LENTIVIRAL VECTORS ENABLING ROUTING ANTIGENS TO MHC-II PATHWAY AND INDUCING CD4+ AND CD8+ T-CELL RESPONSES IN A HOST

Field of the invention

The invention relates to lentiviral vectors designed to provide a new generation of multifunctional vectors leveraged to target and activate Dendritic Cells, to route immunogens to MHC-II pathway, and to induce both $CD4^+$ and $CD8^+$ T-cell responses.

In particular the invention relates to such lentiviral vectors expressing antigen(s) selected for their interest in eliciting an immunological response in a host, in particular a human host in need thereof wherein the immunological response encompassing a CD4+ T cell response. The antigens may be expressed from an insert in the lentiviral backbone of the vector consisting of a polynucleotide encoding a single antigen or from a polynucleotide encoding multiple antigens. The polynucleotide encoding the single or multiple antigens is provided in the vector by means of a scaffold carrier.

The lentiviral vector of the invention is provided for use in the design of immunological compositions, preferably of a vaccine candidate, in particular a vaccine suitable for a human host.

Background of the invention

Lentiviral Vectors (LV) provide one of the most efficient vaccine platforms, relied on their outstanding potential of gene transfer to the nuclei of the host cells, including notably Antigen Presenting Cells (APC). Such nuclear transfer of genes initiates expression of antigens which readily access the Major Histocompatibility Complex Class-I (MHC-I) presentation machinery, i.e., proteasome, for further triggering of $CD8^+$ T cells [1-3]. In net contrast with their substantial ability at routing the endogenously produced antigens into the MHC-I pathway, viral vectors, including LV, are barely effective or inoperative in delivery of non-secreted antigens to the endosomal MHC-II compartment (MIIC) and unable to trigger $CD4^+$ T cells. Although $CD8^+$ T cells contribute largely to the immune control of infectious diseases or tumor growth, $CD4^+$

T cells are the major immune players. In addition to their long lifespan and their own direct effector functions, $CD4^+$ T cells orchestrate the immune system by regulating innate immunity, tailoring B-cell responses and supporting $CD8^+$ T-cell effector functions [4]. Therefore, leveraging the potential of LV to induce $CD4^+$ T cells will maximize their success rate in vaccine strategies.

Summary of the invention

In one aspect, the present invention relates to a recombinant lentiviral vector genome comprising a polynucleotide encoding a fusion polypeptide, wherein said fusion polypeptide comprises arranged from N-terminal to C-terminal ends a first recombinant polypeptide and a second polypeptide, wherein:

(i) said first recombinant polypeptide comprises a multimerization scaffold which comprises at least one collectin or a fragment thereof suitable to enable self-assembly of multimers of the first polypeptide, fused with at least one antigenic polypeptide;

(ii) said second polypeptide comprises a CD40L ectodomain or a receptor binding fragment thereof, in particular the CD40L ectodomain of the human CD40L.

The present invention further relates to a DNA plasmid comprising the recombinant vector genome according to the invention.

The present invention also relates to a recombinant lentiviral vector or a recombinant lentiviral vector particle which comprises the recombinant lentiviral vector genome according to the invention.

The present invention also relates to a fusion polypeptide comprising arranged from N-terminal to C-terminal ends a first recombinant polypeptide and a second polypeptide, wherein:

(i) said first recombinant polypeptide comprises a multimerization scaffold which comprises at least one collectin or a fragment thereof suitable to enable self-assembly of multimers of the first polypeptide, fused with at least one antigenic polypeptide;

(ii) said second polypeptide comprises a CD40L ectodomain or a receptor binding fragment thereof, in particular the CD40L ectodomain of the human CD40L.

The invention further relates to a host cell, preferably a mammalian host cell, in particular a human host, transfected with a DNA plasmid according to the invention, in particular wherein said host cell is a HEK-293T cell line or a K562 cell line.

In another aspect, the invention relates to a pharmaceutical composition, in particular a vaccine composition, suitable for administration to a mammalian host, in particular a human host, comprising a recombinant lentiviral vector of the invention, a recombinant lentiviral vector particle of the invention, or a host cell of the invention together with one or more pharmaceutically acceptable excipient(s) suitable for administration to a host in need thereof, in particular a human host.

In particular, the invention relates to the pharmaceutical composition for use in the elicitation of a protective, preferentially prophylactic, immune response by the elicitation of T-cell responses directed against epitopes contained in the antigenic polypeptide or immunogenic fragments thereof, and/or cellular and/or humoral response in a host in need thereof, in particular a human host.

Another aspect of the invention relates to a method for the preparation of recombinant lentiviral vector particles suitable for the preparation of a pharmaceutical composition, in particular a vaccine, comprising the following steps:

a) transfecting the recombinant lentiviral transfer vector carrying the lentiviral vector genome according to the invention, or the DNA plasmid according to the invention in a host cell, for example a HEK-293T cell line or a K562 cell line;

b) co-transfecting the cell of step a) with: (i) a plasmid vector encoding envelope proteins and with a plasmid vector encoding the lentiviral GAG and POL or mutated POL protein as packaging construct; and (ii) a plasmid encoding VSV-G Indiana or New Jersey envelope ;

c) culturing the host cell under conditions suitable for the production of recombinant lentiviral vector particles expressing the antigenic polypeptide, or an immunogenic fragment thereof;

d) recovering the recombinant lentiviral particles expressing the antigenic polypeptide, or an immunogenic fragment thereof.

Detailed description of the invention

The inventors have designed and prepared a platform of lentiviral vector encoding a recombinant secreted protein monomer based on soluble collagen-containing C-type lectins (collectins), such as Mannan-Binding Lectin (MBL) or Surfactant-associated Protein D (SPD), as scaffold protein carriers. The inventors have discovered that these scaffold protein carriers, when fused with the CD40L ectodomain, could elicit an MHC-II antigen presentation, and a strong CD4-mediated immune response. This was unexpected, since the T-cell immunogenicity of the existing lentiviral platforms were mostly restricted to a $CD8^+$ T-cell-mediated immune response.

The invention hence discloses a recombinant lentiviral vector genome comprising a polynucleotide encoding a fusion polypeptide expressed as a recombinant carrier protein.

The recombinant carrier protein is encoded by a polynucleotide that is recombined in the backbone of the lentiviral transfer vector in order to enable preparing lentiviral vector particles expressing the protein carrier harboring the antigen(s) for elicitation of an immunological response.

The recombinant protein carrier expressed by the lentiviral vector of the invention is obtained as a fusion polypeptide or as a multimer of such fusion polypeptide carrying single or multiple distinct antigens suitable for the elicitation of an immunogenic response, in particular a protective immunogenic response or advantageously a sterile protection against the pathogen providing the antigen(s).

In one aspect, the invention thus relates to a recombinant lentiviral vector genome comprising a polynucleotide encoding a fusion polypeptide, comprising, arranged from N-terminal to C-terminal ends a first recombinant polypeptide and a second polypeptide, wherein:

(i) said first recombinant polypeptide comprises a multimerization scaffold which comprises at least one collectin or a fragment thereof, fused with at least one antigenic polypeptide;

(ii) said second polypeptide comprises a CD40L ectodomain or a receptor binding fragment thereof.

As used herein, a multimerization scaffold refers to a polypeptide chain that is capable of self-assembling, thereby inducing the formation of multimeric proteins. Multimerization of collectins is mediated by the formation of disulfide bonds between their cysteine-rich N-terminal crosslinking region[34, 54], containing one or several cysteine residues.

Accordingly, said at least fragment of collectin suitable to enable self assembly of multimers of the first polypeptide may be determined according to the available knowledge. In particular, it comprises at least a N-terminal crosslinking region or domain of a collectin. Said crosslinking region may preferably comprise at least two cysteine residues separated by up to 6 amino acid residues, e.g. 4 or 5 amino acids. The second cysteine residue is typically preceded by a hydrophobic amino acid residue. The crosslinking region generally comprises between 5 and 50 amino acids, more specifically between 10 and 35 amino acids. In one embodiment, the crosslinking region is the crosslinking region/domain of a collectin selected from mannan-binding lectin (MBL), surfactant protein D (SP-D), surfactant protein A (SP-A), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin of 43 kDa (CL-43), collectin of 46 kDa (CL-46), and collectin kidney 1 (CL-K1), in particular human MBL (SEQ ID No. 17), SP-D (SEQ ID No. 18), CL-L1 (SEQ ID No. 34), SP-A1 (SEQ ID No. 35), SP-A2 (SEQ ID No. 36), CL-P1 (SEQ ID No. 37) and CL-K1 (SEQ ID No. 38), or a sequence with at least 50%, in particular at least 60%, more particularly at least 80% or 90%, even more particularly at least 95% or 99% sequence identity with the crosslinking region/domain of said collectins.

According to the invention, when a polypeptide is fused with another polypeptide, the nucleotide sequences encoding the two polypeptides are ligated to each other in-frame to create a chimeric gene encoding a fusion protein. In the invention, the nucleotide sequence of the antigenic polypeptide may be ligated in 5' or 3' position with respect to the nucleotide sequence of the collectin or fragment thereof. Preferably, it is inserted, i.e. fused in-frame within the nucleotide sequence of the collectin or fragment thereof, such that the collectin or its fragment harbors the antigenic polypeptide within its polypeptide chain. Preferably, the antigenic polypeptide is harbored within the collagen-like domain of the collectin. The fusion between two polypeptides may be direct or indirect. In particular, a linker or spacer peptide or polypeptide chain may be present between the two fused polypeptides.

According to the invention, the second polypeptide comprises CD40 ligand (CD40L) or a receptor binding fragment thereof capable of binding to the CD40 receptor when contained in the fusion polypeptide of the invention, in particular a CD40L ectodomain or a receptor binding fragment thereof. CD40L is preferably human CD40L.

The CD40L ectodomain is preferably the ectodomain of human CD40L. In particular, the CD40L ectodomain has the sequence set forth in SEQ ID No. 19. The second polypeptide may also comprise any receptor binding fragment or region of CD40L or its ectodomain, i.e. a fragment or region capable of binding CD40.

In this fusion protein, the collectin or its fragment acts as a carrier for the antigenic polypeptide. The collectin also acts as a multimerization scaffold which promotes the formation of collectin-CD40L multimers, which are competent to bind CD40 receptor at the surface of the Antigen Presenting Cells (APCs) which will become activated.

The invention also relates to the fusion polypeptide, in a monomer or multimer form. Preferably, the collectin or its fragment is able to induce a trimerization of the fusion polypeptide. Collectin trimers are also able to further multimerize, inducing the formation of dodecamers (tetramers of trimers) or octodecamers (hexamers of trimers). The fusion polypeptide expressed as a multimer is in particular a soluble macromolecule carrier that is able to circulate in the blood of the host or able to be taken up by APCs.

Collectins are typically composed of four distinct regions: (i) a crosslinking region, (ii) a collagen-like region, (iii) a neck region, and (iv) a Carbohydrate-Recognition Domain (CRD). Preferably, the collectin or collectin fragment comprises the following regions of a collectin, from N-terminal to C-terminal ends: at least one crosslinking region; at least one collagen-like region of a collectin; and at least one neck region of a collectin. In one embodiment, the carbohydrate recognition domain (CRD) of the collectin is absent from the fusion protein. In this embodiment, the second polypeptide comprising the ectodomain of CD40L or a receptor binding fragment thereof, may replace the CRD of the collectin, i.e. the second polypeptide may be fused to the neck region of the collectin, optionally with a linker or a spacer. In particular, the CD40L ectodomain or its fragment may be fused with the neck region via a rigid spacer such as a coiled coil region which prevents interaction between CD40L ectodomain and the antigenic polypeptide to be expressed.

The collectin may be selected from mannan-binding lectin (MBL), surfactant protein D (SP-D), surfactant protein A (SP-A), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin of 43 kDa (CL-43), collectin of 46 kDa (CL-46), and collectin kidney 1 (CL-K1). Preferably, the collectin is a human collectin selected from human MBL (UniProtKB - P11226)(SEQ ID No. 17), SP-D (UniProtKB - P35247) (SEQ ID No. 18), CL-L1 (UniProtKB - Q9Y6Z7) (SEQ ID No. 34), SP-A1 (UniProtKB - Q8IWL2) (SEQ ID No. 35), SP-A2 (UniProtKB - Q8IWL1) (SEQ ID No. 36), CL-P1 (UniProtKB - Q5KU26) (SEQ ID No. 37) and CL-K1 (UniProtKB - Q9BWP8) (SEQ ID No. 38). Preferably, the collectin is MBL or SP-D, especially human MBL or human SP-D, such as human MBL of SEQ ID no. 17 or human SP-D of SEQ ID No. 18.

In one embodiment, the collectin or collectin fragment in the fusion polypeptide of the invention is a chimeric collectin or a fragment thereof. A chimeric collectin comprises two or more collectin fragments originating from different collectins. In one embodiment, the chimeric collectin comprises a fragment of human Pulmonary surfactant-associated protein D (SP-D, UniProtKB - P35247) (SEQ ID No. 18) in particular amino acids 1-106 comprising a signal peptide ensuring secretion, a cysteine-rich region and a collagen-like domain, and a fragment of human Mannose-binding protein C (MBL, UniProtKB - P11226) (SEQ ID No. 17), in particular amino acids 65-130 comprising a collagen-like domain and a coiled-coil region. The antigenic polypeptide is preferably fused to the chimeric collectin between the SP-D fragment and the MBL fragment. An exemplary structure of a fusion protein comprising a chimeric cellectin is shown in Figure 13.

Preferably, the polynucleotide(s) encoding the antigenic polypeptide(s) is or are inserted within the nucleotide sequence of the collagen-like region of said collectin, in particular it is fused in frame within this sequence.

The antigenic polypeptide may be linked within the collagen-like region of said collectin via linkers, in particular flexible peptide linkers, such as “GS” linkers made of stretches of glycine and serine, in particular the $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ linker. Suitable linkers are also shown in the Examples, in particular in Tables S2 and S3.

According to the invention the fusion polypeptide carries one or several antigens or antigenic polypeptides. In a particular embodiment the fusion polypeptide provides at least 2, in particular at least 3 or at least 4 or at least 5 and in particular are especially

2, 3, 4 or 5, and accordingly encompass at least 2, at least 3 or at least 4 antigens (and/or antigenic fragments or mutated antigens with respect to a native or wild type determined antigen of a pathogen). In a particular embodiment the antigenic polypeptide contained in the fusion polypeptide comprises or consists of a fusion of up to 6 antigens or antigenic fragments or mutated fragments thereof.

In one embodiment, antigen or immunogenic fragment thereof is selected from a bacterial, parasite or viral pathogen, or is a tumoral antigen or immunogenic fragment thereof, in particular wherein the at least two antigens or immunogenic fragments thereof are selected from distinct pathogens. In one embodiment, the pathogen is selected from *Mycobacterium tuberculosis* (Mtb), an influenza virus in particular a type A, type B or type C influenza virus, more specifically an H1N1, H2N2 or H3N2 influenza virus, or a coronavirus, in particular SARS-CoV-2.

In particular, the antigenic polypeptide may comprise one or more *Mycobacterium tuberculosis* (Mtb) antigens, in particular selected from EsxA (UniProtKB - P9WNK7)(SEQ ID No. 39), EspC (UniProtKB - P9WJD7)(SEQ ID No. 40), EsxH (UniProtKB - P9WNK3)(SEQ ID No. 41), PE19 (UniProtKB - Q79FK4)(SEQ ID No. 42), Hypoxic response protein 1 (Hrp1) (UniProtKB - P9WJA3)(SEQ ID No. 43) and Resuscitation promoting factor D (RpfD) (UniProtKB - P9WG27)(SEQ ID No. 44), or immunogenic fragments thereof, e.g. a fragment lacking the initial methionine. Preferably, the immunogenic fragment of RpfD is the $RpfD_{42-154}$ ectodomain. In one embodiment, the antigenic polypeptide may comprise one of the following Mtb antigenic combinations:

(a) EsxH;
(b) EsxH and EsxA;
(c) EsxH, EsxA and PE19;
(d) EsxH, EsxA, PE19 and EspC;
(e) EsxH, EsxA, PE19, EspC, Hrp1 and RpfD;
or immunogenic fragments thereof.

In one embodiment, the fusion polypeptide further comprises polypeptides, in particular via 2A self-cleaving peptides, ensuring other immuno-modulatory functions like that of CXCL9, CXCL10, CCL3, CCL4 and/or CCL5 as pro-inflammatory Th1-related chemokines, or CXCL20 as Th17-promoting chemokine.

In one embodiment, the fusion polypeptide further comprises a polypeptide comprising CCL20, or a receptor binding domain thereof. CCL20, or the receptor binding domain thereof, may be in particular inserted within the collagen-like domain of the collectin.

In one embodiment, the polypeptide comprises human CCL20 or a receptor binding domain thereof. In particular, the polypeptide comprises the human CCL20 sequence set forth in SEQ ID No. 20.

In one embodiment, the fusion polypeptide has the sequence set forth in SEQ ID NO: 24 and in Figure 13, wherein the sequence of the antigenic polypeptide EsxH may be replaced by another antigenic polypeptide of interest.

The invention accordingly also relates to a nucleic acid molecule encoding the fusion polypeptide defined herein. The nucleic acid may be DNA, in particular cDNA or may be RNA, in particular stabilized RNA. The RNA sequences are deducted from the DNA sequences wherein the Thymine (T) nucleobase is replaced by an Uracile (U) nucleobase. RNA polynucleotides may be obtained by transcription of DNA or cDNA or may be synthesized.

The nucleic acid molecule may further comprise control nucleotide sequences for the transcription or for the expression of the fusion polypeptide comprising the antigen(s). It may also be modified, in order to be operably ligated to a distinct polynucleotide such as a plasmid or a vector genome (transfer plasmid), in particular a lentiviral vector genome. It may also be modified, in particular to be rendered more stable such as for use as RNA. In a further embodiment, the nucleic acid is a mammalian codon-optimized, in particular a human codon-optimized sequence for expression in mammalian, respectively human cells.

The invention also relates to a plasmid vector recombined with a nucleic acid molecule encoding the fusion polypeptide carrying antigen(s) selected for the elicitation of an immune response in a host.

In a further embodiment, the plasmid vector is a transfer vector in particular a lentiviral transfer vector suitable to provide the genome of a lentiviral vector of the invention. The lentiviral vector expresses the selected antigenic polypeptides with their carrier protein as a secreted monomer or multimer when expressed *in vivo* in a host.

An “antigen” or an “antigenic polypeptide” as defined herein as a wild type or native antigen of a pathogenic organism or as a fragment of such wild type a native antigen or as a mutated polypeptide comprising less than 5% of mutated especially substituted amino acid residues with respect to the wild type or native antigen. Mutations are in particular point mutations of 1, 2, 3 or 4 amino acid residues of the amino acid sequence of the wild type or native antigen. A fragment of the wild type or the native antigen advantageously keeps the immunogenic properties of the polypeptide from which it derives or shows improved immunogenic properties when it is expressed by the lentiviral vector of the invention and advantageously shows immune protective properties when expressed in a host. A fragment of an antigen has an amino acid sequence which is sufficient to provide one or several epitope(s) in particular T cell epitopes and more particularly CD4+ or CD8+ T cell epitopes or both and which keeps the immunogenic, especially the protective properties leading to the protective activity of the antigenic polypeptide from which it derives and/or exhibits such protective properties when expressed by the lentiviral vector of the invention.

The expression “*T-cell epitope*” refers to antigenic determinants that are involved in the adaptive immune response driven by T cells. In particular said T-cell epitopes elicit T cells, when delivered to the host in suitable conditions. According to a particular embodiment the antigenic polypeptides targeted according to the invention and the polypeptide derivatives of these antigenic polypeptides comprise epitope(s) mediating CD4+ T cell response and advantageously also $CD8^+$ T cell response.

Polypeptides and antigens described and used in the invention may have at least 50% amino acid identity with the native protein, in particular at least 60%, in particular at least 70%, in particular at least 80%, more particularly at least 90 or 95%, more particularly at least 99% identity.

In a particular embodiment, the nucleic acid molecule containing the genome of the transfer vector is provided as a plasmid comprising the lentiviral backbone vector recombined with a polynucleotide encoding the selected antigen(s) of the pathogen, for their expression as a monomeric fusion polypeptide or as a multimeric, preferably soluble carrier protein when said vector genome is provided in a lentiviral vector particle that is used for administration to a host.

Additionally, the nucleic acid molecule may contain sequences for the control of transcription and/or for the control of expression, and/or may contain sequences for

ligation to a distinct nucleic acid such as for ligation to a plasmid or a vector genome. Hence the nucleic acid may contain one or more of sequences for restriction site(s), Kozak sequence, promoter or other sequences as disclosed herein and illustrated in the examples.

The expression "*vector*" relates to biological or chemical entities suitable for the delivery of the polynucleotides encoding the antigenic polypeptides of the combination of compounds to the cells of the host administered with such vectors. Vectors are well known in the art and may be viral vectors as those described herein such as lentiviruses which infect human. The invention relates in particular to the use of HIV vectors, especially HIV-1 vectors which are illustrated in the Examples. Details for the construction for HIV-1 vectors are known in the art and provided in the examples.

In accordance with the invention, lentiviral vectors expressing antigenic polypeptides are provided wherein the vectors have or comprise in their genome (vector genome) a recombinant polynucleotide which encodes a fusion polypeptide according to the invention, wherein said fusion polypeptide comprises at least one antigenic polypeptide, in particular of a pathogen.

The lentiviral vectors of the invention, especially the preferred HIV-1 based vectors, replication-incompetent pseudotyped lentiviral vector, in particular a replication-incompetent pseudotyped HIV-1 lentiviral vector, wherein said vector contains a genome comprising a mammal codon-optimized synthetic nucleic acid, in particular a human-codon optimized synthetic nucleic acid, wherein said synthetic nucleic acid encodes a fusion polypeptide according to the invention, comprising an antigenic polypeptide, in particular the antigenic polypeptide(s) of a determined pathogen infecting a mammal, in particular a human host.

Use of codon-optimized sequences in the genome of the vector particles allows in particular strong expression of the antigenic polypeptide in the cells of the host administered with the vector, especially by improving mRNA stability or reducing secondary structures. In addition, the expressed antigenic polypeptide undergoes post translational modifications which are suitable for processing of the antigenic polypeptide in the cells of the host, in particular by modifying translation modification

sites (such as glycosylation sites) in the encoded polypeptide. Codon optimization tools are well known in the art, including algorithms and services such as those made available by GeneArt (Life technologies-USA) and DNA2.0 (Menlo Park, California – USA). In a particular embodiment codon-optimization is carried out on the open reading frame (ORF) sequence encoding the antigenic polypeptide and the optimization is carried out prior to the introduction of the sequence encoding the ORF into the plasmid intended for the preparation of the vector genome. In another embodiment additional sequences of the vector genome are also codon-optimized.

The active ingredients consisting of the viral vectors may be integrative pseudotyped lentiviral vectors, especially replication-incompetent integrative pseudotyped lentiviral vectors, in particular a HIV-1 vector. Such lentiviral vectors may in addition contain a genome comprising a mammal-codon optimized synthetic nucleic acid, in particular a human-codon optimized synthetic nucleic acid, wherein said synthetic nucleic acid encodes the antigenic polypeptide(s) of a determined pathogen infecting a mammal such as disclosed herein, in particular a virus or a bacteria or a parasite infecting a human host.

Alternatively, the lentiviral vector and in particular the HIV-1 based vector may be a non-integrative replication-incompetent pseudotyped lentiviral vector.

A particular embodiment of a lentiviral vector suitable to achieve the invention relates to a lentiviral vector whose genome is obtained from the pTRIP vector plasmid, in particular the pTRIP vector plasmid of nucleotide sequence SEQ ID No. 21, wherein the nucleic acid encoding the fusion polypeptide has been cloned under control of a promoter functional in mammalian cells, in particular the CMV promoter, the human beta-2 microglobulin promoter, the composite “BCUAG” promoter as disclosed herein (SEQ ID No. 22) and wherein the vector optionally comprises post-transcriptional regulatory element of the woodchuck hepatitis virus (WPRE), wild type or mutated. In particular, the WPRE is a mutant WPRE as set forth in SEQ ID No. 23.

In a further embodiment of the invention, the lentiviral vector particle expressing the fusion polypeptide according to the features herein described is pseudotyped with the

glycoprotein G from a Vesicular Stomatitis Virus (V-SVG) of Indiana or of New-Jersey serotype.

The particular features of such lentiviral vectors will be further discussed in detail below.

The invention also relates to a DNA plasmid comprising the recombinant lentiviral vector genome according to the definitions provided herein, in particular wherein said genome is inserted within the pTRIP vector plasmid of nucleotide sequence SEQ ID No. 21.

The invention further relates to a host cell, preferably a mammalian host cell, transfected with a DNA plasmid according to the invention. In particular, said host cell is a HEK-293T cell line or a K562 cell line. The invention further relates to a culture of said host cells.

The invention also relates to a formulation or pharmaceutical composition, in particular a vaccine composition, suitable for administration to a mammalian host, comprising a recombinant lentiviral vector of the invention together with one or more pharmaceutically acceptable excipient(s) suitable for administration to a host in need thereof, in particular a human host.

The invention also relates to a formulation suitable for administration to a mammalian host, in particular a human host comprising as an active ingredient lentiviral vector particles as defined herein for protection against a pathogen infection or against the pathogen-induced condition or disease, together with excipient(s) suitable for administration to a host in need thereof, in particular a human host. The disease may be tuberculosis, influenza, in particular caused by a type A, type B or type C influenza virus, more specifically an H1N1, H2N2 or H3N2 influenza virus. The disease may also be a coronavirus disease, in particular caused by SARS-CoV-2.

In another aspect of the invention the active ingredient or the composition or the formulation comprising the same is for use in the protective immunization against a pathogenic infection or against pathogen-induced condition or disease, in a mammalian host, especially a human host, optionally in association with an appropriate

delivery vehicle and optionally with an adjuvant component and/or with an immunostimulant component.

Accordingly, the active ingredient, or the composition, in particular the lentiviral vector particles of the invention, when administered to a host in needs thereof, especially to a mammalian in particular to a human host, elicits an immune response, encompassing activation of naïve lymphocytes and generation of effector T-cell response and generation of immune memory antigen-specific T-cell response against antigen(s) of the pathogen.

The immune response involves the induction of MHC-II restricted presentation of the antigenic polypeptide or immunogenic fragments thereof, by an antigen-presenting cell, in particular a dendritic cell, and the induction of a CD4+-T-cell immune response. The immune response may either prevent the infection by the pathogen or may prevent the onset or the development of a pathological state resulting from infection.

Physiologically acceptable vehicles may be chosen with respect to the administration route of the immunization composition. In a preferred embodiment administration may be carried out by injection, in particular intramuscularly, intradermally, subcutaneously, or, by intranasal administration or topical skin application.

Recombinant lentiviral vector particles of the invention are used for elicitation in a host, in particular a human host, of an immune response against the pathogen providing the antigens expressed by the particles, said use involving an immunization pattern comprising administering an effective amount of the LV vector particles that elicits the cellular immune response of the host, especially as an effective amount of said active ingredients to boost the cellular immune response of the host previously elicited by a priming dose of a selected active ingredient against said pathogen, and optionally repeating (once or several times) said administration step for boosting.

For each step of administration of the lentiviral vector particles, in particular in a regimen that encompasses multiple administration steps, it is preferred that the pseudotyping envelope protein(s) of the vector particles is(are) different from the one used in the other step(s), especially originate from different viruses, in particular

different VSVs. In the prime-boost regimen, the administered combination of compounds of each step comprises lentiviral vectors as defined herein.

Priming and boosting steps are separated in time by at least 2 weeks, in particular 6 weeks, in particular by at least 8 weeks.
Details on the administration regimen will be discussed further below.

The LV particles provides a cellular immune response (T-cell immune response), particularly a CD4+T-cell immune response and advantageously a CD8+- T-cell immune response, *i.e.,* an adaptive immune response which is mediated by activated cells harbouring respectively CD4 or CD8 receptors.

In a particularly advantageous embodiment, the immune response conferred by the LV particles, is a long-lasting immune response *i.e.,* said immune response encompasses memory cells response and in particular central memory cells response; in a particular embodiment it can be still detected at least several months.

In accordance with the invention when the lentiviral particles are used in a prime-boost regimen or a multiple steps administration regimen, lentiviral vector particles are provided which are pseudotyped with a first determined pseudotyping envelope G protein obtained from the VSV, strain Indiana or New-Jersey, and later administered lentiviral vector particles are provided which are pseudotyped with a second determined pseudotyping envelope G protein obtained from a VSV, strain New Jersey or Indiana. The order of use in the prime-boost regimen of the first and second compounds thus described may alternatively be inversed. Thus, the lentiviral vector particles contained in the separate active ingredients/compounds of the combinations or compositions of the invention when intended for use in a prime-boost regiment are distinct from each other, at least due to the particular pseudotyping envelope protein(s) used for pseudotyping the vector particles.

Doses of lentiviral vectors intended for elicitation of the cellular immune response which is used in the administration pattern, may comprise from 10^5 TU to 10^{10} TU of recombinant lentiviral particles especially from 10^5 to 10^8, when integrative vectors are used. The dose intended for administration to the host may comprise from 10^8 to 10^{10}

of each type of recombinant lentiviral vector particles when integrative-incompetent vectors are used.

The invention also concerns a method of providing immunization in a mammalian host, especially in a human host, comprising the step of administering, as a prime or as a boost, the recombinant lentiviral vector particles of the invention to elicit the immune response, and optionally repeating the administration steps one or several times, in particular to boost said response, in accordance with the present disclosure.

Optionally, the recombinant lentiviral vector particles may be used in association with an adjuvant compound suitable for administration to a mammalian, especially a human host, and/or with an immunostimulant compound, together with an appropriate delivery vehicle. In a preferred embodiment, the recombinant lentiviral vector particles of the invention may also be used without adjuvant, since the CD40L ectodomain already acts as a slight adjuvant.

The recombinant lentiviral vector particles can be administered to the host via injection through different routes including subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.) or intravenous (i.v.) injection, or may be administered orally to topically trough mucosal or skin administration, especially intranasal administration or inhalation. The quantity to be administered (dosage) depends on the subject to be treated, including considering the condition of the patient, the state of the individual's immune system, the route of administration and the size of the host. Suitable dosages range may be determined with respect to the content in equivalent transducing units of HIV-1-derived lentiviral vector particles.

Other examples and features of the invention will be apparent when reading the examples and the figures which illustrate the preparation and application of the lentiviral vector particles with features that may be individually combined with the definitions given in the present description.

Detailed description of the lentiviral vectors for use according to the invention

The invention accordingly involves lentiviral vector which are recombinant lentiviral particles (i.e. recombinant vector particles), and which may be replication-incompetent

lentiviral vectors, especially replication-incompetent HIV-1 based vectors characterized in that: (i) they are pseudotyped with a determined heterologous viral envelope protein or viral envelope proteins originating from a RNA virus which is not HIV, and (ii) they comprise in their genome at least one recombinant polynucleotide encoding at least one antigenic polypeptide (or polypeptide derivative thereof) carrying epitope(s) of an antigen of a pathogen wherein the pathogen is capable of infecting a mammalian host, and wherein said epitopes encompass T-cell epitope(s), in particular both CD4+ T-cell epitopes and CD8+ T-cell epitopes.

According to a particular embodiment of the invention, the lentiviral vectors are either designed to express proficient (i.e., integrative-competent) or deficient (i.e., integrative-incompetent) particles. According to a particular embodiment of the invention, the recombinant lentiviral vector particles are both integration-incompetent and replication-incompetent.

The preparation of the lentiviral vectors is well known from the skilled person and has been extensively disclosed in the literature (confer for review Sakuma T. et al (Biochem. J. (2012) 443, 603-618). The preparation of such vectors is also illustrated herein in the Examples.

In a particular embodiment of the invention, the polynucleotide(s) encoding the antigenic polypeptides (ORF) of the lentiviral vector has(have) been mammal-codon optimized (CO) in particular human-codon optimized. Optionally the lentiviral sequences of the genome of said particles have also a mammal-codon optimized nucleotide sequence. In a particular aspect of the invention the codon optimization has been carried out for expression in mouse cells. In another embodiment the sequence of the polynucleotide(s) encoding the antigenic polypeptides of the lentiviral vector has(have) been human-codon optimized (CO).

It has been observed that codon optimized nucleotide sequences, especially when optimized for expression in mammalian and in particular in human cells, enable the production of higher yield of particles in such mammalian or human cells. Production cells are illustrated in the examples. Accordingly, when lentiviral vector particles of the invention are administered to a mammalian, especially to a human host, higher amounts of particles are produced in said host which favour the elicitation of a strong immune response.

The recombinant lentiviral vector (i.e., lentiviral vectors particles or lentiviral-based vector particles) defined in the present invention are pseudotyped lentiviral vectors consisting of vector particles bearing envelope protein or envelope proteins which originate from a virus different from the particular lentivirus (especially a virus different from HIV, in particular HIV-1), which provides the vector genome of the lentiviral vector particles. Accordingly, said envelope protein or envelope proteins, are "heterologous" viral envelope protein or viral envelope proteins with respect to the vector genome of the particles. In the following pages, reference will also be made to "envelope protein(s)" to encompass any type of envelope protein or envelope proteins suitable to perform the invention.

When reference is made to "lentiviral" vectors (lentiviral-based vectors) in the application, it relates in particular, to HIV-based vectors and especially HIV-1-based vectors.

The lentiviral vectors suitable to perform the invention are so-called replacement vectors, meaning that the sequences of the original lentivirus encoding the lentiviral proteins are essentially deleted in the genome of the vector or, when present, are modified, and especially mutated, especially truncated, to prevent expression of biologically active lentiviral proteins, in particular, in the case of HIV, to prevent the expression by said transfer vector providing the genome of the recombinant lentiviral vector particles, of functional ENV, GAG, and POL proteins and optionally of further structural and/or accessory and/or regulatory proteins of the lentivirus, especially of HIV.

In a particular embodiment, the lentiviral vector is built from a first-generation vector, in particular a first-generation of a HIV-based vector which is characterized in that it is obtained using separate plasmids to provide (i) the packaging construct, (ii) the envelope and (iii) the transfer vector genome. Alternatively, it may be built from a second-generation vector, in particular a second-generation of a HIV-based vector which in addition, is devoid of viral accessory proteins (such as in the case of HIV-1, Vif, Vpu, Vpr or Nef) and therefore includes only four out of nine HIV full genes: *gag*, *pol*, *tat* and *rev*. In another embodiment, the vector is built from a third-generation vector, in particular a third-generation of a HIV-based vector which is furthermore devoid of said viral accessory proteins and also is Tat-independent; these third-generation vectors may be obtained using 4 plasmids to provide the functional elements of the vector, including one plasmid encoding the Rev protein of HIV when

the vector is based on HIV-1. Such vector system comprises only three of the nine genes of HIV-1. The structure and design of such generations of HIV-based vectors is well known in the art.

In any of these generations of the vector, modifications are additionally provided according to the invention by insertion in the vector backbone of the collectin scaffold as described herein, fused with the ectodomain of CD40L or a receptor binding fragment thereof, to provide a LV vector leveraged to target and activate APC, in particular dendritic cells to route immunogens to MHC-II pathway and to induce both CD4+ and CD8+ T-cell responses.

The "vector genome" of the vector particles is a recombinant nucleic acid which also comprises as a recombined sequence the polynucleotide or transgene of interest encoding the fusion polypeptide according to the invention comprising one or more antigenic polypeptide(s), in particular of pathogen as disclosed herein. The lentiviral-based sequence and polynucleotide/transgene of the vector genome are borne by a plasmid vector thus giving rise to the "transfer vector" also referred to as "sequence vector". Accordingly, these expressions are used interchangeably in the present description. According to a particular embodiment, a vector genome prepared for the invention comprises a nucleic acid having a sequence selected in the group of SEQ ID No. 21, in which the polynucleotide encoding the fusion polypeptide of the invention is inserted.

The vector genome as defined herein accordingly contains, apart from the so-called recombinant polynucleotide(s) encoding the fusion polypeptide of the invention comprising the antigenic polypeptide(s) placed under control of proper regulatory sequences for its expression, the sequences of the original lentiviral genome which are non-coding regions of said genome, and are necessary to provide recognition signals for DNA or RNA synthesis and processing (mini-viral genome). These sequences are cis-acting sequences necessary for packaging (ψ), reverse transcription (LTRs possibly mutated with respect to the original ones) and transcription and optionally integration (RRE) and furthermore for the particular purpose of the invention, they contain a functional sequence favouring nuclear import in cells and accordingly transgene transfer efficiency in said cells, which element is

described as a DNA Flap element that contains or consists of the so-called central cPPT-CTS nucleotidic domain present in lentiviral genome sequences especially in HIV-1 or in some retroelements such as those of yeasts.

The structure and composition of the vector genome used to prepare the lentiviral vectors of the invention are based on the principles described in the art and on examples of such lentiviral vectors primarily disclosed in (Zennou et al, 2000; Firat H. et al, 2002; VandenDriessche T. et al). Constructs of this type have been deposited at the CNCM (Institut Pasteur, France) as will be referred to herein. In this respect reference is also made to the disclosure, including to the deposited biological material, in patent applications WO 99/55892, WO 01/27300 and WO 01/27304.

According to a particular embodiment of the invention, a vector genome may be a replacement vector in which all the viral protein coding sequences between the 2 long terminal repeats (LTRs) have been replaced by the recombinant polynucleotide encoding the fusion polypeptide of the invention comprising the antigenic polypeptide(s) as disclosed herein, and wherein the DNA-Flap element has been re-inserted in association with the required cis-acting sequences described herein. Further features relating to the composition of the vector genome are disclosed in relation to the preparation of the particles.

In a particular embodiment, a lentiviral vector of the invention may comprise in its genome one or more than one recombinant polynucleotide encoding a fusion polypeptide according to the invention. In particular, said vector genome comprises two polynucleotides which are consecutive or separated on the genome and which encode different polypeptides of either the same or distinct antigens of the pathogen or of distinct pathogens.

Particular features of the lentiviral vectors used in accordance with the various embodiments of the invention are also disclosed in the Examples, such features being either taken alone or in combination to produce the vectors.

According to the invention, the lentiviral vector particles are pseudotyped with a heterologous viral envelope protein or viral polyprotein of envelope originating from an RNA virus which is not the lentivirus providing the lentiviral sequences of the genome of the lentiviral particles.

As examples of typing envelope proteins for the preparation of the lentiviral vector, the invention relates to viral transmembrane glycosylated (so-called G proteins) envelope

protein(s) of a Vesicular Stomatitis Virus (VSV), which is(are) for example chosen in the group of VSV-G protein(s) of the Indiana strain and VSV-G protein(s) of the New Jersey strain.

Other examples of VSV-G proteins that may be used to pseudotype the lentiviral vectors of the invention encompass VSV-G glycoprotein may especially be chosen among species classified in the vesiculovirus genus: Carajas virus (CJSV), Chandipura virus (CHPV), Cocal virus (COCV), Isfahan virus (ISFV), Maraba virus (MARAV), Piry virus (PIRYV), Vesicular stomatitis Alagoas virus (VSAV), Vesicular stomatitis Indiana virus (VSIV) and Vesicular stomatitis New Jersey virus (VSNJV) and/or stains provisionally classified in the vesiculovirus genus as Grass carp rhabdovirus, BeAn 157575 virus (BeAn 157575), Boteke virus (BTKV), Calchaqui virus (CQIV), Eel virus American (EVA), Gray Lodge virus (GLOV), Jurona virus (JURV), Klamath virus (KLAV), Kwatta virus (KWAV), La Joya virus (LJV), Malpais Spring virus (MSPV), Mount Elgon bat virus (MEBV), Perinet virus (PERV), Pike fry rhabdovirus (PFRV), Porton virus (PORV), Radi virus (RADIV), Spring viremia of carp virus (SVCV), Tupaia virus (TUPV), Ulcerative disease rhabdovirus (UDRV) and Yug Bogdanovac virus (YBV).

The envelope glycoprotein of the vesicular stomatitis virus (VSV-G) is a transmembrane protein that functions as the surface coat of the wild type viral particles. It is also a suitable coat protein for engineered lentiviral vectors. Presently, nine virus species are definitively classified in the VSV gender, and nineteen rhabdoviruses are provisionally classified in this gender, all showing various degrees of cross-neutralisation. When sequenced, the protein G genes indicate sequence similarities. The VSV-G protein presents an N-terminal ectodomain, a transmembrane region and a C-terminal cytoplasmic tail. It is exported to the cell surface *via* the trans-Golgi network (endoplasmic reticulum and Golgi apparatus).

Vesicular stomatitis Indiana virus (VSIV) and Vesicular stomatitis New Jersey virus (VSNJV) are preferred strains to pseudotype the lentiviral vectors of the invention, or to design recombinant envelope protein(s) to pseudotype the lentiviral vectors. Their VSV-G proteins are disclosed in GenBank, where several strains are presented. For VSV-G New Jersey strain reference is especially made to the sequence having accession number V01214. For VSV-G of the Indiana strain, reference is made to the sequence having accession number AAA48370.1 in Genbank corresponding to strain JO2428.

Said viral envelope protein(s) are capable of uptake by antigen presenting cells and especially by dendritic cells including by liver dendritic cells by mean of fusion and/or of endocytosis. In a particular embodiment, the efficiency of the uptake may be used as a feature to choose the envelope of a VSV for pseudotyping. In this respect the relative titer of transduction (Titer DC/Titer of other transduced cells e.g. 293T cells) may be considered as a test and envelope having a relatively good ability to fuse with DC would be preferred.

Antigen Presenting Cells (APC) and especially Dentritic cells (DC) are proper target cells for pseudotyped lentiviral vectors which are used as immune compositions accordingly.

The VSV-G envelope protein(s) are expressed from a polynucleotide containing the coding sequence for said protein(s), which polynucleotide is inserted in a plasmid (designated envelope expression plasmid or pseudotyping env plasmid) used for the preparation of the lentiviral vector particles of the invention. The polynucleotide encoding the envelope protein(s) is under the control of regulatory sequences for the transcription and/or expression of the coding sequence (including optionally post-transcriptional regulatory elements (PRE) especially a polynucleotide such as the element of the Woodchuck hepatitis virus, i.e. the WPRE sequence, obtainable from Invitrogen or a mutant sequence of WPRE as set forth in SEQ ID No. 23.

Accordingly, a nucleic acid construct is provided which comprises an internal promoter suitable for the use in mammalian cells, especially in human cells *in vivo* and the nucleic acid encoding the envelope protein under the control of said promoter. A plasmid containing this construct is used for transfection of cells suitable for the preparation of vector particles. Promoters may in particular be selected for their properties as constitutive promoters, tissue-specific promoters, or inducible promoters. Examples of suitable promoters encompass the promoters of the following genes: MHC Class-I promoters, human beta-2 microglobulin gene (β2M promoter), EF1α, human PGK, PPI (preproinsulin), thiodextrin, HLA DR invariant chain (P33), HLA DR alpha chain, Ferritin L chain or Ferritin H chain, Chymosin beta 4, Chymosin beta 10, Cystatin Ribosomal Protein L41, CMVie or chimeric promoters such as GAG(CMV early enhancer / chicken β actin) disclosed in Jones S. et al (Jones S. et al Human Gene Therapy, 20:630-640(June 2009)) or beta-2m-CMV (BCUAG) as disclosed herein.

These promoters may also be used in regulatory expression sequences involved in the expression of *gag-pol* derived proteins from the encapsidation plasmids, and/or to express the antigenic polypeptides from the transfer vector.

Alternatively, when the envelope expression plasmid is intended for expression in stable packaging cell lines, especially for stable expression as continuously expressed viral particles, the internal promoter to express the envelope protein(s) is advantageously an inducible promoter such as one disclosed in Cockrell A.S. et al. (Mol. Biotechnol. (2007) 36:184-204). As examples of such promoters, reference is made to tetracycline and ecdysone inducible promoters. The packaging cell line may be the STAR packaging cell line (ref Cockrell A.S. et al (2007), Ikedia Y. et al (2003) Nature Biotechnol. 21: 569-572) or a SODk packaging cell line, such as SODk0 derived cell lines, including SODk1 and SODk3 (ref Cockrell A.S. et al (2007), Cockrell A;S.et al (2006) Molecular Therapy, 14: 276-284, Xu K. et al. (2001) ,Kafri T. et al (1999) Journal of Virol. 73:576-584).

According to the invention, the lentiviral vectors are the product recovered from co-transfection of mammalian cells, with:

- a vector plasmid comprising (i) lentiviral, especially HIV-1, cis-active sequences necessary for packaging, reverse transcription, and transcription and further comprising a functional lentiviral, especially derived from HIV-1, DNA flap element and (ii) a polynucleotide encoding the fusion polypeptide of the invention, itself comprising one or more antigenic polypeptide(s) of one or more pathogens against which an immune response is sought under the control of regulatory expression sequences, and optionally comprising sequences for integration into the genome of the host cell;
- an expression plasmid encoding a pseudotyping envelope derived from an RNA virus, said expression plasmid comprising a polynucleotide encoding an envelope protein or proteins for pseudotyping, wherein said envelope pseudotyping protein is advantageously from a VSV and is in particular a VSV-G of the Indianan strain or of the New Jersey strain and,
- an encapsidation plasmid, which either comprises lentiviral, especially HIV-1, *gag-pol* packaging sequences suitable for the production of integration-competent vector particles or modified *gag-pol* packaging sequences suitable for the production of integration-deficient vector particles.

The invention thus also concerns lentiviral vector particles as described above, which are the product recovered from a stable cell line transfected with:

- a vector plasmid comprising (i) lentiviral, especially HIV-1, cis-active sequences necessary for packaging, reverse transcription, and transcription and further comprising a functional lentiviral, especially HIV-1, DNA flap element and optionally comprising cis-active sequences necessary for integration, said vector plasmid further comprising, (ii) a polynucleotide of a codon-optimized sequence for murine or for human of the gene encoding the fusion polypeptide of the invention, comprising one or more antigenic polypeptide(s) of one or more pathogens as disclosed herein, under the control of regulatory expression sequences, especially a promoter;
- a VSV-G envelope expression plasmid comprising a polynucleotide encoding a VSV-G envelope protein in particular VSV-G of the Indiana strain or of the New Jersey strain, wherein said polynucleotide is under the control of regulating expression sequences, in particular regulatory expression sequences comprising a promoter, and;
- an encapsidation plasmid, wherein the encapsidation plasmid either comprises lentiviral, especially HIV-1, *gag-pol* coding sequences suitable for the production of integration-competent vector particles or modified *gag-pol* coding sequences suitable for the production of integration-deficient vector particles, wherein said *gag-pol* sequences are from the same lentivirus sub-family as the DNA flap element, wherein said lentiviral *gag-pol* or modified *gag-pol* sequence is under the control of regulating expression sequences.

The stable cell lines expressing the vector particles of the invention are in particular obtained by transduction of the plasmids.

The polynucleotide encodes the fusion polypeptide according to the invention, which comprises at least one antigenic polypeptide of a pathogen according to any embodiment disclosed in the present specification. In particular, it encodes a polypeptide which is a truncated mammalian, especially human, codon-optimized sequence coding for such antigenic polypeptide of a pathogen.

Accordingly, the vector plasmid may comprise one or several expression cassettes for the expression of the various antigenic polypeptides or may comprise bi-cistronic or multi-cistronic expression cassettes where the polynucleotides encoding the fusion polypeptide comprising the antigenic polypeptide(s) and optionally additional various

polypeptides are separated by an IRES sequence of viral origin (Internal Ribosome Entry Site), or it may encode fusion protein(s).

The internal promoter contained in the vector genome and controlling the expression of the polynucleotide encoding an antigenic polypeptide of the pathogen (as a transgene or in an expression cassette) may be selected from the promoters of the following genes: MHC Class I promoters, such as human beta-2 microglobulin gene (β2M promoter), or EF1α, human PGK, PPI (preproinsulin), thiodextrin, HLA DR invariant chain (P33), HLA DR alpha chain, Ferritin L chain or Ferritin H chain, Chymosin beta 4, Chimosin beta 10, or Cystatin Ribosomal Protein L41 CMVie or chimeric promoters such as GAG(CMV early enhancer / chicken β actin) disclosed in Jones S. et al (2009) or BCUAG.

A promoter among the above-cited internal promoters may also be selected for the expression of the envelope protein(s) and packaging (*gag-pol* derived) proteins.

The following particular embodiments may be carried out when preparing the lentiviral vector based on human lentivirus, and especially based on HIV-1 virus.

According to the invention, the genome of the lentiviral vector is derived from a human lentivirus, especially from the HIV lentivirus. In particular, the pseudotyped lentiviral vector is an HIV-based vector, such as an HIV-1, or HIV-2 based vector, in particular is derived from HIV-1M, for example from the BRU or LAI isolates. Alternatively, the lentiviral vector providing the necessary sequences for the vector genome may be originating from lentiviruses such as EIAV, CAEV, VISNA, FIV, BIV, SIV, HIV-2, HIV-O which are capable of transducing mammalian cells.

As stated above, when considering it apart from the recombinant polynucleotide that it finally contains, the vector genome is a replacement vector in which the nucleic acid between the 2 long terminal repeats (LTRs) in the original lentivirus genome have been restricted to cis-acting sequences for DNA or RNA synthesis and processing, including for the efficient delivery of the transgene to the nuclear of cells in the host, or at least are deleted or mutated for essential nucleic acid segments that would enable the expression of lentiviral structure proteins including biological functional GAG polyprotein and possibly POL and ENV proteins.

In a particular embodiment, the 5' LTR and 3' LTR sequences of the lentivirus are used in the vector genome, but the 3'-LTR at least is modified with respect to the 3'LTR of the original lentivirus at least in the U3 region which for example can be deleted or

partially deleted for the enhancer (delta U3). The 5'LTR may also be modified, especially in its promoter region where for example a Tat-independent promoter may be substituted for the U3 endogenous promoter.

In a particular embodiment the vector genome comprises one or several of the coding sequences for Vif-, Vpr, Vpu- and Nef-accessory genes (for HIV-1 lentiviral vectors). Alternatively, these sequences can be deleted independently or each other or can be non-functional (second-generation lentiviral vector).

The vector genome of the lentiviral vector particles comprises, as an inserted cis-acting fragment, at least one polynucleotide consisting in the DNA flap element or containing such DNA flap element. In a particular embodiment, the DNA flap is inserted upstream of the polynucleotide encoding the fusion polypeptide of the invention carrying the antigenic polypeptide(s) and is advantageously - although not necessarily - located in an approximate central position in the vector genome. A DNA flap suitable for the invention may be obtained from a retrovirus, especially from a lentivirus, in particular a human lentivirus especially a HIV-1 retrovirus, or from a retrovirus-like organism such as retrotransposon. It may be alternatively obtained from the CAEV (Caprine Arthritis Encephalitis Virus) virus, the EIAV (Equine Infectious Anaemia Virus) virus, the VISNA virus, the SIV (Simian Immunodeficiency Virus) virus or the FIV (Feline Immunodeficiency Virus) virus. The DNA flap may be either prepared synthetically (chemical synthesis) or by amplification of the DNA providing the DNA Flap from the appropriate source as defined above such as by Polymerase chain reaction (PCR). In a more preferred embodiment, the DNA flap is obtained from an HIV retrovirus, for example HIV-1 or HIV-2 virus including any isolate of these two types.

The DNA flap (also designated cPPT/CTS) (defined in Zennou V. et al. ref 27, 2000, Cell vol 101, 173-185 or in WO 99/55892 and WO 01/27304), is a structure which is central in the genome of some lentiviruses especially in HIV, where it gives rise to a 3-stranded DNA structure normally synthesized during especially HIV reverse transcription and which acts as a cis-determinant of HIV genome nuclear import. The DNA flap enables a central strand displacement event controlled in *cis* by the central polypurine tract (cPPT) and the central termination sequence (CTS) during reverse transcription. When inserted in lentiviral-derived vectors, the polynucleotide enabling the DNA flap to be produced during reverse-transcription, stimulates gene transfer

efficiency and complements the level of nuclear import to wild-type levels (Zennou et al., Cell, 2000 Cell vol 101, 173-185 or in WO 99/55892 and WO 01/27304).

Sequences of DNA flaps have been disclosed in the prior art, especially in the above cited patent applications. These sequences are also disclosed in the sequence of the pTRIP vector herein described. They are preferably inserted as a fragment, optionally with additional flanking sequences, in the vector genome, in a position which is preferably near the centre of said vector genome. Alternatively they may be inserted immediately upstream from the promoter controlling the expression of the polynucleotide(s) encoding the fusion polypeptide of the invention. Said fragments comprising the DNA flap, inserted in the vector genome may have a sequence of about 80 to about 200 bp, depending on its origin and preparation.

According to a particular embodiment, a DNA flap has a nucleotide sequence of about 90 to about 140 nucleotides.

In HIV-1, the DNA flap is a stable 99-nucleotide-long plus strand overlap. When used in the genome vector of the lentiviral vector of the invention, it may be inserted as a longer sequence, especially when it is prepared as a PCR fragment. A particular appropriate polynucleotide comprising the structure providing the DNA flap is a 124-base pair polymerase chain reaction (PCR) fragment encompassing the cPPT and CTS regions of the HIV-1 DNA.

It is specified that the DNA flap used in the genome vector and the polynucleotides of the encapsidation plasmid encoding the GAG and POL polyproteins should originate from the same lentivirus sub-family or from the same retrovirus-like organism.

Preferably, the other cis-activating sequences of the genome vector also originate from the same lentivirus or retrovirus-like organism, as the one providing the DNA flap.

The vector genome may further comprise one or several unique restriction site(s) for cloning the recombinant polynucleotide.

In a preferred embodiment, in said vector genome, the 3' LTR sequence of the lentiviral vector genome is devoid of at least the activator (enhancer) and possibly the promoter of the U3 region. In another particular embodiment, the 3' LTR region is devoid of the U3 region (delta U3). In this respect, reference is made to the description in WO 01/27300 and WO 01/27304.

In a particular embodiment, in the vector genome, the U3 region of the LTR 5' is replaced by a non lentiviral U3 or by a promoter suitable to drive tat-independent

primary transcription. In such a case, the vector is independent of *tat* transactivator (third generation vector).

The vector genome also comprises the psi (ψ) packaging signal. The packaging signal is derived from the N-terminal fragment of the *gag* ORF. In a particular embodiment, its sequence could be modified by frameshift mutation(s) in order to prevent any interference of a possible transcription/translation of gag peptide, with that of the transgene.

The vector genome may optionally also comprise elements selected among a splice donor site (SD), a splice acceptor site (SA) and/or a Rev-responsive element (RRE).

According to a particular embodiment, the vector plasmid (or added genome vector) comprises the following cis-acting sequences for a transgenic expression cassette:

1. The LTR sequence (Long-Terminal Repeat), required for reverse transcription, the sequences required for transcription and including optionally sequences for viral DNA integration. The 3' LTR is deleted in the U3 region at least for the promoter to provide SIN vectors (Self-inactivating), without perturbing the functions necessary for gene transfer, for two major reasons: first, to avoid trans-activation of a host gene, once the DNA is integrated in the genome and secondly to allow self-inactivation of the viral *cis*-sequences after retrotranscription. Optionally, the tat-dependent U3 sequence from the 5'-LTR which drives transcription of the genome is replaced by a non endogenous promoter sequence. Thus, in target cells only sequences from the internal promoter will be transcribed (transgene).
2. The ψ region, necessary for viral RNA encapsidation.
3. The RRE sequence (REV Responsive Element) allowing export of viral messenger RNA from the nucleus to the cytosol after binding of the *Rev* protein.
4. The DNA flap element (cPPT/CTS) to facilitate nuclear import.
5. Optionally post-transcriptional regulatory elements, especially elements that improve the expression of fusion polypeptide and/or antigenic polypeptide in dendritic cells, such as the WPRE *cis*-active sequence (Woodchuck hepatitis B virus Post-Responsive Element) also added to optimize stability of mRNA (Zufferey et al., 1999), the matrix or scaffold attachment regions (SAR and MAR sequences) such as those of the immunoglobulin-kappa gene (Park F. et al Mol Ther 2001; 4: 164-173).

The lentiviral vector of the invention is non replicative (replication-incompetent) *i.e.,* the vector and lentiviral vector genome are regarded as suitable to alleviate concerns regarding replication competent lentiviruses and especially are not able to form new particles budding from the infected host cell after administration. This may be achieved in well-known ways as the result of the absence in the lentiviral genome of the *gag*, *pol* or *env* genes, or their absence as "functional genes". The *gag* and *pol* genes are thus, only provided in *trans.* This can also be achieved by deleting other viral coding sequence(s) and/or cis-acting genetic elements needed for particles formation.

By "*functional*" it is meant a gene that is correctly transcribed, and/or correctly expressed. Thus, if present in the lentiviral vector genome of the invention in this embodiment contains sequences of the *gag*, *pol*, or *env* are individually either not transcribed or incompletely transcribed; the expression "*incompletely transcribed*" refers to the alteration in the transcripts gag, gag-pro or gag-pro-pol, one of these or several of these being not transcribed. Other sequences involved in lentiviral replication may also be mutated in the vector genome, in order to achieve this status. The absence of replication of the lentiviral vector should be distinguished from the replication of the lentiviral genome. Indeed, as described before, the lentiviral genome may contain an origin of replication ensuring the replication of the lentiviral vector genome without ensuring necessarily the replication of the vector particles.

In order to obtain lentiviral vectors according to the invention, the vector genome (as a vector plasmid) must be encapsidated in particles or pseudo-particles. Accordingly, lentiviral proteins, except the envelope proteins, have to be provided *in trans* to the vector genome in the producing system, especially in producing cells, together with the vector genome, having recourse to at least one encapsidation plasmid carrying the *gag* gene and either the *pol* lentiviral gene or an integrative-incompetent *pol* gene, and preferably lacking some or all of the coding sequences for *Vif*-, *Vpr*, *Vpu*- and *Nef*-accessory genes and optionally lacking *Tat* (for HIV-1 lentiviral vectors).

A further plasmid is used, which carries a polynucleotide encoding the envelope pseudotyping protein(s) selected for pseudotyping lentiviral vector particles.

In a preferred embodiment, the packaging plasmid encodes only the lentiviral proteins essential for viral particle synthesis. Accessory genes whose presence in the plasmid could raise safety concerns are accordingly removed. Accordingly, viral proteins brought in *trans* for packaging are respectively as illustrated for those originating from HIV-1:

1. GAG proteins for building of the matrix (MA, with apparent Molecular Weight p17), the capsid (CA, p24) and nucleocapsid (NC, p6).
2. POL encoded enzymes: integrase, protease and reverse transcriptase.
3. TAT and REV regulatory proteins, when TAT is necessary for the initiation of LTR-mediated transcription; TAT expression may be omitted if the U3 region of 5'LTR is substituted for a promoter driving tat-independent transcription. REV may be modified and accordingly used for example in a recombinant protein which would enable recognition of a domain replacing the RRE sequence in the vector genome, or used as a fragment enabling binding to the RRE sequence through its RBD (RNA Binding Domain).

In order to avoid any packaging of the mRNA generated from the genes contained in the packaging plasmid in the viral particles, the ψ region is removed from the packaging plasmid. A heterologous promoter is inserted in the plasmid to avoid recombination issues and a poly-A tail is added 3' from the sequences encoding the proteins. Appropriate promoters have been disclosed above.

The envelope plasmid encodes the envelope protein(s) for pseudotyping which are disclosed herein, under the control of an internal promoter, as disclosed herein.

Any or all the described plasmids for the preparation of the lentiviral vector particles of the invention may be codon optimized (CO) in the segment encoding proteins. Codon optimization according to the invention is preferably performed to improve translation of the coding sequences contained in the plasmids, in mammalian cells, murine or especially human cells. According to the invention, codon optimization is especially suited to directly or indirectly improve the preparation of the vector particles or to improve their uptake by the cells of the host to whom they are administered, or to improve the efficiency of the transfer of the polynucleotide encoding the fusion polypeptide comprising the antigenic polypeptide (transgene) in the genome of the transduced cells of the host. Methods for optimizing codons are well known in the art and codon optimization is especially performed using available programs to that effect. Codon optimization is illustrated for the coding sequences used in the examples.

In a particular embodiment of the invention, the pseudotyped lentiviral vector is also, or alternatively, integrative-competent, thus enabling the integration of the vector genome and of the recombinant polynucleotide which it contains into the genome of the transduced cells or in the cells of the host to whom it has been administered.

In another particular embodiment of the invention, the pseudotyped lentiviral vector is also, or alternatively, integrative-incompetent. In such a case, the vector genome and thus the recombinant polynucleotide which it contains do not integrate into the genome of the transduced cells or in the cells of the host to whom it has been administered.

The present invention relates to the use of a lentiviral vector wherein the expressed integrase protein is defective and which further comprises a polynucleotide especially encoding the fusion polypeptide of the invention, in particular comprising at least one antigenic polypeptide carrying epitope(s) of a pathogen, in an immunogenic composition.

By "integration-incompetent", it is meant that the integrase, preferably of lentiviral origin, is devoid of the capacity of integration of the lentiviral genome into the genome of the host cells *i.e.,* an integrase protein mutated to specifically alter its integrase activity.

Integration-incompetent lentiviral vectors are obtained by modifying the *pol* gene encoding the Integrase, resulting in a mutated *pol* gene encoding an integrative deficient integrase, said modified *pol* gene being contained in the encapsidation plasmid. Such integration-incompetent lentiviral vectors have been described in patent application WO 2006/010834. Accordingly the integrase capacity of the protein is altered whereas the correct expression from the encapsidation plasmid of the GAG, PRO and POL proteins and/or the formation of the capsid and hence of the vector particles, as well as other steps of the viral cycle, preceding or subsequent to the integration step, such as the reverse transcription, the nuclear import, stay intact. An integrase is said defective when the integration that it should enable is altered in a way that an integration step takes place less than 1 over 1000, preferably less than 1 over 10000, when compared to a lentiviral vector containing a corresponding wild-type integrase.

In a particular embodiment of the invention, the defective integrase results from a mutation of class 1, preferably amino acid substitutions (one-amino acid substitution) or short deletions fulfilling the requirements of the expression of a defective integrase. The mutation is carried out within the *pol* gene. These vectors may carry a defective integrase with the mutation D64V in the catalytic domain of the enzyme, which specifically blocks the DNA cleaving and joining reactions of the integration step. The D64V mutation decreases integration of pseudotyped HIV-1 up to 1/10,000 of wild

type, but keep their ability to transduce non dividing cells, allowing efficient transgene expression.

Other mutations in the *pol* gene which are suitable to affect the integrase capacity of the integrase of HIV-1 are the following: H12N, H12C, H16C, H16V, S81 R, D41A, K42A, H51A, Q53C, D55V, D64E, D64V, E69A, K71A, E85A, E87A, D116N, D116I, D116A, N120G, N120I, N120E, E152G, E152A, D-35-E, K156E, K156A, E157A, K159E, K159A, K160A, R166A, D167A, E170A, H171A, K173A, K186Q, K186T, K188T, E198A, R199C, R199T, R199A, D202A, K211A, Q214L, Q216L, Q221 L, W235F, W235E, K236S, K236A, K246A, G247W, D253A, R262A, R263A and K264H.

In a particular embodiment, mutation in the *pol* gene is performed at either of the following positions D64, D116 or E152, or at several of these positions which are in the catalytic site of the protein. Any substitution at these positions is suitable, including those described above.

Another proposed substitution is the replacement of the amino acid residues RRK (positions 262 to 264) by the amino acids residues AAH.

In a particular embodiment of the invention, when the lentiviral vector is integration-incompetent, the lentiviral genome further comprises an origin of replication (ori), whose sequence is dependent on the nature of cells where the lentiviral genome has to be expressed. Said origin of replication may be from eukaryotic origin, preferably of mammalian origin, most preferably of human origin. It may alternatively be of viral origin, especially coming from DNA circular episomic viruses, such as SV40 or RPS.

It is an advantageous embodiment of the invention to have an origin or replication inserted in the lentiviral genome of the lentiviral vector of the invention. Indeed, when the lentiviral genome does not integrate into the cell host genome (because of the defective integrase), the lentiviral genome is lost in cells that undergo frequent cell divisions; this is particularly the case in immune cells, such as B or T cells. The presence of an origin of replication ensures that at least one lentiviral genome is present in each cell, even after cell division, accordingly maximizing the efficiency of the immune response.

The lentiviral vector genome of said lentiviral vectors of the invention may especially be derived from HIV-1 plasmid pTRIPΔU3.CMV-GFP deposited at the CNCM (Paris, France) on October 11, 1999 under number I-2330 (also described in WO01/27300) or variants thereof. The lentiviral vector genome of said lentiviral vectors of the

invention may also be derived from HIV-1 plasmid pFlap-SP1beta2m-GFP-WPREm deposited at the CNCM (Paris, France) on February 16, 2021 under number CNCM I-5657 or variants thereof.

When the vector genome is derived from these particular plasmids, a sequence of a recombinant polynucleotide encoding the fusion polypeptide of the invention, in particular comprising an antigenic polypeptide of a pathogen as disclosed in the present application, is inserted therein, in addition or in replacement of the GFP coding fragment. The CMV promoter may also be substituted by another promoter, especially one of the promoters disclosed above, especially in relation to the expression of the transgene.

The WPRE sequence also contained in the particular deposited vectors may optionally be deleted.

Vector particles may be produced after transfection of appropriate cells (such as mammalian cells or human cells, such as Human Embryonic Kidney cells illustrated by 293 T cells) by said plasmids, or by other processes. In the cells used for the expression of the lentiviral particles, all or some of the plasmids may be used to stably express their coding polynucleotides, or to transiently or semi-stably express their coding polynucleotides.

The concentration of particles produced can be determined by measuring the P24 (capsid protein for HIV-1) content of cell supernatants.

The lentiviral vector of the invention, once administered into the host, infects cells of the host, possibly specific cells, depending on the envelope proteins it was pseudotyped with. The infection leads to the release of the lentiviral vector genome into the cytoplasm of the host cell where the retro-transcription takes place. Once under a triplex form (via the DNA flap), the lentiviral vector genome is imported into the nucleus, where the polynucleotide(s) encoding polypeptide(s) of antigen(s) of the pathogen is (are) expressed via the cellular machinery. When non-dividing cells are transduced (such as DC), the expression may be stable. When dividing cells are transduced, such as B cells, the expression is temporary in absence of origin of replication in the lentiviral genome, because of nucleic acid dilution and cell division. The expression may be longer by providing an origin of replication ensuring a proper diffusion of the lentiviral vector genome into daughter cells after cell division. The stability and/or expression may also be increased by insertion of MAR (Matrix

Associated Region) or SAR (Scaffold Associated Region) elements in the vector genome.

Indeed, these SAR or MAR regions are AT-rich sequences and enable to anchor the lentiviral genome to the matrix of the cell chromosome, thus regulating the transcription of the polynucleotide encoding the fusion polypeptide of the invention comprising at least one antigenic polypeptide, and particularly stimulating gene expression of the transgene and improving chromatin accessibility.

If the lentiviral genome is non integrative, it does not integrate into the host cell genome. Nevertheless, the at least one polypeptide encoded by the transgene is sufficiently expressed and longer enough to be processed, associated with MHC molecules and finally directed towards the cell surface. Depending on the nature of the polynucleotide(s) encoding antigenic polypeptide(s) of a pathogen, the at least one polypeptide epitope associated with the MHC molecule triggers a cellular immune response.

Unless otherwise stated, or unless technically not relevant, the characteristics disclosed in the present application with respect to any of the various features, embodiments or examples of the structure or use of the lentiviral particles, especially regarding their envelope protein(s), or the recombinant polynucleotide, may be combined according to any possible combinations.

The invention further relates to a combination of compounds for separate administration to a mammalian host, which comprises at least:

(i) lentiviral vector particles of the invention which are pseudotyped with a first determined heterologous viral envelope pseudotyping protein or viral envelope pseudotyping proteins; such first pseudotyping protein may be from the New-Jersey strain of VSV;

(ii) provided separately from lentiviral vector particles in (i), lentiviral vector particles of the invention which are pseudotyped with a second determined heterologous viral envelope pseudotyping protein or viral envelope pseudotyping proteins distinct from said first heterologous viral envelope pseudotyping protein(s); such second pseudotyping protein may be from the Indiana strain of VSV.

In another embodiment of the invention, possibly in combination with the above disclosed alternative forms of the nucleic acid, the polynucleotide encoding the fusion polypeptide of the invention, comprising at least one antigenic polypeptide is

structurally modified and/or chemically modified. Illustrative thereof a polynucleotide comprises a Kozak consensus sequence in its 5' region. Other nucleic acid sequences that are not of lentiviral origin may be present in the vector genome are IRES sequence(s) (Internal Ribosome entry site) suitable to initiate polypeptide synthesis WPRE sequence as post-transcriptional regulatory element to stabilize the produced RNA.

According to another embodiment of the invention, if multiple heterologous polypeptides are encoded by one vector genome, the coding sequences may optionally be separated by a linker moiety which is either a nucleic acid-based molecule or a non-nucleic acid-based molecule. Such a molecule may be a functionalized linker molecule aimed at recognizing a 3' functionalized nucleic acid to which it shall be linked. A sequence suitable to function as a linker may alternatively be a nucleic acid which encodes a self-cleaving peptide, such as a 2A peptide.

Further features and properties of the present invention, including features to be used in the embodiments described above will be described in the examples and figures which follow and may accordingly be used to characterise the invention.

SEQUENCE LISTING

SEQ ID No. 1: M40-H amino acid sequence
SEQ ID No. 2: M40-H DNA sequence
SEQ ID No. 3: M40-HA amino acid sequence
SEQ ID No. 4: M40-HA DNA sequence
SEQ ID No. 5: M40-HAP amino acid sequence
SEQ ID No. 6: M40-HAP DNA sequence
SEQ ID No. 7: M40-HAPE amino acid sequence
SEQ ID No. 8: M40-HAPE DNA sequence
SEQ ID No. 9: S40-H amino acid sequence
SEQ ID No. 10: S40-H DNA sequence
SEQ ID No. 11: S40-HAPE amino acid sequence
SEQ ID No. 12: S40-HAPE DNA sequence
SEQ ID No. 13: S40-HAPEHR amino acid sequence
SEQ ID No. 14: S40-HAPEHR DNA sequence
SEQ ID No. 15: S40-HAPEHR-20 amino acid sequence

SEQ ID No. 16: S40-HAPEHR-20 DNA sequence

SEQ ID No. 17: human Mannose-binding protein (UniProtKB - P11226), amino acid sequence

SEQ ID No. 18: human Pulmonary surfactant-associated protein (UniProtKB - P35247), amino acid sequence

SEQ ID No. 19: human homolog of ectodomain CD40L115-260 (UniProtKB – P29965), amino acid sequence

SEQ ID No. 20: human homolog of CCL20 segment 28-97 (UniprotKB - P78556), amino acid sequence

SEQ ID No. 21: pTRIP vector used for subcloning the constructs (pFlapDeltaU3 kpnI to AscI (no promoter, no transgene and no WPRE)

SEQ ID No. 22: BCUAG promoter

SEQ ID No. 23: mutant WPRE

SEQ ID No. 24: hSPD40-ESXH amino acid sequence

SEQ ID No. 25: Hrp-1:77-91 amino acid sequence

SEQ ID No. 26: RpfD:57-71 amino acid sequence

SEQ ID No. 27: RpfD:87-101 amino acid sequence

SEQ ID No. 28: forward primer specific to pFLAP plasmid

SEQ ID No. 29: reverse primer specific to pFLAP plasmid

SEQ ID No. 30: forward primer specific to the host housekeeping gene *gadph*

SEQ ID No. 31: reverse primer specific to the host housekeeping gene *gadph*

SEQ ID No. 32: sequence of Inf cluster

SEQ ID No. 33: linker motif

SEQ ID No. 34: human CL-L1 amino acid sequence

SEQ ID No. 35: human SP-A1 amino acid sequence

SEQ ID No. 36: human SP-A2 amino acid sequence

SEQ ID No. 37: human CL-P1 amino acid sequence

SEQ ID No. 38: human CL-K1 amino acid sequence

SEQ ID No. 39: EsxA amino acid sequence

SEQ ID No. 40: EspC amino acid sequence

SEQ ID No. 41: EsxH amino acid sequence

SEQ ID No. 42: PE19 amino acid sequence

SEQ ID No. 43: Hrp1 amino acid sequence

SEQ ID No. 44: RpfD amino acid sequence

LEGENDS OF THE FIGURES

Figure 1. Schematic structure of MBL or SPD collectin polymers. (**A)** Structural domains of MBL or SPD. CRD = Carbohydrate-Recognition Domain. **(B)** MBL or SPD self-assembled, collagen-like triple helixes, formed by interchain cysteine bonds. (**C**) SPD cross-shaped dodecamer. (**D**) SPD or MBL "tulip-bouquet" octodecamer. Adapted from [34]. (**E-F**) Schematic primary structure of the designed M40 **(E)** or S40 **(F)** monomers, harboring selected Mtb antigens. Crosslinking region (S), Collagen-like region (Coll), Neck region (N).

Figure 2. Properties of LV::M40 at inducing antigenic presentation by both MHC-I and-II pathways. (A) BM-DC from BALB/c (H-2^d) or C57BL/6 (H-2^b) mice were transduced (MOI = 20) with LV::M40-H, -HA, -HAP or -HAPE, under the transcriptional control of BCUAG promoter. Control cells were transduced with LV::EsxH alone. **(B)** BM-DC from BALB/c or C57BL/6 mice were incubated with successive dilutions of supernatants of HEK-293T cells, transduced (MOI = 20) for 48h by each of the indicated LV. **(C)** At day 3 after the addition of LV, or at day 1 after incubation with the HEK-293T cell supernatants or peptides, presentation of MHC-I- or -II-restricted epitopes of the EsxH, EsxA, PE19 or EspC mycobacterial antigens by DC were assessed by their co-culture with T-cell hybridomas specific to EsxH:20-28 (YB8 cell line, restricted by K^d), EsxH:74-88 (1G1 cell line, restricted by I-A^d), EsxA:1-20 (NB11 cell line, restricted by I-A^b), PE:19:1-18 (IF6 cell line, restricted by I-A^b), or EspC:45:54 (IF1 cell line, restricted by I-A^b). **(D)** BM-DC from BALB/c or C57BL/6 mice were incubated with successive dilutions of supernatants of HEK-293T cells, transduced (MOI = 20) for 48h by each of the indicated LV. Results are concentrations of IL-2 produced by T-cell hybridomas 24h after the beginning of the co-cultures. The amounts

of IL-2 produced in the co-culture supernatants are proportional to the efficacy of antigenic presentation by DC and TCR triggering.

Figure 3. Phenotypic Maturation of DC Induced by M40 or S40. (A-B) Phenotypic maturation of BM-DC from C57BL/6 mice infected at MOI of 5 with Mtb, as positive control, or incubated with supernatants from HEK-293T cells transduced (MOI = 20) with LV::EsxH alone (H = Ctrl), LV::M40-H or LV::S40-H. Expression of co-stimulatory or MHC molecules were assessed by cytometry on $CD11b^+$ $CD11c^+$ cells at 24 h p.i. **(B)** Heatmaps recapitulating the Mean Fluorescence Intensity (MFI) of CD40 or CD80 surface expression or percentages of $CD86^{hi}$, MHC-I ^{hi}, or MHC-II ^{hi} DCs.

Figure 4. T-cell immunogenicity of LV encoding for M40-H. (A) IFN-γ- or TNF-α-producing $CD8^+$ (top) or $CD4^+$ (bottom) T-cell responses, as assessed by ELISPOT at day 13 post-immunization, in the spleen of individual BALB/c mice (n = 3), immunized s.c. with 1 × 10^8 TU/mouse of LV::M40-H, harboring β2m, CMV or BCUAG promoters. Frequencies of Spot Forming Cells (SFC) were determined subsequent to in vitro stimulation of splenocytes with EsxH:20-28 (top) or EsxH:74-88 (bottom) synthetic peptide. Shown are Median with two tailed values and 95% confidence. Quantitative differences between various groups were not statistically significant (non-parametric Mann & Whitney test, $p < 0.05$). **(B)** Cytometric gating strategy of $CD4^+$ or $CD8^+$ T splenocytes and Representative IFN-γ^+ or IFN-γ^- $CD8^+$ or $CD4^+$ T cells, expressing TNF-α and/or IL-2. **(C)** Recapitulative percentages of each functional subsets within the $CD8^+$ or $CD4^+$ T-cell population from the mice immunized with LV::M40-H harboring β2m, CMV or BCUAG promoters. Shown are Mean +/- SD Quantitative differences between various groups were not statistically significant (non-parametric Mann & Whitney test, $p < 0.05$).

Figure 5. Immunogenicity of the poly-antigenic LV::M40-HAPE. (A) IFN-γ T-cell responses, as assessed by ELISPOT at day 14 post-immunization, in the spleen of individual C57BL/6 mice (n = 3), immunized s.c. with 1 × 10^8 TU/mouse of LV::M40-HAPE harboring β2m, CMV or BCUAG promoters. The frequencies of responding T cells were determined subsequent to in vitro stimulation with EsxH:3-11 (containing MHC-I-restricted epitope) or EsxA:1-20 (containing MHC-II-restricted epitope), PE10:-

1-18 (containing MHC-II-restricted epitope), or EspC:45-54 (containing MHC-I and II-restricted epitopes) synthetic peptide. Shown are Median with two tailed values and 95% confidence. Quantitative differences among the groups of mice immunized with LV::M40-HAPE, harboring β2m, CMV or BCUAG promoter, were not statistically significant (non-parametric Mann & Whitney, $p < 0.05$). **(B)** Representative gating strategy **(C)** and dot plots of TNF-α^+ *vs* IFN-γ^+ or IL-2$^+$ *vs* IFN-γ^+ inside the CD8$^+$ T splenocyte subset, subsequent to stimulation with a negative control, EsxH:3-11, or EspC:45-54 peptide. **(D-E)** Heatmap CD8$^+$ **(D)** recapitulating percentages of each CD4$^+$ **(E)** or functional subsets specific to EsxH or EspC antigens in mice immunized with LV::M40-HAPE, harboring β2m, CMV or BCUAG promoters or injected with PBS. Quantitative differences among the groups of mice immunized with LV::M40-HAPE, harboring distinct promoter were not statistically significant (non-parametric Mann & Whitney, $p < 0.05$). The immunized C57BL/6 mice were those detailed in the Figure 7.

Figure 6. Immunogenicity of the Multi-Antigenic LV::S40-HAPEHR or LV::S40-HAPEHR-20. (A) IFN-γ T-cell responses, as assessed by ELISPOT at day 13 post-immunization, in the spleen of individual C57BL/6 mice ($n = 3$), immunized s.c. with 1 × 10^8 TU/mouse of LV::S40-HAPEHR or LV::S40-HAPEHR-20. The frequencies of responding T cells were determined following in vitro stimulation with the indicated synthetic peptides. Shown are Median with two tailed values and 95% confidence. Quantitative differences among the groups of mice immunized with LV::S40-HAPEHR or LV::S40-HAPEHR-20, were not statistically significant (non-parametric Mann & Whitney, $p < 0.05$). **(B)** Epitope mapping of Hrp-1 and RfpD as determined by the pooled splenocytes from C57BL/6 mice, injected with PBS or immunized s.c. with 1 × 10^8 TU/mouse of LV::S40-HAPEHR, subsequent to stimulation with each of the individual peptides from the Hrp-1- or RfpD-derived overlapping 15-mers offset by 5 a.a.. (C) Cytometric analysis of intracellular IFN-γ versus IL-2 staining of CD4+ T splenocytes after stimulation with 10 µg/ml of the indicated peptides encompassing the immunodominant epitopes, identified in (B).

Figure 7. Features of mucosal CD4$^+$ T cells triggered by i.n. immunization with LV::S40-HAPEHR or LV::S40-HAPEHR-20. C57BL/6 mice were immunized i.n. with 1 × 10^8 TU of LV::S40-HAPEHR or LV::S40-HAPEHR-20. At 14 dpi, lung CD4$^+$ T cells were distinguished for their location within the interstitium (CD45$_{i.v}^-$) or in the

vasculature ($CD45_{i.v}^+$) by an i.v. PE-anti-CD45 mAb injection. **(A)** Profile of CD27 versus CD62L or CD45RB, and **(B)** CD103 vs CD69, or CD44 vs CXCR3 of the lung $CD4^+$ T cells of the interstitium or vasculature. **(C)** Heatmap recapitulating percentages of (poly)functional $CD4^+$ T cells specific to EsxA, PE19 or EspC in the lung interstitium or vasculature, as determined by ICS. Results, representative of 2 independent experiments, are from pooled lungs per group to reach enough cells for accurate cytometric analyses.

Figure 8. Features of mucosal $CD8^+$ T cells induced by i.n. immunization with LV::S40-HAPEHR or LV::S40-HAPEHR-20. The immunized C57BL/6 mice are those studied in the Figure 7. **(A)** Shown are lung $CD8^+$ T cells, distinguished for their location within the interstitium ($CD45_{i.v}^-$) or in the vasculature ($CD45_{i.v}^+$). Profile of CD27 versus CD62L or CD45RB, and **(B)** CD103 vs CD69, or CD44 vs CXCR3 of the lung $CD8^+$ T cells from the interstitium or vasculature. **(C)** Heatmap recapitulating percentages of (poly)functional $CD8^+$ T cells specific to EsxH or EspC in the lung interstitium or vasculature, as determined by ICS. Results, representative of 2 independent experiments, are from pooled lungs per group to reach enough cells for accurate cytometric analyses.

Figure 9. Protective potential of an optimized poly-antigenic LV as a booster against Mtb. (A) Time line of prime-boost-challenge performed in C57BL/6 mice (n = 5-9/group). **(B)** Mtb burdens as quantitated by CFU counting in the lungs and spleen of mice at week 5 post challenge. NS = not significant, *, **, *** = statistically significant, as determined by One Tail Mann Whitney test, p = 0.0415, p = 0.0040, p = 0.00105, respectively.

Figure 10. Sensitivity of the T-cell hybridomas used in the Mtb antigen presentation assays. BM-DC from BALB/c ($H\text{-}2^d$) or C57BL/6 ($H\text{-}2^b$) mice were incubated with various concentrations of homologous or negative control peptides. At day 1, presentation of MHC-I- or -II-restricted epitopes were assessed by use of T-cell hybridomas specific to EsxH:20-28 (YB8, restricted by K^d), EsxH:74-88 (1G1, restricted by $I\text{-}A^d$), EsxA:1-20 (NB11, restricted by $I\text{-}A^b$), PE:19:1-18 (IF6, restricted by $I\text{-}A^b$), or EspC:45:54 (IF1, restricted by $I\text{-}A^b$). Results are concentrations of IL-2 produced by T-cell hybridomas 24h after the T-cell addition.

Figure 11 (A) Schematic description of the three used promoters. β2m promoter (RefSeq: LRG-1215; from 4556 to 5070), hCMV promoter (RefSeq: MN920393.1; from 174188 to 174714), BCUAG is a combination of β2m and hCMV promoters with the addition of "Inf" (Inflammation-related) cluster, a set of cis-regulating motifs associated with inflammation. **(B)** Sequence of Inf Cluster. Cis regulating motifs and associated transcriptional factor are indicated under the sequence.

Figure 12. Map of the segment of the pFLAP plasmid containing M40 or S40 harboring the selected mycobacterial antigens, under β2m, CMV or BCUAG promoter. The codon-optimized cDNA sequences, encoding for EsxH variants or poly-antigenic fusion proteins of vaccine interest, were inserted under the SP1-β2m promoter [36] in a pFLAP backbone plasmid.

Figure 13. Structure of a human SPD-40 polypeptide harboring the antigen EsxH. P1-106: fragment of Pulmonary surfactant-associated protein D (UniProtKB - P35247 p1-106), ensures secretion via signal peptide (p1-20), enables antigen access to APC surrounding transduced cells and 2 levels of multimerization: collagen-like domain (p46-106), generates a trimerization with hydrogen bounds and cystein-rich region (p21-45), generates a covalent multimerization (n>3) via disulfide bounds. The scaffold is expected to increased bioavailability and CD40L functionality mimicking its natural trimerization. P208-273: fragment from Mannose-binding protein C (UniProtKB - P11226 p65-130). Multimerization via collagen-like domain (p208-243) and coiled-coil region (p244-273). Coiled-coil region is also a rigid spacer between transgene and CD40L ectodomain preventing deleterious interactions. P277-422: fragment of CD40L ectodomain (UniProtKB - P29965 p116-261). Ensures APC targeting and maturation through interaction with its native receptor CD40. Peptide sequence starts after EMQK motif preventing the natural methionine cleavage that occurs in wild type CD40L full-length.

EXAMPLES

Introduction

Lentiviral Vectors (LV) provide one of the most efficient vaccine platforms, relied on their outstanding potential of gene transfer to the nuclei of the host cells, including notably Antigen Presenting Cells (APC). Such nuclear transfer of genes initiates expression of antigens which readily access the Major Histocompatibility Complex Class-I (MHC-I) presentation machinery, i.e., proteasome, for further triggering of $CD8^+$ T cells [1-3]. In net contrast with their substantial ability at routing the endogenously produced antigens into the MHC-I pathway, viral vectors, including LV, are barely effective or inoperative in delivery of non-secreted antigens to the endosomal MHC-II compartment (MIIC) and unable to trigger $CD4^+$ T cells. Although $CD8^+$ T cells contribute largely to the immune control of infectious diseases or tumor growth, $CD4^+$ T cells are the major immune players. In addition to their long lifespan and their own direct effector functions, $CD4^+$ T cells orchestrate the immune system by regulating innate immunity, tailoring B-cell responses and supporting $CD8^+$ T-cell effector functions [4]. Therefore, leveraging the potential of LV to induce $CD4^+$ T cells will maximize their success rate in vaccine strategies.

Implication of $CD4^+$ T cells is notably of utmost importance in the immune protection against the leading cause of death from a single infectious agent, *Mycobacterium tuberculosis* (Mtb), the etiologic agent of human pulmonary tuberculosis (TB) [5]. During the chronic infection, this intracellular bacillus is lodged inside the phagosomes of infected phagocytes, which results in the presentation of its antigens essentially by MHC-II molecules. Consequently, it is via MHC-II that the adaptive immune effector cells can recognize the infected cells to eradicate them or to strengthen their intracellular microbicidal arsenal [6, 7]. In order to develop a poly-antigenic and multistage anti-TB subunit vaccine, we engineered a new generation of LV able to induce MHC-II antigen presentation, resulting in $CD4^+$ T-cell initiation. In our rational design, we also took into the account the fact that direct delivery of antigens to APC, by their addressing to appropriate co-stimulatory cell surface receptors, substantially increases the immunogenicity by lowering the threshold of required antigen amounts and by providing a slight and local adjuvant effect [8, 9].

We generated a platform of LV encoding for secreted monomers of collagen-containing C-type lectins (collectins), i.e., Mannan-Binding Lectin (MBL) or *Surfactant-associated* Protein D (SPD) [10], as scaffold protein carriers. The latter are engineered

to harbor multiple Mtb immunogens and the ectodomain of the Tumor Necrosis Factor (TNF) family member CD40 ligand (CD40L, CD154), with the perspective of targeting and activating APC via the co-stimulatory receptor CD40 [11]. Transduction of host cells with such LV results in secretion of antigen-bearing MBL-CD40L ("M40") or SPD-CD40L ("S40") monomers. Such monomers can spontaneously self-assemble into helicoidal trimers, as the first structural units. This leads potentially to a CD40L homo-trimeric configuration, required to cluster CD40. In their turn, the trimers can further tetra- or hexamerize to form soluble macromolecule carriers, able to circulate in biological fluids or be locally taken up by bystander APC. Therefore, such macromolecule carriers can be more efficiently delivered to $CD40^+$ APC and notably dendritic cells (DC), in which they gain access to MIIC. Moreover, the C-ter CD40L trimeric motifs will mimic the trivalent membrane CD40L on $CD4^+$ T cells to activate DC, similar to an adjuvant. Our results provided proof-of-concept evidences that, in contrast to conventional LV, this new generation of LV encoding for M40 or S40 carriers which bear multistage Mtb immunogens: (i) induces MHC-II-restricted antigen presentation, (ii) triggers $CD4^+$ — and $CD8^+$ — T cells when used in systemic or intranasal (i.n.) immunization, and (iii) displays a significant booster protective effect in the TB mouse model. This innovating approach can be largely extended to LV-based vaccine candidates against numerous other bacterial or viral infectious diseases or cancers, with the critical advantage of inducing robust $CD4^+$ T-cell responses, a rare property for a viral vector vaccine.

Results

Rational selection of antigens

To generate an LV-based vaccine vector encoding for M40 or S40 antigen carriers and potentially usable in an infectious disease controllable by $CD4^+$ T cells, we selected EsxA, EspC (ESX-1 secretion-associated protein C), EsxH, PE19, Hypoxic response protein 1 (Hrp1), and Resuscitation promoting factor D (RpfD) immunogens from Mtb **(Table 1)**. EsxA and EspC virulence factors are strongly immunogenic, due to their small size and their active secretion through the ESX-1 Type VII Secretion System (T7SS), which favor their access to MHC presentation machineries of the host phagocytes [12, 13]. The highly immunogenic EsxH [14-16], secreted through the ESX-3 T7SS, has shown protective potential in several subunit vaccine candidates. EsxH,

and its close relative EsxR, are present in all the Mtb clinical isolates so far studied [17]. Inclusion of PE19 from the large family of PE/PPE Mtb proteins, secreted through ESX-5 T7SS [13, 18-23], was based on its contents in T-cell epitopes, shared by numerous homologous members of the large PE multigenic family, with various expression profiles at the distinct stages of infection, which may generate a constant display of such shared T-cell epitopes during the course of infection [20, 23-25].

As Mtb evolves from acute to persistence phase, its adaptation to starvation, hypoxia, nitrogen stress or host immune pressure, is regulated by the *dosRS* two-component regulatory system [26], which initiates transcription of 48 genes, including *rv2626c*, among the most strongly induced [27]. The resulted Hrp1 can be target of the host adaptive immunity when the bacilli are quiescent within the granuloma [28]. Immunity to Hrp1 may dampen latent TB reactivation. In addition, Mtb possesses five Resuscitation promoting factors (RpfA-E). Rpf are cell-wall associated or secreted enzymes with peptidoglycan hydrolase, trans-glycosylase and lytic activities. These enzymes contribute to biosynthesis of muropeptides, involved in cell wall remodeling during the bacterial division, at both acute or reactivation phases [29-31]. We selected RpfD because of its demonstrated immunogenicity in both mice [32] and latent TB humans [33]. Both Hrp-1 and Rpf lack human homologs.

Design of an LV encoding for collectin scaffolds harboring Mtb antigens and a DC targeting segment

Collectins are soluble Pattern Recognition Receptors (PRRs), able to bind oligosaccharides or lipids at the surface of microorganisms and thereby contributing to their elimination by opsonization or complement activation [34]. Among collectins, MBL and SPD are composed of four distinct segments: (i) N-terminal cysteine-rich crosslinking domain, (ii) collagen-like domain, (iii) α-helical neck domain, and (iv) Carbohydrate-Recognition Domain (CRD) **(Figure 1A)**. A self-assembled collagen-like triple helix forms the first structural MBL or SPD unit **(Figure 1B)**. SPD triple-subunits themselves can tetramerize to form cross-shaped dodecamer **(Figure 1C)**. SPD or MBL triple-subunit can also hexamerize to form “tulip-like nano-bouquet” octodecamer **(Figure 1D)**. The resulted secreted polymers are soluble [34]. We first engineered the murine MBL to harbor complete sequences of: (i) EsxH alone, (ii) EsxH and EsxA, (iii) EsxH, EsxA, and PE19, or (iv) EsxH, EsxA, PE19 and EspC within the collagen-like

region, while replacing its CRD by the murine $CD40L_{115-260}$ ectodomain. The prospective MBL polymers will be referred to as "M40-H", "M40-HA", "M40-HAP", or "M40-HAPE", respectively **(Figure 1E, Table S1)**.

In parallel, we engineered SPD to harbor: (i) EsxH alone, (ii) EsxH, EsxA, PE19, EspC, or (iii) EsxH, EsxA, PE19, EspC, Hrp1 and $RpfD_{42-154}$ within the collagen-like region and we substituted its CRD by $CD40L_{115-260}$ **(Figure 1F, Table S1)**. The expected SPD polymers are referred to as "S40-H", "S40-HAPE", or "S40-HAPEHR", respectively. To confer some chemo-attracting properties to the resultant fusion protein, we also designed an S40-HAPEHR, which harbors the murine $CCL20_{28-97}$ segment within the collagen-like domain ("S40-HAPEHR-20"). CCL20 is the CCR6 ligand, largely involved in the migration and recruitment of DC and lymphocytes [35].

Induction of MHC-II-restricted antigen presentation by LV::M40

DCs (H-2^d or H-2^b) were directly transduced with LV::M40-H, -HA, -HAP or -HAPE, using the BCUAG promoter. Control DC were transduced with a conventional LV encoding for EsxH without being inserted into an engineered scaffold. Three days post-transduction, the DCs were co-cultured with T-cell hybridomas, specific to the immunodominant epitopes of each of the Mtb antigens. The DCs transduced with either LV were largely able to induce presentation of EsxH via MHC-I **(Figure 2A)**. In contrast to the conventional LV::EsxH, the LV encoding for M40-H, -HA, -HAP or -HAPE induced the presentation of EsxH, EsxA and PE19, when these antigens were included. No MHC-II presentation of EspC was detected in this context, which can be explained by the relatively weak sensitivity of the anti-EspC T-cell hybridoma **(Figure 10)**. To evaluate whether M40 or S40 carriers secreted by transduced cells induce presentation through MHC-II, DC were incubated with successive dilutions of M40-H-, -HA-, -HAP-, or -HAPE-containing supernatants from transduced HEK-293T cells **(Figure 2B)**. At day 1 after incubation, co-culture of the DCs with T-cell hybridomas, showed that these DCs were unable to present EsxH via MHC-I, strongly suggesting that endocytosis/micropinocytosis or CD40-mediated cell entry of the M40 carrier does not allow their access to MHC-I machinery, in contrast to observations made by others [38]. In net contrast, DCs incubated with M40-H-, -HA-, -HAP-, or -HAPE-containing supernatants were very efficient at inducing presentation of the respective antigens via MHC-II, including EspC. It was noticed that the intensity of antigen presentation had a

propensity to decrease with growing number of antigens carried by the M40 scaffold **(Figure 2A, B)**. In a mutually non-exclusive manner, this may result from: (i) a slight structural instability of the carriers with the insertion of increasing number of antigens, (ii) a competition among the multiple T-cell epitopes for the available MHC presentation sites.

Direct transduction of DCs with LV::S40-HAPE, -HAPEHR or -HAPEHR-20 induced also efficacious MHC-I- or -II-restricted presentation of the selected Mtb antigens **(Figure 2C)**. Incubation of DC with successive dilutions of supernatants from HEK-293T cells transduced with LV::S40-HAPE, -HAPEHR or -HAPEHR-20 induced MHC-II-restricted presentation of the Mtb antigens **(Figure 2D)**. In the absence of identified T-cell epitope or T-cell hybridoma specific to Hrp1 or RpfD, the immunogenicity of these antigens in the context of the developed vectors was studied in vivo, as detailed below. Notably, the addition of Hrp1 and RpfD and CCL20 to the S40 scaffold did not impact the efficacy of the presentation of the other antigens. The assay performed with DC incubated with synthetic peptides harboring the homologous T-cell epitopes showed the sensitivity of the T-cell hybridoma-based presentation assay **(Figure 10)**.

These results showed that, in net opposition to conventional LV, this new generation of LV encoding for secreted scaffolds which can incorporate numerous antigens and immune mediators, possess a strong capacity at inducing MHC-II-restricted antigen presentation and thus provide a valuable platform for both $CD4^+$ and $CD8^+$ T cell induction.

M40 and S40 potential at inducing DC maturation

To evaluate the potential of M40 and S40 carriers to induce DC maturation, BM-DCs were incubated with supernatants from HEK-293T cells transduced with LV::M40-H or LV::S40-H. In parallel, DCs were incubated with supernatants from HEK-293T cells transduced with the conventional LV::H, as a negative control or were infected with Mtb, as a positive control. The expression of surface co-stimulatory and MHC molecules was assessed on $CD11b^+$ $CD11c^+$ cells at day 1 post incubation **(Figure 3A, B)**. On DCs incubated with M40-H or S40-H, no increase of CD40 surface expression was detected, probably as a consequence of direct interaction of CD40 with M40-H or S40-H **(Figure 3A, B)**. CD80 upregulation was only detected on DCs

incubated with S40-H, while CD86 upregulation and increase in the percentages of MHC-I^{hi} or MHC-II^{hi} cells was detected on DCs incubated with M40-H or S40-H. Therefore, through induction of M40 or S40 secretion, this new generation of LV is able to induce DC maturation, instrumental for appropriate T-cell activation.

T-cell immunogenicity of LV encoding for M40 or S40 carrying a single or multiple Mtb immunogens

To assess the immunogenicity of this new generation of LV, BALB/c mice (*n* = 3/group) were immunized s.c. with LV::M40-H harboring human β2-microglobulin (β2m) promoter [36], human CytoMegaloVirus (CMV) immediate early enhancer and promoter (CMV) [37], or a composite β2m-CMV promoter ("BCUAG") to get insights on possible consequences of distinct antigen carrier transcription profile on the induction of immune responses **(Figure 11)**. At day 13 post injection (dpi), stimulation of the splenocytes with EsxH:20-28 (MHC-I) or EsxH:74-88 (MHC-II) peptides [15, 16] detected both CD8$^+$ T and CD4$^+$ T cells by ELISPOT **(Figure 4A)**. Intracellular Cytokine Staining (ICS) showed the multifunctional properties of these CD8$^+$ or CD4$^+$ **(Figure 4B, C)** T cells. Functional CD8$^+$ T cells effectors were mainly distributed among IFN-γ$^+$ (single positive), IFN-γ$^+$ TNF-α$^+$ (double positive), or IFN-γ$^+$ TNF-α$^+$ IL-2$^+$ (triple positive) subsets, while CD4$^+$ T cells were essentially IFN-γ$^+$ (single positive), or IFN-γ$^+$ TNF-α$^+$ IL-2$^+$ (triple positive) **(Figure 4C)**. It is noteworthy that conventional LV encoding EsxH as single do not induce such CD4$^+$ T-cell responses [39].

To evaluate the immunogenic potential of the developed poly-antigenic LV::M40-HAPE, C57BL/6 mice (*n* = 3/group) were immunized s.c. with LV::M40-HAPE harboring β2m, CMV or BCUAG promoter. At 14 dpi, CD8$^+$ and CD4$^+$ T splenocyte responses, specific to EsxH:3-11 (MHC-I), EsxA:1-20 (MHC-II), PE19:1-18 (MHC-II), or EspC:45-54 (MHC-I and -II) [39], were detected in all mice, as assessed by ELISPOT **(Figure 5A)**. ICS analysis of the splenocytes from the same mice showed the multifunctional properties of the induced CD8$^+$ **(Figure 5B)** or CD4$^+$ **(Figure 5C)** T cells. Functional CD8$^+$ T cell effectors were again mainly distributed among IFN-γ$^+$ single positive, IFN-γ$^+$ TNF-α$^+$ double positive, or IFN-γ$^+$ TNF-α$^+$ IL-2$^+$ triple positive subsets. CD4$^+$ T cells specific to EsxA, PE10 or EspC antigen were preferentially distributed among IFN-γ$^+$ single positive, IFN-γ$^+$ TNF-α$^+$ double positive or IFN-γ$^+$ TNF-

α^+ IL-2^+ triple positive subsets **(Figure 5D, E)**. No consistent quantitative or qualitative differences were detected in the T-cell responses in the mice immunized with LV::M40 harboring each of the distinct promoters. Again, conventional LV encoding for these Mtb proteins as a poly-antigen, is unable to induce $CD4^+$ T-cell responses [39].

We further established the induction of both $CD8^+$ and $CD4^+$ T cells specific to EsxH, EsxA, PE19 and EspC in C57BL/6 mice (*n* = 3/group) immunized s.c. with LV::S40-HAPEHR or LV::S40-HAPEHR-20 **(Figure 6A)**. The immunogenicity of Hrp-1 and RpfD was assessed by their epitope mapping by use of splenocytes from LV::S40-HAPEHR-immunized mice in ELISPOT assay **(Figure 6B)**. Hrp-1:77-91 (SIYYVDANASIQEML), RpfD:57-71 (IAQCESGGNWAANT) and RpfD:87-101 (SNGGVGSPAAASPQQ) immunogenic regions were identified.

Altogether, these results provide evidence of the induction of robust, polyfunctional $CD4^+$ T-cell responses by immunization with the developed new generation of LV.

Immunogenicity of the poly-antigenic multistage LV::S40 at the mucosal level

We then evaluated the immunogenicity of LV::S40-HAPEHR or LV::S40-HAPEHR20 in C57BL/6 mice immunized (i.n.) with 1×10^8 TU. At 14 dpi, intravenous (i.v.) injection of the immunized mice with PE-anti-CD45 mAb, 3 min before sacrifice, allowed detection of massive T-cell recruitment to the lung interstitium distinct from those in the vasculature [40]. The lung interstitial ($CD45_{i.v.-}$) $CD4^+$ **(Figure 7A)** or $CD8^+$ **(Figure 8A)** T cells of these LV::S40-HAPEHR- or LV::S40-HAPEHR20-vaccinated mice contained increased frequencies of $CD27^-$ $CD45RB^-$ $CD62L^-$ migrant effectors and $CD69^+$ $CD103^+$ resident cells **(Figure 7B, Figure 8B)** compared to their PBS-injected counterparts. Most of the $CD69^+$ $CD103^+$ $CD4^+$ or $CD8^+$ T cells were $CD44^+$ $CXCR3^+$. ICS analysis of these cells indicated the presence of (poly)functional $CD4^+$ **(Figure 7C)** or $CD8^+$ **(Figure 8C)** T cells specific to EsxA, EspC, EsxH or PE19, and essentially located in the lung interstitium.

Booster protective effect of LV::S40-HAPEHR-20 against Mtb infection

Prime-boost strategies using BCG or an improved live-attenuated vaccine for priming, and subunit vaccine candidates for boosting, is a promising approach to

improve the incomplete efficacy of BCG. To assess the booster potential of LV::S40-HAPEHR-20, C57BL/6 mice were left unvaccinated or were immunized s.c. at week 0 with 1×10^6 CFU of a genetically improved BCG, i.e., BCG::ESX-1^{Mmar} vaccine candidate [41] **(Figure 9A)**. The latter provide the opportunity to perform a prime-boost with the developed LV vaccine as this live-attenuated vaccine actively secretes EsxA and EspC. A group of BCG::ESX-1^{Mmar}-primed mice was boosted s.c. with 1×10^8 TU of LV::S40-HAPEHR-20 at week 5, and then again boosted i.n. at week 10 with the same LV to recruit the induced immune effectors to the lung mucosa. At week 12, mice were challenged with ≈ 200 CFU of Mtb H37Rv via aerosol and lung and spleen mycobacterial burdens were determined at week 17 **(Figure 9B)**. The average lung Mtb load in the primed-boosted mice was decreased by ≈ 2.5 $\log_{10}$ compared to unvaccinated controls (Mann-Whitney test, p value = 0,0005), and by ≈ 1 $\log_{10}$ compared to their BCG::ESX-1^{Mmar}-vaccinated counterparts (Mann-Whitney test, p value = 0,0415). The LV::S40-HAPEHR-20 boost resulted in a tendency to reduce in the spleen Mtb loads, which was however not statistical significance. An explanation for this is the particularly strong protective effect of ESX-1-complemented BCG strains against dissemination to the spleen [12, 42, 43].

Discussion

We developed a new generation of multifunctional LV which, in comparison to the conventional vector, has been leveraged to: (i) facilitate poly-antigen delivery, (ii) target antigens to APC that it activates, (iii) route antigens through MHC-II pathway, and (iv) induce, in addition to $CD8^+$ T cells, robust and polyfunctional $CD4^+$ T-cell response. Such LV are tailored to induce secretion of multimeric protein carriers formed by truncated collectin-based scaffolds, able to harbor several antigens, as well as protein components with adjuvant or chemo-attracting properties. This is achieved by insertion of potent immunogens within the collagen-rich regions of MBL or SPD, substituted with CD40L ectodomain at their CRD region. Self-assemblage and polymerization of the monomers produced in the LV-transduced cells in vitro or in vivo, results in secreted multimeric carriers able to interact with $CD40^+$ cells, including APC and notably DC. It is known that antigen delivery to appropriate surface receptors of DC improves the efficacy of antigenic presentation by *several orders of* magnitude [8, 44, 45]. The conventional LV per se, as prepared in our conditions, is barely inflammatory and

induces almost no DC phenotypic or functional maturation, even used at very high doses. The capacity of LV to induce transitorily minute levels of IFN-I in vivo and IFN-I signaling in DC in vivo is not linked either to its outstanding T-cell immunogenicity [39]. Unlike the conventional LV, the new generation of LV described here, induces some degrees of DC maturation, via CD40 clustering by the trimeric extremities of M40 or S40 carriers. Therefore, these vectors assemble: (i) the intrinsic and outstanding $CD8^+$ T-cell immunogenicity of the conventional LV and (ii) the properties of slight adjuvantation, antigen delivery to DC surface receptors, antigen routing to MHC-II and $CD4^+$ T-cell immunogenicity of the secreted multimeric scaffolds that they encode.

Since TB is a disease primarily controlled by $CD4^+$ T responses, as a first application, we investigated these optimized LV for their potential at inducing T-cell responses against selected Mtb antigens with preferential expression at distinct infection phases. We demonstrated in vitro a slight DC activating property of the secreted M40 or S40 carriers and their large efficiency at MHC-II- (and -I)-restricted presentation of the Mtb antigens inserted within their collagen-like domains. We then evidenced in vivo efficient induction of both (poly)functional $CD8^+$ and $CD4^+$ T-cell effectors, at the both systemic or mucosal levels, following s.c. or i.n. immunization. Notably only one shot of i.n. immunization generates high quality $CD8^+$ and $CD4^+$ T-cell effectors, with activated/effector/resident memory phenotype and located at the pulmonary interstitium. In future experiments, it will be informative to determine whether such T cells are located in the lung tertiary lymphoid organs [46].

One of the multimeric carriers, i.e., S40-HAPEHR also harbors a segment of CCL20, a strong chemo-attracting chemokine. The receptor for CCL20, CCR6 is expressed on lymphocytes and DC, thus S40-HAPEHR-20 should reinforce the recruitment of immune cells. It is admitted that prime immunization with improved live-attenuated vaccine candidates and boosting with subunit vaccines is a promising approach. We used LV::S40-HAPEHR-20 as subunit booster in the mouse TB model after prime with BCG::ESX-1^{Mmar} vaccine candidate, with largely improved protective potential compared to the parental BCG [41]. We observed that the lung Mtb burdens were statistically reduced by ~1 $\log_{10}$ after LV::S40-HAPEHR-20 boosting.

A plasmid DNA encoding SPD-CD40L has been already used as an adjuvant when mixed with another plasmid DNA encoding for the HIV-1 Gag protein and led to a

significant enhancement of $CD8^+$ T cell responses (43). In net contrast to the LV platform developed here, this plasmid adjuvant was unable to induce $CD4^+$ T-cell proliferative or cytokine production. Insertion of SPD-Gag-CD40L into an adenoviral vector serotype 5 (Ad5) has been more recently demonstrated to elicit much stronger Gag-specific $CD8^+$ T-cell responses and a protection from a Gag-expressing vaccinia virus in the mouse model [47]. Co-immunization with a plasmid DNA encoding for SPD-gp100-CD40L, bearing the tumor gp100 antigen, and plasmids encoding for IL-12p70 and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), increased immune control of the melanoma cells in mice [48]. However, induction of MHC-II-restricted antigenic presentation or $CD4^+$ T-cell initiation have not been addressed in these studies. Compared to these previous studies, our project used polymers of M40 or S40 to generate, not only $CD8^+$ T cells, but also notably (poly)functional $CD4^+$ T-cell responses and some constructs harbor the CCL20 chemoattractant component, without need for additional other adjuvant or immune-stimulatory molecules. This new property of LV at inducing $CD4^+$ T cells is of utmost importance, as $CD4^+$ T cells are major immune players, based on their: (i) long lifespan, (ii) direct effector functions, (iii) capacity at orchestrating the immune system by regulating innate immunity, (iv) helper functions at tailoring B-cell responses, and (v) helper functions at supporting $CD8^+$ T-cell effector pathways [4].

Altogether, we set up a new generation of LV vector leveraged to target and activate DCs, to route immunogens to MHC-II pathway and to induce both $CD4^+$ and $CD8^+$ T-cell responses. The applications of this innovating strategy are much larger and can be extended to vaccine LV against multiple other bacterial, viral, parasitic infectious diseases or cancers.

Material and Methods

Construction of transfer pFLAP plasmids encoding for MBL or SPD collectin scaffolds, harboring selected mycobacterial antigens, CD40L and/or CCL20

Genes encoding for *Mus musculus* Mannan-Binding Lectin (MBL) or *Surfactant-associated* Protein D (SPD), engineered to harbor selected mycobacterial antigens and/or CCL20 within their collagen-like domains, and murine CD40L ectodomain instead of their CRD, were synthetized by GenScript after codon optimisation. Each of

these genes were inserted into the sites BamHI and XhoI of the transfer pFLAPΔU3 plasmid [49]. Transcription is under control of the native human CMV, human β2-microglobulin β2m or BCUAG promoters, the two latter replacing CMV promoter after insertion between MluI and BamHI sites. The human β2-microglobulin promoter has been previously described [50]. The BCUAG promoter is a hybrid promoter comprising CMV enhancer, inflammation-related cis-regulating region and β2m core promoter **(Figure 11)**. The pFLAPΔU3 plasmid contains also a mutated WPRE (Woodchuck Posttranscriptional Regulatory Element) sequence to improve protein expression.

Plasmid amplification and purification

Plasmid DNA were amplified in DH5α *Escherichia coli* in Lysogeny Broth (LB) completed with 50 µg/ml of kanamycin. The plasmid DNA was then purified by use of the NucleoBond Xtra Maxi EF Kit (Macherey Nagel). After drying, the DNA pellets were resuspended in Tris-EDTA Endotoxin-Free (TE-EF) buffer overnight, quantitated in a NanoDrop 2000c spectrophotometer (Thermo Scientific), adjusted to 1 µg/µl in TE-EF buffer, aliquoted and stored at -20°C. The quality of the plasmid DNA was controlled: (i) either undigested or subsequent to digestion with a mixture of 2 plasmid-specific appropriate restriction enzymes prior to gel electrophoresis, and (ii) by sequencing the inserts in each pFLAP plasmid.

Production and titration of LV

Non-replicative integrative LV were produced in Human Embryonic Kidney (HEK)-293T cells, as previously detailed (Zennou et al., 2000). Briefly, 1×10^7 cells/Petri dish were cultured in DMEM and were co-transfected in a tripartite manner with 1 ml of a mixture of: (i) 2.5 µg/ml of the pSD-GP-NDK packaging plasmid, encoding for codon optimized *gag-pol-tat-rre-rev*, (ii) 10 µg/ml of VSV-G Indiana envelop plasmid, and (iii) 10 µg/ml of "transfer" pFLAP plasmid in Hepes 1X containing 125 mM of $Ca(ClO_3)_2$. Supernatants were harvested at 48h post-transfection, clarified by 6-minute centrifugation at 2500 rpm and concentrated by 1-hour ultracentrifugation at 22,000 rpm at 4°C. LV were then aliquoted in PBS 1X, PIPES 20 mM, sucrose 2.5%, NaCl 75 mM and conserved at -80°C.

To determine the titers of the produced LV, HEK-293T were distributed at 4 × 10^4 cell/well in flat-bottom 96-well-plates in complete DMEM in the presence of 8 µM aphidicolin (Sigma) to blocks the cell growth. The cells were then transduced with serial dilutions of concentrated LV. The titers, proportional to efficacy of the nuclear gene transfer, were determined as "Transduction Unit" (TU)/ml by quantitative real-time PCR on total lysates at day 3 post-transduction, by use of forward 5'-TGG AGG AGG AGA TAT GAG GG-3' and reverse 5'-CTG CTG CAC TAT ACC AGA CA-3' primers, specific to pFLAP plasmid and forward 5'-TCT CCT CTG ACT TCA ACA GC-3' and reverse 5'-CCC TGC ACT TTT TAA GAG CC-3' primers specific to the host housekeeping gene *gadph*, as described elsewhere [51].

Mice, immunization

C57BL/6JRj or BALB/cJ mice (Janvier, Le Genest Saint Isle, France) were used between the age of 7 and 10 weeks. Experimentation on mice was performed in accordance with the European and French guidelines (Directive 86/609/CEE and Decree 87-848 of 19 October 1987) subsequent to approval by the Institut Pasteur Safety, Animal Care and Use Committee, under local ethical committee protocol agreement # CETEA 2013–0036, # CETEA DAP180030, and CETEA 2012–0005 (APAFIS#14638-2018041214002048). Mice were immunized subcutaneously (s.c.) at the basis of the tail with the indicated amounts of LV contained in 200 µl. When indicated, mice were immunized intranasally (i.n.) with the indicated amounts of LV contained in 20 µl, as previously detailed [52]. The i.n. administration was realized under anesthesia, obtained by peritoneal injection of a mixture of Xylazine (Rompun, 10 mg/kg) and Ketamine (Imalgene, 100 mg/kg).

T-cell assay by ELISPOT

At day 11-14 post-immunization, splenocytes from individual mice (*n* = 3/group) were homogenized and filtered through 100 µm-pore filters and centrifuged at 1300 rpm during 5 minutes. Cells were then treated with Red Blood Cell Lysing Buffer (Sigma), washed twice in PBS and counted in a MACSQuant10 cytometric system (Miltenyi Biotec). Splenocytes were then seeded at 0.5 — 1 × 10^5 cells /well in 200 µl of RPMI-GlutaMAX, containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 × 10^{-4} M non-essential amino-acids, 1% vol/vol HEPES, 1 × 10^{-}

^{3} M sodium pyruvate and 5 × 10^{-5} M of β-mercapto-ethanol in the wells of IFN-γ or TNF-α ELISPOT plates (Mouse ELISPOT^{PLUS}, Mabtech). Cells were left unstimulated or were stimulated with 2 µg/ml of synthetic peptide (Proteogenix, Strasbourg, France), harboring the well-defined MHC-I-, or -II-restricted T-cell epitopes of each mycobacterial antigen. In parallel, splenocytes were stimulated with 2.5 µg/ml of Concanavalin A (Sigma), as a functionality control. For each individual, the assays were run in technical triplicates, following Mabtech's recommendations. Spots were quantified in an ELR04 ELISPOT reader (AID, Strassberg, Germany).

T-cell assay by intracellular cytokine staining, lung T-cell phenotyping

Splenocytes from immunized mice were obtained by tissue homogenization and passage through 100 µm-pore filter and were cultured during 6h at 8 × 10^{6} cells/well in 24-well plates in the presence of 10 µg/ml of homologous or control peptide, 1 µg/ml of anti-CD28 (clone 37.51) and 1 µg/ml of anti-CD49d (clone 9C10-MFR4.B) mAbs (BD Pharmingen). During the last 3h of incubation, cells were added with a mixture of Golgi Plug and Golgi Stop (BD Pharmingen). Cells were then collected, washed with PBS containing 3% heat-inactivated FBS and 0.1% NaN_3 (FACS buffer) and incubated for 25 minutes at 4°C with a mixture of FcγII/III receptor blocking anti-CD16/CD32 (clone 2.4G2), APC eF780-anti-CD3ε (clone 17A2), eFluor450-anti-CD4 (RM4-5) and BV711-anti-CD8α (53-6.7), mAbs (BD Pharmingen and eBioscience). Cells were then washed twice in FACS buffer, permeabilized by use of Cytofix/Cytoperm kit (BD Pharmingen). Cells were then washed twice with PermWash 1x buffer from the Cytofix/Cytoperm kit and incubated with a mixture of FITC-anti-IL-2 (clone JES6-5H4, eBioscience), PE-Dazzle-anti-TNF-α (MP6-XT22, Biolegend) and APC-anti-IFN-γ (clone XMG1.2, BD Pharmingen) mAbs or a mixture of appropriate control Ig isotypes, during 30 minutes at 4°C. Cells were then washed twice in PermWash and once in FACS buffer and then fixed with Cytofix (BD Pharmingen) overnight at 4°C. The cells were acquired in an Attune NxT cytometer system (Invitrogen). Data were analyzed by FlowJo software (Treestar, OR, USA). Lung T-cell phenotyping was performed as recently described [39].

Antigenic presentation assay

Bone-marrow derived DC were plated at 5×10^5 cells/well in 24-well plates in RPMI 1640 containing 5% FBS. Cells were transduced with LV or were loaded homologous or control synthetic peptides. At 24 h post infection 5×10^5 appropriate T-cell hybridomas [53] were added and the culture supernatants were quantitated for IL-2 production at 24h by ELISA. Synthetic peptides were synthesized by Proteogenix (Schiltigheim, France).

Protection assay

Mtb H37Rv strain or BCG::ESX-1^{Mmar} [41], were cultured in Dubos broth, complemented with Albumine, Dextrose and Catalase (ADC, Difco, Becton Dickinson, Le Pont-de-Claix, France). Experiments with pathogenic mycobacteria were performed in BSL3, following the hygiene and security recommendations of Institut Pasteur. C57BL/6 mice were primed s.c. with 1×10^6 CFU/mouse of BCG::ESX-1^{Mmar} [41] at day 0, boosted s.c. with 5×10^8 TU of SPD40-HAPEHR-20 at week 5, and boosted i.n. with 5×10^8 TU of SPD40-HAPEHR-20 at week 10. The mice were challenged 2 weeks after the mucosal boost by use of a homemade nebulizer via aerosol, as previously described [52]. Briefly, 5 ml of a suspension of 1.7×10^6 CFU/ml of Mtb H37Rv strain were aerosolized in order to deliver an inhaled dose of ≈ 200 CFU/mouse. The mice were then placed in isolator. Five weeks later, lungs or spleen of the infected mice were homogenized by using a MillMixer homogenizer (Qiagen, Courtaboeuf, France) and serial 5-fold dilutions prepared in PBS were plated on 7H11 Agar complemented with ADC (Difco, Becton Dickinson). CFU were counted after 3 weeks of incubation at 37°C. Statistical significance of inter-group Mtb load differences was determined by Mann-Whitney t-test by use of Prism v8.01 (GraphPad Software, Inc.).

Tables

Table 1. Mtb proteins rationally selected as target antigens to be incorporated in the prospective multistage anti-TB LV.

Mtb immunogen	Locus in H37Rv	Size a.a	Major characteristics

EsxA	*rv3875*	95	Early Secreted Antigenic Target 6 kDa (ESAT-6) secreted by ESX-1 T7SS
EspC	*rv3615c*	103	ESX-1 secretion-associated proteins C secreted by ESX-1 T7SS
EsxH	*rv0288*	96	Virulence-related factor (TB10.4) secreted by ESX-3 T7SS
PE19	*rv1791*	99	Virulence-related factor, with numerous homologous secreted by ESX-5 T7SS
Hrp1	*rv2626c*	143	Dormancy-related Hypoxic response protein 1
RpfD	*rv2389c*	154 (42-154)*	Reactivation-related Resuscitation promoting factor D (mb)

*Only the $RpfD_{42-154}$ ectodomain was included to minimize the hydrophobicity of the resulted protein.

Supplemental Table 1. Various M40 and S40 scaffolds designed to harbor the selected Mtb antigens and/or CCL20.

Carrier	**Antigens**	**Chemo-attractant**	**Nomenclature**	**Monomer Lenght (a.a)**
MBL40	EsxH	—	M40-H	414
	EsxH-EsxA	—	M40-HA	556
	EsxH-EsxA-PE19	—	M40-HAP	721
	EsxH-EsxA-PE19-EspC	—	M40-HAPE	885
SPD40	EsxH	—	S40-H	xx
	EsxH-EsxA-PE19-EspC	—	S40-HAPE	736
	EsxH-EsxA-PE19-EspC-Hrp1-RpfD	—	S40-HAPEHR	1004

	EsxH-EsxA-PE19-EspC-Hrp1-RpfD	CCL20	S40-HAPEHR-20	1128

Table S2. Sequences of MBL fused with selected Mtb antigens and CCL20 as coded by LV

Poly-antigenic LV	Insert length (a.a.)	Sequence
LV::M40-**EsxH** (LV::M40-H)	414	MSIFTSFLLLCVVTVVYAETLTEGVQNSCPVVTCSSPGLNGFPGKDGRDGAK GEKGEPGQGLRGLQGPPGAVGPTGPPGNPGLKGAVGPKGDRGDRGGG**SQIM YNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQA WQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG**GS GLRGLQGPPGALGPPGSVGSPGSPGPKGQKGDHGDNRAIEEKLANMEAEIRI LKSKLQLTNKLHAFSMGGGSGDEDPQIAAHVVSEANSNAASVLQWAKKGYY TMKSNLVMLENGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKP SSGSERILLKAANTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHR VGFSSFGLLKL
LV::M40-**EsxH**-***EsxA*** (LV::M40-HA)	556	MSIFTSFLLLCVVTVVYAETLTEGVQNSCPVVTCSSPGLNGFPGKDGRDGAK GEKGEPGQGLRGLQGPPGAVGPTGPPGNPGLKGAVGPKGDRGDRGGG**SQIM YNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQA WQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG**GS GFPGPPGPKGEPGSPAGRGERGFQGSPGKMGPAGSKGEPGGGSG***TEQQWNF AGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWD ATATELNNALQNLARTISEAGQAMASTEGNVTGMFA***GGGSGLRGLQGPPGA LGPPGSVGSPGSPGPKGQKGDHGDNRAIEEKLANMEAEIRILKSKLQLTNKL HAFSMGGGSGDEDPQIAAHVVSEANSNAASVLQWAKKGYYTMKSNLVMLE NGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKPSSGSERILLKAA NTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHRVGFSSFGLLKL
LV::M40-**EsxH**-***EsxA****-PE19* (LV::M40-HAP)	721	MSIFTSFLLLCVVTVVYAETLTEGVQNSCPVVTCSSPGLNGFPGKDGRDGAK GEKGEPGQGLRGLQGPPGAVGPTGPPGNPGLKGAVGPKGDRGDRGGG**SQIM YNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQA WQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG**GS GFPGPPGPKGEPGSPAGRGERGFQGSPGKMGPAGSKGEPGGGSG***TEQQWNF AGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWD ATATELNNALQNLARTISEAGQAMASTEGNVTGMFA***GGGSGLPGRDGRDG REGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERGLSG GGG*SFVTTQPEALAAAAANLQGIGTTMNAQNAAAAAPTTGVVPAAADEVSALT AAQFAAHAQMYQTVSAQAAAIHEMFVNTLVASSGSYAATEAANAAAAG*GGSG LRGLQGPPGALGPPGSVGSPGSPGPKGQKGDHGDNRAIEEKLANMEAEIRIL KSKLQLTNKLHAFSMGGGSGDEDPQIAAHVVSEANSNAASVLQWAKKGYYT MKSNLVMLENGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKPS SGSERILLKAANTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHRV GFSSFGLLKL
LV::M40-**EsxH**-***EsxA****-PE19*-**<u>EspC</u>** (LV::M40-HAPE)	885	MSIFTSFLLLCVVTVVYAETLTEGVQNSCPVVTCSSPGLNGFPGKDGRDGAK GEKGEPGQGLRGLQGPPGAVGPTGPPGNPGLKGAVGPKGDRGDRGGG**SQIM YNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQA WQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG**GS GFPGPPGPKGEPGSPAGRGERGFQGSPGKMGPAGSKGEPGGGSG***TEQQWNF AGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWD ATATELNNALQNLARTISEAGQAMASTEGNVTGMFA***GGGSGLPGRDGRDG REGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERGLSG GGG*SFVTTQPEALAAAAANLQGIGTTMNAQNAAAAAPTTGVVPAAADEVSALT AAQFAAHAQMYQTVSAQAAAIHEMFVNTLVASSGSYAATEAANAAAAG*GCPG LPGAAGPKGEAGAKGDRGESGLPGIPGKEGPTGPKGNQGEKGIRGEKGDSGP SGGGS**<u>TENLTVQPERLGVLASHHDNAAVDASSGVEAAAGLGESVAITHGP YCSQFNDTLNVYLTAHNALGSSLHTAGVDLAKSLRIAAKIYSEADEAWR</u>**

	KAIDGLFTGGGSGLRGLQGPPGALGPPGSVGSPGSPGPKGQKGDHGDNRAIE EKLANMEAEIRILKSKLQLTNKLHAFSMGGGSGDEDPQIAAHVVSEANSNAA SVLQWAKKGYYTMKSNLVMLENGKQLTVKREGLYYVYTQVTFCSNREPSS QRPFIVGLWLKPSSGSERILLKAANTHSSSQLCEQQSVHLGGVFELQAGASVF VNVTEASQVIHRVGFSSFGLLKL

Table S3. Sequences of SPD fused with selected Mtb antigens and CCL20 as coded by LV

Polyantigenic LV	Insert length (a.a.)	Sequence
LV::S40-**EsxH** (LV::S40-H)	506	MLPFLSMLVLLVQPLGNLGAEMKSLSQRSVPNTCTLVMCSPTENGLPGRDG RDGREGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERG LSGGSG**SQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAW QGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDT AEAAKWGG**GSGPPGLPGIPGPAGKEGPSGKQGNIGPQGKPGPKGEAGPKGE VGAPGMQGSTGAKGSTGPKGERGAPGVQGAPGNAGAAGPAGPAGPQGAPG SRGPPGLKGDRGVPGDRGIKGESGLPDSAALRQQMEALKGKLQRLEVAFSH YQKAALFPDGGGSGDEDPQIAAHVVSEANSNAASVLQWAKKGYYTMKSNL VMLENGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKPSSGSERI LLKAANTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHRVGFSSF GLLKL
LV::S40-**EsxH**-***EsxA***-*PE19*-**EspC** (LV::S40-HAPE)	736	MLPFLSMLVLLVQPLGNLGAEMKSLSQRSVPNTCTLVMCSPTENGLPGRDG RDGREGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERG LSGGSG**SQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAW QGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDT AEAAKWGG**GSGG***TEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLA AAWGGSGSEAYQGVQQKWDATATELNNALQNLARTISEAGQAMASTEGN VTGMFA***GGGSGG*SFVTTQPEALAAAAANLQGIGTTMNAQNAAAAAPTTGVVP AAADEVSALTAAQFAAHAQMYQTVSAQAAAIHEMFVNTLVASSGSYAATEAAN AAAAG*GGSGG**TENLTVQPERLGVLASHHDNAAVDASSGVEAAAGLGESV AITHGPYCSQFNDTLNVYLTAHNALGSSLHTAGVDLAKSLRIAAKIYSEA DEAWRKAIDGLFT**GSGGSGGLRGLQGPPGALGPPGSVGSPGSPGPKGQKGD HGDNRAIEEKLANMEAEIRILKSKLQLTNKLHAFSMGGGSGDEDPQIAAHVV SEANSNAASVLQWAKKGYYTMKSNLVMLENGKQLTVKREGLYYVYTQVTF CSNREPSSQRPFIVGLWLKPSSGSERILLKAANTHSSSQLCEQQSVHLGGVFE LQAGASVFVNVTEASQVIHRVGFSSFGLLKL
LV::S40-**EsxH**-***EsxA***-*PE19*-**EspC** ***-Hrp1-RpfD*** LV::S40-HAPEHR	1004	MLPFLSMLVLLVQPLGNLGAEMKSLSQRSVPNTCTLVMCSPTENGLPGRDG RDGREGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERG LSGGSG**SQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAW QGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDT AEAAKWGG**GSGG***TEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLA AAWGGSGSEAYQGVQQKWDATATELNNALQNLARTISEAGQAMASTEGN VTGMFA***GGGSGG*SFVTTQPEALAAAAANLQGIGTTMNAQNAAAAAPTTGVVP AAADEVSALTAAQFAAHAQMYQTVSAQAAAIHEMFVNTLVASSGSYAATEAAN AAAAG*GGSGG**TENLTVQPERLGVLASHHDNAAVDASSGVEAAAGLGESV AITHGPYCSQFNDTLNVYLTAHNALGSSLHTAGVDLAKSLRIAAKIYSEA DEAWRKAIDGLFT**GSGGSGG*TTARDIMNAGVTCVGEHETLTAAAQYMREHD IGALPICGDDDRLHGMLTDRDIVIKGLAAGLDPNTATAGELARDSIYYVDANASI QEMLNVMEEHQVRRVPVISEHRLVGIVTEADIARHLPEHAIVQFVKAICSPMAL AS*GGGSGSGG***LSTISSKADDIDWDAIAQCESGGNWAANTGNGLYGGLQISQA TWDSNGGVGSPAAASPQQQIEVADNIMKTQGPGAWPKCSSCSQGDAPLGS***

		LTHILTFLAAETGGCSGSRDDGGSGGLRGLQGPPGALGPPGSVGSPGSPGPKG QKGDHGDNRAIEEKLANMEAEIRILKSKLQLTNKLHAFSMGGGSGDEDPQI AAHVVSEANSNAASVLQWAKKGYYTMKSNLVMLENGKQLTVKREGLYYVY TQVTFCSNREPSSQRPFIVGLWLKPSSGSERILLKAANTHSSSQLCEQQSVHL GGVFELQAGASVFVNVTEASQVIHRVGFSSFGLLKL
LV::S40-**EsxH**-***EsxA***-*PE19*-**EspC** *Hrp1*-***RpfD***-**CCL20** LV::S40 HAPEHR-20	1128	MLPFLSMLVLLVQPLGNLGAEMKSLSQRSVPNTCTLVMCSPTENGLPGRDG RDGREGPRGEKGDPGLPGPMGLSGLQGPTGPVGPKGENGSAGEPGPKGERG LSGGSG**SQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAW QGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAMMARDT AEAAKWGG**GSGG***TEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLA AAWGGSGSEAYQGVQQKWDATATELNNALQNLARTISEAGQAMASTEGN VTGMFA***GGGSGG*SFVTTQPEALAAAAANLQGIGTTMNAQNAAAAAPTTGVVP AAADEVSALTAAQFAAHAQMYQTVSAQAAAIHEMFVNTLVASSGSYAATEAAN AAAAG*GGSGG**TENLTVQPERLGVLASHHDNAAVDASSGVEAAAGLGESV AITHGPYCSQFNDTLNVYLTAHNALGSSLHTAGVDLAKSLRIAAKIYSEA DEAWRKAIDGLFT**GSGGSGG*TTARDIMNAGVTCVGEHETLTAAAQYMREHD IGALPICGDDDRLHGMLTDRDIVIKGLAAGLDPNTATAGELARDSIYYVDANASI QEMLNVMEEHQVRRVPVISEHRLVGIVTEADIARHLPEHAIVQFVKAICSPMAL AS*GGGSGSGG***LSTISSKADDIDWDAIAQCESGGNWAANTGNGLYGGLQISQA TWDSNGGVGSPAAASPQQQIEVADNIMKTQGPGAWPKCSSCSQGDAPLGS LTHILTFLAAETGGCSGSRDD***GGSGGG**FPGPPGPKGEPGSPAGRGERGFQG SPGKMGPAGSKGEPGGSGSGGASNYDCCLSYIQTPLPSRAIVGFTRQMAD EACDINAIIFHTKKRKSVCADPKQNWVKRAVNLLSLRVKKM**GSGSGSG**G** LRGLQGPPGALGPPGSVGSPGSPGPKGQKGDHGDNRAIEEKLANMEAEIRIL KSKLQLTNKLHAFSMGGGSGDEDPQIAAHVVSEANSNAASVLQWAKKGYYT MKSNLVMLENGKQLTVKREGLYYVYTQVTFCSNREPSSQRPFIVGLWLKPS SGSERILLKAANTHSSSQLCEQQSVHLGGVFELQAGASVFVNVTEASQVIHRV GFSSFGLLKL

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0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	B14346WO AD/PPT

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	**page**	32
1-2	**line**	31-32
1-3	**Identification of deposit**	
1-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
1-3-2	Address of depositary institution	Institut Pasteur, 25-28, Rue du Dr. Roux, 75724 Paris Cédex 15, France
1-3-3	Date of deposit	11 October 1999 (11.10.1999)
1-3-4	Accession Number	CNCM I-2330
1-5	**Designated States for Which Indications are Made**	All designations
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2-1	**page**	33
2-2	**line**	1-3
2-3	**Identification of deposit**	
2-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes (CNCM)
2-3-2	Address of depositary institution	Institut Pasteur, 25-28, Rue du Dr. Roux, 75724 Paris Cédex 15, France
2-3-3	Date of deposit	16 February 2021 (16.02.2021)
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Claims

1. A recombinant lentiviral vector genome comprising a polynucleotide encoding a fusion polypeptide, wherein said fusion polypeptide comprises arranged from N-terminal to C-terminal ends a first recombinant polypeptide and a second polypeptide, wherein:

(i) said first recombinant polypeptide comprises a multimerization scaffold which comprises at least one collectin or a fragment thereof suitable to enable self-assembly of multimers of the first polypeptide, fused with at least one antigenic polypeptide ;

(ii) said second polypeptide comprises a CD40L ectodomain or a receptor binding fragment thereof, in particular the CD40L ectodomain of the human CD40L, preferably the CD40L ectodomain of SEQ ID No. 19.

2. The recombinant lentiviral vector genome according to claim 1, wherein said collectin or fragment thereof comprises arranged from N-terminal to C-terminal ends:

- at least one crosslinking region of a collectin;
- at least one collagen-like region of a collectin; and
- at least one neck region of a collectin.

3. The recombinant lentiviral vector genome according to claim 2, wherein the polynucleotide encoding the said at least one antigenic polypeptide is fused in-frame within the nucleotide sequence of the collagen-like region of said collectin.

4. The recombinant lentiviral vector genome according to any one of claims 1 to 3, wherein said collectin or fragment thereof is a carbohydrate recognition domain (CRD)-truncated form of collectin, wherein said at least one antigenic polypeptide is inserted within the collagen-like region of the collectin.

5. The recombinant lentiviral vector genome according to any one of claims 1 to 4, wherein said fusion polypeptide further comprises a polypeptide comprising a

chemokine, in particular selected from CCL20, a pro-inflammatory Th1-related chemokine such as CXCL9, CXCL10, CCL3, CCL4 and/or CCL5, and/or a Th17-promoting chemokine such as CXCL20, or a receptor binding domain thereof, preferably wherein said chemokine is the CCL20 domain of SEQ ID No. 20, in particular wherein said chemokine or fragment thereof is inserted within the collagen-like domain of said collectin.

6. The recombinant lentiviral vector genome according to any one of claims 1 to 5, wherein said collectin is selected from mannan-binding lectin (MBL), surfactant protein D (SP-D), surfactant protein A (SP-A), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin collectin of 43 kDa (CL-43), collectin of 46 kDa (CL-46),and collectin kidney 1 (CL-K1), preferably wherein said collectin is a human collectin selected from MBL (SEQ ID No. 17), SP-D (SEQ ID No. 18), CL-L1 (SEQ ID No. 34), SP-A1 (SEQ ID No. 35), SP-A2 (SEQ ID No. 36), CL-P1 (SEQ ID No. 37) and CL-K1 (SEQ ID no. 38).

7. The recombinant lentiviral vector genome according to any one of claims 1 to 6, wherein said antigenic polypeptide is a mono-antigenic polypeptide comprising one antigen or immunogenic fragment thereof, or is a poly-antigenic polypeptide comprising at least two antigens or immunogenic fragments thereof.

8. The recombinant lentiviral vector genome according to any one of claims 1 to 7, wherein the at least one antigen or immunogenic fragment thereof is selected from a bacterial, parasite or viral pathogen, in particular from *Mycobacterium tuberculosis*, an influenza virus or a coronavirus such as SARS-CoV-2 or is a tumoral antigen or immunogenic fragment thereof.

9. The recombinant lentiviral vector genome according to any one of claims 1 to 8, wherein said antigenic polypeptide comprises one or more *Mycobacterium tuberculosis* (Mtb) antigens selected from EsxA, EspC, EsxH, PE19, Hypoxic response

protein 1 (Hrp1) and Resuscitation promoting factor D (RpfD), or an immunogenic fragment thereof, in particular one of the following Mtb antigenic combinations:

(a) EsxH;
(b) EsxH and EsxA;
(c) EsxH, EsxA and PE19;
(d) EsxH, EsxA, PE19 and EspC;
(e) EsxA, PE19, EspC, HRp1 and RpfD;
or an immunogenic fragment thereof.

10. The recombinant lentiviral vector genome, wherein said genome is obtained from the pTRIP vector plasmid of nucleotide sequence SEQ ID No. 21, wherein the polynucleotide encoding the fusion polypeptide has been cloned under control of a promoter functional in mammalian cells, in particular the CMV promoter, the human beta-2 microglobulin promoter, the composite BCUAG promoter of SEQ ID No. 22 and wherein the vector optionally comprises post-transcriptional regulatory element of the woodchuck hepatitis virus (WPRE).

11. A DNA plasmid comprising the recombinant vector genome according to any one of claims 1 to 10, in particular wherein said genome is inserted within the pTRIP vector plasmid of nucleotide sequence SEQ ID No.21 or within the pFlap-SP1beta2m-GFP-WPREm deposited at the CNCM (Paris, France) on February 16, 2021 under number CNCM I-5657 or variants thereof.

12. A recombinant lentiviral vector particle which comprises the recombinant lentiviral vector genome according to any one of claims 1 to 10.

13. The recombinant lentiviral vector particle according to claim 12 which is a recombinant integration-deficient lentiviral vector particle, in particular the recombinant integration-deficient lentiviral vector is a HIV-1 based vector and is integrase deficient as a result of a mutation of the integrase gene encoded in the genome of the lentivirus in such a way that the integrase is not expressed or not functionally expressed, in

particular the mutation in the integrase gene leads to the expression of an integrase substituted on its amino acid residue 64, in particular the substitution is D64V in the catalytic domain of the HIV-1 integrase encoded by Pol.

14. The recombinant lentiviral vector particle according to any one of claim 12 or 13, wherein said recombinant lentiviral vector genome is the genome of a replication-incompetent pseudotyped lentiviral vector, in particular a replication-incompetent pseudotyped HIV-1 lentiviral vector, in particular wherein the vector is pseudotyped with the glycoprotein G from a Vesicular Stomatitis Virus (V-SVG) of Indiana or of New-Jersey serotype.

15. A host cell, preferably a mammalian host cell, transfected with a DNA plasmid according to claim 11, in particular wherein said host cell is a HEK-293T cell line or a K562 cell line.

16. A pharmaceutical composition, in particular a vaccine composition, suitable for administration to a mammalian host, comprising a recombinant lentiviral vector particle of any one of claims 12 to 14 together with one or more pharmaceutically acceptable excipient(s) suitable for administration to a host in need thereof, in particular a human host.

17. The pharmaceutical composition of claim 16, for use in the elicitation of a protective, preferentially prophylactic, immune response by the elicitation of antibodies directed against the antigenic polypeptide or immunogenic fragments thereof, and/or cellular and/or humoral response in a host in need thereof, in particular a human host.

18. The pharmaceutical composition of claim 16 or 17, wherein the immune response involves the induction of MHC-II restricted presentation of the antigenic polypeptide or immunogenic fragments thereof, by an antigen-presenting cell, in particular a dendritic cell, and the induction of a CD4-mediated cellular immune response.

19. The pharmaceutical composition of any one of claims 16 to 18, for preventing and/or treating an infection by a pathogen in a mammalian host in need thereof, in particular a human host.

20. A method for the preparation of recombinant lentiviral vector particles suitable for the preparation of a pharmaceutical composition, in particular a vaccine, comprising the following steps:

a) transfecting the recombinant lentiviral transfer vector carrying the lentiviral vector genome according to any one of claims 1 to 10, or the DNA plasmid according to claim 11 in a host cell, for example a HEK-293T cell line or a K562 cell line;

b) co-transfecting the cell of step a) with: (i) a plasmid vector encoding envelope proteins and with a plasmid vector encoding the lentiviral GAG and POL or mutated POL protein as packaging construct; and (ii) a plasmid encoding VSV-G Indiana or New Jersey envelope,

c) culturing the host cell under conditions suitable for the production of recombinant lentiviral vector particles expressing the antigenic polypeptide, or an immunogenic fragment thereof;

d) recovering the recombinant lentiviral particles expressing the antigenic polypeptide, or an immunogenic fragment thereof.