



(51) International Patent Classification:

A61K 47/48 (2006.01) A61P 37/04 (2006.01)
 A61K 39/00 (2006.01) A61P 31/14 (2006.01)
 A61K 39/29 (2006.01) A61P 35/00 (2006.01)
 A61K 39/02 (2006.01) A61P 31/04 (2006.01)
 A61P 37/00 (2006.01)

(21) International Application Number:

PCT/EP2012/053633

(22) International Filing Date:

2 March 2012 (02.03.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

11382065.8 11 March 2011 (11.03.2011) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to the identity of the inventor (Rule 4.17(i))
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: IMMUNOGENIC COMPOSITIONS AND METHODS FOR THEIR USE

(57) Abstract: The invention relates to immunogenic compositions and kits-of-parts comprising a first conjugate comprising fibronectin EDA and a first member of a binding pair and a second conjugate comprising an antigenic entity and a second member of a binding pair. The invention relates as well to therapeutic methods using said compositions and kits-of-parts as well as to mature and activated antigen presenting cells obtained using the immunogenic compositions and kits-of-parts of the invention.



WO 2012/123269 A1

IMMUNOGENIC COMPOSITIONS AND METHODS FOR THEIR USE

FIELD OF THE INVENTION

The present invention relates to the fibronectin Extra Domain A (EDA), a natural ligand for TLR4, as a means for antigen (Ag) delivery to TLR4 expressing cells. EDA is capable of inducing appropriate selection and maturation of antigen presenting cells (APCs) while delivering the antigen of choice to antigen presenting cells finally leading to an effective specific cytotoxic T lymphocytes (CTLs)-mediated response.

10 BACKGROUND OF THE INVENTION

Pathogens and cancer remain the leading causes of death worldwide. The development of vaccines to prevent diseases for which no vaccine currently exists, such as AIDS or malaria, or to treat chronic infections or cancers, as well as the improvement of efficacy and safety of existing vaccines, remains a high priority. In most cases, the development of such vaccines requires strategies capable of specifically stimulating CD8+ CTLs.

CTLs are activated by the presentation to T-cell receptors (TCRs) of short peptides associated with MHC class I molecules. These peptide-MHC class I complexes are present on the surface of APCs, which are also capable of providing co-stimulatory signals required for optimal CTL activation.

Dendritic cells (DC) are the most potent APCs, with a unique capacity to interact with naive T lymphocytes and initiate primary immune responses, activating helper CD4+ and cytotoxic CD8+ T lymphocytes. Antigen presentation and T cell stimulation by DC is reviewed by Guermonprez *et al.* ("Antigen presentation and T cell stimulation by DC". *Annu Rev Immunol* 2002, 20: 621-627), which is here included by reference. These cells orchestrate a repertoire of immune responses from tolerance to self-antigens to resistance to infectious pathogens depending on their maturation status (Reis e Sousa, C., *Nat Rev Immunol* 2006;6(6):476-83). Thus, it is generally accepted that efficient activation of T-cell immune responses are dependent on DC maturation triggered by a combination of stimuli derived from microbial products or inflammatory signals.

For these reasons, several vaccination strategies incorporate ligands for pattern recognition receptors (PRRs), agonistic antibodies against co-stimulatory molecules or

cytokines able to trigger maturation of DC. However, if a DC encounters the maturation stimuli before seeing the antigen, it will undergo an activation program which will reduce its antigen capture capacity, processing and presentation and will fail to present subsequently encountered antigens (Young LJ, *et al. Proc. Natl. Acad. Sci. USA* 5 2007;104(45):17753-8.26). Thus, a strategy of targeting the antigen to the same DC which is receiving the toll-like receptor (TLR) stimulation may have advantages over the use of mixtures of antigens and TLR ligands not associated within the same particle or molecule. However, engagement of TLR on DC loaded with the antigen may induce DC activation, expression of cytokines and DC migration to draining lymph nodes for 10 an efficient presentation of the processed antigen to T cells. Interestingly, this TLR engagement may modify the maturation of the phagosome containing the antigen in a way which allows antigen presentation in a highly immunogenic manner (Blander JM, *et al., Nature*, 2006; 440: 808-1227).

WO2006134190 describes that a fusion protein comprising an antigen and the 15 extra domain A of fibronectin (EDA) leads to antigen targeting to TLR4-expressing DC, enhancing cross-presentation and immunogenicity. Moreover, Mansilla *et al. (J Hepatol* 2009;51:520-527) have described that a fusion protein comprising the extra domain A of fibronectin (EDA) and the hepatitis C virus (HCV) NS3 protein induced strong and long lasting NS3-specific CD4⁺ and CD8⁺ T-cell responses and, in combination with 20 poly(I:C) and anti-CD40, down regulated intrahepatic expression of NS3 RNA.

However, there is still a need of additional strategies for the strengthening the immune response to an antigen.

SUMMARY OF THE INVENTION

25 In a first aspect, the invention relates to a composition or kit-of-parts comprising

(i) a conjugate comprising

a. the fibronectin EDA domain or a functionally equivalent variant thereof and

b. a first member of a binding pair; and

30 (ii) an antigenic entity which is modified by a second member of said binding pair.

In another aspect, the invention relates to an avidin or streptavidin oligomer comprising a plurality of avidin or streptavidin monomers wherein each of the avidin or streptavidin monomers is conjugated to a fibronectin EDA domain or a functionally equivalent variant thereof and wherein at least one of the monomers in the oligomer is
5 connected to a biotinylated antigenic entity through the biotin binding site in said at least one monomer.

In another aspect, the invention relates to a method for the generation of a composition according to the invention which comprises the steps of

- (i) contacting a conjugate comprising
 - 10 i. the fibronectin EDA domain or a functionally equivalent variant thereof and
 - ii. a first member of a binding pairwith an antigenic entity which is modified with a second member of the binding pair
15 wherein the contacting is carried out under conditions adequate for the formation of complexes between the conjugate and the antigenic entity via the interaction between said first and second members of the binding pair and
- (ii) recovering the complexes obtained in step (i).

20 In yet another aspect, the invention relates to a method for obtaining an immunogenic antigen presenting cell specific for a given antigenic entity comprising the steps of:

- (i) contacting an immature antigen presenting cell with a composition, kit-of-parts, oligomer or immunogenic composition according to the
25 invention and
- (ii) recovering the immunogenic antigen presenting cell.

In further aspects, the invention relates to a composition comprising an antigen presenting cell according to the invention.

In another aspects, the invention relates to a pharmaceutical composition
30 comprising a composition, a kit-of-parts, oligomer or immunogenic composition according to the invention and a pharmaceutically acceptable carrier and to a composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical

composition according to the invention for use in medicine and for use in the treatment of diseases which require the generation of an immune response towards said antigenic entity.

In another aspect, the invention relates to a conjugate comprising

- 5 (i) the fibronectin EDA or a functionally equivalent variant thereof
 and
 (ii) a first member of a binding pair.

In further aspects, the invention relates to a polynucleotide encoding a conjugate according to the invention wherein said conjugate is a fusion protein, to a vector
10 comprising said polynucleotide and to a host cell comprising a conjugate, a polynucleotide according or a vector according to the invention.

LEGENDS TO FIGURES

Figure 1. Recombinant EDavidin tetramerizes and binds biotinylated proteins with high affinity. (A) Recombinant EDavidin was produced, purified, refolded, detoxified and analyzed by SDS-PAGE. Boiled and not boiled protein samples were run in loading buffer and gels were stained with Coomassie Blue. (B) Biomolecular interaction analysis: Biotinylated BSA (Pierce) was immobilized covalently on a GLM chip (Biorad) and different concentrations of EDavidin were injected three times over
20 the protein bound surfaces and analyzed by using a surface plasmon resonance optical biosensor. The sensor chip was regenerated with a pulse of biotin and then different concentrations of streptavidin were passed through the surface. Results represented as RU (relative units) after the subtraction of the signal obtained in the reference channel. (C) ELISA-based binding assay of EDavidin to biotinylated proteins: biotinylated OVA or
25 BSA protein-coated wells were incubated with EDavidin or EDA, washed, incubated with anti-EDA antibodies and developed by using anti-rabbit IgG-HPRO antibodies. (D) SDS-PAGE-based binding assay of EDavidin to biotinylated OVA protein: EDavidin, biotinylated OVA (OVA-Biot) or EDavidin incubated for 15 minutes at room temperature with biotinylated OVA were loaded into a SDS-PAGE and stained
30 with Coomassie Blue. (E) Binding of EDavidin to biotinylated proteins in Western-Blot. A molecular weight marker containing biotinylated proteins (Biot) or the High-Range Rainbow Molecular Weight Marker (RB), were loaded into a 10% SDS-PAGE

followed by electrophoretic transfer to nitrocellulose membranes. The membranes were incubated with EDAvidin, EDA or streptavidin horseradish peroxidase and subsequently with anti-EDA polyclonal antibodies. The bands were detected with ECL chemoluminescence.

- 5 **Figure 2. Recombinant EDAvidin improves antigen capture by bone marrow derived dendritic cells (BMDC) and activates monocyte THP-1 cells** (A) BMDC obtained from C57/Bl6 mice were incubated during 15 minutes with or without 5 mg/ml of the indicated products. Cells were washed and analyzed by flow cytometry for visualization of GFP. (B) THP-1 cells were incubated for 15 hours with 0,1 µg/ml LPS,
10 0,25 µM EDAvidin or culture medium (Neg). After culturing, supernatants were harvested and the released TNF-α was measured by ELISA.

- Figure 3. EDAvidin binds to NS3-biotinylated protein and induces anti NS3 protein immune responses *in vivo*.** (A) Biotinylated or not biotinylated NS3 proteins from HCV were coated onto an ELISA plate, incubated with EDAvidin or EDA, and
15 developed as described in methods. (B, C) HHD mice were immunized i.v. with 2 nmols of EDAvidin plus biotinylated NS3 (NS3Biot), EDANS3, biotinylated NS3 protein, EDA plus biotinylated NS3 protein or streptavidin plus biotinylated NS3 protein. Seven days after immunization, mice spleens were obtained and the number of IFN-γ producing spots in response to CTL epitope p1073 from NS3 or NS3 protein (B)
20 or the CTL activity against target cells loaded with p1073 (C) were measured by ELISPOT and by *in vivo* killing assay, respectively. (D) EDAvidin binds to TRP2(180-188)-biotinylated peptides and induces anti-TRP2(180-188) immune responses *in vivo*. C57BL/6 mice were immunized sc. with 2 nmols of EDAvidin plus melanoma TRP-2(180-188) peptide biotinylated at the amino or carboxy terminus. Seven days after
25 immunization, mice spleens were obtained and the number of IFN-γ producing spots in response to CTL epitope TRP-2(180-188) were measured by ELISPOT.

- Figure 4. Protection conferred to BALB/c mice by Hot-Saline antigenic extracts (HS) or formalin inactivated bacterins obtained from *S. Enteritidis* rough mutants SEΔ*waaL* and SEΔ*Gal*.** Mice (n=5) were intraperitoneally immunized with the
30 corresponding antigenic preparation, i.e. Hot Saline (HS) or Bacterin (B) from SEΔ*waaL* or SEΔ*Gal* rough mutants, alone or in combination with EDA (+EDA) or MEDA (+MEDA). Additional groups of mice (n=5) receiving a bacterin obtained from

S. Enteritidis parental strain (B-SEwt) or PBS were used as controls. Four weeks after vaccination, all mice were intraperitoneally (i.p.) infected with 2.3×10^2 CFU/animal of SE-wt virulent strain and protection determined by $\log_{10}$ CFU/spleen of the challenging strain, at day 4 after challenge. Statistical comparisons were performed by ANOVA and Bonferroni's test. § $P < 0.01$ with respect to the homologous HS extract. * $P < 0.0005$ with respect to B-SE Δ Gal administered alone.

Figure 5. Detection of biotinylated bacterins by EDAvidin in ELISA. Biotinylated and not biotinylated bacterins were used to coat ELISA plates and, then, incubated with EDAvidin or EDA (as a negative control). The binding was monitored using a rabbit polyclonal anti-EDA antibody followed by an anti-rabbit whole IgG horseradish-peroxidase-conjugated antibody (Sigma). The optical density (O.D.₄₀₅) is represented.

Figure 6. Protection conferred to BALB/c mice by biotinylated bacterins bound to EDAvidin (BEDA-SE Δ waal and BEDA-SE Δ Gal), compared to SE Δ waal and SE Δ Gal live rough mutants. Mice (n=4) were immunized intraperitoneally (i.p.) with BEDA-SE Δ waal, BEDA-SE Δ Gal (20 μ g/mouse of bacterin total protein content) or *S. Enteritidis* SE Δ waal or SE Δ Gal live rough mutants (1×10^4 CFU/mouse). Animals (n=5) inoculated i.p. with bacterin obtained from *S. Enteritidis* wild type (wt) parental strain (B-SEwt) or PBS were used as controls. Four weeks after vaccination, all mice were challenged i.p. with 2.3×10^2 CFU/animal and spleen bacterial counts spleen determined 4 days later. Protection is expressed as counts ($\log_{10}$ CFU/spleen) of the SE-wt challenging strain.

DETAILED DESCRIPTION OF THE INVENTION

The authors of the present invention have taken advantage of the uniquely tenacious affinity of streptavidin protein to bind biotin or biotinylated compounds to facilitate the preparation of EDA-Ag complexes. Thus, by constructing a recombinant fusion protein (EDAvidin) containing EDA fused to the N terminus of streptavidin, it was observed that EDAvidin bound biotinylated proteins very efficiently, that it retained the proinflammatory activities of EDA and that it favoured the capture of biotinylated antigens by dendritic cells. These results indicate the usefulness of EDAvidin for preparing immunogenic compositions.

1. Conjugate of the invention

In a first aspect, the invention relates to a conjugate comprising

- (i) fibronectin EDA or a functionally equivalent variant thereof and
- (ii) a first member of a binding pair.

5 The term “conjugate”, as used herein, refers to two or more compounds which are covalently linked together so that the function of each compound is retained in the conjugate.

1.A. Fibronectin EDA or a functionally equivalent variant thereof

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The terms “extracellular domain A”, “fibronectin extra domain A”, “fibronectin EDA”, “EDA” are indistinctly used herein to refer to a region of the fibronectin molecule resulting from the transcription/translation of an exon of the fibronectin gene which is capable of specifically binding to Toll-like receptors 4 (TLR4). This domain
15 was originally described by Muro A. F. *et al.*, (*J Cell Biol* 2003;162:149-160). The EDA region may be derived from fibronectin obtained from different species such as human (SwissProt P02751), mouse (SwissProt P11276), bovine (SwissProt P07589) or rat (SwissProt P04937).

As used herein, “fibronectin” is understood as a multifunctional high molecular
20 weight glycoprotein present in blood and in the extracellular matrix of tissues. Fibronectin is a dimer formed by two identical polypeptide chains bound by C-terminal disulfide bonds. Each monomer has an approximate molecular weight of 230-250 kDa. Each monomer contains three types of modules: type I, type II and type III. Each of these modules is formed by two anti-parallel β -helices.

25 The term “functionally equivalent variant”, when referred to the fibronectin EDA, is understood as all those polypeptides derived from the EDA sequence by means of modification, insertion and/or deletion of one or more amino acids, provided that the function of binding to TLR4 receptors, of delivering antigens to dendritic cells and/or of activating dendritic cells is substantially preserved. Functionally equivalent variants are
30 those showing a degree of identity with respect to the fibronectin EDA domain higher than at least 25%, at least 40%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%. As used herein, “%

sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the polypeptide or polynucleotide sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue or nucleic acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window and multiplying the result by 100 to provide the percentage of sequence identity. Algorithms to align sequences are known in the art. Exemplary algorithms include, but are not limited to, the local homology algorithm of Smith and Waterman (Add APL Math, 2:482, 1981); the homology alignment algorithm of Needleman and Wunsch (*J Mol Biol* 1970;48:443); the search for similarity method of Pearson and Lipman (*Proc Natl Acad Sci USA* 1988;85:2444); and computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis.). In one embodiment, two sequences may be aligned using the "Blast 2 Sequences" tool at the NCBI website at default settings (Tatusova and Madden. *FEMS Microbiol Lett* 1999;174:247-250). Alternatively, amino acid sequences or nucleic acids sequences may be aligned by human inspection.

The capacity of the functionally equivalent variant to interact with TLR4 can be determined by means of using conventional methods known by the person skilled in the art. For example, by way of illustration, the capacity of the fibronectin EDA region variant to bind to TLR4 can be determined using co-immunoprecipitation experiments, in which the protein of interest (e.g. EDA variant) is isolated with a specific antibody and the molecules which interact with the protein (e.g. TLR4) are subsequently identified by means of a western blot. Assays for determining the capacity of the functionally equivalent variants of EDA to promote the maturation of dendritic cells are known by a person skilled in the art, such as for example incubation of BMDC with the recombinant proteins in vitro during 24-48 h and then testing by flow cytometry the upregulation of maturation markers, such as surface molecules CD54 and CD86, or by measuring the production of proinflammatory cytokines (such as IL-12 or TNF-alpha)

by commercial ELISA. In addition, the assay described in Example 2 of the present application based on determining the production of TNF- α by the THP1 monocyte cell line in response to the treatment the fibronectin EDA variant could also be used. Assays for determining the capacity of the functionally equivalent variants of EDA to deliver
5 antigens to dendritic cells are known by a person skilled in the art, such as for example the assay described in Example 2 of the present application based on determining the amount of marker protein (e.g. GFP) incorporated into dendritic cells contacted with a fusion protein comprising the EDA variant and the marker protein.

The person skilled in the art understands that the mutations in the nucleotide
10 sequence encoding the EDA domain sequences that give rise to conservative substitutions of amino acids in non-critical positions for the functionality of the protein, are evolutionary neutral mutations which do not affect its overall structure or its functionality.

In a preferred embodiment, the fibronectin EDA of the conjugate of the
15 invention corresponds to amino acids 1,631 to 1,721 of human fibronectin as shown in the UniProt database with accession number FINC_HUMAN and which corresponds to the polypeptide of sequence SEQ ID NO:1.

NIDRPKGLAFTDVDVDSIKIAWESPQGQVSRVRYVTYSSPEDGIRELFPAPDGEDDTAEL
20 QGLRPGSEYTVSVVALHDDMESQPLIGIQST (SEQ ID NO:1)

1.B. First member of a binding pair

The term "first member of a binding pair", as used herein, refers to a molecule which has affinity for and "binds" to another (hereinafter known as "second member of
25 the binding pair") under certain conditions, referred to as "binding conditions". The first and/or second members of the binding pair can be of a peptide (protein) or non-peptide nature.

Without being bound by theory, it is believed in the art that these kinds of non-covalent bonds result in binding, in part due to complementary shapes or structures of
30 the molecules involved in the binding pair. The term "binding" according to the invention refers to the interaction between affinity binding molecules or specific binding pairs (e.g., between biotin as an affinity tag molecule and streptavidin as an

affinity-tag-binding molecule) as a result of non-covalent bonds, such as, but not limited to, hydrogen bonds, hydrophobic interactions, van der Waals bonds, and ionic bonds. Based on the definition of "binding," and the wide variety of affinity binding molecules or specific binding pairs, it is clear that "binding conditions" vary for different specific binding pairs. Those skilled in the art can easily determine conditions whereby, in a sample, binding occurs between the affinity binding molecules. In particular, those skilled in the art can easily determine conditions whereby binding between affinity binding molecules that would be considered in the art to be "specific binding" can be made to occur. As understood in the art, such specificity is usually due to the higher affinity between the affinity binding molecules than for other substances and components (e.g., vessel walls, solid supports) in a sample. In certain cases, the specificity might also involve, or might be due to, a significantly more rapid association of affinity binding molecules than with other substances and components in a sample.

The term "binding pair" does not involve any particular size or any other technical structural characteristic other than that said binding pair can interact and bind to the other member of the binding pair resulting in a conjugate wherein the first and second components are bound to each other by means of the specific interaction between the first and second member of a binding pair.

The binding pair includes any type of immune interaction such as antigen/antibody, antigen/antibody fragment, hapten/anti-hapten as well as non-immune interactions such as avidin/biotin, avidin/biotinylated molecules, folic acid/folate-binding protein, hormone/hormone receptor, lectin/carbohydrate, lectin/molecule modified with carbohydrates, enzyme/enzyme substrate, enzyme/enzyme inhibitor, protein A/antibody, protein G/antibody, complementary nucleic acids (including sequences of DNA, RNA and peptide nucleic acids (PNA)), polynucleotide/polynucleotide-binding protein and the like.

As used in the present invention, the expression "specific binding" refers to the capacity of a first molecule to bind specifically to a second molecule by means of the existence of complementarity between the three-dimensional structures of the two molecules with a substantially higher affinity for non-specific binding such that the binding between said first and second molecule preferably takes place before the binding of any of said molecules with respect to the other molecules present in the

reaction mixture. It is understood that there is high affinity in the binding of two molecules when the complex resulting from said binding has a dissociation constant (KD) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-10} M, less than 10^{-11} M, less than 10^{-12} M, less than 10^{-13} M, less than 10^{-14} M or less than 10^{-15} M.

The terms "bond" and "binding" are used indistinctly to refer to an interaction between two or more entities. In those cases in which two entities are bound to one another, they can be directly bound (for example, by means of covalent bonds, ionic forces, hydrogen bonds, electrostatic interactions, Van der Waals forces or a combination of the above) or they can be indirectly bound, for example, by means of a linker.

In a preferred embodiment, the first member of a binding pair is a biotin-binding molecule. More preferably, the biotin-binding molecule is avidin. As used herein, the term "avidin" refers to a glycoprotein found in egg white and in tissues of birds, reptiles and amphibian and which has the capacity to bind to biotin with high affinity as well as any expressed or engineered form of the avidin biotin-binding molecule, such as streptavidin, neutravidin and the like. The term avidin includes both avidin found naturally in the eggs of *Gallus gallus* (NCBI accession numbers NM_205320.1 / GL45384353en) as well as the orthologues of said protein in other species. The term streptavidin, as used herein, corresponds to the protein from *Streptomyces avidinii* (accession number CAA00084.1 in GenBank), as well as the orthologues, homologues and fragments of streptavidin defined in the same manner as avidin. Streptavidin comprises 4 subunits each of which contains a binding site for biotin. Streptavidin or avidin fragments which retain substantial binding activity for biotin, such as at least 50 percent or more of the binding affinity of native streptavidin or avidin, respectively, may also be used. Preferably, the affinity of the avidin variant for biotin is of at least 10^{15} M^{-1} , 10^{14} M^{-1} , 10^{13} M^{-1} , 10^{12} M^{-1} , 10^{10} M^{-1} or 10^9 M^{-1} .

For convenience, in the instant description, the terms "avidin" and "streptavidin" as used herein are intended to encompass biotin-binding fragments, mutants and core forms of these binding pair members. Avidin and streptavidin are available from commercial suppliers. Moreover, the nucleic acid sequences encoding streptavidin and avidin and the streptavidin and avidin amino acid sequences can be found, for example,

in GenBank Accession Nos. X65082; X03591; NM --205320; X05343; Z21611; and Z21554.

Avidin and streptavidin variants suitable for use in the present invention include, without limitation

- 5 - "Core streptavidin", which is a truncated version of the full-length streptavidin polypeptide which may include streptavidin residues 13-138, 14-138, 13-139 and 14-139. See, e.g., Pahler *et al.*, (*J Biol Chem* 1987;262:13933-37).
- 10 - Truncated forms of streptavidin and avidin that retain strong binding to biotin (See, e.g. Sano *et al.*, (*J Biol Chem* 1995;270:28204-09) (describing core streptavidin variants 16-133 and 14-138) (U.S. Pat. No. 6,022,951).
- 15 - Mutants of streptavidin and core forms of streptavidin which retain substantial biotin binding activity or increased biotin binding activity. See Chilcoti *et al.*, *Proc Natl Acad Sci USA* 1995;92(5):1754-8; Reznik *et al.*, *Nat Biotechnol* 1996;14(8):1007-1011.
- 20 - Mutants with reduced immunogenicity, such as mutants modified by site-directed mutagenesis to remove potential T cell epitopes or lymphocyte epitopes. See Meyer *et al.*, *Protein Sci* 2001;10:491-503.
- 25 - Mutants of avidin and core forms of avidin which retain substantial biotin binding activity or increased biotin binding activity also may be used. See Hiller *et al.*, *J Biochem* 1991;278:573-85; Livnah *et al.* *Proc Natl Acad Sci USA* 1993;90:5076-80.
- 30 - Variants resulting from the chemical modification of avidin such as those resulting from the complete or partial modification of glycosylation and fragments thereof as well as the completely deglycosylated avidin variant known as neutravidin.
- Avidin mutants as described in WO05047317A1
- Avidin-like proteins as described in WO06045891,
- Recombinant avidin as described in WO0198349,
- Avidin variants as described in WO0027814,
- Monomeric streptavidin as described in WO06084388,

- Modified streptavidin dimers such as those described in WO06058226,
- The protein with biotin binding capacity as described in WO04018509,
- 5 - Streptavidin having a higher affinity for biotin as described in WO9840396,
- The modified streptavidin and avidin molecules as described in WO9640761,
- The streptavidin mutants as described in WO9711183,
- 10 - The streptavidin with modified affinity as described in WO9624606.

Different avidin variants are commercially available, such as Extravidin (Sigma-Aldrich), NeutrAvidin (Thermo Scientific), NeutrAvidin (Invitrogen) and NeutraLite (Belovo).

In a preferred embodiment, the first member of the binding pair comprises the
15 sequence

```

1  AEAGITGTWY NQLGSTFIVT AGADGALTGT YESAVGNAES RYVLTGRYDS APATDGSGTA
61  LGWTVAWKNN YRNAHSATTW SGQYVGGAEA RINTQWLLTS GTTEANAWKS TLVGHDTFTK
121 VKPSAAS (SEQ ID NO:2)

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20

The interaction between biotin and its binding partner, avidin or streptavidin, offers several advantages in the context of the present invention. For example, biotin has an extremely high affinity for both streptavidin (10^{13} M^{-1}) and avidin (10^{15} M^{-1}). Additionally, both streptavidin and avidin are tetrameric polypeptides that each binds
25 four molecules of biotin. Conjugates comprising streptavidin or avidin therefore have a tendency to form tetramers and higher structures. As a result, they can cross-link their corresponding immune cell receptors for more potent signal transduction, such as through aggregation of receptors.

The fibronectin EDA or a functionally equivalent variant thereof and the first
30 member of a binding pair may be directly connected, i.e. by means of a specific direct interaction between both components. Alternatively, the fibronectin EDA or a functionally equivalent variant thereof and the first member of a binding pair may be indirectly connected i.e. by means of using a “connector”.

In a preferred embodiment, the first and second components of the conjugate form a single polypeptide chain (hereinafter the fusion protein of the invention).

A person skilled in the art will appreciate that the different elements of the fusion protein of the invention can be placed in any order provided that the fibronectin EDA maintains its dendritic cell activating properties and that the first member of the binding pair maintains its capacity of binding to the second member of the binding pair.

Thus, examples of arrangement of the elements of the conjugate of the invention, always referring to the placement of elements in the N-terminal to C-terminal direction, are, among others:

- first member of a binding pair - fibronectin EDA,
- fibronectin EDA - first member of a binding pair,

In a preferred embodiment, the fibronectin EDA region is connected to the C-terminal end of the first member of the binding pair.

Moreover, the invention also contemplates fusion proteins comprising more than one fibronectin EDA as well as more than one first member of a binding pair. These fusion proteins may also contain a variety of arrangements, which are shown in the N- to C-terminal regions, such as:

- fibronectin EDA - first member of a binding pair - fibronectin EDA,
- first member of a binding pair - fibronectin EDA - first member of a binding pair,
- repeats of the last two.

In a preferred embodiment, the conjugate of the invention further comprises a “tag”. The term “tag”, as used herein, relates to any amino acid sequence for which specific binding molecules are available, thus allowing the detection/purification of any polypeptide carrying said tag. The tag is generally placed at the amino- or the carboxyl-terminus of the polypeptide. The presence of such tag allows the adapter molecule to be detected using an antibody against the tag polypeptide. Also, the provision of the tag enables the adapter polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity reagent that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.*, Mol Cell Biol, 1988;8:2159-2165); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Molecular and Cellular Biology* 1985;5:3610-3616); the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.*, *Protein Engineering* 1990;3:547-553). Other tag polypeptides include the Flag-peptide (Hopp *et al.*, *BioTechnology* 1988;6:1204-1210); the KT3 epitope peptide [Martin *et al.*, *Science* 1993;255:192-194); tubulin epitope peptide (Skinner *et al.*, *J Biol Chem* 1991;266:15163-15166); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, *Proc Natl Acad Sci USA* 1990;87:6393-6397). In a preferred embodiment, the purification tag is a polyhistidine tag. In a still more preferred embodiment, the purification tag is an hexahistidine tag.

In a preferred embodiment, the fusion protein of the present invention has the sequence:

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1  MNIDRPKGLA FTDVDVDSIK IAWESPQGQV SRYRVITYSSP EDGIRELFPA PDGEDDTAEL
61  QGLRPGSEYT VSVVALHDDM ESQPLIGIQS THMAEAGITG TWYNQLGSTF IVTAGADGAL
121 TGTYESAVGN AESRYVLTGR YDSAPATDGS GTALGWTVAW KNNYRNAHSA TTWSGQYVGG
181 AEARINTQWL LTSGTTEANA WKSTLVGHDT FTKVKPSAAS HHHHHH (SEQ ID NO:3)

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2. Polynucleotides, vectors and host cells of the invention

In another aspect, the invention relates to a polynucleotide encoding a conjugate of the invention. A person skilled in the art will understand that the polynucleotides of the invention will only encode the conjugates in which component (ii) has a peptide nature and which forms a single peptide chain with component (i), regardless of both the relative orientation and the fact that both components are directly connected or separated by a spacer region.

In a preferred embodiment, the polynucleotide according to the invention has the sequence

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1  ATGAACATTG ATCGCCCTAA AGGACTGGCA TTCACTGATG TGGATGTCGA TTCCATCAAA
61  ATTGCTTGGG AAAGCCCACA GGGGCAAGTT TCCAGGTACA GGGTGACCTA CTCGAGCCCT
121 GAGGATGGAA TCCGGGAGCT TTTCCCTGCA CCTGATGGTG AAGACGACAC TGCAGAGCTG

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181 CAGGGCCTCA GGCCGGGGTC TGAGTACACA GTCAGTGTGG TTGCCTTGCA CGATGATATG
 241 GAGAGCCAGC CCCTGATTGG AATCCAGTCC ACACATATGG CTGAAGCTGG TATCACCGGC
 301 ACCTGGTACA ACCAGCTGGG ATCCACCTTC ATCGTTACCG CTGGTGCTGA CGGTGCTCTG
 361 ACCGGTACCT ACGAATCCGC TGTTGGTAAC GCTGAATCTA GATACGTTCT GACCGGTCGT
 5 421 TACGACTCCG CTCCGGCTAC CGACGGTTCC GGAACCGCTC TGGGTTGGAC CGTTGCTTGG
 481 AAAACAACCT ACCGTAACGC TCACTCCGCT ACCACCTGGT CTGGCCAGTA CGTTGGTGGT
 541 GCTGAAGCTC GTATCAACAC CCAGTGGTTG TTGACCTCCG GCACCACCGA AGCTAACGCG
 601 TGGAAATCCA CCCTGGTTGG TCACGACACC TTCACCAAAG TTAAACCGTC CGCTGCTTCC
 661 CATCACCATC ACCACCATTA A (SEQ ID NO:4)

10

In another aspect, the invention relates to a gene construct comprising a polynucleotide of the invention. The construct preferably comprises the polynucleotide of the invention located under the operative control of sequences regulating the expression of the polynucleotide of the invention. A person skilled in the art will

15 understand that the polynucleotides of the invention must access the nucleus of a target tissue and there be transcribed and translated to give rise to the biologically active fusion protein.

In principle, any promoter can be used for the gene constructs of the present invention provided that said promoter is compatible with the cells in which the

20 polynucleotide is to be expressed. Thus, promoters suitable for the embodiment of the present invention include, without being necessarily limited to, constitutive promoters such as the derivatives of the genomes of eukaryotic viruses such as the polyoma virus, adenovirus, SV40, CMV, avian sarcoma virus, hepatitis B virus, the promoter of the metallothionein gene, the promoter of the herpes simplex virus thymidine kinase gene,

25 retrovirus LTR regions, the promoter of the immunoglobulin gene, the promoter of the actin gene, the promoter of the EF-1alpha gene as well as inducible promoters in which the expression of the protein depends on the addition of a molecule or an exogenous signal, such as the tetracycline system, the NFκB/UV light system, the Cre/Lox system and the promoter of heat shock genes, the regulatable promoters of RNA polymerase II

30 described in WO/2006/135436 as well as tissue-specific promoters. Preferably, the promoter used for expressing the polynucleotides of the invention are promoters functional in dendritic cells such as the fascin gene promoter as described by Bros *et al.* (*J Immunol* 2003;171:1825-1834), the DC-CK1, DC-STAMP and DC-SIGN gene promoters, the Dectin-2 promoter described in Morita *et al.*, (*Gene Ther* 2001;8:1729-

37), the CD11c gene promoter as described in Masood, R., *et al.* (*Int J Mol Med* 2001;8:335-343) and Somia, N. V., *et al.* (*Proc Acad Sci USA* 1995;92:7570-7574).

Other examples of promoters which are tissue-specific include the promoter of the albumin gene (Miyatake *et al.*, *J Virol* 1997;71:5124-32), the core promoter of hepatitis virus (Sandig *et al.*, *Gene Ther* 1996;3:1002-9), the promoter of the alpha-fetoprotein gene (Arbuthnot *et al.*, *Hum. Gene Ther* 1996;7:1503-14), and the promoter of the globulin-binding protein which binds to thyroxine (Wang, L., *et al.*, *Proc Natl Acad Sci USA* 1997;94:11563-11566).

The polynucleotides of the invention or the gene constructs forming them can form part of a vector. Thus, in another aspect, the invention relates to a vector comprising a polynucleotide or a gene construct of the invention. A person skilled in the art will understand that there is no limitation as regards the type of vector which can be used because said vector can be a cloning vector suitable for propagation and for obtaining the polynucleotides or suitable gene constructs or expression vectors in different heterologous organisms suitable for purifying the conjugates. Thus, suitable vectors according to the present invention include expression vectors in prokaryotes such as pUC18, pUC19, Bluescript and their derivatives, mp18, mp19, pBR322, pMB9, CoIE1, pCRI, RP4, phages and shuttle vectors such as pSA3 and pAT28, expression vectors in yeasts such as vectors of the type of 2 micron plasmids, integration plasmids, YEP vectors, centromeric plasmids and the like, expression vectors in insect cells such as the pAC series and pVL series vectors, expression vectors in plants such as vectors of expression in plants such as pIBI, pEarleyGate, pAVA, pCAMBIA, pGSA, pGWB, pMDC, pMY, pORE series vectors and the like and expression vectors in superior eukaryotic cells based on viral vectors (adenoviruses, viruses associated to adenoviruses as well as retroviruses and lentiviruses) as well as non-viral vectors such as pSilencer 4.1-CMV (Ambion), pcDNA3, pcDNA3.1/hyg pHCMV/Zeo, pCR3.1, pEF1/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1, pZeoSV2, pCI, pSVL and pKSV-10, pBPV-1, pML2d and pTDT1.

The vector of the invention can be used to transform, transfect or infect cells which can be transformed, transfected or infected by said vector. Said cells can be prokaryotic or eukaryotic. By way of example, the vector wherein said DNA sequence is introduced can be a plasmid or a vector which, when it is introduced in a host cell, is

integrated in the genome of said cell and replicates together with the chromosome (or chromosomes) in which it has been integrated. Said vector can be obtained by conventional methods known by the persons skilled in the art (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2001), mentioned above).

5 Therefore, in another aspect, the invention relates to a cell comprising a conjugate, a polynucleotide, a gene construct or a vector of the invention, for which said cell has been able to be transformed, transfected or infected with a construct or vector provided by this invention. The transformed, transfected or infected cells can be obtained by conventional methods known by persons skilled in the art (Sambrook *et al.*,
10 2001, mentioned above). In a particular embodiment, said host cell is an animal cell transfected or infected with a suitable vector.

Host cells suitable for the expression of the conjugates of the invention include, without being limited to, mammal, plant, insect, fungal and bacterial cells. Bacterial cells include, without being limited to, Gram-positive bacterial cells such as species of
15 the *Bacillus*, *Streptomyces*, *Listeria* and *Staphylococcus* genus and Gram-negative bacterial cells such as cells of the *Escherichia*, *Salmonella* and *Pseudomonas* genus. Fungal cells preferably include cells of yeasts such as *Saccharomyces*, *Pichia pastoris* and *Hansenula polymorpha*. Insect cells include, without being limited to, *Drosophila* and Sf9 cells. Plant cells include, among others, cells of crop plants such as cereals,
20 medicinal, ornamental or bulbous plants. Suitable mammal cells in the present invention include epithelial cell lines (human, ovine, porcine, etc.), osteosarcoma cell lines (human, etc.), neuroblastoma cell lines (human, etc.), epithelial carcinomas (human, etc.), glial cells (murine, etc.), hepatic cell lines (from monkey, etc.), CHO (Chinese Hamster Ovary) cells, COS cells, BHK cells, HeLa cells, 911, AT1080, A549, 293 or
25 PER.C6, NTERA-2 human ECC cells, D3 cells of the mESC line, human embryonic stem cells such as HS293, BGV01, SHEF1, SHEF2, HS181, NIH3T3 cells, 293T, REH and MCF-7 and hMSC cells.

3. Methods for obtaining the conjugate of the invention

30

The conjugate of the invention can be obtained using any method known for a person skilled in the art. It is thus possible to obtain the EDA peptide or the variant of

said protein by any standard method. For example, the EDA peptide can be obtained from cDNA by means of expression in a heterologous organism such as, for example, *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris* or from tobacco chloroplasts as described by Farran *et al.* (*Planta* 2010;231:977-90).

5 Once a sufficient amount of the purified EDA peptide is available, the latter must be conjugated to the first member of the binding pair. The conjugation of said first member of the binding pair to the EDA molecule can be carried out in different ways. One possibility is the direct conjugation of a functional group to the therapeutically active component in a position which does not interfere with the activity of said
10 component. As understood in the present invention functional groups refer to a group of specific atoms in a molecule which are responsible for a characteristic chemical reaction of said molecule. Examples of functional groups include, without limitation, hydroxy, aldehyde, alkyl, alkenyl, alkynyl, amide, carboxamide, primary, secondary, tertiary and quaternary amines, aminoxy, azide, azo (diimide), benzyl, carbonate, ester, ether,
15 glyoxylyl, haloalkyl, haloformyl, imine, imide, ketone, maleimide, isocyanide, isocyanate, carbonyl, nitrate, nitrite, nitro, nitroso, peroxide, phenyl, phosphine, phosphate, phosphono, pyridyl, sulfide, sulfonyl, sulfinyl, thioester, thiol and oxidized 3,4-dihydroxyphenylalanine (DOPA) groups. Examples of said groups are maleimide or glyoxylyl groups, which react specifically with thiol groups in the Apo A molecule and
20 oxidized 3,4-dihydroxyphenylalanine (DOPA) groups which react with primary amino groups in the EDA molecule and of component (ii).

 Another possibility is to conjugate the first member of the binding pair to the EDA molecule by means of the use of homo- or hetero- bifunctional groups. The bifunctional group can first be conjugated to the therapeutically active compound and,
25 then, conjugated to the EDA peptide or, alternatively, it is possible to conjugate the bifunctional group to the EDA peptide and, then, conjugate the latter to component (ii). Illustrative examples of this type of conjugates include the conjugates known as ketone-oxime (described in US20050255042) in which the first component of the conjugate comprises an aminoxy group which is bound to a ketone group present in a
30 heterobifunctional group which, in turn, is bound to an amino group in the second component of the conjugate.

 In another embodiment, the agent used to conjugate components (i) and (ii) of

the conjugates of the invention can be photolytically, chemically, thermally or enzymatically processed. In particular, the use of linking agents which can be hydrolyzed by enzymes that are in the target cell, such that the therapeutically active compound is only released into the cell, is of interest. Examples of linking agent types
5 that can be intracellularly processed have been described in WO04054622, WO06107617, WO07046893 and WO07112193.

In a preferred embodiment, wherein the first member of a binding pair is a compound of a peptide nature (e.g., streptavidin), including both oligopeptides, peptides and proteins, it is possible to chemically modify a polypeptide chain using widely
10 known methods to the person skilled in the art so that the protein can be covalently coupled to a second polypeptide. Thus, suitable methods for the covalent coupling of two polypeptides include methods based on the conjugation through the thiol groups present in the cysteine moieties, methods based on the conjugation through the primary amino groups present in the lysine moieties (US6809186), methods based on the
15 conjugation through the N- and C-terminal moieties can be used. Reagents suitable for the modification of polypeptides to allow their coupling to other compounds include: glutaraldehyde (allows binding compounds to the N-terminal end of polypeptides), carbodiimide (allows binding the compound to the C-terminal end of a polypeptide), succinimide esters (for example MBS, SMCC) which allow activating the N-terminal
20 end and cysteine moieties, benzidine (BDB), which allows activating tyrosine moieties, and periodate, which allows activating carbohydrate moieties in those proteins which are glycosylated.

In the particular case in which the fibronectin EDA region and the first member of a binding pair form a single peptide chain, it is possible to express the conjugate in a
25 single step using a gene construct of the invention encoding said conjugate, for which said construct is introduced in a vector suitable for its expression in a heterologous organism together with transcription and, optionally, translation control elements. The transcription and, optionally, translation control elements present in the expression cassette of the invention include promoters, which direct the transcription of the
30 nucleotide sequence to which they are operatively linked and other sequences which are necessary or suitable for the transcription and its suitable regulation in time and place, for example, initiation and termination signals, cleavage sites, polyadenylation signal,

replication origin, transcriptional enhancers, transcriptional silencers, etc. Said elements, as well as the vectors used for constructing the expression cassettes and the recombinant vectors according to the invention are generally chosen according to the host cells to be used.

5

4. Compositions and kits-of-parts of the invention

The conjugates of the invention can be contacted with an antigenic entity which is modified with a second member of a binding pair. This will lead to the formation of a complex between the conjugates and the antigenic entity via the specific interaction
10 between said first and second member of the binding pair. This complex may then be used as immunogenic composition in order to obtain an immune response against said antigenic entity. Thus, in another aspect, the invention relates to a composition or kit-of-parts comprising

- (i) a conjugate comprising
 - 15 i. the fibronectin EDA or a functionally equivalent variant thereof
and
 - ii. a first member of a binding pair; and
- (ii) an antigenic entity which is modified by a second member of said binding pair.

20 The term “composition”, as used in this invention, refers to a material composition that comprises the above-mentioned components, that is, a conjugate comprising the fibronectin EDA or a functionally equivalent variant thereof and a first member of a binding pair; and an antigenic entity which is coupled to a second member of said binding pair as well as any product resulting, directly or indirectly, from the
25 combination of the different components in any quantity thereof. Those skilled in the art will observe that the composition may be formulated as a single formulation or may be presented as separate formulations of each of the components, which may be combined for joint use as a combined preparation. The composition may be a kit-of-parts wherein each of the components is individually formulated and packaged.

30 The components of the compositions according to the invention are described in detail below.

4.A. Conjugate comprising the fibronectin EDA or a functionally equivalent variant thereof and a first member of a binding pair

The conjugate comprising fibronectin EDA or a functionally equivalent variant thereof and a first member of a binding pair has been described in detail above. In a preferred embodiment, fibronectin EDA or the functionally equivalent variant thereof and the first member of a binding pair form a single polypeptide chain. In another preferred embodiment, the first member of the binding pair is a biotin-binding molecule. Even more preferably, the biotin-binding molecule is avidin or streptavidin.

4.B. Antigenic entity modified by a second member of the binding pair

The second component of the composition or kit-of-parts according to the invention is an antigenic entity modified by a second member of a binding pair. The term "modified", as used herein, means that the antigenic entity contains one or more covalently or high-affinity coupled second members of the binding pair. It will be understood that, when the antigenic entity is formed by a plurality of different molecules, a substantial percentage of the molecules will be modified by a second member of the binding pair.

4.B.I. Second member of a binding pair

The term "second member of a binding pair", as used herein, refers to a molecule which is capable of binding with high affinity to the first member of the binding pair. In a preferred embodiment, the first and second members of the binding pair are, respectively, a biotin binding molecule and biotin. Even more preferably, the biotin-binding molecule is avidin or streptavidin.

4.B.II. Antigenic entity

An "antigenic entity" is herein defined to encompass any cell, microorganism, soluble molecule, mixture of molecules or cell-surface bound molecule (including a protein, lipid or carbohydrate), that are at least capable of binding to an antibody or of generating a T-cell response and may also contribute to the development of an immune response. The present invention is not particularly limiting with regard to the type of antigenic entity that can be modified with the second member of the binding pair. Thus,

the antigenic entity can be a whole cell which has been modified on its surface by the second member of the binding pair and that, upon being contacted with the conjugate containing the first member of the binding pair, becomes “decorated” with said conjugates. The whole cell may be a cell of a microorganism or a tumour cell.

5 Alternatively, the antigenic entity may be a heterogeneous composition comprising different antigenic molecules such as a cell lysate or extract. Moreover, the antigenic entity may be an isolated molecule.

4.B.III. Microorganism as antigenic entity

10 In a preferred embodiment, the antigenic entity is a microorganism. As used herein, the terms "microorganism" or "microbe" refer to an organism of microscopic size, to a single-celled organism, and/or to any virus particle. The term, as used herein, includes Bacteria, Archaea, single-celled Eukaryotes (protozoa, fungi, and ciliates), and viral agents.

15 In a preferred embodiment, the microorganism is a pathogenic microorganism. "Pathogenic microorganism", as used herein, refers to any disease-causing microorganism as defined above. The pathogen may be an "attenuated pathogen", which refers to a live microorganism that is less virulent in its natural host but which preferably, when introduced said host, causes a protective immunological response such

20 that resistance to infection will be enhanced and/or the clinical severity of the disease reduced. Suitable pathogens for use in the present invention include, without limitation, bacteria, viruses, protozoa, fungi and the like.

In another embodiment, the microorganism is an inactivated microorganism. As used herein, the term inactivated form of a microorganism refers to a dead or inactivated

25 cell of such a microorganism which is no longer capable to form a single colony on a plate specific for said microorganism. The term "inactivated form of the microorganism", as described herein, also encompasses lysates, fractions or extracts of the microorganism.

30 4.B.III.1. *Bacteria*

In a preferred embodiment, the microorganism is a bacterial cell, which can be, without limitation, a whole-inactivated bacterial cell (known as bacterin), a subcellular

bacterial fraction of a mixture of subcellular bacterial fractions or a live attenuated bacterial cell.

A bacterin useful in vaccines may be obtained by culturing the bacterium of interest, and then killing the bacterium to produce a bacterin containing a variety of bacterial components, including cell wall components. The bacteria may be killed by a variety of methods including those to expose them to a compound such as merthiolate, formalin, formaldehyde, diethylamine, binary ethylenamine (BEI), beta propiolactone (BPL), and glutaraldehyde. Combinations of these compounds may be used. In addition, it is possible to kill the bacteria by sterilizing radiation, heat, ultrasounds (e.g. sonication), cell rupture (e.g. French press) or other procedures. Combinations of these crude, purified or structurally modified compounds as well as synthesized fractions may be used, individually or combined.

Suitable bacteria that can be used as antigenic entities either as whole-inactivated bacteria or as attenuated live bacteria include, without limitation, *Neisseria* spp., including *N. gonorrhoeae* and *N. meningitidis*; *Streptococcus* spp., including *S. pyogenes*; *Bordetella* spp., including *B. pertussis*; *Mycobacterium* spp., including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M. smegmatis*; *Legionella* spp., including *L. pneumophila*; *Escherichia* spp, including enterotoxigenic *E. coli*, enterohemorrhagic *E. coli* and enteropathogenic *E. coli*; *Vibrio* spp, including *V. cholera*, *Shigella* spp., including *S. sonnei*, *S. dysenteriae*, *S. flexnerii*; *Yersinia* spp., including *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*; *Campylobacter* spp., including *C. jejuni*, *Salmonella* spp., including *S. bongori*, and *S. enterica* subspp. *enterica* (serogroups A, B, C, D and E), *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*; *Listeria* spp., including *L. monocytogenes*; *Helicobacter* spp., including *H. pylori*; *Pseudomonas* spp, including *P. aeruginosa*; *Staphylococcus* spp., including *S. aureus* and *S. epidermidis*; *Enterococcus* spp., including *E. faecalis* and *E. faecium*; *Clostridium* spp., including *C. tetani*, *C. botulinum* and *C. difficile*; *Bacillus* spp., including *B. anthracis*; *Corynebacterium* spp., including *C. diphtheriae*; *Borrelia* spp., including *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. andersonfi* and *B. hermsii*; *Ehrlichia* spp., including *E. equi*, *Rickettsia* spp, including *R. rickettsii*; *Chlamydia* spp., including *C. trachomatis*, *Chlamydia* *C. pneumonia* and *C. psittaci*; *Leptospira* spp.,

including *L. interrogans*; *Treponema spp.*, including *T. pallidum*, *T. denticola* and *T. hyodysenteriae*; *Streptococcus spp.*, including *S. pneumonia*, and *Haemophilus spp.*

In a preferred embodiment, the bacterin has been obtained by formalin treatment of the bacteria. In a still more preferred embodiment, the bacterin is a *Salmonella* bacterin.

In those cases wherein the antigenic entity is an attenuated pathogen, said attenuated pathogen can be obtained by numerous methods including but not limited to chemical mutagenesis, genetic insertion, deletion (Miller, J., 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) or recombination using recombinant DNA methodology (Maniatis, T., *et al.*, 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), laboratory selection of natural mutations, etc.

In a preferred embodiment, the antigenic entity is an attenuated *Salmonella* strain. Methods for obtaining attenuated *Salmonella* strains which are non-reverting non-virulent auxotrophic mutants suitable for use as live vaccines are described in U.S. Pat. No. 4,735,801 issued on Apr. 5, 1988 and copending U.S. patent application Ser. No. 798,052, filed Nov. 14, 1985, by Stocker, which are incorporated by reference herein in their entirety. A reliable method to achieve attenuation of *Salmonella* has been described (Hoiseth, S. K., and Stocker, B. A. D., *Nature* 1981;291:238; Stocker B. A. D., *et al.*, *Develop Biol Standard* 1982;53:47; and U.S. Pat. No. 4,550,081) and can be used in a particular embodiment of the invention.

Attenuated *Salmonella* which can be used in the live vaccine formulations of the invention include but are not limited to *S. typhi*, *S. enterica* sbsp. *enterica* serotypes *typhimurium* (*S. Typhimurium*), *enteritidis* (*S. Enteritidis*), *paratyphi* (*S. Paratyphi*).

Salmonella may be attenuated by modification of the genome structure of the bacteria, such as by deletion of part or parts of a *Salmonella* gene, by insertion of heterologous nucleotide sequence into at least one *Salmonella* gene, and/or by substitution of part or parts of a *Salmonella* gene by heterologous nucleotide sequence. It is possible to attenuate *Salmonella* by introducing mutations that (i) confer auxotrophy, (ii) interfere with sugar metabolism and lipopolysaccharide biosynthesis or (iii) affect some global means of regulating genes needed for a full display of virulence.

Attenuation can be determined by performing virulence studies in recipient and determining lethality and splenic infections in surviving mice.

Several genes have been identified, which when mutated, will attenuate *Salmonella*. In particular, *Salmonella* strains harboring non-reverting mutations in genes involved in the pre-chorismate biosynthetic pathway which make excellent oral vaccines triggering strong humoral, local and cellular immune responses in the host (Chatfield S, N. *et al.*, *Vaccine*, 1989;7(6): 495-8; Chatfield S, N. *et al.*, *FEMS Immunol Med Microbiol* 1993;7(1): 1-7), in *aro* genes of the aromatic biosynthetic pathway (EP-B1-0322237), or the transcriptional regulator RfaH of *Salmonella Typhimurium*, mutants which are efficient as attenuated oral vaccines against salmonellosis in mice (Nagy G. *et al.*, *Infect. Immun* 2006;74(10): 5914-25).

In specific embodiments, *Salmonella* bacteria that have been attenuated by chromosomal deletion of gene(s) for aromatic compound biosynthesis (*aro*), mutation in the *galE* gene, or that are *cva*-, *crp*- vir plasmid-, etc., deletion or inactivation in a gene located within the *Salmonella* pathogenicity island 2 (SPI2), deletion or inactivation of at least one gene associated with pathogenesis selected from *ssaV*, *ssaJ*, *ssaU*, *ssaK*, *ssaL*, *ssaM*, *ssaO*, *ssaP*, *ssaQ*, *ssaR*, *ssaS*, *ssaT*, *ssaU*, *ssaD*, *ssaE*, *ssaG*, *ssaL*, *ssaC* (*spiA*) and *ssaH* genes, by deletion or inactivation of at least one gene selected from *sseA*, *sseB*, *sseC*, *sseD*, *sseE*, *sseF*, *sseG*, *sseL* and *spiC* (*ssaB*) and the like. Some attenuated *Salmonella* vaccines and some inactivated *Salmonella* vaccines are commercially available (see U.S. Pat. Nos. 7,045,122; 6,923,957; 6,905,691; 6,605,285; 5,843,426; 5,733,760; 5,424,065; 5,389,368; and 6,592,869 relate to *Salmonella* vaccines, including attenuated and inactivated vaccines). In a preferred embodiment, the *Salmonella* is attenuated as a result of a defect in the LPS.

Strains carrying a deletion in LPS are known in the art and include, *inter alia*, strains carrying a deletion or inactivation in the *wbaP* gene, encoding the phosphogalactosyltransferase starting O-antigen biosynthesis as described in Ilg *et al.* (*Infect Immun* 2009;77: 2568-75); strains carrying a deletion or inactivation of the *wzy* gene which codes for the O-antigen polymerase as described by Piao *et al.* (*J Microbiol* 2010;48:486-95), strains carrying a deletion or an inactivation in the *waaL* gene encoding the O-antigen ligase as described by Nagy *et al.* (*Infect Immun* 2006;74:5914–5925), strains carrying a deletion or an inactivation in the *waaG* gene showing a LPS

truncated at the level of inner core as described by Nagy *et al.* (*Infect Immun* 2006;74:5914–5925), strains carrying a deletion or an inactivation in the *waaP* gene as described by Nagy *et al.* (*Infect Immun* 2006;74:5914–5925), strains carrying a deletion or an inactivation in the *rfaH* gene as described by Nagy *et al.* (*J Infect Dis* 2008;198:1699–706), strains carrying a deletion or an inactivation in the *wzzST* and or the *wzzfepE* genes, as described by Murray *et al.*, (*Mol Microbiol* 2003;47:1395-1406), strains carrying a deletion in the *galE* gene as described by Hone *et al.* (*J Infect Dis* 1987;156:167-174). The determination of whether a bacterial strain carries a defect in the LPS gene can be carried out using standard methods such as by detecting the presence and size of the LPS components by SDS-PAGE alkaline-silver staining as described by Tasi *et al.* (*Anal Biochem* 1982;119:115-9) or by using specific monoclonal antibodies or by characterizing the rough phenotype by analyzing the surface topology by susceptibility to the a battery of *Salmonella* Enteritidis Typing Phages as described by Ward *et al.* (*Epidemiol Infect* 1987;99:291-294) and De Lappe *et al.* (*Med Microbiol* 2009;58:86-93) or *Salmonella* Typhimurium Typing Phages.

4.B.III.2. Viruses

In another preferred embodiment, the microorganism is a virus, which can be either an inactivated virus or an attenuated virus. Examples of infectious pathogens include viruses such as, but not limited to dengue virus, rotavirus, viral meningitis virus, rhinovirus, respiratory syncytial virus (RSV), parainfluenza virus, rotavirus, tick borne encephalitis virus, coronaviridae, rhabdoviridae, VZV, human papilloma virus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), retroviruses such as human immunodeficiency virus (HIV- 1 and HIV-2), herpes viruses such as Epstein Barr Virus (EBV), cytomegalovirus (CMV), Herpesvirus type 8 (Kaposi sarcoma agent), HSV-1 and HSV-2, SARS, EDBV, FeLV, FIV, HTLV-I, HTLV-II, Ebola virus, Marburg virus and influenza virus.

4.B.III.3. Fungi

Fungi for use with the compositions and methods of the invention include, but are not limited to, *Candida* species (including *C.albicans*, *C.glabrata* and *C.tropicalis*),

Aspergillus, Fusarium, Basidiomycetes, Blastomyces, Coccidioides, Cryptococcus, Histoplasma, Microsporium, Trichophyton, Zygomycetes, and Scedosporium.

4.B.III.4. Protozoa

5 Protozoa for use with the compositions and methods of the present invention include, but are not limited to, *Plasmodium spp.*, including *P. falciparum*, *Toxoplasma spp.* and *T. gondii*; schistosomes, *Leishmania major*; *Trypanosoma cruzi*; *Entamoeba spp.*, including *E. histolytica*; *Babesia spp.*, including *B. microti*; *Trypanosoma spp.*, including *T. cruzi*; *Giardia spp.*, including *G. lamblia*; *leishmania spp.*, including *L. major*; *Pneumocystis spp.*, including *P. carinii*; *Trichomonas spp.*, including *T. vaginalis*; *Schistosoma spp.*, including *S. mansoni*.

4.B.IV. Tumor cells as antigenic entity

In another preferred embodiment, the antigenic entity is a tumor cell.

15 Representative tumor cells which can be incorporated in the composition of the invention include, without limitation, carcinomas, which may be derived from any of various body organs including lung, liver, breast, skin, bladder, stomach, colon, pancreas, and the like. Carcinomas may include adenocarcinoma, which develop in an organ or gland, and squamous cell carcinoma, which originate in the squamous

20 epithelium. Other cancer cells that can be used in the present invention include sarcomas, such as osteosarcoma or osteogenic sarcoma (bone), chondrosarcoma (cartilage), leiomyosarcoma (smooth muscle), rhabdomyosarcoma (skeletal muscle), mesothelial sarcoma or mesothelioma (membranous lining of body cavities), fibrosarcoma (fibrous tissue), angiosarcoma or hemangioendothelioma (blood vessels),

25 liposarcoma (adipose tissue), glioma or astrocytoma (neurogenic connective tissue found in the brain), myxosarcoma (primitive embryonic connective tissue), melanoma (melanocytes), an mesenchymous or mixed mesodermal tumor (mixed connective tissue types).

In addition cells from liquid tumors are also susceptible to treatment. A "liquid

30 tumor," which refers to neoplasia that is diffuse in nature, as they do not typically form a solid mass. Particular examples include neoplasia of the reticuloendothelial or hematopoietic system, such as lymphomas, myelomas and leukemias. Non- limiting

examples of leukemias include acute and chronic lymphoblastic, myeloblastic and multiple myeloma. Typically, such diseases arise from poorly differentiated acute leukemias, e.g., erythroblastic leukemia and acute megakaryoblastic leukemia. Specific myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML),
5 acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). Lymphoid malignancies include, but are not limited to, acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocyte leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Specific malignant lymphomas include non-
10 Hodgkin's lymphoma and variants, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T- cell lymphoma (CTCL), large granular lymphocytic leukemia (LGL), Hodgkin's disease and Reed-Sternberg's disease.

The tumor, cancer, malignancy or neoplasia cell may derived from a tumor in any stage, e.g., early or advanced, such as a stage I, II, III, IV or V tumor. The tumor
15 may have been subject to a prior treatment or be stabilized (non-progressing) or in remission.

4.B.V. Cell lysates or extracts as antigenic entity

In another embodiment, the antigenic entity is a heterogeneous mixture of
20 molecules derived from an extract of a microorganism (bacterial, virus, fungi, protozoa and the like) or from a tumor cell. The skilled person will appreciate that when the antigenic entity is formed by different types of molecules, the second member of the binding pair modifies a substantial percentage of the molecules found in the antigenic entity and that, therefore, the expression "antigenic entity modified by a second member
25 of the binding pair" has to be understood as the components of the antigenic entity which are modified by a second member of the binding pair and not the antigenic entity. Wherein the antigenic entity is a bacterial extract, it may be prepared by bacteriological culture followed by heat inactivation, concentration and harvest of biomass, alkaline lysis of single bacterial biomass or alkaline lysis of mixtures of bacterial biomass under
30 defined conditions. The alkaline lysates under different conditions may be mixed prior to purification by filtration. The obtained filtrate may be further purified, such as to remove particulate matter, and may also be lyophilized and/or formulated. Suitable

bacterial preparations for obtaining an extract or lysate for use in the present invention are essentially those as described above in the context of the antigenic entity being a bacterial cell.

In a preferred embodiment, the lysate is from a bacterium of the *Salmonella* genus. In a still more preferred embodiment, the bacterial extract is a hot saline extract (HS). This type of treatment releases the antigen complex in a saline medium and with heat. This HS contains phospholipids, surface proteins and lipopolysaccharide and can be obtained essentially as described in the prior art (Gamazo *et al.*, *Infect Immun* 1989;57:1419-1426). In another embodiment, the extract is a chaotropic extract (ChE) obtained essentially as described by Altman *et al.* (*Biochem J* 1982;201:505-513).

Wherein the antigenic entity is a tumor cell extract, the term includes tumor cell extracts, tumor cell sonicates, tumor cell hot water extracts and tumor sub-cellular fractions.

Extracts can be obtained by any method known to disrupt the cells such as by mechanical disruption with glass beads, a Dounce homogenizer, French press, sonication, freeze-thawing, shearing, osmotic disruption, irradiation or exposure to microwaves or a combination of these methods. Typically, tumor cells are treated with collagenase in order to dissociate them prior to the extraction.

A variety of detergents may be used to solubilize cells, including anionic, cationic, zwitterionic and non-ionic detergents. By virtue of their amphipathic nature, detergents are able to disrupt bipolar membranes. In selecting a detergent, consideration will be given to the nature of the target antigen(s), and the fact that anionic and cationic detergents are likely to have a greater effect on protein structure than zwitterionic or non-ionic detergents. However, non-ionic detergents tend to interfere with charge-bases analyses like mass spectroscopy, and are also susceptible to pH and ionic strength. Zwitterionic detergents provide intermediate properties that, in some respects, are superior to the other three detergent types. Offering the low-denaturing and net-zero charge characteristics of non-ionic detergents, zwitterionics also efficiently disrupt protein aggregation without the accompanying drawbacks. Exemplary anionic detergents include chenodeoxycholic acid, N-lauroylsarcosine sodium salt, lithium dodecyl sulfate, 1-octanesulfonic acid sodium salt, sodium cholate hydrate, sodium deoxycholate, sodium dodecyl sulfate and glycodeoxycholic acid sodium salt. Cationic

detergents include cetylpyridinium chloride monohydrate and hexadecyltrimethylammonium bromide. Zwitterionic detergents include CHAPS, CHAPSO, SB3-10 and SB3-12. Non-ionic detergents may be selected from N-decanoyl-N-methylglucamine, digitonin, n-dodecyl beta -D-maltoside, octyl a-D-glucopyranoside, Triton X- 100, Triton X-114, Tween 20 and Tween 80.

A tumor cell extract useful in the invention can be a fractionated extract. As an example, an extract can be fractionated by centrifugation to remove insoluble material such as membranes and large cellular structures. Fractionation of the extract can include, without limitation, centrifugation, protein precipitation, liquid-liquid extraction, solid-phase extraction, or chromatography such as reverse phase chromatography, ion pairing chromatography or ion exchange chromatography, as described, for example, in Rubino, J. *Chromatog* 2001;764:217-254. Additional methods that can be used to obtain and fractionate cellular extracts are well known in the art, as described, for example, in Scopes, supra, 1994, and Coligan et al., supra, 2000.

Commercial sources of tumor lysates are also available. For example, Protein Biotechnologies (www.proteinbiotechnologies.com) sells lung, breast, colon, uterine, cervical, ovarian and stomach tumor lysates.

Suitable tumor cells for obtaining an extract or lysate for use in the present invention are essentially those as described above in the context of the antigenic entity being a tumor cell.

4.B.VI. Antigenic molecule as antigenic entity

In another preferred embodiment, the antigenic entity is an isolated antigenic molecule. The antigenic molecule can be, for example, but is not limited to, a viral antigen, a bacterial antigen, a fungal antigen, a protozoal antigen, an allergen or environmental antigen, a differentiation antigen, a tumor antigen, an embryonic antigen, an antigen of oncogenes and mutated tumor-suppressor genes, a unique tumor antigen resulting from chromosomal translocations and/or derivatives thereof. It is also possible that the antigenic polypeptide is an immunogenic fragment of a viral antigen, bacterial antigen, a fungal antigen, a protozoal antigen, an allergen or environmental antigen, a differentiation antigen or a tumor antigen. As used herein, the expression "immunogenic fragment" refers to a peptide molecule which comprises one or more epitopes capable

of stimulating the immune system of an organism to generate an antigen-specific cell or humoral immune response. As a result of contacting the antigenic peptide with the suitable cells in a subject, the antigen generates a state of sensitivity or capacity for immune response in said subject such that both antibodies and immune cells obtained
5 from said subject are capable of specifically reacting with the antigen.

4.B.VI.1. Viral antigens

Viral antigens which are capable of eliciting an immune response against the virus include animal and human retro- and lentiviral antigens such as those of HIV-1,
10 namely HIV-1 antigens, (such as tat, nef, gp120 or gp160, gp40, p24, gag, env, vif, vpr, vpu, rev), human herpes viruses, (such as gH, gL gM gB gC gK gE or gD or derivatives thereof or Immediate Early protein such as ICP27, ICP47, ICP4, ICP36 from HSV1 or HSV2, cytomegalovirus, especially Human, (such as gB or derivatives thereof), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpl, II,
15 Ill and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or Hepatitis core antigen), hepatitis C virus (for example core, E1, NS3 or NS5 antigens), from paramyxoviruses such as Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), from parainfluenza virus, from rubella virus (such as proteins E1 and E2), measles virus, mumps virus, human papilloma viruses (for
20 example HPV6, 11, 16, 18, eg LI, L2, E1, E2, E3, E4, E5, E6, E7), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus cells, such as HA, NP, NA, or M proteins, or combinations thereof), rotavirus antigens (such as VP7sc and other rotaviral components), and the like (see Fundamental Virology, Second Edition, eds. Fields, B.
25 N. and Knipe, D. M. (Raven Press, New York, 1991) for additional examples of viral antigens).

In a preferred embodiment, the antigenic peptide derives from hepatitis C virus. In a more preferred embodiment, the peptide derives from the hepatitis C virus NS3 protein. In a still more preferred embodiment, the antigenic peptide corresponds to the
30 amino acids 1073-1081 of the HLA-A2-restricted NS3 peptide, whose sequence is CVNGVCWTV (SEQ ID NO:5).

In a preferred embodiment, the antigenic peptide derives from human papillomavirus. In a more preferred embodiment, the antigenic peptide derives from human papillomavirus of a serotype selected from the groups 6, 11, 16 and 18. In a still more preferred embodiment, the peptide derives from the human papillomavirus E6 or E7 proteins.

As used herein, the expressions "HPV E7 protein-derived antigenic peptide" or "HPV E6 protein-derived antigenic peptide" means a fragment of the HPV E7 or E6 proteins which is capable of stimulating the immune system of a mammal such that an immune response against said protein capable of inhibiting the growth of tumors caused by the expression of E7 or E6 or of inhibiting the proliferation of HPV is generated.

It will be observed that the antigen or the antigens can be the complete E6 or E7 proteins, as well as isolated domains of said protein, peptide fragments of the E6 or E7 protein or polyepitope fusion proteins comprising multiple epitopes (for example from 5 to 100 different epitopes). In a particular embodiment of the composition of the invention, the latter comprises an HPV16 E7 protein-derived antigenic peptide corresponding to or containing amino acids 1 to 29 of E7 (MHGDTPTLHEYMLDLQPETTDLYCYEQLNH SEQ ID NO: 6), a peptide corresponding to or containing amino acids 43 to 98 of E7 (GQAEPDRAHYNIVTFCKKCDSTLRRLCVQSTHVDIRLTLEDLLMGTLGIVCPICSQKP SEQ ID NO: 7) or a peptide comprises the sequence RAHYNIVTF (SEQ ID NO:8).

Moreover, the viral antigenic entity can be individual or combined preparations of one or more of the above viral protein as well as fragments thereof prepared as synthetic peptides, purified peptidic or proteic extracts or recombinant proteins of the virus.

4.B.VI.2. Bacterial antigens

The invention contemplates the use of bacterial antigens such as antigens from *Neisseria spp*, including *N. gonorrhea* and *N. meningitidis* (transferrin-binding proteins, lactoferrin binding proteins, PilC and adhesins); antigens from *Streptococcus pyogenes* (such as M proteins or fragments thereof and C5A protease); antigens from *Streptococcus agalactiae*, *Streptococcus mutans*; *Haemophilus ducreyi*; *Moraxella spp*, including *M catarrhalis*, also known as *Branhamella catarrhalis* (such as high and low

molecular weight adhesins and invasins); antigens from *Bordetella* spp., including *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (such as pertactin, pertussis toxin or derivatives thereof, filamentous hemagglutinin, adenylate cyclase, fimbriae); antigens from *Mycobacterium* spp., including *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M. smegmatis*; *Legionella* spp, including *L. pneumophila*; (for example ESAT6, Antigen 85A, -B or -C, MPT 44, MPT59, MPT45, HSP10, HSP65, HSP70, HSP 75, HSP90, PPD 19kDa [Rv3763], PPD 38kDa [Rv0934]); antigens from *Escherichia* spp, including enterotoxigenic *E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), antigens from enterohemorrhagic *E. coli* and enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); antigens from *Vibrio* spp, including *V. cholera* (for example cholera toxin or derivatives thereof); antigens from *Shigella* spp, including *S. sonnei*, *S. dysenteriae*, *S. flexnerii*; *Yersinia* spp, including *Y. enterocolitica* (for example a Yop protein); antigens from *Y. pestis*, *Y. pseudotuberculosis*; *Campylobacter* spp, including *C. jejuni* (for example toxins, adhesins and invasins); antigens from *Salmonella* spp, including *S. typhi*, *S. enterica* and *S. bongori*; *Listeria* spp., including *L. monocytogenes*; *Helicobacter* spp, including *H. pylori* (for example urease, catalase, vacuolating toxin); antigens from *Pseudomonas* spp, including *P. aeruginosa*; *Staphylococcus* spp., including *S. aureus*, *S. epidermidis*; *Enterococcus* spp., including *E. faecalis*, *E. faecium*; *Clostridium* spp., including *C. tetani* (for example tetanus toxin and derivative thereof); antigens from *C. botulinum* (for example botulinum toxin and derivative thereof), antigens from *C. difficile* (for example clostridium toxins A or B and derivatives thereof); antigens from *Bacillus* spp., including *B. anthracis* (for example anthrax toxin and derivatives thereof); *Corynebacterium* spp., including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); antigens from *Borrelia* spp., including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB); antigens from *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), antigens from *B. andersoni* (for example OspA, OspC, DbpA, DbpB and antigens from *B. hermsii*; antigens from *Ehrlichia* spp., including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia* spp, including *R. rickettsii*; *Chlamydia* spp., including *C. trachomatis* (for example MOMP, heparin-binding proteins); antigens from *Chlamydia pneumoniae* (for example MOMP,

heparin-binding proteins), antigens from *C. psittaci*; *Leptospira spp.*, including *L. interrogans*; *Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), antigens from *T. denticola*, *T. hyodysenteriae*, antigens from *M. tuberculosis* (such as Rv2557, Rv2558, RPFs: Rv0837c, Rv1884c, Rv2389c, Rv2450, Rv1009, aceA (Rv0467), PstS1, (Rv0932), SodA (Rv3846), Rv2031c 16kDal., Tb Ra12, Tb H9, Tb Ra35, Tb38-1, Erd 14, DPV, MTI, MSL, mTTC2 and hTCC1);
 5 antigens from Chlamydia (such as the High Molecular Weight Protein (HWMP), ORF3 (EP 366 412), and putative membrane proteins (Pmps); antigens from *Streptococcus spp.*, including *S. pneumoniae* (PsaA, PspA, streptolysin, choline-binding proteins, the protein antigen Pneumolysin, and mutant detoxified derivatives thereof);
 10 antigens derived from *Haemophilus spp.*, including *H. influenzae type B* (for example PRP and conjugates thereof); antigens from non typeable *H. influenzae* (such as OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides, or multiple copy variants or fusion proteins thereof).
 15 The bacterial antigens may be purified or recombinant bacterial proteins or synthetic bacterial peptides as well as combinations and mixtures of any of the above.

4.B.VI.3. Fungal antigens

Fungal antigens for use with the compositions and methods of the invention
 20 include, but are not limited to, e.g., antigens from *Candida spp.*, including *C. albicans*; histoplasma fungal antigens such as heat shock protein 60 (HSP60) and other histoplasma fungal antigen components; antigens from *Cryptococcus spp.*, including *C. Neoformans* such as capsular polysaccharides and other cryptococcal fungal antigen components; coccidioides fungal antigens such as spherule antigens and other
 25 coccidioides fungal antigen components; and tinea fungal antigens such as trichophytin and other coccidioides fungal antigen components.

4.B.VI.4. Protozoal antigens

Protozoal antigens include, but are not limited to, antigens from *Plasmodium*
 30 *spp.*, including *P. falciparum* such as merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, blood-stage antigen pf, 55/RESA and other plasmodial antigen components (for example RTS.S,

TRAP, MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in *Plasmodium* spp.); antigens from *Toxoplasma* spp. and *T. gondii* (for example SAG2, SAGS, Tg34, p30 and other toxoplasma antigen components); schistosomae antigens such as glutathione- S- transferase, paramyosin, and other schistosomal antigen components; leishmania major and other leishmaniae antigens such as gp63, lipophosphoglycan and its associated protein and other leishmanial antigen components; and *Trypanosoma cruzi* antigens such as the 75-77 kDa antigen, the 56 kDa antigen and other trypanosomal antigen components, antigens from *Entamoeba* spp., including *E. histolytica*; *Babesia* spp., including *B. microti*; *Trypanosoma* spp., including *T. cruzi*; *Giardia* spp., including *G. lamblia*; *leishmania* spp., including *L. major*; *Pneumocystis* spp., including *P. carinii*; *Trichomonas* spp., including *T. vaginalis*; *Schistosoma* spp., including *S. mansoni*.

15 4.B.VI.5. Allergen or environmental antigen

The antigen can be an allergen or environmental antigen, such as, but not limited to, an antigen derived from naturally occurring allergens such as pollen allergens (tree-, herb, weed-, and grass pollen allergens), insect allergens (inhalant, saliva and venom allergens), animal hair and dandruff allergens, and food allergens. Important pollen allergens from trees, grasses and herbs originate from the taxonomic orders of Fagales, Oleales, Pinales and platanaceae including La birch (*Betula*), alder (*Alnus*), hazel (*Corylus*), hornbeam (*Carpinus*) and olive (*Olea*), cedar (*Cryptomeria* and *Juniperus*), Plane tree (*Platanus*), the order of Poales including i.e. grasses of the genera *Lolium*, *Phleum*, *Poa*, *Cynodon*, *Dactylis*, *Holcus*, *Phalaris*, *Secale*, and *Sorghum*, the orders of
 25 Asterales and Urticales including i.a. herbs of the genera *Ambrosia*, *Artemisia*, and *Parietaria*. Other allergen antigens that may be used include allergens from house dust mites of the genus *Dermatophagoides* and *Euroglyphus*, storage mite e.g. *Lepidoglyphus*, *Glycyphagus* and *Tyrophagus*, those from cockroaches, midges and fleas e.g. *Blattella*, *Periplaneta*, *Chironomus* and *Ctenocephalides*, those from
 30 mammals such as cat, dog and horse, birds, venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of *Hymenoptera* including bees (superfamily Apidae), wasps and ants (superfamily Formicoidae). Still

other allergen antigens that may be used include inhalation allergens from fungi such as from the genus *Alternaria* and *Cladosporium*.

4.B.VI.6. Tumoral antigens

5 Examples of tumor antigens include MAGE, MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-0017-1A/GA733, Carcinoembryonic Antigen (CEA) and its antigenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its antigenic epitopes PSA-1, PSA-2, and
 10 PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3- ζ chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGEA4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGEC5), GAGE-
 15 family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, 13-catenin, γ -catenin, p120ctn, gp100^{Pme1117}, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin,
 20 Connexin 37, Immunoglobuline-idiotype (Ig-idiotype), p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, Imp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL40), SSX-3, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2, acute lymphoblastic leukemia (etv6, aml1, cyclophilin b), B cell
 25 lymphoma (Ig-idiotype), glioma (E-cadherin, α -catenin, 13-catenin, 7-catenin, p120ctn), bladder cancer (p21ras), biliary cancer (p21ras), breast cancer (MUC family, HER2/neu, c-erbB-2), cervical carcinoma (p53, p21ras), colon carcinoma (p21ras, HER2/neu, c-erbB-2, MUC family), colorectal cancer (Colorectal associated antigen (CRC)-0017-1A/GA733, APC), choriocarcinoma (CEA), epithelial cell cancer (cyclophilin b),
 30 gastric cancer (HER2/neu, c-erbB-2, ga733 glycoprotein), hepatocellular cancer, Hodgkins lymphoma (Imp-1, EBNA-1), lung cancer (CEA, MAGE-3, NY-ESO-1), lymphoid cell-derived leukemia (cyclophilin b), melanoma (p15 protein, gp75,

oncofetal antigen, GM2 and GD2 gangliosides, MelanA/MART-1, cdc27, MAGE-3, p21ras, gp100^{Pmel117}), myeloma (MUC family, p21ras), non-small cell lung carcinoma (HER2/neu, c-erbB-2), nasopharyngeal cancer (lmp-1, EBNA-1), ovarian cancer (MUC family, HER2/neu, c-erbB-2), prostate cancer (Prostate Specific Antigen (PSA) and its
 5 antigenic epitopes PSA-1, PSA-2, and PSA-3, PSMA, HER2/neu, c-erbB-2, ga733 glycoprotein), renal cancer (HER2/neu, c-erbB-2), squamous cell cancers of the cervix and esophagus (viral products such as human papilloma virus proteins), testicular cancer (NY-ESO-1), and T cell leukemia (HTLV-1 epitopes).

In a preferred embodiment, the tumor antigen is mesothelin or an immunogenic
 10 fragment thereof. Reference to mesothelin herein refers to both the isolated full-length polypeptide and isolated polypeptide fragments of at least 10 contiguous amino acids from the full-length sequence wherein the fragment binds to antisera raised against the full-length polypeptide.

In a preferred embodiment, the tumor antigen is a melanoma-associated antigen
 15 such as TRP1/gp75, TRP2, Tyrosinase, gp100 (Pmel17), Melan-A/MART-1, COA1, RAB38/NY-MEL-1, a Melanoma Antigen Gene (MAGE) family member, in particular, MAGE-1, -2, -3, -4, -6 or -12), a B Melanoma Antigen (BAGE) family member, a GAGE family member (GAGE-1 to 7, 7b and 8), a LAGE-1/NY-ESO-1 family
 20 member, GnTV, CDK4 and catenin or an immunogenic fragment thereof.

In a preferred embodiment, the melanoma-associated antigen is TRP2. The term “TRP2” or “tyrosinase-related protein 2” is used herein to refer to a protein showing specific expression in melanocytes and having DOPAchrome tautomerase activity. In preferred embodiments, the TRP2-derived antigen is selected from the group of
 25 SVYDFFVWL (SEQ ID NO: 9), TLDSQVMSL (SEQ ID NO: 10), LLGPGRPYR (SEQ ID NO:11), LLGPGRPYR, (SEQ ID NO:12), ANDPIFVVL (SEQ ID NO: 13), QCTEVRADTRPWSGP (SEQ ID NO: 14) and ALPYWNFATG (SEQ ID NO: 15). In a preferred embodiment, the TRP2-derived antigen corresponds to amino acids 59 to 257 of the TRP2 protein and has the sequence

30

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1  GQCTEVRADT RPWSGPYILR NQDDRELWPR KFFHRTCKCT GNFAGYNCGD CKFGWTGPNC
61  ERKKPPVIRQ NIHSLSPQER EQFLGALDLA KKRVPDYVI TTQHWLGLLG PNGTQPQFAN
121 CSVYDFFVWL HYYSVRDTLL GPGRPYRAID FSHQGPFAVT WHRYHLLCLE RDLQRLIGNE

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181 SFALPYWNFA TGRNECDVC (SEQ ID NO: 16)

In another preferred embodiment, the tumor antigen is an immunogenic molecule capable of inducing an immune response against a tumor cell idio-
5 type derived from the same subject to which it is administered. As used herein, the term "idiotype," refers to an epitope in the hypervariable region of an immunoglobulin. Typically, an idiotype or an epitope thereof is formed by the association of the hypervariable or complementarity determining regions (CDRs) of VH and VL domains. The immunogenic molecule capable of inducing an immune response against a tumor cell
10 idiotype is a Id protein or a mixture thereof isolated from a sample of the patient or a molecule comprising the CD3 region of an hybridoma obtained resulting from the tumor cells of the patient.

15 4.C. Modification of the antigenic entity with a second member of a binding pair

In order for the complexes between EDAvidin and the antigenic entity to be formed, the antigenic entity is coupled to a second member of binding pair. The antigenic entity may contain a second member of a binding pair or may be reacted in the presence of a
20 modifying agent which couples said second member of the binding pair to the antigenic entity. In a preferred embodiment, the second member of a binding pair is biotin.

Incorporation of biotin into an antigenic entity may be carried out using biotinylating agents. As used herein a "biotinylating agent" refers to any molecule which is capable of adding a biotin molecule to a reactive group in a target molecule. A
25 biotinylating agent can react with amino groups, carboxyl groups or thiol groups in the target molecule. Suitable biotinylating agents include, without limitation, Biotin-PEO-Amine (reactive with carboxyl groups), PEO-Iodoacetyl-Biotin (reactive with thiol groups), biotin-NHS, biotin-sulfoNHS, biotin-LC-NHS, biotin-LC-sulfoNHS, biotin-LC-LC-NHS, and biotin-LC-LC-sulfoNHS (reactive with amino groups). "LC" stands
30 for "long chain," which represents a seven atom spacer between biotin and the NHS ester. Another example of a preferred biotinylating agent biotin derivative is tetrafluorophenyl polyethylene oxide biotin (TFP-PEO-biotin).

The active ester reagents are preferably used at about 0.05 - 0.5 M concentrations, and more preferably at 0.1 M concentrations, in phosphate buffered saline ("PBS") or in a solution of 9:1 PBS to dimethylsulfoxide ("DMSO"), if necessary for solubilization.

5 Alternatively, biotin can be added to the antigenic moiety by enzymatic means using, for instance, the Biotin AviTag technology from Avidity, Inc. (Denver, Colo.). The Biotin AviTag is comprised of a unique 15 amino acid peptide that is recognized by biotin ligase, BirA that attaches biotin to a lysine residue in the peptide sequence. (Schatz, Biotechnology 1993; 11: 1138-43. The Biotin AviTag can be genetically fused
10 to any protein of interest, allowing the protein to be tagged with a biotin molecule.

One potential drawback to the Biotin AviTag technology is the possibility of a low degree of biotinylation, because the system biotinylates the protein at a single, unique lysine residue in the tag region. To overcome any such problem, the purified tagged proteins can be modified *in vitro* using purified biotin ligase. Because the biotinylation
15 is performed enzymatically, the reaction conditions are gentler, the labeling is highly specific, and the reaction is more efficient than chemical modification of the protein using biotin derivatives. Alternatively, the methods described in Jordan, *et al.* (Clin Diag Lab Immunol 2003;10: 339-44), can be used to produce a genetically engineered biotinylated protein.

20 4.D. Co-estimulatory molecules

In another aspect, the compositions or kits-of-parts according to the invention may further comprise at least one immune co-estimulatory molecule.

The term "immune costimulatory molecule", as used herein, includes any
25 molecule which is able to either enhance the stimulating effect of an antigen- specific primary T cell stimulant or to raise its activity beyond the threshold level required for cellular activation, resulting in activation of naive T cells and the resulting increase in an individual's immune response against the first component.

Suitable immune co-stimulatory molecule for use in the compositions according
30 to the present invention include, without limitation, TLR ligands, soluble forms of any of B7, CD137-L, CD134-L, GITR-L and CD40-L. The soluble form of a co-stimulatory molecule derived from an antigen presenting cell retains the ability of the native co-

stimulatory molecule to bind to its cognate receptor/ligand on T cells and stimulate T cell activation.

In a preferred embodiment, the immune co-stimulatory agent is a TLR ligand. In the present invention “TLR receptor ligand” is understood as a molecule which
5 specifically binds to at least one of the TLR (toll-like receptor) receptors and which upon binding is capable of stimulating some of the signals of co-stimulation signals characteristic of the binding of said receptor with its natural ligand or other signals which result from the binding of said receptor with a TLR agonist.

Toll-like receptors (or TLRs) are a family of type I transmembrane proteins
10 forming part of the innate immune system. In vertebrates they also enable the adaptation of the immune system. TLRs together with interleukin receptors form a superfamily known as the Interleukin-1/toll-like receptor superfamily. All the members of this family have in common the domain called the Toll-IL-1 receptor (TIL) domain.

It has been estimated that most mammals have between 10 and 15 types of
15 TLRs. Thirteen types of TLRs have been identified so far in humans and mice (Du X, *et al. Eur Cytokine Netw* 2000;11:362–71; Chuang TH. *et al., Eur Cytokine Netw* 2000;11:372–378; Tabeta K, *et al., Proc. Natl. Acad. Sci. U.S.A.* 2004;101:3516–3521).

TLR ligands induce several immune responses depending on the cells in which the TLR is expressed as well as depending on the origin of TLR ligand. For example, in
20 the case of microbial ligands, immune cells can produce cytokines that will cause inflammation. In the case of a viral factor, the cells can undergo apoptosis.

In a particular embodiment, the ligands are agonist ligands. Agonist ligands of TLR receptors are (i) natural ligands of the actual TLR receptor, or a functionally equivalent variant thereof which conserves the capacity to bind to the TLR receptor and
25 induce co-stimulation signals thereon, or (ii) an agonist antibody against the TLR receptor, or a functionally equivalent variant thereof capable of specifically binding to the TLR receptor and, more particularly, to the extracellular domain of said receptor, and inducing some of the immune signals controlled by this receptor and associated proteins. The binding specificity can be for the human TLR receptor or for a TLR
30 receptor homologous to the human one of a different species.

Examples of ligands of the different TLRs are summarized in Table 1.

TLR (No.)	Ligand
TLR 1	Multiple triacyl Lipopeptides.
TLR 2	Multiple glycopeptides, lipopeptides and lipoproteins. Lipoteichoic acid, HSP70, zymosan from host cells.
TLR 3	double-stranded RNA, poly(I:C) (polyinosinic-polycytidylic acid or polyinosinic-polycytidylic acid sodium salt)
TLR 4	Lipopolysaccharides (Gram-negative bacteria), different heat shock proteins (bacteria and host cells), fibrinogen from host cells, heparan sulfate fragments from host cells, hyaluronic acid fragments from host cells, many others.
TLR 5	Flagellin
TLR 6	multiple diacyl lipopeptides (mycoplasmas)
TLR 7	Imidazoquinoline, loxoribine (guanosine analog), small synthetic compounds of broprimine and single-stranded RNA.
TLR 8	Small synthetic compounds, single-stranded RNA.
TLR 9	Unmethylated CpG DNA (bacteria)
TLR 11	Profilin (<i>Toxoplasma gondii</i>)

As the person skilled in the art understands, there is a wide variety of immunoassays available to detect the activity of agonist ligands and generally ligands of the TLR receptor, such as the *in vitro* co-stimulation of dendritic cells. Briefly, said assay consists of contacting a culture of dendritic cells with a TLR agonist ligand and measuring the activation of said cells. Said activation can be determined by means of the detection of any marker, for example poly(I:C) in the event that the receptor is TLR3. The activated dendritic cells express different proteins such as CD80 (B7.1), CD86 (B7.2) and CD40. It is thus possible to detect the agonistic activity of a TLR agonist ligand by means of detecting changes in the expression levels of said proteins in the dendritic cells after being exposed to said ligand, as described for example by Chen X.Z. *et al.* (*Arch Dermatol Res* 2010; 302: 57-65)

In another embodiment, the co-stimulatory molecule is a CD40 agonist. The term “CD40 agonist”, as used herein, refers to a compound that binds to the CD40

receptor and triggers signaling in a manner similar to the endogenous CD40 ligand. Assays adequate for determining whether a compound is capable of acting as a CD40 ligand are those based on the detection of either the increase in the expression of more CD40 and TNF receptors in macrophages or the activation of B cells and their
5 transformation into plasma cells. The activation of B-cells in response to a CD40 ligand can be assayed by measuring the increase in Inositol 1,4,5-Trisphosphate levels or the activation of tyrosine kinases as described by Uckun *et al.* (*J Biol Chem* 1991;26:17478-17485). Alternatively, the determination of whether a compound is a CD40 agonist can be carried out for example, in macrophages that expressed CD40 on
10 the membrane. In said macrophages, when a CD40-agonist-bearing-Tcell interacts with the macrophage, the macrophage expresses more CD40 and TNF receptors on its surface which helps to increase the level of activation. The increase in activation results in the introduction of potent microbicidal substances in the macrophage, including reactive oxygen species and nitric oxide.

15 Suitable CD40 agonists for use in the present invention include, without limitation, soluble CD40 Ligand (CD40L), a functionally equivalent variant of the CD40 ligand, CD40L fragments (such as the ones described in WO2009141335), conjugates and derivatives thereof such as oligomeric CD40L polypeptides, e.g., trimeric CD40L polypeptides, the C4BP Core protein (the C-terminal domain of the
20 alpha chain of C4BP) as described in WO05051414 and a CD40 agonistic antibody.

In a preferred embodiment, the CD40 agonist is a CD40 agonistic antibody (such as those described in US2008286289, US2007292439, US2005136055).

The compositions and kits-of-parts according to the present invention may be used alone or may be delivered in combination with other antigens or with other
25 compounds such as cytokines that are known to enhance immune stimulation of CTL responses, such as, GM-CSF, IL-12, IL-2, TNF, IFN, IL-18, IL-3, IL-4, IL-8, IL-9, IL-13, IL-10, IL-14, IL-15, G-SCF, IFN alpha, IFN beta, IFN gamma, TGF- α , TGF- β , and the like. The cytokines are known in the art and are readily available in the literature or commercially.

30 In another embodiment, the co-stimulatory molecule is an universal DR binding peptide (a PADRE peptide) as described in WO9726784 and having the structure selected from the group consisting of aAXAAAKTAAAAa, aAXAAAATLKAAa,

aAXVAAATLKAAa, aAXVAAATLKAAa, aAXIAAATLKAAa, aKXVAAWTLKAAa, and aKFVAAWTLKAAa wherein a is D-Alanine, A is L-Alanine, X is cyclohexylalanine, K is lysine, T is threonine, L is leucine, V is valine, I is isoleucine, W is tryptophan, and F is phenylalanine.

5 In yet another embodiment, the co-stimulatory molecules may also be modified with the second member of the binding pair which may form complexes with the conjugate of the invention by means of the interaction between the first member of the binding pair in the conjugate according to the invention and the second member of the binding pair in the co-stimulatory molecule. Thus, the compositions of the invention
10 may be formed by a mixture of different complexes, wherein some of the complexes are formed by the conjugate of the invention and the antigenic entity modified with a second member of the binding pair and part of the complexes are formed by the conjugate of the invention and the co-stimulatory molecule modified with a second member of the binding pair. The skilled person will appreciate that the ratio of the two different kinds
15 of complexes may vary depending on the specific circumstances.

In a preferred embodiment, the first member of the binding pair is avidin or a functionally equivalent variant thereof, thus resulting in that the conjugate of the invention appears as a tetramer carrying four biotin binding regions. In this particular case, part of the biotin-binding sites may be occupied by the biotinylated antigenic
20 entity and part of the biotin-binding sites may be occupied by the biotinylated co-stimulatory molecule. In a preferred embodiment, the tetrameric EDAvidin is bound to two biotinylated antigenic entities and to two biotinylated co-stimulatory molecules.

25 5. Method for the generation of compositions according to the invention

In another aspect, the invention relates to a method for the generation of a composition according to the invention which comprises the steps of

- (i) contacting a conjugate comprising
 - i. the fibronectin EDA domain or a functionally equivalent variant thereof and
 - 30 ii. a first member of a binding pair

with an antigenic entity which is modified with a second member of the binding pair

wherein the contacting is carried out under conditions adequate for the formation of complexes between the conjugate and the antigenic entity via the interaction between said first and second members of the binding pair and

(ii) recovering the complexes obtained in step (i).

In yet another aspect, the invention relates to an immunogenic composition obtainable using the method according to the invention or “immunogenic composition of the invention”.

6. Oligomers according to the invention

The authors of the present invention have found that avidin or streptavidin acquire their quaternary structure also when conjugated to the fibronectin EDA domain or to a functionally equivalent variant thereof (see e.g. example 1). These oligomers may further contain biotinylated antigenic entities connected to the different monomers by the biotin binding sites giving rise to oligomers which are another aspect of the present invention. Thus, in another aspect, the invention relates to an avidin or streptavidin oligomer comprising a plurality of avidin or streptavidin monomers wherein each of the avidin or streptavidin monomers is conjugated to a fibronectin EDA domain or a functionally equivalent variant thereof and wherein at least one of the monomers in the oligomer is connected to a biotinylated antigenic entity through the biotin binding site in said at least one monomer.

The term “oligomer”, as used herein, refers to a protein complex with multiple components. Preferably, the oligomer is formed by four identical subunits in which case it is a homotetramer.

The term “antigenic entity”, “fibronectin EDA domain”, “functionally equivalent variant of the fibronectin EDA domain” have been described above in the context of the compositions and kits-of-parts of the invention.

It will be appreciated that the different biotin binding sites in the oligomers of the invention need not be all of them occupied by the biotinylated antigenic entity. Thus, in preferred embodiments, the biotinylated antigenic entity is connected to one, to two, to

three or to four monomers. The oligomers according to the invention may also be modified, in addition by one or more biotinylated antigenic entities, by one or more biotinylated immune coestimulatory molecule or molecules.

The term “coestimulatory molecule” has been described above in the context of the compositions according to the invention. In a preferred embodiment, the biotinylated coestimulatory molecule is selected from the group consisting of a biotinylated TLR ligand, a biotinylated CD40 agonist and a biotinylated PADRE peptide.

In a still more preferred embodiment, the oligomer according to the invention comprises two monomers which are connected to biotinylated antigenic entities and two monomers which are connected to biotinylated immune coestimulatory molecules.

The skilled person will appreciate that the oligomers according to the present invention can be prepared by contacting the monomers under conditions adequate for the formation of the oligomers followed by contacting with the biotinylated antigenic entities, or with a mixture of the biotinylated antigenic entities and the biotinylated immune coestimulatory molecules. Alternatively, the purified monomers can be contacted in separate batches with the biotinylated antigenic entities and the biotinylated immune coestimulatory molecules and then contacted in the adequate ratios so that the oligomers are formed with the desired proportion of monomers connected to biotinylated antigenic entities to monomers connected to biotinylated immune coestimulatory molecules.

7. Pharmaceutical compositions and medical uses of the compositions of the invention

The authors of the present invention have observed that the compositions and oligomers of the invention, besides being capable of inducing the maturation of dendritic cells, are capable of inducing the activation of an immune response *in vivo* against the antigenic entity forming part of the composition. In particular, it was found that *in vivo* administration of EDAvidin mixed with biotinylated NS3 protein was able to induce strong anti-NS3 T cell immune responses and that *in vivo* administration of EDAvidin mixed with biotinylated bacterins of *Salmonella* rough mutants improved significantly ($p < 0.001$) the efficacy of the corresponding bacterin administered alone (see Examples 3 to 6 of the invention). These results show that EDAvidin can be used to prepare immunoreactive complexes with biotinylated antigens which could be included

in prophylactic or therapeutic vaccine formulations against viral infections such as HCV, against bacterial infections such as salmonellosis or against cancer.

Thus, in another aspect, the invention relates to a pharmaceutical composition, or pharmaceutical composition of the invention, comprising an immunologically effective
5 amount of a composition, a kit-of-parts, an oligomer or an immunogenic composition of the invention and at least one pharmacologically acceptable carrier or adjuvant.

In yet another aspect, the invention relates to a composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical composition according to the invention for use in medicine.

10 The term "immunogenically effective amount" has its usual meaning in the art, *i.e.* an amount of an immunogen which is capable of inducing a significant immune response against pathogenic agents that share immunological features with the immunogen.

"Adjuvant" is understood as any substance intensifying the effectiveness of the
15 pharmaceutical composition of the invention. Suitable adjuvants include, without limitation, adjuvants formed by aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc, formulations of oil-in-water or water-in-oil emulsions such as complete Freund's Adjuvant (CFA) as well as the incomplete Freund's Adjuvant (IFA); mineral gels; block copolymers, Avridine™, SEAM62,
20 adjuvants formed by components of the bacterial cell wall such as adjuvants including liposaccharides (e.g., lipid A or Monophosphoryl Lipid A (MLA), trehalose dimycolate (TDM), and components of the cell wall skeleton (CWS), heat shock proteins or the derivatives thereof, adjuvants derived from ADP-ribosylating bacterial toxins, which include diphtheria toxin (DT), pertussis toxin (PT), cholera toxin (CT), *E.coli* heat-
25 labile toxins (LT1 and LT2), *Pseudomonas* Endotoxin A and exotoxin, *B. cereus* exoenzyme B, *B. sphaericus* toxin, *C. botulinum* toxins C2 and C3, *C. limosum* exoenzyme as well as the toxins of *C. perfringens*, *C. spiriforma* and *C. difficile*, *S. aureus*, EDIM and mutants of mutant toxins such as CRM-197, non-toxic mutants of diphtheria toxin; saponins such as ISCOMs (immunostimulating complexes),
30 chemokines, quimiokines and cytokines such as interleukins (IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12, etc), interferons (such as the interferon gamma) macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), defensins 1 or 2,

RANTES, MIPI- α , and MEP-2, muramyl peptides such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-s-n-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) etc; adjuvants derived
5 from the family of CpG molecules, CpG dinucleotides and synthetic oligonucleotides which comprise CpG motifs, *C. limosum* exoenzyme and synthetic adjuvants such as PCPP, the cholera toxin, *Salmonella* toxin, alum and the like, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, MTP-PE and RIBI, containing three components extracted from
10 bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a squalene emulsion at 2% Tween 80. Other examples of adjuvants include DDA (dimethyl dioctadecyl ammonium bromide) and QuilA.

The term "carrier" refers to a diluent or excipient with which the active ingredient is administered. Such pharmaceutical carriers can be sterile liquids, such as
15 water and oils, including those of petroleum, animal, plant or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solutions of saline solution and aqueous dextrose and glycerol solutions, particularly for injectable solutions, are preferably used as carriers. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin, 1995. Preferably,
20 the carriers of the invention are approved by a regulatory agency of the Federal or a state government or listed in the United States Pharmacopoeia or another generally recognized pharmacopeia for use in animals, and more particularly in humans.

For use in medicine, the pharmaceutical compositions of the invention can be administered by any route, including, without limitation, oral, intravenous,
25 intramuscular, intrarterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual or rectal route. A review of the different forms for the administration of active ingredients, of the excipients to be used and of the processes for manufacturing them can be found in Tratado de Farmacia Galénica, C. Faulí i Trillo, Luzán 5, S.A. de Ediciones, 1993 and
30 in Remington's Pharmaceutical Sciences (A.R. Gennaro, Ed.), 20th edition, Williams & Wilkins PA, USA (2000). Examples of pharmaceutically acceptable carriers are known in the state of the art and include phosphate buffered saline (PBS) solutions, water,

emulsions, such as oil/water emulsions, different types of wetting agents, sterile solutions, etc. The compositions comprising said carriers can be formulated by conventional methods known in the state of the art.

In another aspect, the invention relates to a composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical composition according to the invention for use in a method of treatment or prevention of a disease which requires the generation of an immune response against the antigenic entity. In another aspect, the invention relates to the use of a composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical composition according to the invention for the manufacture of a medicament for the treatment or prevention of a disease which requires the generation of an immune response against the antigenic entity. Alternatively, the invention relates to a method for the treatment or prevention of a disease which requires the generation of an immune response against an antigenic entity in a subject in need thereof which comprises the administration to said subject a composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical composition according to the invention.

The skilled person will appreciate that the disease to be treated or prevented will require an adequate composition, kit-of-parts, oligomer, immunogenic composition or pharmaceutical composition which comprises the antigenic entity derived from the agent which is causing the disease (pathogenic microorganism or tumor cell) and against which an immune response is desired. Thus, in further aspects, the invention relates to therapeutic or prophylactic uses of the compositions, kits-of-parts, oligomers, immunogenic composition and pharmaceutical composition according to the invention wherein:

- (i) wherein the antigenic entity is selected from the group consisting of a pathogenic microorganism, an extract of a pathogenic microorganism or an antigen from a pathogenic microorganism, then the disease which requires the generation of an immune response against said antigenic entity is an infectious disease caused by the pathogenic microorganism;
- (ii) wherein the antigenic entity is selected from the group consisting of a pancreatic tumor cell, a pancreatic tumor cell extract, mesothelin or an immunogenic fragment thereof, then the disease which requires the

generation of an immune response against said antigenic entity is pancreatic cancer;

(iii) wherein the antigenic entity is selected from the group consisting of a pancreatic tumor cell, a pancreatic tumor cell extract, mesothelin or an immunogenic fragment thereof, then the disease which requires the generation of an immune response against said antigenic entity is pancreatic cancer;

(iv) wherein the antigenic entity is selected from the group consisting of a leukemia cell, a leukaemia cell extract, an Ig-idiotypic or an immunogenic fragment thereof, then the disease which requires the generation of an immune response against said antigenic entity is leukemia;

(v) wherein the antigenic entity is selected from the group consisting of a melanoma cell, a melanoma cell extract, TRP2 or an immunogenic fragment thereof then the disease which requires the generation of an immune response against said antigen is melanoma;

(vi) wherein the antigenic entity is selected from the group consisting of HCV, an HCV extract, HCV NS3 or an immunogenic fragment thereof, then the disease which requires the generation of an immune response against said antigenic entity is a disease caused by HCV infection;

(vii) wherein the antigenic entity is selected from the group consisting of HPV, an HPV extract, HPV E7 or an immunogenic fragment thereof, then the disease which requires the generation of an immune response against said antigen is a disease caused by HPV infection.

The term “infectious disease”, as used herein, refers to diseases caused by pathogens such as viruses, bacteria, fungi, protozoa, and parasites. Infectious diseases may be caused by viruses including adenovirus, cytomegalovirus, dengue, Epstein-Barr, hanta, hepatitis A, hepatitis B, hepatitis C, herpes simplex type I, herpes simplex type II, human immunodeficiency virus (HIV), human papilloma virus (HPV), influenza, measles, mumps, papova virus, polio, respiratory syncytial virus, rinderpest, rhinovirus, rotavirus, rubella, SARS virus, smallpox, viral meningitis, and the like. Infections diseases may also be caused by bacteria including *Bacillus anthracis*, *Borrelia burgdorferi*, *Campylobacter jejuni*, *Chlamydia trachomatis*, *Clostridium botulinum*,

Clostridium tetani, *Diphtheria*, *E. coli*, *Legionella*, *Helicobacter pylori*, *Mycobacterium rickettsia*, *Mycoplasma nesissaria*, *Pertussis*, *Pseudomonas aeruginosa*, *S. pneumonia*, *Streptococcus*, *Staphylococcus*, *Vibria cholerae*, *Yersinia pestis*, and the like. Infectious diseases may also be caused by fungi such as *Aspergillus fumigatus*, *Blastomyces*
 5 *dermatitidis*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Penicillium marneffeii*, and the like. Infectious diseases may also be caused by protozoa and parasites such as chlamydia, kokzidioa, leishmania, malaria, rickettsia, trypanosoma, and the like.

In a preferred embodiment, the infectious disease is caused by a bacterium. In a
 10 still more preferred embodiment, the infectious disease is caused by a bacterium of the family Enterobacteriaceae.

As used herein, "Enterobacteriaceae" and "enterobacteria" refer to a large family of bacteria, including many of the more familiar pathogens, such as *Salmonella* and *Escherichia coli*. Genetic studies place them among the Proteobacteria, and they are
 15 given their own order (Enterobacteriales).

Examples of Enterobacteriaceae include, but are not limited to, *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophomus*, *Azotivirga*, *Blochmannia*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia* (e.g. *Erwinia amylovora*), *Escherichia* (e.g.
 20 *Escherichia coli*), *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella* (e.g. *Klebsiella pneumoniae*), *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Candidatus Phlomobacter*, *Photorhabdus* (e.g. *Photorhabdus luminescens*), *Plesiomonas* (e.g. *Plesiomonas shigelloides*), *Pragia*, *Proteus* (e.g. *Proteus vulgaris*), *Providencia*, *Rahnella*, *Raoultella*, *Salmonella* (e.g.
 25 *Salmonella enterica*), *Samsonia*, *Serratia* (e.g. *Serratia marcescens*), *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia* (e.g. *Yersinia pestis*), and *Yokenella*.

Examples of enterobacterial infections include, but are not limited to, Anthrax (by the bacterium *Bacillus anthracis*), bacterial acute gastroenteritis (caused by a variety of
 30 bacteria including, among others, *Salmonella enterica*, *Listeria monocytogene* and *Campylobacter spp.*), Bacterial Meningitis (caused by a variety of bacteria, including, but not limited to, *Neisseria meningitides*, *Streptococcus pneumoniae*, *Listeria*

monocytogenes, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Haemophilus influenzae*), Botulism (caused by bacterium *Clostridium botulinum*), Brucellosis (caused by bacteria of the genus *Brucella*), Campylobacteriosis (caused by bacteria of the genus *Campylobacter*), Cat Scratch Disease (caused by
5 *Bartonella henselae* and *Bartonella clarridgeiae*), Cholera (caused by the bacterium *Vibrio cholerae*), Diphtheria (caused by *Corynebacterium diphtheriae*), Epidemic Typhus (causative organism is *Rickettsia prowazekii*), Impetigo (caused by several bacteria, including, *Staphylococcus aureus* and *Streptococcus pyogenes*), Legionellosis (caused by bacteria belonging to the genus *Legionella*), Leprosy or Hansen's Disease)
10 (caused by the bacterium *Mycobacterium leprae*), Leptospirosis (caused by spirochaetes of the genus *Leptospira*), Listeriosis (caused by the bacterium *Listeria monocytogenes*), Lyme Disease (caused by spirochete bacteria from the genus *Borrelia*), Melioidosis (caused by the bacterium *Burkholderia pseudomallei*), MRSA infection (caused by *Staphylococcus aureus*), Nocardiosis (bacterium of the genus *Nocardia*, most
15 commonly *Nocardia asteroides* or *Nocardia brasiliensis*), Pertussis or Whooping Cough) (caused by the bacterium *Bordetella pertussis*), Plague (caused by the enterobacteria *Yersinia pestis*), Pneumococcal pneumonia (caused by a variety of bacteria, including, but not limited to, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*,
20 *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*), Psittacosis (caused by a bacterium called *Chlamydophila psittaci*), Q fever (caused by infection with *Coxiella burnetii*), Rocky Mountain Spotted Fever (RMSF) (by *Rickettsia rickettsii*), Salmonellosis (caused by bacteria of the genus *Salmonella*), Scarlet Fever, Shigellosis
25 (caused by bacteria of the genus *Shigella*), Syphilis (caused by *Treponema pallidum*), Tetanus (*Clostridium tetani*), Trachoma (caused by *Chlamydia trachomatis*), Tuberculosis (caused by bacteria of genus *Mycobacterium*, mainly *Mycobacterium tuberculosis*), Tularemia (by the bacterium *Francisella tularensis*), Typhoid Fever (caused by the bacterium *Salmonella typhi*), and Urinary Tract Infections (caused by
30 bacteria such as *Escherichia coli*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Pseudomonas* and *Enterococcus*). In a preferred embodiment, the disease is caused by an infection by including infections

caused by *Salmonella bongori* y *Salmonella enterica* (including all subspecies and serotypes).

The term “pancreatic cancer” refers to a group of malignant or neoplastic cancers originating in the pancreas of an individual and includes primary and metastatic cancers
5 of the exocrine pancreas, including adenocarcinoma, serous and mucinous cystadenocarcinomas, acinar cell carcinoma, undifferentiated carcinoma, pancreatoblastoma and endocrine pancreatic cancers such as insulinoma.

The term “leukemia” refers to neoplastic diseases of the blood and blood forming organs and include, without limitation, acute leukemias (such as acute lymphocytic
10 leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblasts, promyelocytic, myelomonocytic, monocytic and erythroleukemia) and chronic leukemias (such as chronic myelocytic -granulocytic- leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia).

The term “melanoma” includes, but is not limited to, melanomas, metastatic
15 melanomas, melanomas derived from either melanocytes or melanocyte related nevus cells, melanocarcinomas, melanoepitheliomas, melanomasarcomas, melanoma *in situ*, superficial spreading melanoma, modular melanoma, lentigo malignant melanoma, acral lentiginous melanoma, invasive melanoma and familial atypical mole and melanoma (FAM-M) syndrome. Moreover, the term “melanoma” refers not only to primary
20 melanomas but also to "melanoma metastasis" which, as used herein, refers to the spread of melanoma cells to regional lymph nodes and/or distant organs. This event is frequent given that melanomas contain multiple cell populations characterized by diverse growth rates, karyotypes, cell-surface properties, antigenicity, immunogenicity, invasion, metastasis, and sensitivity to cytotoxic drugs or biologic agents. Melanoma
25 shows frequent metastasis to brain, lungs, lymph nodes, and skin. Thus, the compositions of the present invention are also adequate for the treatment of melanoma metastasis.

The term “disease caused by HCV infection” includes, without limitation, progressive liver fibrosis, inflammation and necrosis leading to cirrhosis, end-stage liver
30 disease, and hepatocellular carcinoma.

The term “disease caused by HPV infection” includes warts (such as foot warts), genital warts, recurrent respiratory papillomatosis (such as laryngeal papillomas) and

cancers associated with papilloma infections. Cancers that have been associated with the papilloma virus include anogenital cancers (e.g. cervical, perianal, vulvar, vaginal, penile cancers, etc), head and neck cancers (e.g. oropharyngeal cavity cancer, esophageal cancer, etc) and skin cancers (e.g., basal cell carcinoma, squamous cell carcinoma).

In another aspect, the invention relates to the use of a composition, kit-of-parts of the invention, an oligomer of the invention or a pharmaceutical composition of the invention for the manufacture of a vaccine.

As used herein, the term "vaccine" refers to a formulation which contains a composition according to the present invention and which is in a form that is capable of being administered to a vertebrate and which induces a protective immune response sufficient to prevent and/or ameliorate an infection and/or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of composition of the invention. As it will be understood, the immune response generated by the vaccine may be a humoral or a cellular immune response.

The expression "cellular immune response", is used herein to describe an immune response against foreign antigen(s) that is mediated by T-cells and their secretion products.

The "humoral immune response", is used herein to describe an immune response against foreign antigen(s) that is mediated by antibodies produced by B-cells.

The vaccine is systemically or locally administered. The vaccine can be administered by means of a single administration, or with a boost by means of multiple administrations as has been previously described for the administration of the compositions of the invention.

The composition and vaccines according to the invention can be presented as a single formulation (for example, as a tablet or capsule comprising a fixed amount of each component) or, otherwise, can be presented as separate formulations for subsequent combination for joint, sequential or separate administration. The compositions of the invention also contemplate the formulation as a kit of parts wherein the components are formulated separately but are packaged in the same container.

The expert in the art will appreciate that the formulation of the first and second component of the compositions of the invention can be similar, in other words,

formulated in a similar way (for example, in tablets or in pills), allowing administration by the same route. In an embodiment wherein the different components of the invention are formulated separately, the two components can be presented in a blister pack. Each blister will contain the medicaments to be consumed throughout the day. If the medicaments need to be administered several times a day, the medicaments corresponding to each administration can be arranged in separate sections of the blister pack, preferably noting on each section of the blister pack the time of day when they need to be administered. Alternatively, the components of the composition of the invention can be formulated in a different manner so that the different components are administered differently. Thus, it is possible, for example, for the first component to be formulated as a tablet or capsule for oral administration and for the second component to be formulated for intravenous administration.

8. Further compositions of the invention

The authors of the present invention have shown that a composition comprising *Salmonella* antigens and fibronectin EDA results in an improved protection to the infection by *Salmonella* than that conferred by these antigens administered alone (see e.g. example 4 of the present invention). Thus, in another aspect, the invention relates to a composition (hereinafter second composition of the invention) comprising

- (i) fibronectin EDA or a functionally equivalent variant thereof and
- (ii) an antigenic entity.

The terms “fibronectin EDA”, “functionally equivalent variant of fibronectin EDA” and “an antigenic entity” have been described in detail above and are used in the context of the present compositions in the same meaning as the compositions of the invention.

In an embodiment, the antigenic entity is a bacterial extract. In a more preferred embodiment, the bacterial extract is a *Salmonella* extract. In a still more preferred embodiment, the bacterial extract has been obtained from an attenuated bacterium. In another embodiment, the attenuated bacterium carries a defect in the LPS. In a more preferred embodiment, the antigenic entity is an extract of an attenuated *Salmonella* strain carrying a defect in LPS caused by a deletion in the galM-galK-galT-galE genes. The bacterial extract can be a hot saline extract of the bacterial cell.

The second composition of the invention may be used in medicine for the prevention and treatment of diseases that require an immune response against an antigenic entity. Thus, the invention relates to a second composition of the invention as well as to a pharmaceutical composition comprising said second composition for use in
5 medicine.

In the particular case that the antigenic entity is a bacterial cell, an attenuated bacterial cell or an extract of a bacterial cell, then the second composition of the invention may be used for the prevention and treatment of diseases caused by bacterial infections. Diseases that can be treated with the second composition of the invention are
10 mentioned above in the context of the pharmaceutical compositions and medical uses of the compositions of the invention.

In a preferred embodiment, the second component of the second composition of the invention is a bacterin from a *Salmonella* strain and the second composition of the invention is used for prevention and the treatment of a *Salmonella* infection. In a more
15 preferred embodiment, the bacterin has been isolated from a *Salmonella* strain carrying a defect in the galM-galK-galT-galE genes.

Immunogenic cell-based compositions of the invention

Another therapy approach is to take advantage of the normal role of antigen
20 presenting cells as immune educators. As has been previously mentioned, antigen presenting cells capture virus antigens among others and present them to T cells to recruit their help in an initial T cell immune response. Due to the fact that an antigen alone may not be enough to generate an immune response, it is possible to contact an immature antigen presenting cells with a composition, kit-of-parts, oligomer or
25 immunogenic composition according to the invention which results in the activation and maturation of the antigen presenting cells, the capture of the antigen or antigens found in the antigenic entity and the presentation thereof in the surface associated to the major histocompatibility antigen. These cells thus activated and matured can be administered to the patient, such that the presentation of the antigens to the immune system of the
30 patient occurs, which eventually results in the generation of an immune response mediated by T cells.

Thus, in another aspect, the invention relates to an *in vitro* method for obtaining an immunogenic antigen presenting cell specific for a given antigenic entity comprising the steps of:

- 5 (i) contacting an immature antigen presenting cell with a composition, kit-of-parts, oligomer or immunogenic composition according to the invention under conditions adequate for stimulation of the antigen presenting cell and
- (ii) recovering the immunogenic antigen presenting cell.

The term "antigen presenting cell" (APC), as used herein, refers to a class of
10 cells capable of presenting one or more antigens in the form of peptide-MHC complex recognizable by specific effector cells of the immune system, and thereby inducing an effective cellular immune response against the antigen or antigens being presented. APC suitable for use in the present invention include both professional APC such as dendritic cells (DC), macrophages and B-cells as well as non-professional APC such as
15 fibroblasts, thymic epithelial cells, thyroid epithelial cells, glial cells, pancreatic beta cells and vascular endothelial cells. In a preferred embodiment, the APC are dendritic cells. Preferably, the APC for use in the methods of the inventions are dendritic cells, since these are the only APC having the capacity to present antigens in an efficient amount to activate naive T-cells for cytotoxic T-lymphocyte (CTL) responses.

20 The term "immunogenic", when used herein to refer to the APCs, refers to phenotypically mature antigen presenting cell with and effector function of immunogenicity (Reis e Sousa C., *Nature reviews*, 2006; 6:476-483). A phenotypically mature antigen presenting cell is an APC expressing high cell-surface levels of MHC molecules, CD40, CD80, CD83 and CD86. An effector function of immunogenicity
25 refers to the ability to prime an immune response. These cells have to be distinguished from tolerogenic APCs, which are cells that, in the absence of deliberate exposure to maturation signals, can tolerize peripheral CD4⁺ and CD8⁺ T cells by inducing deletion, anergy or regulation, depending on the model system studied.

The term "dendritic cells (DCs)" refers to a diverse population of
30 morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues (Steinman *Ann. Rev Immunol* 1991;9:271-296). Dendritic cells constitute the most potent and preferred APCs in the organism. While the dendritic cells can be

differentiated from monocytes, they possess distinct phenotypes. For example, a particular differentiating marker, CD14 antigen, is not found in dendritic cells but is possessed by monocytes. Also, mature dendritic cells are not phagocytic, whereas the monocytes are strongly phagocytotic cells. It has been shown that mature DCs can provide all the signals necessary for T cell activation and proliferation.

Dendritic cells can be isolated or generated from blood or bone marrow, or secondary lymphoid organs of the subject, such as but not limited to spleen, lymph nodes, tonsils, Peyer's patches of the intestine, and bone marrow, by any of the methods known in the art. Preferably, DCs used in the methods of the invention are (or terminally differentiated) dendritic cells. The source of dendritic cells is preferably human blood monocytes.

Immune cells obtained from such sources typically comprise predominantly recirculating lymphocytes and macrophages at various stages of differentiation and maturation. Dendritic cell preparations can be enriched by standard techniques (see e.g., Current Protocols in Immunology, 7.32.1-7.32.16, John Wiley and Sons, Inc., 1997) such as by depletion of T cells and adherent cells, followed by density gradient centrifugation. DCs may optionally be further purified by sorting of fluorescence-labeled cells, or by using anti-CD83 MAb magnetic beads. Alternatively, a high yield of a relatively homogenous population of DCs can be obtained by treating DC progenitors present in blood samples or bone marrow with cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4). Under such conditions, monocytes differentiate into dendritic cells without cell proliferation. Further treatment with agents such as TNF alpha stimulates terminal differentiation of DCs.

Alternatively, the antigen-presenting cells (APCs), including but not limited to macrophages, dendritic cells and B-cells, can be obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba *et al.* (*J Exp Med*;1992:176 1693-1702).

APCs and, in particular, dendritic cells, obtained in this way characteristically express the cell surface marker CD83. In addition, such cells characteristically express high levels of MHC class II molecules, as well as cell surface markers CD1 alpha, CD40, CD86, CD54, and CD80, but lose expression of CD14. Other cell surface

markers characteristically include the T cell markers CD2 and CD5, the B cell marker CD7 and the myeloid cell markers CD13, CD32 (Fc gamma R II), CD33, CD36, and CD63, as well as a large number of leukocyte-associated antigens.

Optionally, standard techniques, such as morphological observation and immunochemical staining, can be used to verify the presence of APCs and, in particular, dendritic cells. For example, the purity of APCs and, in particular, dendritic cells, can be assessed by flow cytometry using fluorochrome-labeled antibodies directed against one or more of the characteristic cell surface markers noted above, e.g., CD83, HLA-ABC, HLA-DR, CD1 alpha, CD40, and/or CD54. This technique can also be used to distinguish between immature and mature DCs, using fluorochrome-labeled antibodies directed against CD 14, which is present in immature, but not mature, DCs.

APCs and, in particular, dendritic cell precursors may be obtained from a healthy subject or a subject known to be suffering from a disease associated with the expression of a particular antigen. Such DC precursors may be allogeneic or autologous.

Once APCs precursors are obtained, they are cultured under appropriate conditions and for a time sufficient to expand the cell population and maintain the APCs in a state for optimal antigen uptake, processing and presentation. In one preferred approach to culture of APC precursors, APCs are generated from such APCs precursors by culture *ex vivo* in serum free or protein-free medium for 40 hours, in the absence of exogenously added cytokines, as detailed in WO0127245.

Preferred aspects of APC isolation and culture include the use of culture medium lacking exogenously supplied cytokines and culture under serum-free conditions in a manner effective to result in the generation of antigen-loaded superactivated APCs, which are cells that have already processed an antigen and have the ability to present the antigen to the immune cells and quickly generate antigen-specific immune responses, e.g., CTL-mediated T cell responses to tumor antigens.

APCs can be preserved by cryopreservation either before or after exposure to the composition, kit-of-parts oligomer or immunogenic composition of the invention.

In step (i) of the method of obtaining immunogenic APC of the invention, the immature APC are contacted with a composition, kit-of-parts, oligomer or immunogenic composition according to the invention. Typically, the contacting step comprises the contacting/incubating of the immature APC with the compositions, with

the components of the kit-of-parts or the oligomer for sufficient time. In one embodiment, sensitization may be increased by contacting the APCs with heat shock protein(s) (hsp) noncovalently bound to the composition. It has been demonstrated that hsps noncovalently bound to antigenic molecules can increase APC sensitization.

5 The activation of the APC can be detected by contacting the APC with T cell clones expressing a T-cell receptor specific for the antigenic peptide present in the composition and measuring the proliferation of the T-cells, usually by measuring the incorporation of a labeled nucleotide analog.

10 Once the APC have been sensitized with the antigen, the cells are isolated in order to obtain the antigen-primed APC. Cell surface markers can be used to isolate the cells necessary to practice the methods of this invention. For example, DCs express MHC molecules and costimulatory molecules (e.g., B7-1 and B7-2). The expression of surface markers facilitates identification and purification of these cells. These methods of identification and isolation include FACS, column chromatography and the like.

15 Labeling agents which can be used to label cell antigen include, but are not limited to monoclonal antibodies, polyclonal antibodies, proteins, or other polymers such as affinity matrices, carbohydrates or lipids. Detection proceeds by any known method, such as immunoblotting, western blot analysis, tracking of radioactive or bioluminescent markers, capillary electrophoresis, or other methods which track a
20 molecule based upon size, charge or affinity.

 Thus, in another aspect, the invention relates to an APC obtainable by the method previously mentioned.

 The antigen-loaded immunogenic APC obtained using the method of the present invention can be used to activate CD8⁺ T cells and/or CD4⁺ T-cells *in vitro* or can be
25 introduced directly in a subject to activate the T cells *in vivo*. Thus, in further aspects, the invention relates to an APC obtainable by the method of the invention for use in medicine as well as to a vaccine or pharmaceutical composition comprising the APC obtainable by the method of the invention. It will be understood that, for the purposes of medical uses, the cells may originate from the same individual which is to be treated
30 (autologous transplantation) or from a different individual (allogeneic transplantation). In allogeneic transplantation, the donor and recipient are matched based on similarity of

HLA antigens in order to minimize the immune response of both donor and recipient cells against each other.

The APCs according to the invention or the pharmaceutical compositions comprising said APCs or DCs can be used in a method for the prevention and treatment
5 of a disease which requires the generation of an immune response against the antigenic entity.

In particular, the APCs can be used wherein the disease to be treated or prevented is pancreatic cancer in which case the antigenic entity used in the method for obtaining the immunogenic antigen presenting is selected from the group consisting of a
10 pancreatic tumor cell, a pancreatic tumor cell extract, mesothelin or an immunogenic fragment thereof. The APCs can also be used for the treatment of leukemia, in which case the antigenic entity used in the method for obtaining the immunogenic antigen presenting is selected from the group of a leukemia cell, a leukemia cell extract, an Ig-idiotype or an immunogenic fragment thereof. Moreover, the APCs according to the
15 invention can also be used for the treatment of melanoma, in which case the antigenic entity used in the method for obtaining the immunogenic antigen presenting is selected from the group consisting of a melanoma cell, a melanoma cell extract, TRP2 or an immunogenic fragment thereof. Moreover, the APCs according to the invention can also be used for the treatment of a disease caused by HCV infection, in which case the
20 antigenic entity used in the method for obtaining the immunogenic antigen presenting is selected from the group consisting of HCV, an HCV extract, HCV NS3 or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigenic entity is a disease caused by HCV infection. Moreover, the APCs according to the invention can also be used for the treatment of a
25 disease caused by HPV infection, in which case the antigenic entity is selected from the group consisting of HPV, an HPV extract, HPV E7 or an immunogenic fragment thereof.

CD8⁺ T cells educated *in vitro* can be introduced into a mammal where they are cytotoxic against target cells bearing antigenic peptides corresponding to T cells are
30 activated to recognize them on class I MHC molecules. These target cells are typically cancer cells, or pathogen-infected cells which express unique antigenic peptides on their MHC class I surfaces.

Similarly, CD4⁺ helper T cells, which recognize antigenic peptides in the context of MHC class II, can also be stimulated by the APCs of the invention, which present antigenic peptides both in the context of class I and class II MHC. Helper T cells also stimulate an immune response against a target cell. As with cytotoxic T cells, helper T cells are stimulated with the antigen-loaded APCs *in vitro* or *in vivo*.

Thus, in another aspect, the invention relates to an antigen-presenting cell according to the invention, for use in a method for induction of a cytotoxic cell response against the cytotoxic antigen. In another aspect, the invention relates to an antigen-presenting cell according to the invention for use in a method for induction of a cytotoxic cell response against an infectious, allergic or neoplastic disease.

The immunogenicity of the antigen-presenting cells or educated T cells produced by the methods of the invention can be determined by well known methodologies including, but not limited to the following:

- ⁵¹Cr-release lysis assay (or equivalent) for CTL function,
- Assay for cytokines released by T cells upon contacting modified APCs
- *In vitro* T-cell education
- Transgenic animal models
- Proliferation Assays which measures the capacity of T cells to proliferate in response to reactive compositions.
- Monitoring TCR Signal Transduction Events.

The APC cells can be used for the treatment of different diseases depending on the type of antigenic entity which form part of the compositions used for the sensitization of the antigen-presenting cells. Suitable antigenic entities for sensitization have been described previously and therefore, the cells are suitable for the treatment of infectious diseases, allergic diseases or neoplastic diseases. Thus, in another aspect, the invention relates to the antigen-presenting cells of the invention for use in the treatment of a disease which requires the generation of an immune response against the antigenic entity. Alternatively, in another aspect, the invention relates to the use of an antigen-presenting cell of the invention in the manufacture of a medicament for the treatment of a disease which requires the generation of an immune response against the antigenic entity. Alternatively, the invention relates to a method for the treatment of a disease that

requires the generation of an immune response against the antigenic entity in a subject which comprises the administration to said subject of an antigen-presenting cell of the invention.

Preferably, the methods of treatment or prevention of the present invention
5 comprise the so-called adoptive immunotherapy. The term “adoptive immunotherapy” refers to a therapeutic approach for treating cancer or infectious diseases in which immune cells are administered to a host with the aim that the cells mediate either directly or indirectly specific immunity to (i.e., mount an immune response directed against) the undesired cells. In preferred embodiments, the immune response results in
10 inhibition of tumor and/or metastatic cell growth and/or proliferation and most preferably results in neoplastic cell death and/or resorption. The immune cells can be derived from a different organism/host (exogenous immune cells) or can be cells obtained from the subject organism (autologous immune cells)

The immune cells are typically activated *in vitro* by a particular antigen (in this
15 case the antigenic entity used in the compositions of the invention) applying any of the techniques mentioned above for the activation of APC *in vitro*. Methods of performing adoptive immunotherapy are well known to those of skill in the art (see, e.g., US Pat Nos 5,081,029, 5,985,270, 5,830,464, 5,776,451, 5,229,115, 690,915, and the like). The invention contemplates numerous modalities of adoptive immunotherapy. In one
20 embodiment, the DC (e.g. isolated from the patient or autologous dendritic cells) are pulsed with the compositions of the invention and then injected back into the subject where they present and activate immune cells *in vivo*. In yet another embodiment, the DC are pulsed with the compositions of the invention and then used to stimulate peripheral blood lymphocytes or tumor-infiltrating lymphocytes (TIL) in culture and
25 activate CTLs targeted against the antigenic entity that are then infused into the patient. Similarly, fibroblasts, and other APCs, or tumor cells are pulsed with the compositions of the invention and used to activate tumor cells or PBLs *ex vivo* to produce CTLs directed against the antigenic entity that can then be infused into a patient.

Inoculation of the activated cells is preferably through systemic administration.
30 The cells can be administered intravenously through a central venous catheter or into a large peripheral vein. Other methods of administration (for example, direct infusion into an artery) are within the scope of the invention.

The APCs of the invention and, in particular, the dendritic cells of the invention, can be provided in a formulation which is suitable for administration to a patient, e.g., intravenously. APCs and, in particular, DCs of the invention, that are suitable for administration to a patient are referred to herein as a "vaccine", "APC vaccine" or "DC vaccine." A vaccine or DC vaccine may further comprise additional components to help modulate the immune response, or it may be further processed in order to be suitable for administration to a patient. Methods of intravenous administration of dendritic cells are known in the art, and one of skill in the art will be able to vary the parameters of intravenous administration in order to maximize the therapeutic effect of the administered DCs.

Thus, APCs or DCs are administered to a subject in any suitable manner, often with at least one pharmaceutically acceptable carrier. The suitability of a pharmaceutically acceptable carrier is determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Most typically, quality control tests (e.g., microbiological assays, clonogenic assays, viability tests), are performed and the cells are reinfused back to the subject, in some cases preceded by the administration of diphenhydramine and hydrocortisone. See, e.g., Korbling *et al. Blood* 1986;67:529-532 and Haas *et al. Exp. Hematol* 1990;18:94-98.

Formulations suitable for parenteral administration, such as, for example, intravenous administration, include aqueous isotonic sterile injection solutions which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, as well as aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Generally, APCs of the invention can be administered to a subject at a rate determined by the effective dose, the toxicity of the cell type (e.g., the LD-50), and the side-effects of the cell type at various concentrations, as appropriate to the mass and overall health of the subject as determined by one of skill in the art. Administration can be accomplished via single or divided doses. The APCs of the invention can supplement other treatments for a disease or disorder, including, for example, conventional radiation therapy, cytotoxic agents, nucleotide analogues and biologic response modifiers.

The dose of the APCs administered to a patient, in the context of the present invention should be sufficient to trigger a beneficial therapeutic response in the patient over time, or to inhibit growth of cancer cells, or to inhibit infection. Thus, cells are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and/or to alleviate, reduce, cure or at least partially arrest symptoms and/or complications from the disease or infection. An amount adequate to accomplish this is defined as a "therapeutically effective dose." The dose will be determined by the activity of the APC produced and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular cell in a particular patient. In determining the effective number of cells to be administered in the treatment or prophylaxis of diseases such as cancer (e.g., metastatic melanoma, prostate cancer, etc.), the physician needs to evaluate circulating plasma levels, CTL toxicity, progression of the disease, and the induction of immune response against any introduced cell type.

Prior to infusion, blood samples are obtained and saved for analysis. Generally at least about 10^4 to 10^6 and typically, between 10^8 and 10^{10} cells are infused intravenously or intraperitoneally into a 70 kg patient over roughly 60-120 minutes. Preferably, cell numbers of at least 10^7 for each vaccination point are used. The injections may be e.g. 4 times repeated in a 2 weeks interval and should be given preferably near lymph nodes by intradermal or subcutaneous injections. Booster injections may be performed after a 4-week pause. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for analysis. Cell reinfusion is repeated roughly every month for a total of 10-12 treatments in a one year period. After the first treatment, infusions can be performed on an outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4 hours following the therapy. For administration, cells of the present invention can be administered at a rate determined by the LD-50 (or other measure of toxicity) of the cell type, and the side-effects of the cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses. In some regimens, patients may optionally receive in addition a suitable dosage of a biological

response modifier including but not limited to the cytokines IFN- α , IFN- γ , IL-2, IL-4, IL-6, TNF or other cytokine growth factor, antisense TGF- β , antisense IL-10, and the like.

In the case that the immunogenic cells are used to treat tumors, the cells can be used alone or in conjunction with other therapeutic regimens including but not limited to administration of IL-2, other chemotherapeutics (e.g. doxorubicin, vinblastine, vincristine, etc), radiotherapy, surgery, and the like. As indicated above, the cells may, optionally, be expanded in culture. This expansion can be accomplished by repeated stimulation of the T cells with the compositions of the invention with or without IL-2 or by growth in medium containing IL-2 alone. Other methods of T cell cultivation (for example with other lymphokines, growth factors, or other bioactive molecules) are also within the scope of the invention.

The invention is described herein by way of the following examples which are to be construed as merely illustrative and not limitative of the scope of the invention.

EXAMPLES

MATERIALS AND METHODS

Construction and purification of the EDavidin

Plasmid pET21a-Streptavidin-Alive (Howarth *et al.*, *Nat Methods* 2006;3:267-273), expressing wild-type subunit of streptavidin with a 6His tag was used for the construction of the expression plasmid pET21a-EDA-Streptavidin.

The extra domain A from fibronectin was obtained from plasmid pET20b-EDA (Lasarte *et al.*, *J.Immunol.*, 2007,178:748-756) by PCR amplification using primers CATATGAACATTGATCGCCCTAAAGGACT (SEQ ID NO:17) (Upper EDA-NdeI) and CATATG TGTGGACTGGATTCCAATCAGGGG (SEQ ID NO:18) (Lower EDA-NdeI). The resulting PCR product was cloned in pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen Life Technologies). This plasmid was digested with *NdeI* and the obtained DNA fragment was subcloned in the *NdeI* digested and dephosphorylated plasmid pET21a-Streptavidin-Alive. All constructs were verified by DNA sequencing. The resulting plasmid expressing EDA in the C-terminal end of streptavidin was

transformed into BL21(DE3) cells that were induced at OD₆₀₀ 0.9 with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for the expression of the recombinant protein. After incubating the cells overnight at room temperature, we obtained the recombinant protein and confirmed that it was expressed mainly in the insoluble
5 fraction as inclusion bodies by SDS-PAGE and western-blotting against Histidine tag. Samples were also loaded without boiling onto the SDS-polyacrylamide gels in order to visualize tetramers.

EDAAvidin was purified from inclusion bodies by affinity chromatography (HisTrap, GE Healthcare, Uppsala, Sweden) and eluted with an imidazol gradient.
10 Resulting proteins were refolded in a sepharose G25 column using a urea gradient size-exclusion chromatography followed by dialysis. Purified recombinant proteins were analyzed by SDS-PAGE and stained with Coomassie blue (Bio-Safe Coomassie reagent, Bio-Rad, Hercules, CA). Non boiled samples were also loaded into the SDS-PAGE confirming the presence of tetrameric forms of the EDAAvidin fusion protein.

15

Binding assay of EDAAvidin to biotinylated proteins by SDS-PAGE

A molecular weight marker containing biotinylated proteins (M.W. 6,500-180,000, Sigma), or the High-Range Rainbow Molecular Weight Marker (12000-
20 225000, GE Healthcare) as negative control, were loaded into a 10% SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked overnight with PBS containing 0.5% Tween 20 and 5% milk (blocking buffer). The detection of biotinylated proteins was carried out by incubating the membranes with 1.33 nmols of EDAAvidin or EDA protein in blocking buffer, for 90 minutes. After
25 washing, the membranes were incubated for 2 hours with blocking buffer containing a 1/2000 dilution of polyclonal anti-EDA antibody produced in rabbit at CIMA. After that, another incubation with anti rabbit IgG horseradish peroxidase linked (Cell Signalling) in blocking buffer, at a dilution of 1/5000 was carried out. And finally the protein bands were detected by ECL chemoluminescence (Amersham). As positive
30 control, one of the membranes was incubated with a dilution 1/500 of horseradish peroxidase conjugated streptavidin for 45 minutes, and also detected with ECL chemoluminescence.

In some cases, EDavidin was incubated with a biotinylated protein (i.e. OVA-biot) and loaded into a 10% SDS-PAGE to analyze the molecular size of the complex formed in comparison with the monomer OVA or the EDavidin tetramer. Gels were
5 stained with Coomassie blue (Bio-Safe Coomassie, Hercules, CA).

ELISA-based Binding assays of EDavidin to biotinylated proteins.

OVA protein (Ovalbumin, chicken egg, Grade III, Sigma) or NS3 protein from HCV were biotinylated using Sulfo-NHS-SS-Biotin (Thermo Scientific). Briefly, 2 mg
10 of protein were incubated with Sulfo-NHS-SS-Biotin for 30 minutes at room temperature and at a Biotin:protein ratio of 20:1. Then, the non-reacted Sulfo-NHS-SS-Biotin was removed by dialysis using a Slide-a-Lyzer Dialysis cassette (3,500 MWCO, Thermo Scientific). Immunopure biotinylated Bovine Serum Albumin (Biotin-LC-BSA) was purchased from Thermo Scientific.

15 Microtitre plates (Nunc maxisorp, Roskilde, Denmark) were coated with 0,1 µg /well of biotinylated proteins. Plates were incubated with PBS containing 10% FCS (Blocking buffer) during 1 hour at room temperature to block non-specific antibody binding. After removing the blocking buffer, a 1/500 dilution of EDavidin or EDA protein were added and incubated at 37 °C for 90 minutes, then washed three times with
20 PBS 0,05% Tween 20 and incubated at 37 °C for 1 hour with blocking buffer containing a 1/500 dilution of a rabbit polyclonal anti EDA antibody produced at Institution. After washing three times with PBS 0.05% Tween 20, wells were incubated with a 1/2500 dilution of anti-rabbit whole IgG horseradish peroxidase conjugated antibody (Sigma) at 37 °C for 45 minutes. After washing three times with PBS 0.05% Tween 20, the colour
25 reaction was started by adding 100 µl of TMB (BD Biosciences). The reaction was stopped with 2 N H₂SO₄. and the plates were read at 450 nm in a Multiskan Ascent (Thermo Electron Corporation).

Biomolecular interaction analysis

30 Binding capacity of EDavidin to biotinylated proteins was also analyzed by surface plasmon resonance (SPR) using ProteOn XPR36 (Bio-Rad, Hercules CA, USA) optical biosensor. BSA and BSA-biotinylated proteins (Pierce, IL, USA) were

covalently immobilized onto the surface of a GLC sensor chips (Bio-Rad) using the coupling reagents sulfo-NHS and EDC (Bio-Rad). After protein immobilization, chip surface was treated with ethanolamine to deactivate the excess of reactive esters. Then, different concentrations of EDAvidin were injected in running buffer (Phosphate buffered saline, 0.005% (v/v) Tween 20, pH 7.4) at a flow of 30 µl/minute. The signal obtained in the channel immobilized with OVA protein was used as reference. Association and dissociation phases were monitored for 300 and 3700 seconds, respectively. After this period, the chip surface was regenerated by the injection of free biotin, to remove the EDAvidin coated protein to the chip. After this regeneration process, different concentrations of streptavidin were injected in running buffer (Phosphate buffered saline, 0.005% (v/v) Tween 20, pH 7.4) at a flow of 30 µl/minute. The signal obtained in the channel immobilized with OVA protein was used as reference.

15 ***Binding assays to dendritic cells.***

To study the capacity of EDAvidin to favour antigen capture by dendritic cells, we biotinylated recombinant Green Fluorescent protein (GFP) as described above and mixed it with EDAvidin or with EDA. These mixtures were incubated with bone marrow-derived dendritic cells (BMDC) for 15 minutes at 4°C, washed and analyzed by flow cytometry. BMDC were generated from mouse femur marrow cell cultures. After lysing erythrocytes with ACK lysing buffer, bone marrow cell were washed and subsequently depleted of lymphocytes and granulocytes by incubation with a mixture of antibodies against CD4 (GK1; ATCC, Manassas, VA), CD8 (53.6.72; ATCC), Ly-6G/Gr1 (BD-Pharmingen; San Diego, CA) and CD45R/B220 (BD-Pharmingen) followed by addition of rabbit complement. Remaining cells were grown at 10^6 cells/ml in 12-well plates in CM (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol) supplemented with 20 ng/ml of mGM-CSF and 20 ng/ml of mIL-4 (both from Peprotech; London, UK). Every two days, two thirds of medium was replaced with fresh medium containing cytokines. Non adherent dendritic cells were harvested at day 7 and cultured in the presence or absence of different stimuli at 37 °C and 5% CO₂.

In vitro analysis on monocyte activation

THP-1 cells were plated at 2×10^5 cells/well and cultured in the presence of different concentrations of the indicated antigens in culture medium. After 15 hours of incubation, culture supernatants were harvested. The concentration of human TNF- α released to the medium by THP-1 cells was quantified using a commercial ELISA assay (BD-Pharmingen), according to manufacturer's instructions.

In vivo induction of anti-NS3 immune responses after immunization with EDAAvidin plus Biotinylated NS3 protein.

C57BL/6 mice were immunized i.v. with 200 μ l of a saline solution containing (i) 2 nanomoles of EDAAvidin plus biotinylated NS3 (ii) 2 nanomoles of EDANS3, (iii) 2 nanomoles of biotinylated NS3 protein (NS3Biot), (iv) 2 nanomoles of EDA plus 2 nanomoles of biotinylated NS3 and (v) 2 nanomoles of streptavidin plus 2 nanomoles of biotinylated NS3 protein. Seven days after immunization, CTL activity was measured by an *in vivo* killing assay as previously described (Mouries J, *et al.*, *Blood* 2008;112(9):3713-22. T-cells producing IFN- γ were enumerated by ELISPOT using a kit from BD-Biosciences (San Diego, CA) according to manufacturer's instructions culturing 8×10^5 splenocytes from the immunized mice in the absence/presence of the indicated peptides p1073 and p1038 (10 μ M), NS3 protein (0,1 μ g/ml) or culture medium (negative control). The numbers of spots were counted using an automated ELISPOT reader (CTL, Aalen, Germany).

Bacterial strains

Salmonella Enteritidis 3934 (Solano *et al.*, *Mol Microbio.* 2002;43:793-808) was used as: (i) parental wild type (SE-wt) smooth strain for constructing the SE Δ *waaL* and SE Δ *Gal* rough mutants; (ii) virulent strain in mice experiments; and (iii) smooth control strain in all the experiments. The SE Δ *Gal* mutant was constructed by deleting the 4378 bp *gal*/ETKM operon using the plasmid pKO3blue as reported previously (*Proc Natl Acad Sci USA* 2009;106:7997-8002) and the *galE*-Fw/*galE*-Rv and *galM*-Fw/*galM*-Rv primers described in Table 1. The SE Δ *waaL* mutant was obtained by replacing the *waaL* gene with a chloramphenicol resistance cassette using a previously described one step inactivation technique (Datsenko *et al.*, *Proc Natl Acad Sci USA* 2000;97:6640-

6645) with some modifications (Garcia *et al.*, *Mol Microbiol* 2004;54:264-277). The chloramphenicol antibiotic resistance cassette was amplified by PCR from the *MudQ* transposon, using the *waal-Clo* Fw and *waal-Clo* Rv primers described in Table 2.

5 **Table 2.** Oligonucleotides designed and used to generate the bacterial strains.

Oligonucleotide	Sequence (5' to 3')	SEQ ID NO:
<i>waal-Clo</i> Fw	TCACCAGAACAGAACCTGGCGAATTTAGATGCCACAAGC	19
	GTATTTGGAAAGATTTCATTAAGTGTAGGCTGGAGCTGCT	
	<u>TC^A</u>	
<i>waal-Clo</i> Rv	AGTTGGGAAAATGTAGCGCAGCGTTTCGAGGAACAAATG	20
	AAAACTGGTTTGATAAGTGACATATGAATATCCTCCTT	
	<u>AG^A</u>	
<i>GalE</i> Fw	GCGGCCGCATTCAGCCCCCTGCAACG	21
<i>GalE</i> Rv	CTCGAGGCCGCTACATGCCCGA	22
<i>GalM</i> Fw	CTCGAGCTCCGTTAAGCCTATGGT	23
<i>GalM</i> Rv	AGATCTAATCTGGTGACCGACAGA	24

^a Priming sequence for the chloramphenicol resistance cassette underlined.

Production and characterization of antigenic preparations

Hot-saline extracts (HS) and formalin-inactivated bacterins (B) from *S. enteritidis*. SEΔ*Gal* (HS-SEΔ*Gal* and B-SEΔ*Gal*), SEΔ*waaL* (HS-SEΔ*waaL* and B-SEΔ*waaL*), and SE-wt (HS-SEwt and B-SEwt) strains were obtained as described previously by Estevan M. *et al.* (*Vet Microbiol*, 2006; 118: 124-32) and Grilló MJ. *et al.* (*Vaccine* 2006;24(15):2910-6). These antigenic preparations were mixed with EDA, either obtained in *E. coli* (named EDA) or in plant chloroplasts (named MEDA), as immunomodulating recombinant proteins. EDA was expressed in *E. coli* BL21(DE3) cells (Amershan Pharmacia Biotech) by pET20b1-2 plasmid transfection, purified by HiTrap[®] affinity chromatography system (Pharmacia), concentrated using an Amicon Ultra 4-5000 MWCO centrifugal filter device (Millipore), and purified from endotoxins, using Profos EndoTrap[®] columns (Hyglos GmbH), as described previously (Lasarte JJ, *et al.*, *J Immunol* 2007;178(2):748-756). The MEDA protein was obtained from leaves of transformed tobacco plants and purified to near homogeneity and characterized as described previously (Planta, 2010, 231:977-90), filter-sterilized using a 20 μm

membrane (Millipore®). Absence of contamination was checked in both protein preparations by plating onto Luria-Bertani medium supplemented with 5% agar (LA).

Then, bacterins were biotinylated and decorated with a recombinant fusion protein between EDA and streptavidin (named EDAvidin), in order to obtain BEDA
5 antigenic preparations. For biotinylation of bacterins, the Sulfo-NHS-SS-Biotin® system (Thermo Scientific Pierce Protein Research Products) was used according to the manufacturer's instructions. The non-reacted Sulfo-NHS-SS-Biotin molecules were removed by dialysis using a Slide-a-Lyzer® Dialysis cassette (3,500 MWCO, Thermo Scientific).

10 Both, the absence of O-PS and the differences in Core structure of LPS in bacterial preparations were assessed by SDS-PAGE and alkaline silver staining, as described above (see point 2.2). Quantification of LPS was based on the detection of 2-keto-3-deoxyoctonate (Kdo) corrected for 2-deoxyaldoses and was done as described previously (Osborn *et al.*, *Proc Natl Acad Sci USA* 1962;48: 1831-1838). Protein and
15 antigenic profiles of both bacterial and recombinant protein preparations were analyzed by Coomassie Brilliant Blue method (King *et al.*, *Anal. Biochem.* 1976;71:223-230) (Bio-Safe Coomassie reagent, Bio-Rad) and immunoblotting, respectively. When indicated, samples were also loaded without boiling onto the SDS-polyacrylamide gels in order to visualize the presence of tetrameric forms of the EDAvidin fusion protein.
20 Immunoblotting was performed with sera from smooth SE-wt experimentally infected mice or EDA hyperimmunized rabbit as primary antibodies and horseradish anti-mouse IgG or goat anti-rabbit IgG (Thermo Fisher Scientific Inc., USA) as secondary antibodies, and the reaction was revealed by chemiluminescence or colorimetrically with diaminobenzidine. Protein was quantified by the Bradford method (Bio-Rad
25 Laboratories Inc.) and expressed as percentage of protein per mg of HS extract or as mg of protein per mL of bacterin, as indicated.

Finally, the capacity of EDAvidin to bind biotinylated and non-biotinylated bacterins was assessed by ELISA in 96-well microtitre plates (Nunc Maxisorp®; eBioscience) coated with 0.1 µg/well of biotinylated bacterins or conventional bacterins
30 as control. After incubation (1 hour, room temperature) with 10% fetal calf serum (Invitrogen) in PBS as blocking buffer, 3 µg/mL of EDA or EDAvidin proteins were added, and the reaction was revealed with 1:500 anti-EDA rabbit polyclonal antibody

diluted 1:500, anti-rabbit whole IgG horseradish peroxidase conjugated antibody (Sigma) at a 1:2,500 dilution, and 100 μ L of TMB (BD Biosciences). After stopping the reaction with 2N H₂SO₄, O.D. was read at 450 nm in a Multiskan Ascent apparatus (Thermo Electron Corporation).

5

Protection assays in mice

The efficacy of HS-SE Δ *waaL*, HS-SE Δ *Gal*, B-SE Δ *waaL* and B-SE Δ *Gal* mixed with EDA or MEDA and BEDA-SE Δ *waaL* and BEDA-SE Δ *Gal* preparations were studied in 8 to 10-week old female BALB/c mice (Charles River International, France).

10 Animals were accommodated in cages, with water and food *ad libitum*, at the animal facilities of the Institution (registration code ES/31-2016-000002-CR-SU-US). Mice handling and experimental procedures were carried out in compliance with the current European, national and local (RD 1201/2005) regulations, with the approval of the Institution animal experimentation Committee. In all cases, mice were inoculated i.p.
15 with 0.1 mL of bacterial suspensions or antigenic preparations, prepared in sterilized PBS (pH 6.85). Bacterial suspensions were adjusted by spectrophotometry in PBS (in our conditions, a suspension of O.D._{600nm}=0.150 contained approximately 2×10^8 CFU/mL) and serially diluted to the concentration specified. The exact number of CFU contained in each suspension was retrospectively assessed by plating (0.1 mL by
20 triplicate) on LA, and subsequently incubating of plates for 24 h, at 37°C.

HS and bacterins immunization assays.

BALB/c mice (n=5) were i.p. immunized with a single dose of HS or bacterin (20 μ g of protein/mouse) preparations, alone or in combination with EDA or MEDA
25 (40 μ g/mouse). Additional control groups of mice (n=5) were inoculated i.p. with: (i) HS-SEwt or B-SEwt preparations containing 20 μ g of protein (protected controls); (ii) sterilized PBS (non-immunized controls); or (iii) EDA or MEDA (40, 100 or 200 μ g/mouse), in order to test for the absence of unspecific protection and the innocuousness of both immunoadjuvants. Four weeks after immunization, all mice were
30 challenged i.p. with the optimal sub-lethal dose (i.e., the minimal dose able to induce a moderate and homogeneous level of splenic infection in all the infected mice), being 2.3×10^2 CFU of SE-wt, as estimated in a previous dose-response experiment (data not

shown). Four days after challenge, the mean (n=5) number of log₁₀ CFU/spleen of the challenging strain were determined for each group of mice, previous logarithmic transformation of individual data (*Vaccine* 2006;24(15):2910-6).

5 ***Immunization with biotinylated bacterins bound to EDavidin molecule (BEDA) or live rough mutants***

The efficacy of BEDA-SEΔ*Gal* and BEDA-SEΔ*waaL* (i.e. biotinylated bacterins obtained from SEΔ*Gal* or SEΔ*waaL*, allowing the reaction with EDavidin) was determined in BALB/c mice (n=5), following the protocol described above. In this case, 10 40 μg of biotinylated bacterin protein bound to EDavidin/mouse were used for immunization. Besides the positive (B-SEwt) and negative (PBS) controls used above, mice (n=5) inoculated with SEΔ*waaL* or SEΔ*Gal* live rough mutants (1×10⁴ CFU/animal) were used as reference. Eventually persistent SEΔ*waaL* colonies and SE-wt challenging strain were distinguished from each other by double plating in LA and 15 LA supplemented with chloramphenicol (20 mg/L).

Statistical analysis

In general, Kolmogorov-Smirnov test was applied to assess the normal distribution of data obtained in each experiment. Then, statistical comparisons of means 20 were performed by a one-way ANOVA test, followed by the Fisher's Protected Least Significant Difference (PLSD) test (when four or less groups were compared) or Bonferroni's test (when more than four groups were compared). Results on protection were graphically represented by the box-plot method, where boxes represent 50% of central data, line inside boxes represents the median of log₁₀ CFU/spleen and whiskers 25 delimit at least 90% of the data obtained. Protection was determined by statistical comparison of mean (n=5) level of virulent infection (log₁₀ SE-wt CFU/spleen) obtained in immunized mice, with respect to that obtained in both, the reference group vaccinated with SE-wt antigens and the unvaccinated control group. In mice survival assays, the percentage of cumulative survivals was calculated and graphically represented by the 30 Kaplan-Meier test, and statistically compared by the LogRank (Mantel-Cox) test. In all cases, the Statview[®] Graphics 5.0 for Windows (SAS Institute Inc[®]) statistical package was applied.

EXAMPLE 1**Recombinant EDavidin tetramerizes and binds to biotinylated proteins.**

Recombinant protein EDavidin was produced by linking EDA to the C-terminal
5 end of streptavidin. The recombinant EDavidin protein was expressed in *E.coli* as
6xHis fusion protein, purified from inclusion bodies by affinity chromatography,
desalted and contaminant endotoxins were removed as described in methods. The
resulting protein was characterized by SDS-PAGE and western blot using anti-His
antibodies (data not shown). A band corresponding to the putative molecular weight of
10 an EDavidin tetramer was observed after the Coomassie blue staining (Figure 1A, lane
1). However, when the sample was boiled before the SDS-PAGE analysis, a band
corresponding to the monomer was found, suggesting that the fusion protein was able to
tetramerize spontaneously in solution. The capacity of EDavidin to bind to biotinylated
ovalbumin was studied by Surface Plasmon Resonance. Thus, different concentrations
15 of EDavidin (6.5-100 nM) were injected into system (Figure 1B). It was found that
EDavidin binds with high affinity to the chip surface coated with biotinylated OVA
protein. In order to disrupt the interaction of EDavidin to the chip surface, free biotin
was injected into the SPR. After this regeneration step, the same concentration of
recombinant streptavidin was injected to study its interaction with biotinylated
20 ovalbumin. This comparative study showed that EDavidin had a lower affinity to bind
biotin. However, the affinity of EDavidin for biotin is still in the range of the very high
affinity constant showed by streptavidin ($K_d 10^{-15}$ M).

Next, the capacity of EDavidin to bind to biotinylated OVA or BSA coated onto the
ELISA plates was studied. Plates were incubated with EDavidin or with EDA and, after
25 extensive washes, the binding of the proteins was developed by using anti EDA
antibodies. It was found that EDavidin, but not free EDA was able to bind to the plates
coated with the biotinylated proteins (Fig. 1C).

The binding capacity of EDavidin to bind to biotinylated proteins was also studied
by SDS-PAGE. Accordingly, when EDavidin was mixed with biotinylated ovalbumin
30 (OVABiot) the tetrameric EDavidin was converted in a larger molecular complex
which may correspond to the putative EDavidin-OVABiot association (Fig. 1D).

Binding of EDAvidin to biotinylated proteins was also studied by western-blot using a molecular weight marker mixture consisting in biotinylated proteins. Thus, the biotinylated protein mixture was run is SDS-PAGE, transferred to nitrocellulose membranes and incubated with EDAvidin or with EDA as control. Membranes were then incubated with anti-EDA antibodies and developed using a secondary anti-IgG antibody conjugated with HRP. It was found that only EDAvidin was able to bind to the biotinylated proteins (Fig. 1E).

EXAMPLE 2

10 **EDAvidin retains the proinflammatory activity of EDA and targets biotinylated antigens to dendritic cells.**

With the aim to study the capacity of EDAvidin to target an antigen to DC, green fluorescence protein (GFP) was biotinylated, mixed with EDAvidin and added to BMDC. After 15 min of incubation, cells were washed and the cell uptake of GFP analyzed by flow cytometry. It was found that a significant proportion of BMDC incubated with EDAvidin plus biotinylated GFP were highly labelled with the fluorescent protein (Figure 2A). This was not observed with biotinylated GFP alone or in combination with EDA, suggesting that EDAvidin was improving the targeting of the biotinylated protein to the dendritic cell surface.

It was next studied whether the fusion protein EDAvidin was able to retain its proinflammatory activity using the human derived monocyte cell line THP1 which expresses TLR4. It was found that EDAvidin, although less efficiently than free EDA, was also able to trigger the production of TNF- α by THP1 cells (Figure 2B).

EXAMPLE 3

25 **EDAvidin plus biotinylated NS3 induce strong anti-NS3 cellular immune responses *in vivo*.**

To study the potential use of EDAvidin to target antigens to dendritic cells and induce strong cellular immune responses specific for the antigen *in vivo*, NS3 protein was biotinylated (NS3Biot) and the capacity of EDAvidin to bind to NS3Biot by ELISA was studied. It was found that EDAvidin, but not free EDA was able to associate with

NS3Biot coated onto the ELISA plate (Figure 3A). We then tested *in vivo* the capacity of a mixture of EDAvidin and NS3Biot to induce anti-NS3 cellular immune responses. HHD mice were injected intravenously (i.v.) with (i) EDAvidin plus NS3Biot, (ii) EDA-NS3, (iii), NS3Biot, (iv) EDA plus NS3Biot or (v) streptavidin plus NS3Biot. One week after immunization mice were sacrificed and spleen cells were cultured in the presence or absence of the HLA-A2 restricted cytotoxic T cell epitope from NS3 (p1073) or the NS3 protein to measure the number of IFN- γ producing cells by ELISPOT. It was found that EDAvidin plus NS3Biot was as good as EDA-NS3 protein to induce anti-NS3 specific T cell immune responses. NS3Biot alone or mixed with streptavidin or with free EDA were unable to induce such responses (Figure 3B). Similar responses were found when we analyzed by *in vivo* killing assay the capacity to induce cytotoxic T cells able to kill target cells pulsed with peptide p1073 (Figure 3C). Indeed, only those animals immunized with EDAvidin plus NS3Biot, or with EDA-NS3 induced strong CTL activity specific against NS3.

We also tested the capacity of EDAvidin to bind biotinylated peptide antigens and to induce *in vivo* T cell specific against the antigen. Thus, EDAvidin was incubated with melanoma TRP-2(180-188) peptide biotinylated at the amino or carboxy terminus. C57/BL6 mice were immunized sc. with 2 nmols of EDAvidin plus the biotinylated melanoma TRP-2(180-188) peptides and seven days after immunization, mice were sacrificed and the number of IFN- γ producing spots in response to CTL epitope TRP-2(180-188) were measured by ELISPOT. It was found that EDAvidin incubated with TRP-2(180-188) peptide biotinylated at the N-terminus was very efficient on the induction of such specific T cell responses against the antigenic peptide suggesting that this strategy based on EDAvidin and biotinylated peptides can be implemented for vaccination purposes (Figure 3D).

EXAMPLE 4

Protection conferred by HS extracts and bacterins, alone or physically mixed with EDA or MEDA, in BALB/c mice.

In order to investigate the potential role of EDA as an adjuvant in a simple physical mix with *Salmonella* antigens from rough strains, mice were immunized with *S. Enteritidis* HS or bacterins, alone or mixed with EDA (HS and bacterins) or MEDA

(only bacterins), and challenged with a sub-lethal dose of smooth virulent SE-wt. As expected, animals vaccinated with HS-SEwt or B-SEwt (vaccinated controls) displayed a total protection, whereas non-vaccinated mice (PBS controls) reached high levels of the virulent SE-wt infection (Fig. 4). Moreover, it was found that after challenge, control groups inoculated with increasing doses of EDA or MEDA (40, 100 or 200 µg per mouse) were not protected and did not show harmful signs, at any dose (not shown). Challenged mice that had been immunized with HS-SE Δ *waaL*, HS-SE Δ *Gal* or B-SE Δ *Gal* alone displayed a severe infection, statistically equivalent to that displayed by PBS controls (Fig. 4). In contrast, B-SE Δ *waaL* alone induced in mice a significant protection ($p=0.001$ vs. PBS control), higher than that induced by B-SE Δ *Gal* ($p<0.0001$) in mice (Fig. 4). Both HS extracts and bacterins from rough mutants protected mice to a lower extent ($p=0.0001$) than smooth HS-SEwt or B-SEwt (Fig. 4). The combined administration of EDA immunoadjuvants with rough HS extracts did not improve the protection conferred by these antigens administered alone (Fig. 4). Highly variable responses were obtained when EDA or MEDA were administered with B-SE Δ *waaL*, and only a tendency of improved protection was observed with MEDA (Fig. 4A). However, the effect of EDA and MEDA as immunoadjuvant was observed with B-SE Δ *Gal*, improving significantly the protection conferred when EDA ($p=0.004$) or MEDA ($p=0.001$) were administered with this bacterin (Fig. 4B).

EXAMPLE 5

Protection conferred by bacterins linked to EDAAvidin

Although the mixture of soluble EDA and B-SE Δ *Gal* improved significantly the protection against challenge with SE-wt, it was further investigated whether a strong linkage of EDA to the surface of bacterins would improve their immunogenicity. For this reason, biotinylated bacterins were prepared and mixed with EDAAvidin, a novel recombinant chimeric molecule by fusing EDA to streptavidin, to take the advantage of high affinity interaction between streptavidin and biotin. ELISA with EDAAvidin reveals that this molecule but not free EDA could bind biotinylated bacterins (Fig. 5)

The results of protection obtained after a single immunization of mice with these antigenic compounds (named BEDA-SE Δ *waaL* and BEDA-SE Δ *Gal*) against a virulent challenge (Fig. 6) indicate that both BEDA preparations improved significantly

($p < 0.001$) the efficacy of the corresponding bacterin administered alone (Fig. 6). Strikingly, the levels of protection conferred by BEDA-SE $\Delta waaL$ and BEDA-SE ΔGal bacterins were equivalent to those observed with live rough mutants (Fig. 6). Moreover, while both live rough mutants showed a lower protection ($p < 0.05$) than B-SEwt
5 immunized control mice, but the protection conferred by these control mice was similar to that conferred by BEDA-SE $\Delta waaL$. In fact, the spleens of two mice immunized with BEDA-SE $\Delta waaL$ were completely cleared from virulent infection.

CLAIMS

1. A composition or kit-of-parts comprising
 - (i) a conjugate comprising
 - a. the fibronectin EDA domain or a functionally equivalent variant thereof and
 - b. a first member of a binding pair; and
 - (ii) an antigenic entity which is modified by a second member of said binding pair.
2. A composition or kit-of-parts according to claim 1 wherein the fibronectin EDA domain or a functionally equivalent variant thereof and the first member of a binding pair of the conjugate form a single polypeptide chain.
3. A composition according to claims 1 or 2 wherein the components (i) and (ii) are forming a complex via the binding between said first and second members of a binding pair.
4. A composition or kit-of-parts according to any of claims 1 to 3 wherein the first and second member of the binding pair are, respectively, a biotin binding molecule and biotin.
5. A composition or kit-of-parts according to claim 4 wherein the biotin-binding molecule is avidin or streptavidin.
6. A composition or kit-of-parts according to any of claims 1 to 5 further comprising at least one immune coestimulatory molecule.
7. A composition or kit-of-parts according to claim 6 wherein the coestimulatory molecule is selected from the group consisting of a TLR ligand, a CD40 agonist and a PADRE peptide.

8. A composition or kit-of-parts according to claims 6 or 7 wherein the coestimulatory molecule is modified with the second member of the binding pair.
9. An avidin or streptavidin oligomer comprising a plurality of avidin or streptavidin monomers wherein each of the avidin or streptavidin monomers is conjugated to a fibronectin EDA domain or a functionally equivalent variant thereof and wherein at least one of the monomers in the oligomer is connected to a biotinylated antigenic entity through the biotin binding site in said at least one monomer.
10. An oligomer according to claim 9 wherein at least one of the monomers in the oligomer is connected to a biotinylated immune coestimulatory molecule through the biotin binding site in said at least one monomer.
11. An oligomer according to claim 10 wherein the immune coestimulatory molecule is selected from the group consisting of a TLR ligand, a CD40 agonist and a PADRE peptide.
12. An oligomer according to any of claims 9 to 11 which is a tetramer.
13. An oligomer according to claim 12 wherein two of the monomers are connected to biotinylated antigenic entities and wherein two of the monomers are connected to biotinylated immune coestimulatory molecules.
14. A composition or a kit-of-parts according to any of claims 1 to 8 or an oligomer according to any of claims 9 to 13 wherein the antigenic entity is selected from the group consisting of a microorganism, a tumor cell, a composition comprising a plurality of molecules from a microorganism and a composition comprising a plurality of molecules from a tumor cell.
15. A composition, a kit-of-parts or an oligomer according to claim 14 wherein the microorganism is selected from the group consisting of a bacterial cell, a virus, a protozoon and a fungus.

16. A composition, a kit-of-parts or an oligomer according to claim 15 wherein the virus is HCV or HPV.
- 5 17. A composition, a kit-of-parts or an oligomer according to claim 15 wherein the bacterial cell is an inactivated cell, an extract of an inactivated cell, an attenuated cell or an extract of an inactivated cell.
18. A composition, a kit-of-parts or an oligomer according to claim 17 wherein the
10 inactivated cell is a formalin-inactivated cell.
19. A composition, a kit-of-parts or an oligomer according to any of claims 17 or 18 wherein the bacterial cell is a *Salmonella* cell.
- 15 20. A composition, a kit-of-parts or an oligomer according to claim 19 wherein the bacterial cell is an attenuated cell and the attenuation results from a defect in LPS.
21. A composition, a kit-of-parts or an oligomer according to claim 14 wherein the
20 tumor cell is selected from a melanoma cell, a leukemia cell or a pancreatic cancer cell.
22. A composition or kit-of-parts according to any of claims 1 to 8 or an oligomer according to any of claims 9 to 13 wherein the antigenic entity is an antigenic molecule.
- 25 23. A composition, a kit-of-parts or an oligomer according to claim 22 wherein the antigenic molecule is selected from the group consisting of a viral antigen, a bacterial antigen, a fungal antigen, a protozoal antigen, an allergen and a tumor antigen.
- 30 24. A composition, a kit-of-parts or an oligomer according to claim 23 wherein the antigenic molecule is a polypeptide or an immunogenic fragment thereof.

25. A composition, a kit-of-parts or an oligomer according to claim 24 wherein the polypeptide is an HCV polypeptide.
- 5 26. A composition, a kit-of-parts or an oligomer according to claim 25 wherein the HCV polypeptide is NS3 or an immunogenic fragment thereof.
27. A composition, a kit-of-parts or an oligomer according to claim 24 wherein the polypeptide is an HPV polypeptide or an immunogenic fragment thereof.
- 10 28. A composition, a kit-of-parts or an oligomer according to claim 27 wherein the HPV polypeptide is E7 or an immunogenic fragment thereof.
- 15 29. A composition, a kit-of-parts or an oligomer according to claim 23 wherein the tumor antigen is selected from the group consisting of mesothelin, Ig-idiotypic, TRP2 or an immunogenic fragment thereof.
30. A method for the generation of an composition according to any of claims 1 to 5 or 14 to 29 which comprises the steps of
- 20 (i) contacting a conjugate comprising
- i. the fibronectin EDA domain or a functionally equivalent variant thereof and
- ii. a first member of a binding pair
- 25 with an antigenic entity which is modified with a second member of the binding pair
- wherein the contacting is carried out under conditions adequate for the formation of complexes between the conjugate and the antigenic entity via the interaction between said first and second members of the binding pair and
- 30 (ii) recovering the complexes obtained in step (i).
31. An immunogenic composition obtainable using the method according to claim 30.

32. A method for obtaining an immunogenic antigen presenting cell specific for a given antigenic entity comprising the steps of:
- 5 (i) contacting an immature antigen presenting cell with a composition, kit-of-parts or oligomer according to any of claims 1 to 29 or the immunogenic composition according to claim 31 under conditions adequate for stimulation of the antigen presenting cell and
- (ii) recovering the immunogenic antigen presenting cell.
- 10 33. A method according to claim 32 wherein the antigen presenting cell is a dendritic cell.
34. An antigen presenting cell obtainable using the method according to claims 32 or 33.
- 15 35. A composition comprising an antigen presenting cell according to claim 34.
36. A pharmaceutical composition comprising a composition, a kit-of-parts or oligomer according to any of claims 1 to 29 or a composition according to claims 20 31 or 35 and a pharmaceutically acceptable carrier.
37. A composition, kit-of-parts or oligomer according to any of claims 1 to 29, a composition according to claims 31 or 35 or a pharmaceutical composition according to claim 36 for use in medicine.
- 25 38. A composition, kit-of-parts or oligomer according to any of claims 1 to 29, a composition according to claims 31 or 35 or a pharmaceutical composition according to claim 36 for use in a method of prevention or treatment of a disease which requires the generation of an immune response against the antigenic entity.
- 30 39. A composition, kit-of-parts, oligomer or a pharmaceutical composition for use according to claim 38 wherein

- 5 (i) the antigenic entity is selected from the group consisting of a pathogenic microorganism, an extract of a pathogenic microorganism or an antigen from a pathogenic microorganism and the disease which requires the generation of an immune response against said antigenic entity is an infectious disease caused by said pathogenic microorganism;
- (ii) the antigenic entity is selected from the group consisting of a pancreatic tumor cell, a pancreatic tumor cell extract, mesothelin or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigenic entity is pancreatic cancer;
- 10 (iii) the antigenic entity is selected from the group consisting of a leukemia cell, a leukemia cell extract, Ig-idiotypic or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigenic entity is leukemia;
- (iv) the antigenic entity is selected from the group consisting of a melanoma cell, a melanoma cell extract, TRP2 or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigen is melanoma;
- 15 (v) the antigenic entity is selected from the group consisting of HCV, an HCV extract, HCV NS3 or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigenic entity is a disease caused by HCV infection; or
- 20 (vi) the antigenic entity is selected from the group consisting of HPV, an HPV extract, HPV E7 or an immunogenic fragment thereof; and the disease which requires the generation of an immune response against said antigen is a disease caused by HPV infection.
- 25

40. A conjugate comprising

- (i) the fibronectin EDA or a functionally equivalent variant thereof and
- (ii) a first member of a binding pair.

30

41. A conjugate according to claim 40 wherein the first member of a binding pair is a biotin-binding molecule.

42. A conjugate according to claim 41 wherein the biotin-binding molecule is avidin or streptavidin.

5 43. A conjugate according to any of claims 40 to 42 wherein the fibronectin EDA domain or a functionally equivalent variant thereof and the first member of a binding pair form a single polypeptide chain.

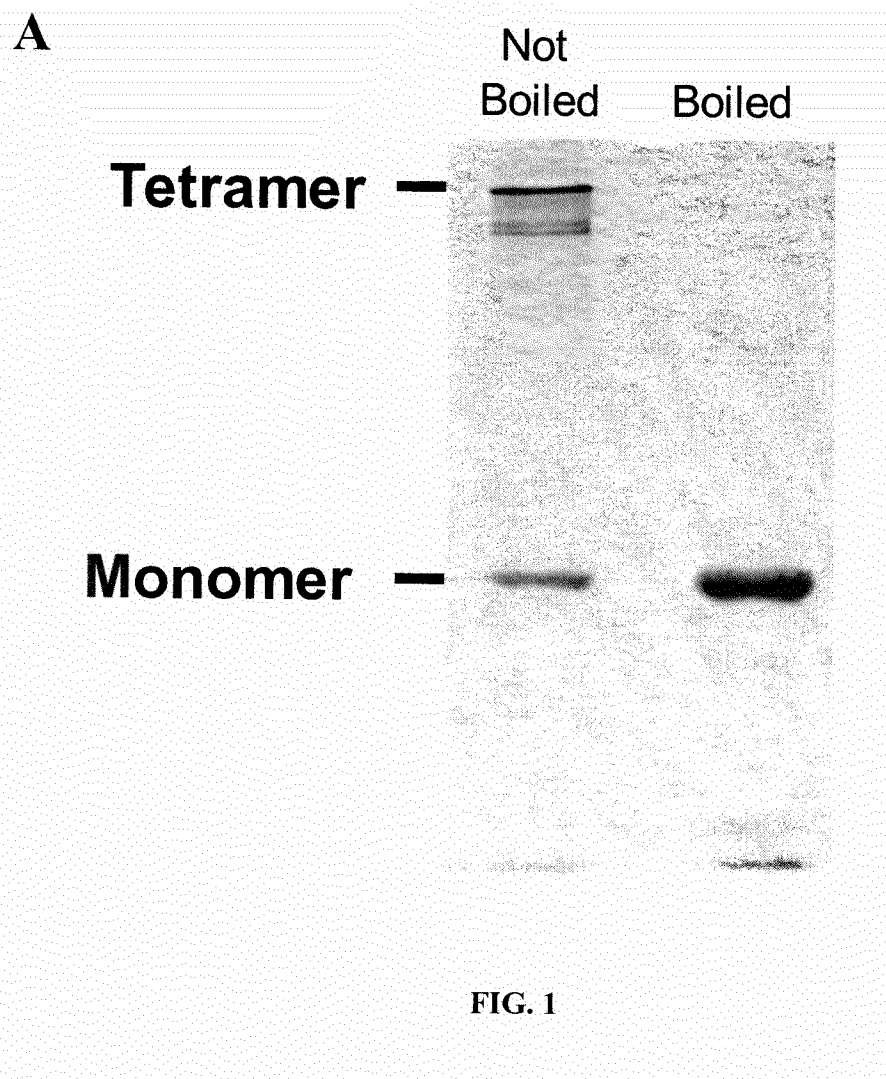
44. A polynucleotide encoding a fusion protein according to claim 43.

10

45. A vector comprising a polynucleotide according to claim 44.

46. A host cell comprising a conjugate according to any of claims 40 to 43, a polynucleotide according to claim 44 or a vector according to claim 45.

15



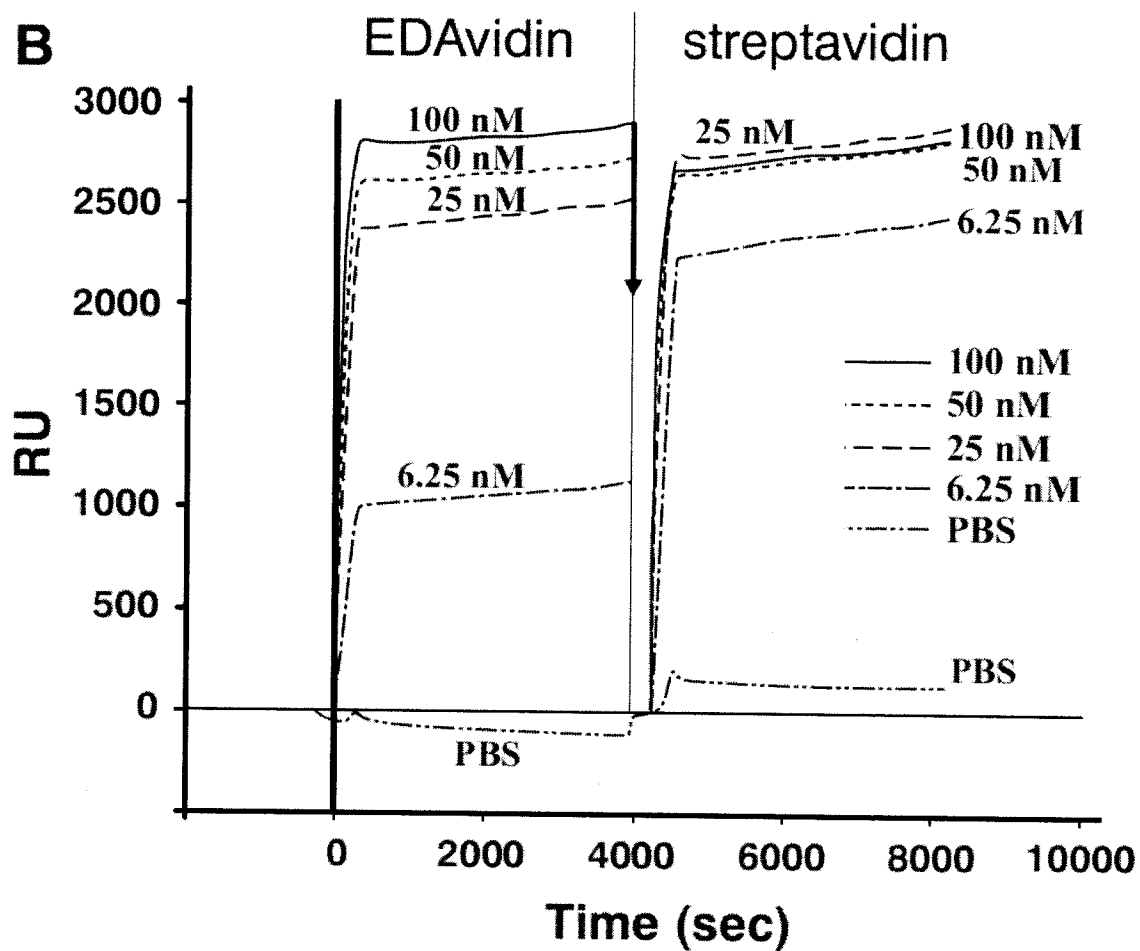


FIG. 1 (cont.)

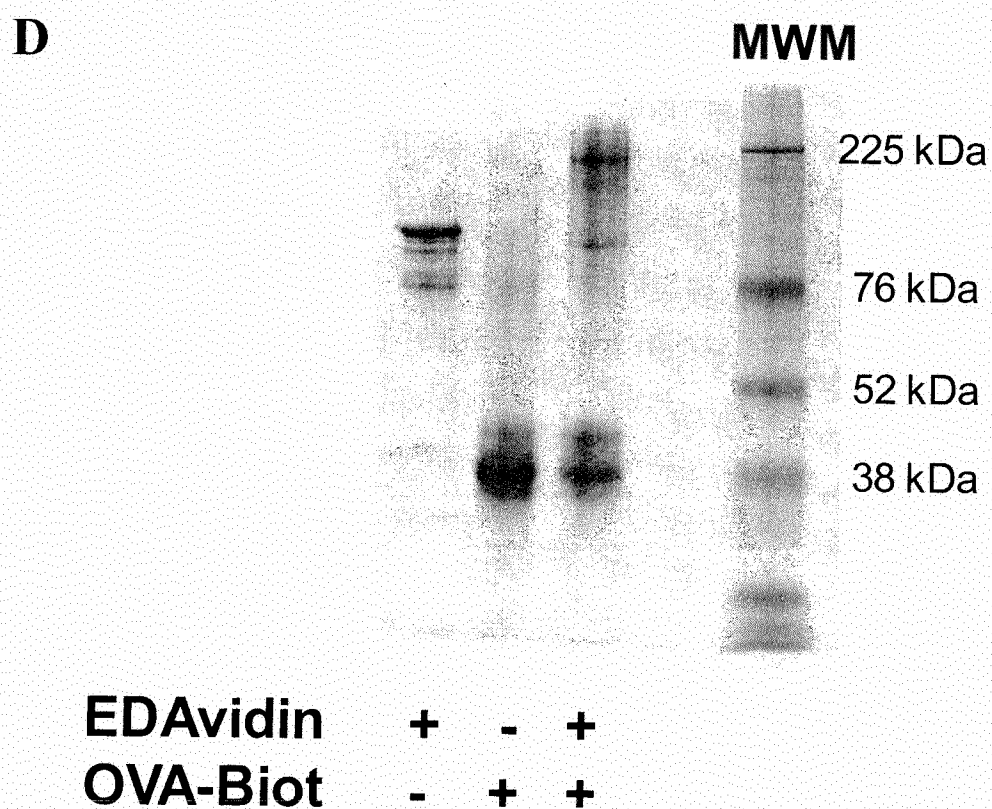
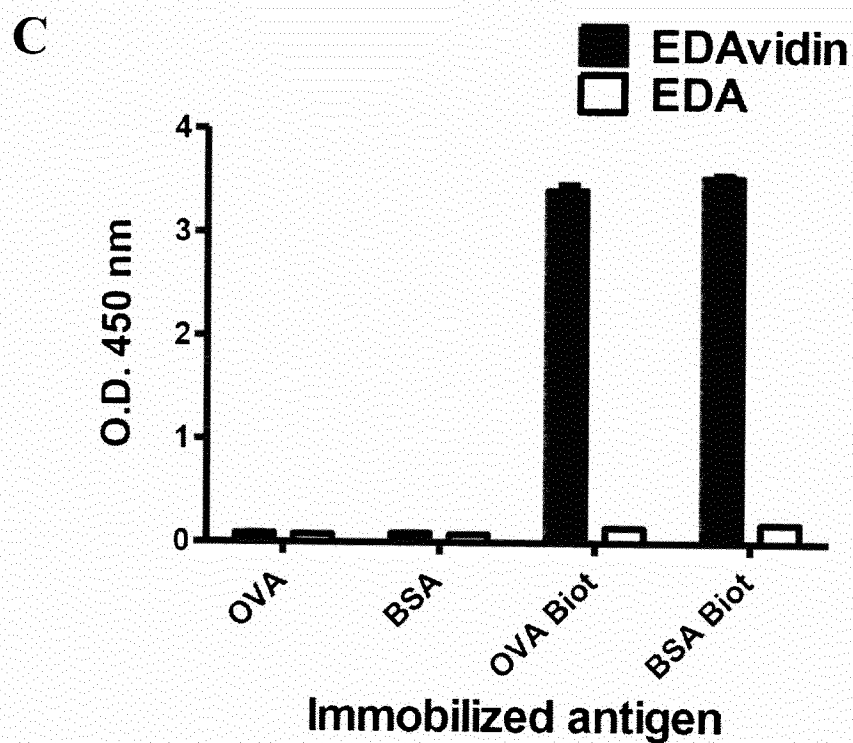
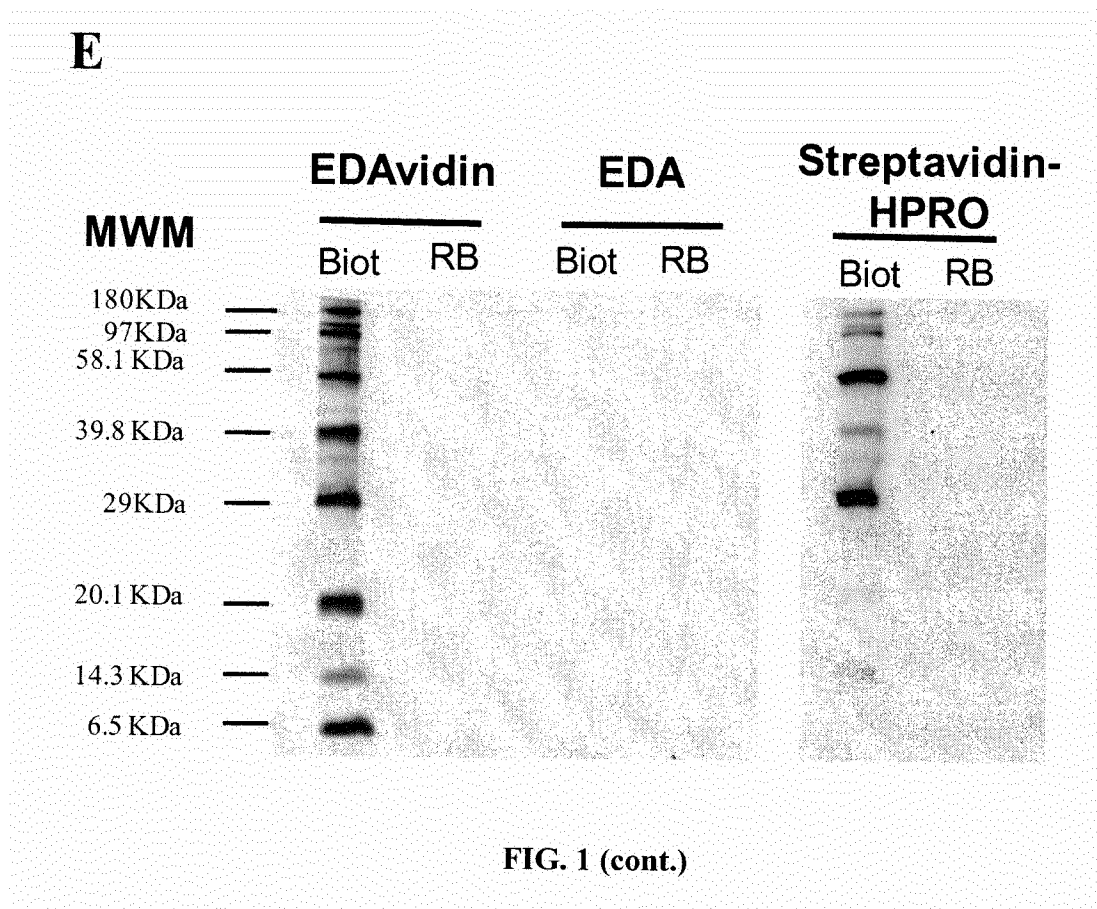


FIG. 1 (cont.)



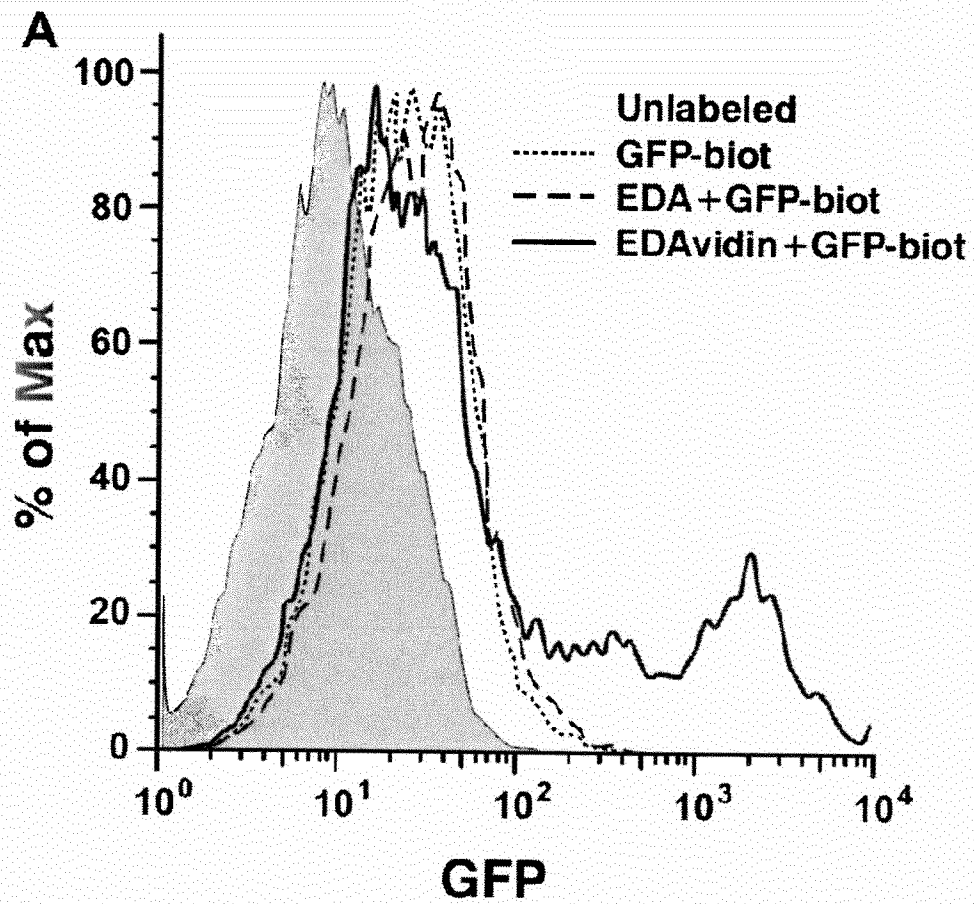
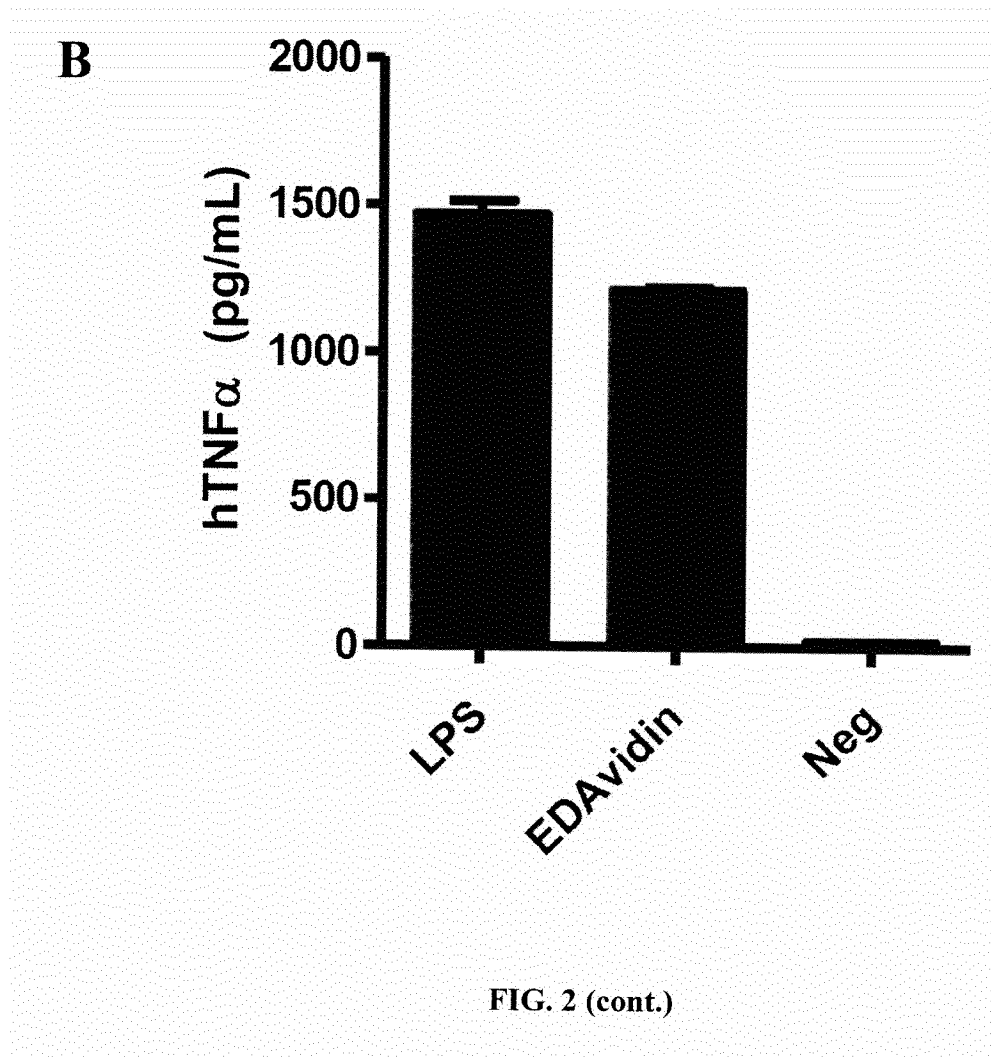


FIG. 2



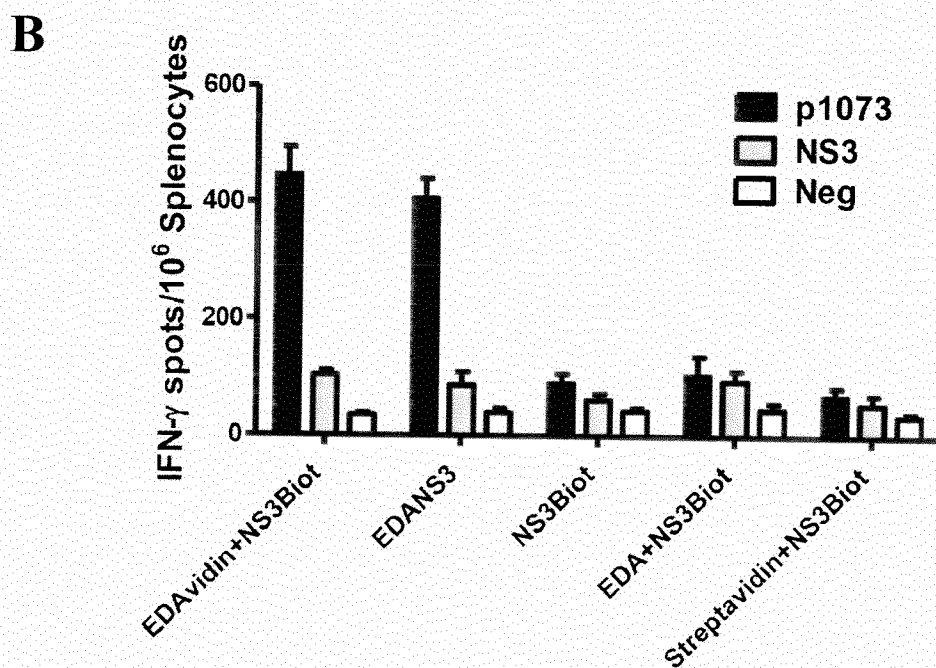
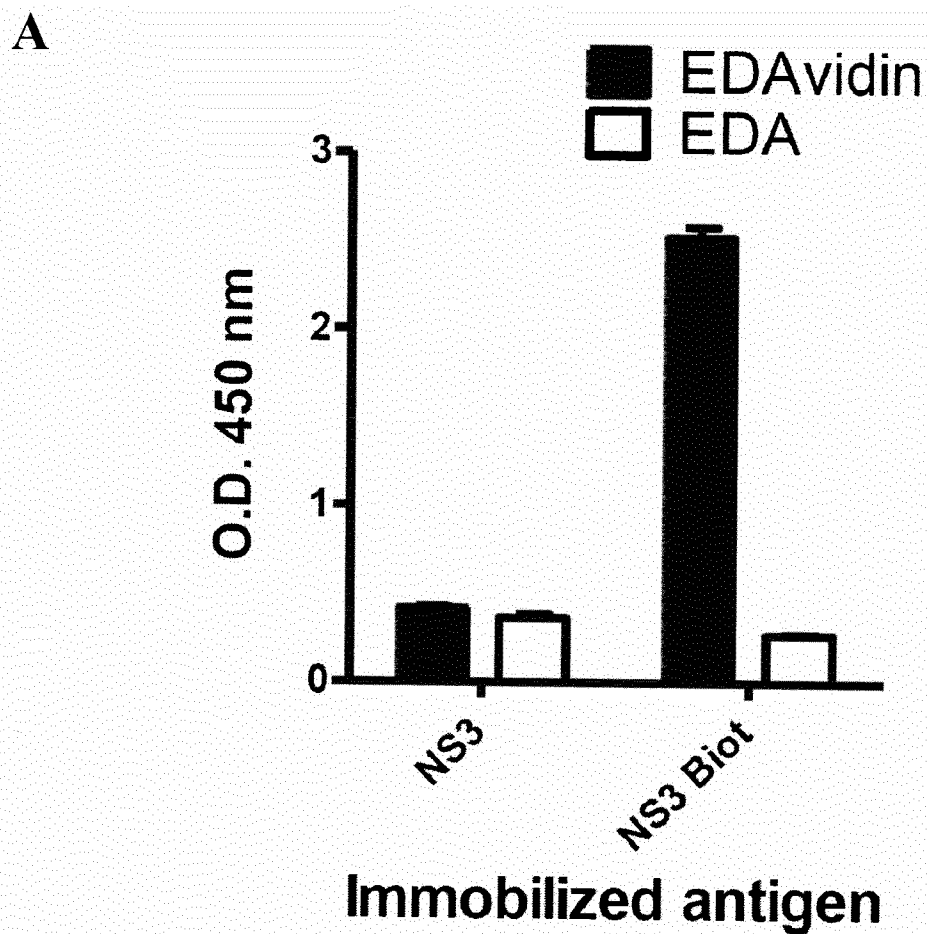


FIG. 3

C

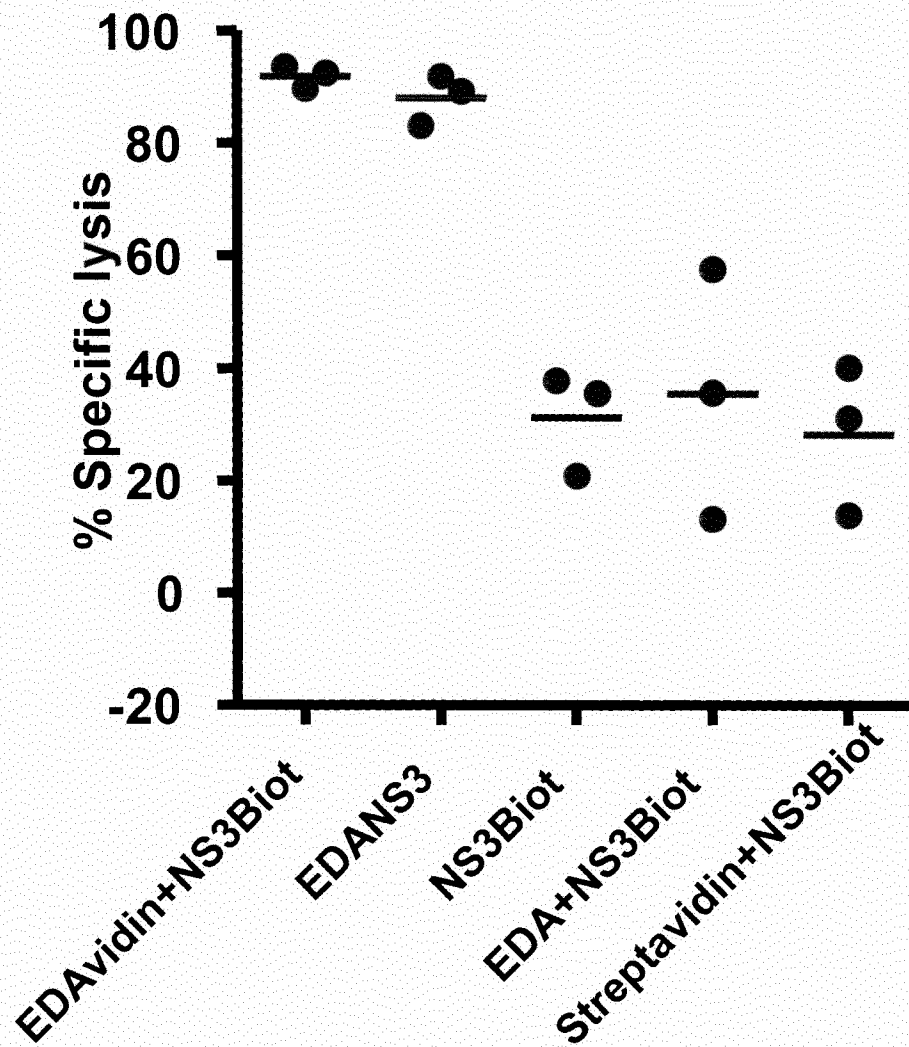
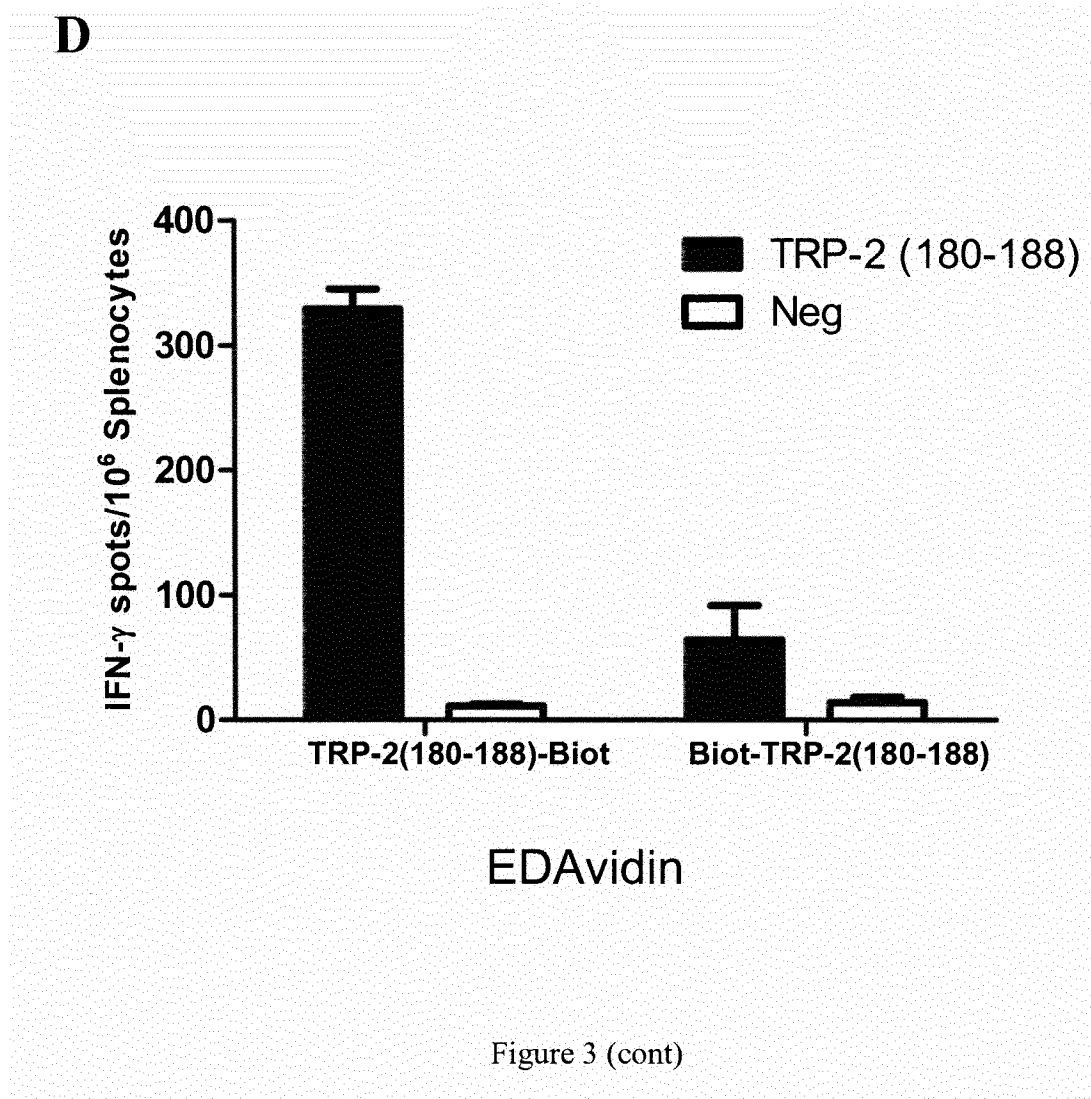


FIG. 3 (cont.)



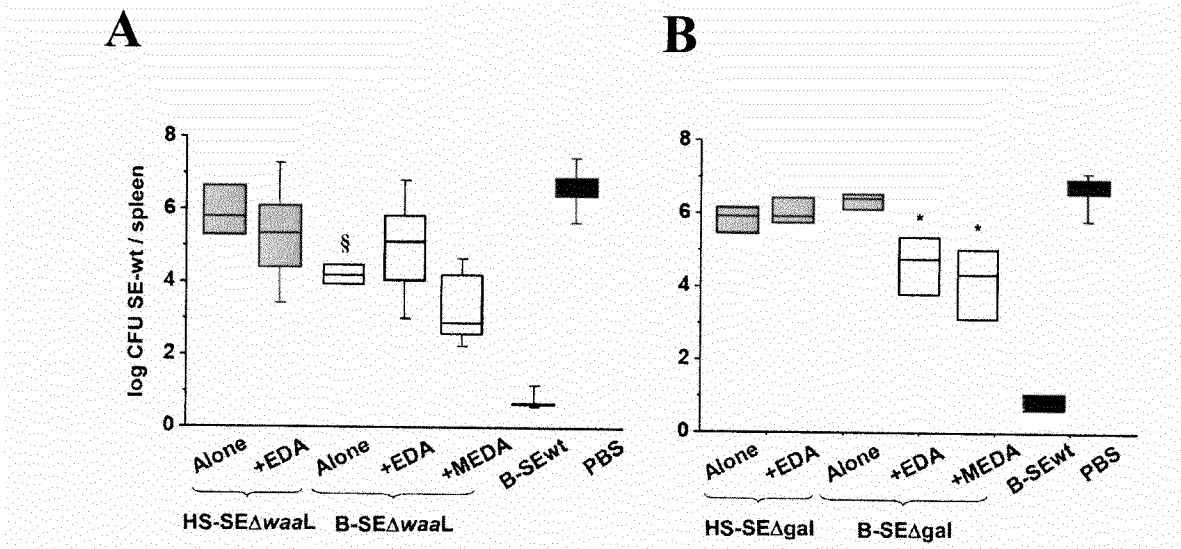


FIG. 4

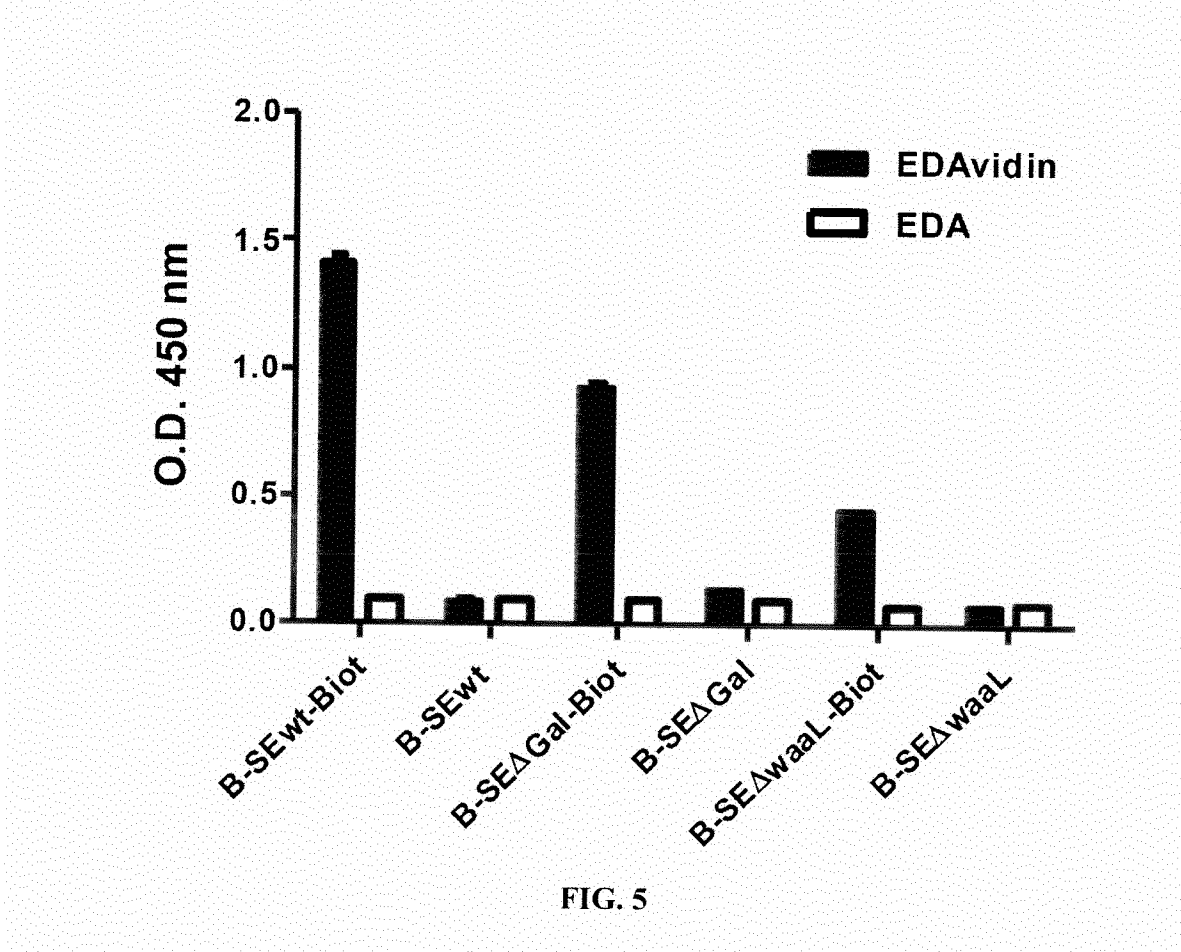


FIG. 5

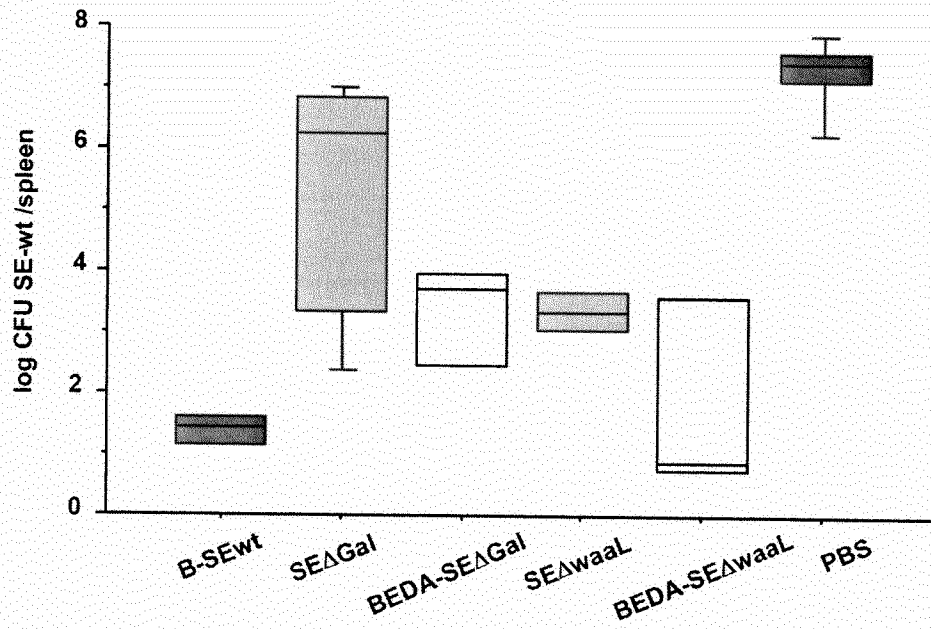


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/053633

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K47/48 A61K39/00 A61K39/29 A61K39/02 A61P37/00 A61P37/04 A61P31/14 A61P35/00 A61P31/04 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, EMBASE, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 913 954 A2 (PROYECTO BIOMEDICINA CIMA SL [ES]) 23 April 2008 (2008-04-23) cited in the application	40-46
A	examples 1-3	1-39

X	LASARTE JUAN J ET AL: "The extra domain A from fibronectin targets antigens to TLR4-expressing cells and induces cytotoxic T cell responses in vivo", JOURNAL OF IMMUNOLOGY, AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 178, no. 2, 1 January 2007 (2007-01-01), pages 748-756, XP002633848, ISSN: 0022-1767	40-46
A	abstract page 749, column 1, paragraph 5 - column 2, paragraph 1	1-39

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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
24 May 2012	04/06/2012	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Monami, Amélie	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/053633

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MANSILLA C ET AL: "Immunization against hepatitis C virus with a fusion protein containing the extra domain A from fibronectin and the hepatitis C virus NS3 protein", JOURNAL OF HEPATOLOGY, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, DK, vol. 51, no. 3, 1 September 2009 (2009-09-01), pages 520-527, XP026541557, ISSN: 0168-8278, DOI: 10.1016/J.JHEP.2009.06.005 [retrieved on 2009-06-23] abstract page 525, column 1, paragraph 2 - page 526, column 2, paragraph 3	1-46
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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