII or E-ectodomain recombinant proteins were not reactive in western blots on denatured proteins.

Example 3 - Anti-DIII antibodies have virus neutralising activity

The neutralising activity of the serotype specific serum was tested implementing the plaque reduction neutralisation test (PRNT) for each virus serotype on Vero cells. Figure 5a shows a representative set of plates with plaques for dengue virus serotype 2 (DENV-2), treated with a mouse pre-immune serum (centre) or a mouse anti-2DIII serum (right).

- 5 The neutralisation activities of each group of animal sera against the homologous dengue serotype were determined. As exemplified in Figure 5b for serotype 2 and summarized in Table 2 for all serotypes, in each group all animals showed high neutralisation titres (taken as fold dilution producing 50% reduction), that were higher than 1:400 for the first three groups and 1:200 for animals immunized with serotype 4. Even though neutralization titres
- 10 showed some variability among different serotypes, they resulted anyway higher than previously described, and this indicated that all four constructs were efficient in inducing neutralising responses against the homologous dengue serotype.

**PRNT titres of sera from mice immunized
with monovalent vaccines**

Serotype 1	>1:400
Serotype 2	>1:800
Serotype 3	>1:400
Serotype 4	>1:200

- 15 Table 2. Plaque Reduction Neutralization Test (PRNT) titres of sera from mice immunized against the E-DIII- γ CH3 of each serotype, assayed on the corresponding dengue virus serotype. Titre is calculated as the dilution producing a 50% reduction in the number of plaques in comparison with the preimmune serum at the same dilution.

Example 4 - Neutralising antibodies of a tetravalent formulation

- 20 A tetravalent formulation containing the four genetic serotype-specific constructs was used to vaccinate a group of animals. The total amount of DNA per cartridge was increased to 2 micrograms. After 3 immunisations, sera from the vaccinated animals were then tested by ELISA and PRNT.

As for the individual constructs, the tetravalent vaccine was able to induce total Ab titres (measured by ELISA, Table 3) and neutralising activity (PRNT, Table 4) against all four

serotypes with titres comparable to the individual ones and, more importantly, similar for the four serotypes.

**ELISA titres of sera from mice immunized
with tetravalent vaccine**

Serotype 1	>1:3000
Serotype 2	>1:4000
Serotype 3	>1:4000
Serotype 4	>1:2000

Table 3. Titres of sera from mice immunized with the tetravalent vaccine, tested in ELISA on E-DIII-εCH4 from each serotype. Titre is calculated as the dilution giving an OD₄₅₀ 10 times higher than the preimmune serum at the same dilution.

**PRNT titres of sera from mice immunized
with tetravalent vaccine**

Serotype 1	>1:400
Serotype 2	>1:400
Serotype 3	>1:200
Serotype 4	>1:200

Table 4. Plaque Reduction Neutralization Test (PRNT) titres of sera from mice immunized with the tetravalent vaccine, assayed on each dengue virus serotype. Titre is calculated as the dilution producing a 50% reduction in the number of plaques in comparison with the preimmune serum at the same dilution.

The present invention provides industrial application for the manufacturing of a vaccine against Flaviviridae viruses, in particular dengue virus. In the latter case, the vaccine can neutralise all 4 serotypes with comparable activity.

CLAIMS

1. A genetic construct comprising a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding any domain of envelope protein E of dengue virus and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.
5
2. The genetic construct according to claim 1, wherein said domain of envelope protein E is anyone of the domains DI, DII and DIII, preferably DIII.
3. The genetic construct according to claim 2, wherein said DIII domain is selected from the group consisting of: SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14.
10
4. A genetic construct comprising a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding domain DIII of envelope protein E of a virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.
15
5. The genetic construct according to anyone of claims 1-4, wherein said carboxyterminal domain is selected from the group consisting of γ CH3, α CH3, ϵ CH4, μ CH4, C κ and C λ domains from any immunoglobulin subclass.
6. The genetic construct according to anyone of claims 1-5 further comprising a nucleotide sequence encoding a tag.
20
7. The genetic construct according to anyone of claims 1-6, which comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding domain III of envelope protein E of dengue virus and a nucleotide sequence encoding one or more carboxyterminal domains from the constant regions of an immunoglobulin.
25
8. The genetic construct according to anyone of claims 1-7, which comprises a nucleotide sequence encoding a secretion leader peptide, a nucleotide sequence encoding domain III of envelope protein E of dengue virus and a nucleotide sequence encoding one or more γ CH3 domain from immunoglobulin G.
9. The genetic construct according to anyone of claims 1-8, which comprises a polynucleotide sequence selected from the group consisting of: SEQ ID NO: 1, 2, 3 and 4.
30

10. The genetic construct according to claim 9, wherein said polynucleotide sequence does not contain a tag.
11. An expression vector comprising the genetic construct of anyone of claims 1-10.
12. A host cell comprising the genetic construct of anyone of claims 1-9 or the expression vector of claim 11.
13. A protein codified by the genetic construct of anyone of claims 1-10.
14. The protein according to claim 13 comprising an amminoacidic sequence selected from the group consisting of SEQ ID NO: 5, 6, 7 and 8.
15. A protein codified by the genetic construct of anyone of claims 4-6.
16. Antibodies raised against the protein of anyone of claims 13-14 or claim 15.
17. The genetic construct of anyone of claims 1-10 or the protein of anyone of claims 13-14 or claim 15 or the antibodies of claim 16 for use as a medicament.
18. The genetic construct of anyone of claims 1-10 or the protein of claim 13 or 14 or the antibodies of claim 16 when dependent on anyone of claims 13 or 14 for use as a medicament for the prevention of dengue infections of any of the four serotypes.
19. The genetic construct of anyone of claims 4-6 or the protein of claim 15 or the antibodies of claim 16 when dependent on claim 15 for use as a medicament for the prevention of infections of a virus belonging to the Flavivirus genus.
20. A vaccine against any of the four dengue virus serotypes comprising at least one genetic construct of anyone of claims 1-10 or at least one protein of claims 13-14, provided that said genetic construct or protein induces neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants.
21. A vaccine against any of the four dengue virus serotypes comprising at least one genetic construct of anyone of claims 1-10 or at least one protein of claims 13-14, provided that said genetic construct or protein induces cross-neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants.
22. The vaccine according to claim 20 or 21 which comprises at least one construct comprising the polynucleotide sequence selected from the group consisting of

SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 SEQ ID NO. 4, SEQ ID NO. 11, SEQ ID NO. 12, SEQ ID NO. 13 and SEQ ID NO. 14.

23. A vaccine against all four dengue virus serotypes which comprises a construct comprising the polynucleotide sequence of SEQ ID NO. 1, a construct comprising the polynucleotide sequence of SEQ ID NO. 2, a construct comprising the polynucleotide sequence of SEQ ID NO. 3 and a construct comprising the polynucleotide sequence of SEQ ID NO. 4; or which comprises a protein comprising the aminoacidic sequence of SEQ ID NO. 5, a protein comprising the aminoacidic sequence of SEQ ID NO. 6, a protein comprising the aminoacidic sequence of SEQ ID NO. 7 and a protein comprising the aminoacidic sequence of SEQ ID NO. 8.

24. A vaccine against a virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, comprising at least one genetic construct of anyone of claims 4-6 or at least one protein of claim 15, provided that said genetic construct or protein induces neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants.

25. A vaccine against a virus belonging to the Flavivirus genus, provided that the envelope protein of said virus is subdivided in three subdomains, comprising at least one genetic construct of anyone of claims 4-6 or at least one protein of claim 15, provided that said genetic construct or protein induces cross-neutralizing antibodies, and pharmaceutically acceptable carriers, excipients and optionally adjuvants.

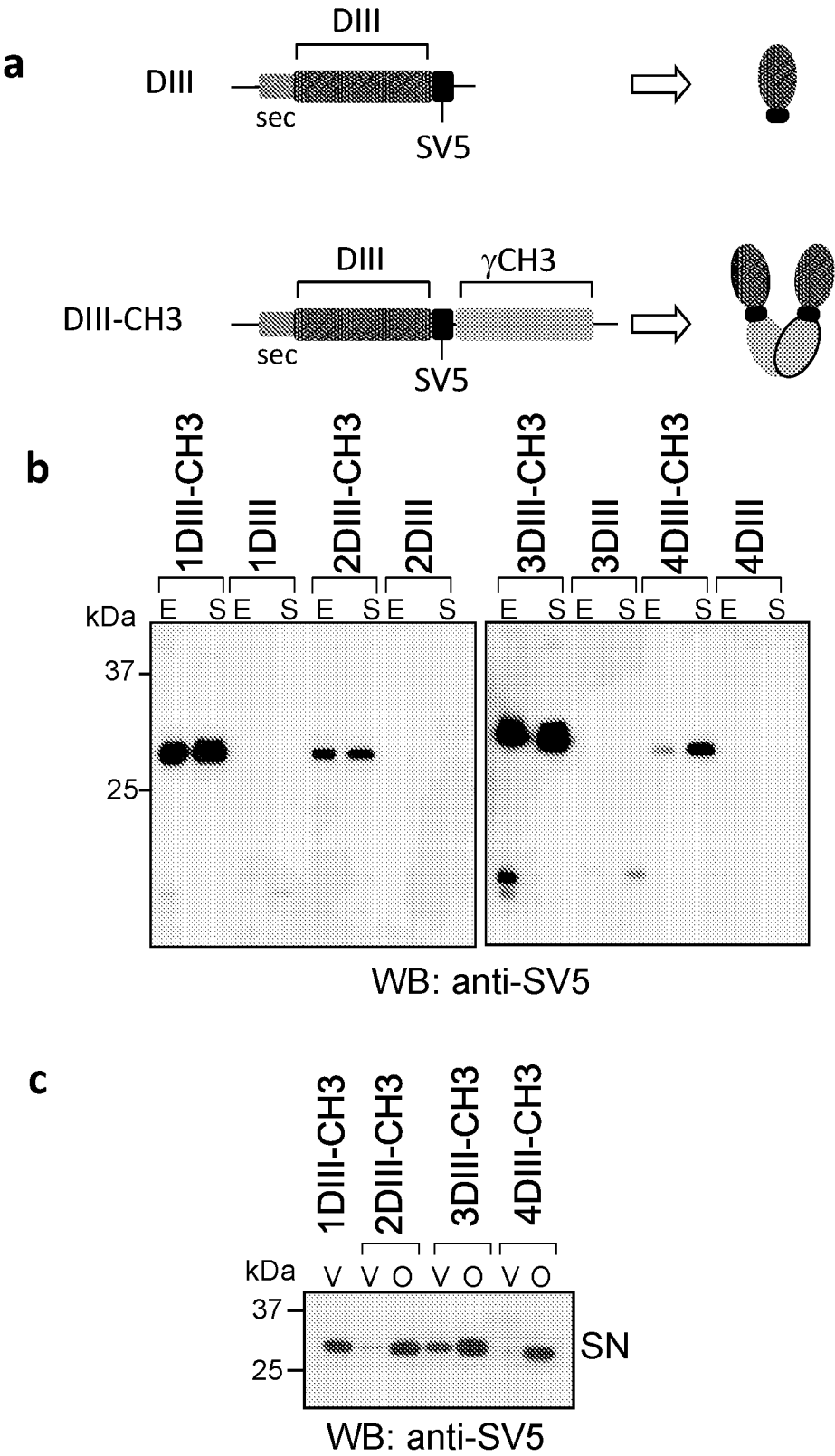
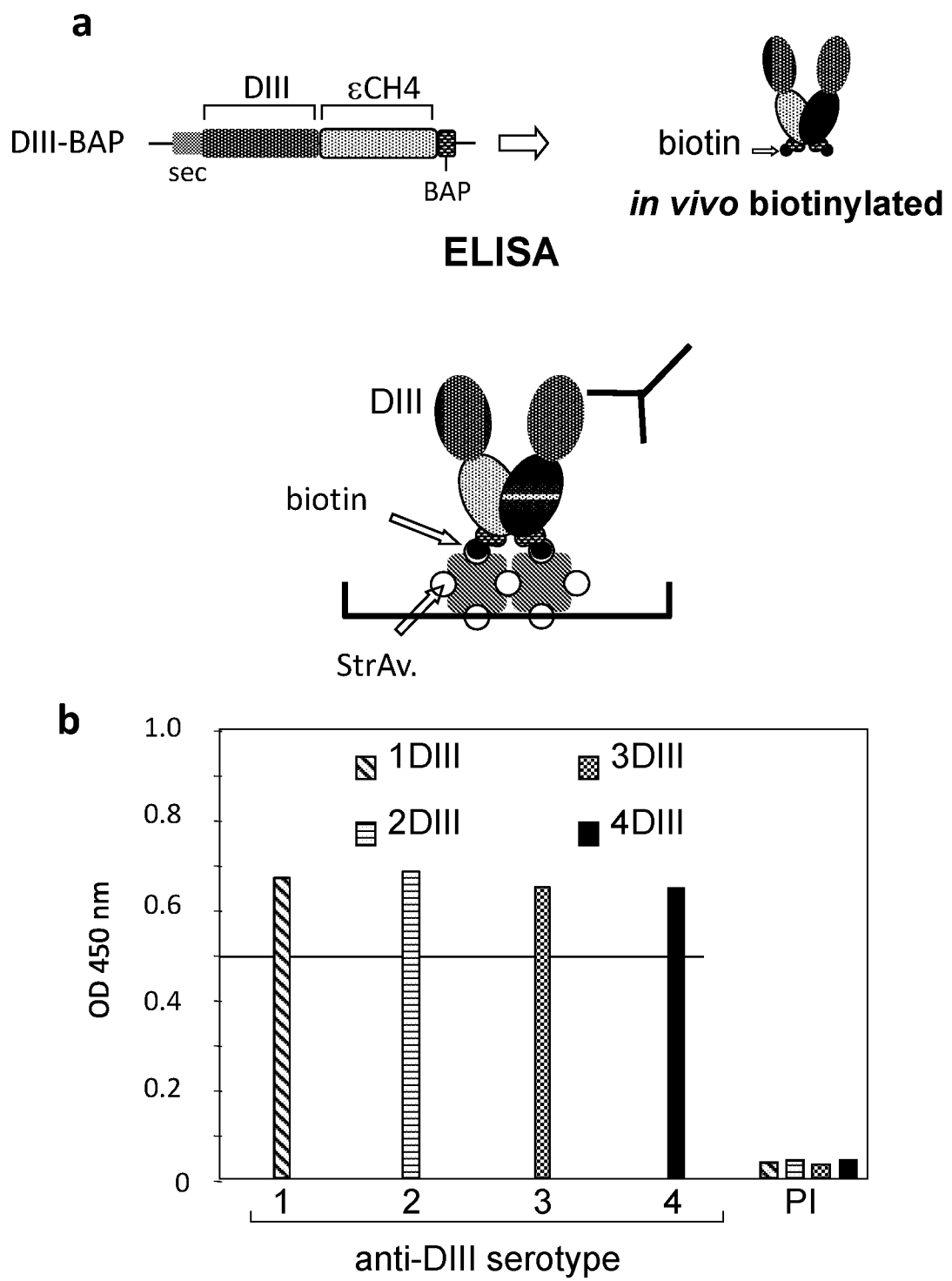
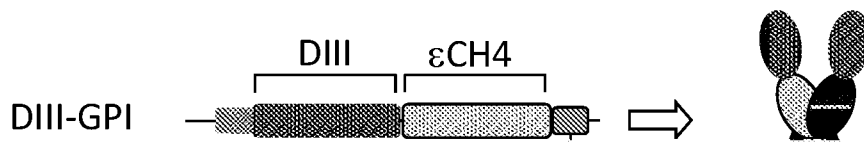
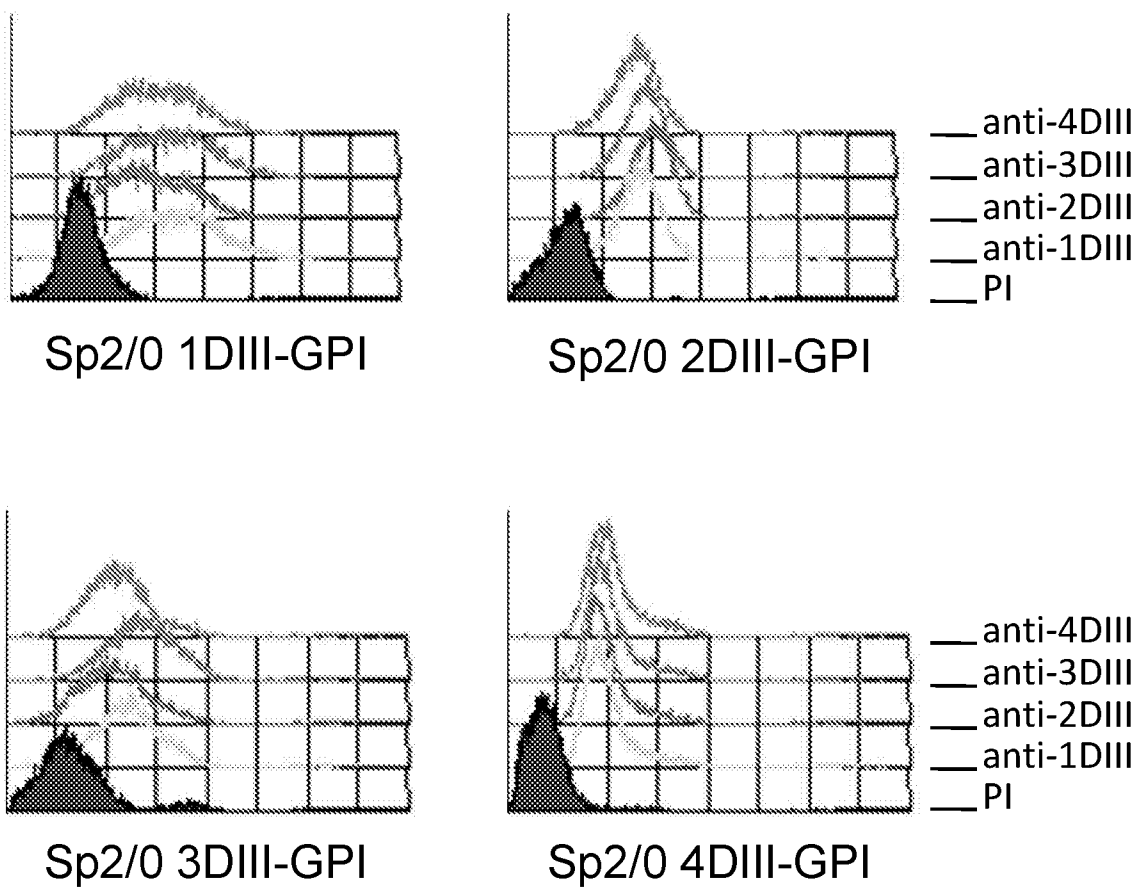


Figure 1

**Figure 2**

a**b****Figure 3**

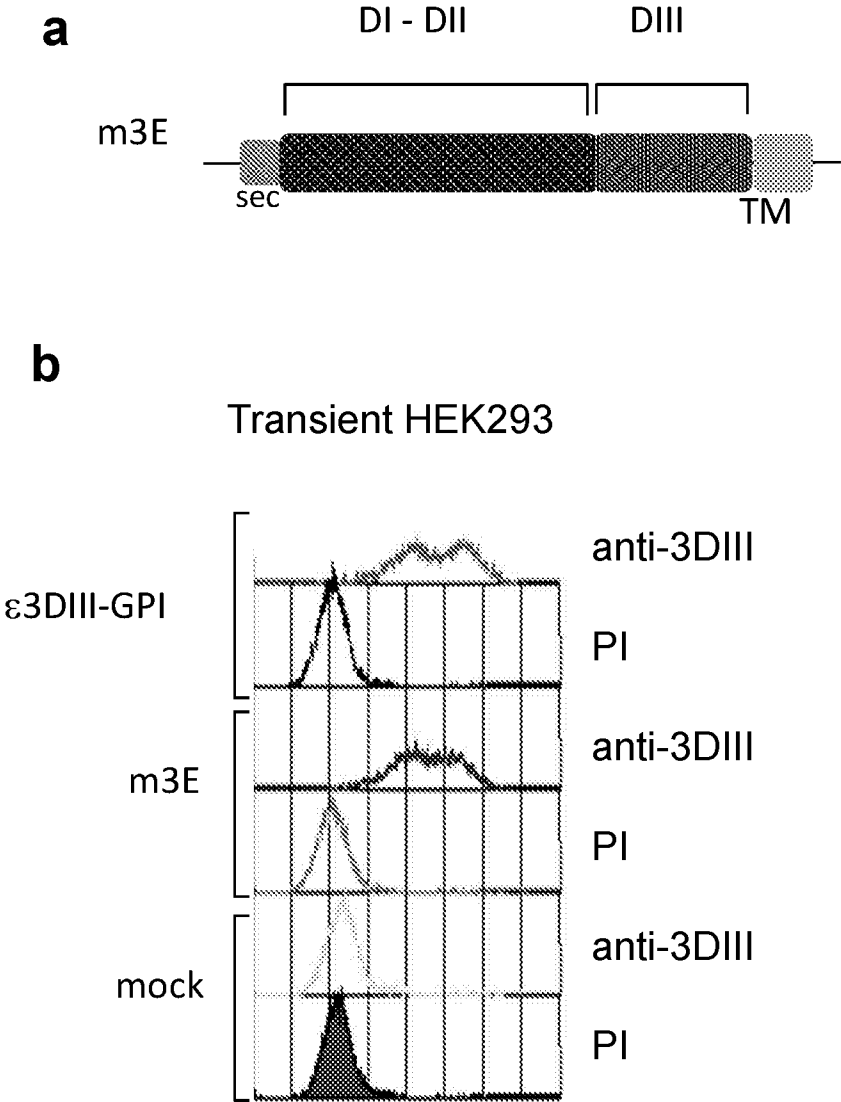
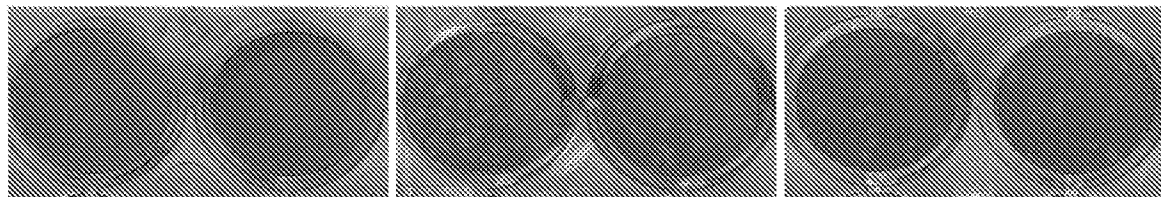


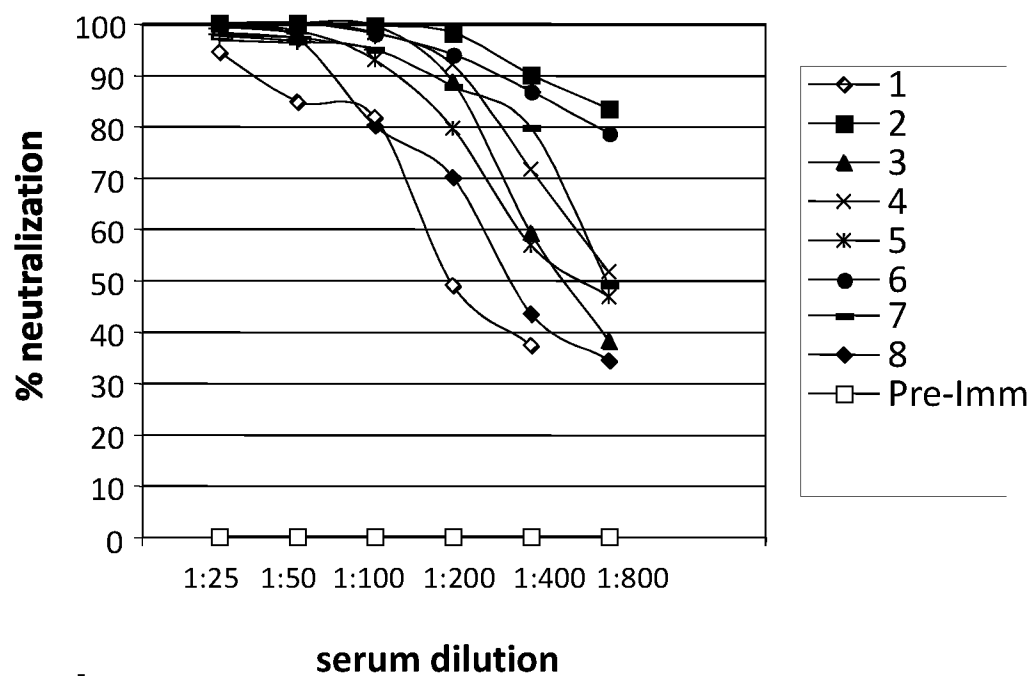
Figure 4

PRNT on Vero cells DENV-2 / anti-2DIII pool sera

Non-infected

Pre-Imm (1:400)

anti-2EDIII (1:400)

a**PRNT DENV-2 / anti-2DIII****b****Figure 5**