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(54) **Title**: LENTIVIRAL VECTOR BASED IMMUNOLOGICAL COMPOUNDS AGAINST MALARIA

(57) **Abstract**: The invention relates to lentiviral vector particles pseudotyped with a determined heterologous viral envelope protein or viral envelope proteins originating from a RNA virus and which comprise in its genome at least one recombinant polynucleotide encoding at least one polypeptide(s) carrying epitope(s) of an antigen of a Plasmodium parasite capable of infecting a mammalian host. The lentiviral vector particles are used in order to elicit an immunological response against malaria parasites.

LENTIVIRAL VECTOR BASED IMMUNOLOGICAL COMPOUNDS AGAINST MALARIA

[001] The invention relates to a lentiviral vector based immunological compounds against malaria.

[002] In view of the impediments which have been observed in the design of vaccine strategies, many diseases that lead to high mortality and morbidity such as malaria still necessitate the development of new vaccine platforms capable of eliciting strong T-cell mediated immunity and advantageously potent humoral immune responses. Among parasitic infections affecting humans, malaria is a disease for which numerous attempts for such vaccines have been proposed.

[003] However, concerning malaria, only vaccines containing radiation attenuated sporozoites consistently induce sterile immunity in rodents (Nussenzweig R.S. et al, Nature 216, 160-162 (1967)), monkeys (Gwadz; R.W. et al, Bull World Health Organ 57 Suppl 1, 165-173 (1979)) and humans (Clyde, D.F. et al, Am J. Med Sci 266, 169-177 (1973). Albeit very interesting, the irradiated sporozoite vaccine approach still needs to overcome numerous challenges, in particular related to safety, production, storage and distribution, before it can be considered promising for mass vaccination.

[004] In a Review Article, Limbach K.J. & Richie T. L. (Parasit immunology, 2009, **31**, 501-519) have considered different available vaccine platforms against malaria, which are based on the use of viral vectors as delivery means for the antigens eliciting an immune response. Such platforms include vaccines designed on the basis of poxvirus-vectored malaria antigens, or adenovirus-vectored malaria antigens, both types of vectors being *inter alia* proposed in approaches involving heterologous prime-boost regimens for administration. Apart from these poxvirus or adenovirus based technologies, the authors of the review

disclose that novel vector systems may be promising in animal models considering accordingly yellow fever vectored strategies or alpha virus replicon vectored strategies. They also envisage various potentially interesting ways to overcome the persisting difficulties in designing vaccine vectors that may be efficient in providing long-lasting immunity in mammalian hosts and fulfil the requirements of safety for administration to human hosts. Such ways would include combination of heterologous delivery means for the antigens, use of adjuvants or immunomodulatory components.

[005] In an attempt to overcome at least some of the drawbacks observed when assaying the proposed vaccine compositions of the prior art, the inventors have considered the approach of lentiviral vectors as a basis for a new vaccination platform with a view to develop prophylactic vaccines against malaria.

[006] Malaria is a pathology which is transmitted to the host by A*nophele* mosquitoes and is endemic in many countries where it generates the death of many millions of people each year. Apart from mortality, malaria causes morbidity in a large proportion of the population in countries where it is endemic, thereby causing medical and economic major concerns for these countries.

[007] Five species of *Plasmodium* parasites are known to infect human: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae, Plasmodium knowlesi* (17) and *Plasmodium falciparum*, the latter being the agent of malaria which causes almost all mortality cases. Infection in humans begins with inoculation of sporozoites forms of the parasite that originate from female mosquitoes. These forms of the parasite are rapidly transferred from the blood flow to the liver of the human host where they proceed with invasion of hepatocytes. Depending on the strain of *Plasmodium*, the duration of the intra-hepatic cycle of the parasite is from 5 to 15 days, the shorter (around 5.5 days) being for *P. falciparum*. The

parasite amplifies in the liver as a result of asexual replication in the infected cells, giving rise to merozoite forms of the parasite. After the merozoites have been liberated from the hepatocytes, they progress toward a blood-stage infection which corresponds to the symptomatic phase of the infection in the human host. Accordingly, the merozoites rapidly penetrate into red blood cells (erythrocytes) through a specific membrane receptor. The merozoite invasion of the erythrocytes corresponds to the erythrocytic stage of the cycle which lasts for 48 to 72 hours depending on the strain of *Plasmodium*. During this stage, merozoites undergo multiple nuclear divisions, giving rise to the liberation of further merozoites, which are able to perform invasion of additional red blood cells and thus to repeat the cycle. In humans, the symptomatic disease is the result of the effects of invasion of erythrocytes, of their destruction and of the response of the hosts. During the infection, some of the parasital forms differenciate as gametocytes which are then ingested by mosquitoes where they undergo a sporogonic cycle, giving rise to sporozoites which then infect humans.

[008] Due to the cycle of the infection encompassing distinct stages in the human body, and different forms of the parasite, various strategies have been proposed in order to deliver vaccine principles and various antigens of *Plasmodium* have been proposed as targets for the immune response, especially when addressing humoral antibody response.

[009] Target candidates suitable for causing or improving the immune response may encompass various antigens with a view to design a vaccine and accordingly encompass “liver-stage antigens” (also designated as “pre-erythrocytic stage antigens”) and/or “blood-stage antigens”.

[010] Within the frame of the present invention, the inventors have primarily considered that pre-erythrocytic stage antigens of malaria parasite may be advantageously used to elicit a protective immune response which would be uniformly effective at the level of a population, because they show less variability than antigens appearing at a later stage in the cycle of the

parasite in humans. The inventors have also considered that for a long-term protection against malaria, means suitable to elicit cellular responses would be necessary, and would advantageously be supplemented by humoral responses.

[011] The inventors have accordingly determined that a protective immune response would require the onset and development of efficient effector cells and memory cells and that said response should be strong enough to outperform the efficiency of the immune response which has been observed to natural infection.

[012] The invention thus provides a novel lentiviral-based vector, as a new platform for the preparation or development of malaria vaccine which, in addition to the delivery means of the active principle for the immune composition, enables the determination of an appropriate immunization patterns suitable for eliciting a strong and long-lasting immune response when adapted to humans, as proved in a murine model commonly used for malaria.

[013] The invention accordingly relates to lentiviral vector particles which are lentiviral vector particles, in particular replication-incompetent lentiviral vector particles, especially replication-incompetent HIV-based vector particles characterized in that (i) they are pseudotyped with a determined heterologous viral envelope protein or viral envelope proteins originating from a RNA virus and (ii) they comprise in their genome at least one recombinant polynucleotide encoding at least one polypeptide(s) carrying epitope(s) of a pre-erythrocytic stage antigen of a *Plasmodium* parasite capable of infecting a mammalian host, wherein said epitope(s) encompass(es) T-epitope(s).

[014] In a particular embodiment of the invention, the encoded polypeptide of a pre-erythrocytic stage antigen of a *Plasmodium* parasite further comprises B-epitope(s).

[015] According to the invention, the lentiviral vector particles are either designed to express proficient (i.e., integrative-competent) or deficient (i.e., integrative-incompetent) particles.

[016] The expressions "*malaria parasite*" and "*Plasmodium parasite* » are used interchangeably in the present application. They designate every and all forms of the parasite that are associated with the various stages of the cycle in the mammalian, especially human host, including in particular sporozoites, especially spororoites present in the blood flow after inoculation, or sporozoites developing in the hepatocytes, merozoites, including especially merozoites produced in the hepatocytes (forms of the pre-erythrocytic stage and including forms of the erythrocytic stage of the cycle such as merozoïtes contained in red-blood cells of the cycle). These forms of the parasite are characterized by various specific antigens that are well known and identified in the art, and can also be designated by reference to the stage of the infection.

[017] The expressions "*T-epitope*" and "*B-epitope*" refer to antigenic determinants that are involved respectively in the adaptive immune response driven by T cells and in the immune response driven by B cells. In particular said T-epitopes and respectively B-epitopes elicit T cell, respectively B cell immune response when delivered to the host in suitable conditions.

[018] The lentiviral vector particles (or lentiviral vectors 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 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.

[019] When reference is made to "lentiviral" vectors (lentiviral-based vectors) in the application, it encompasses, in a particular embodiment, HIV-based vectors and especially HIV-1-based vectors.

[020] The lentiviral vectors according to the invention are 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, 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.

[021] The "vector genome" of the vector particles is a recombinant vector which also comprises the polynucleotide or transgene of interest encoding the polypeptide(s) of malaria parasite. 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 application.

[022] The vector genome as defined herein accordingly contains, apart from the so-called recombinant polynucleotide 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, reverse transcription and transcription 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 and contains or consists of the so-called central cPPT-CTS nucleotidic domain present in lentiviral genome sequences or in some retroelements such as those of yeasts.

[023] 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.

[024] 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 polypeptide of the malaria parasite, 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.

[025] A lentiviral vector particle of the invention may comprise in its genome more than one recombinant polynucleotide encoding at least one polypeptide carrying epitope(s) of a pre-erythrocytic stage antigen as disclosed herein. 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 pre-erythrocytic stage of a *Plasmodium* parasite or different polypeptides of distinct antigens of different forms of the malaria parasite, especially antigens of the pre-erythrocytic stage and antigens of the erythrocytic stage of the parasite.

[026] In a particular embodiment, the vector genome contains two recombinant polynucleotides, each of them encoding a distinct polypeptide and each polypeptide originating from a different antigen of the same stage.

[027] By the expression "polypeptide carrying epitope(s) of an antigen ", it is intended according to the present invention a polypeptide which may be a native antigen of a *Plasmodium* parasite, a mutated version thereof and in particular a fragment of such a native antigen and especially a truncated version of such a native antigen. A polypeptide has an amino acid sequence which is sufficient to provide one or several epitope(s), and may accordingly have a length of at least about 4 amino acid residues and especially from about 4 to about 8 amino acid residues for conformational B epitopes or at least about 9 amino acid residues and in particular from about 9 to about 19 amino acid residues for sequential T epitopes.

[028] In a particular embodiment of the invention, the recombinant polynucleotide of the lentiviral vector particles encodes a truncated version of an antigen of the malaria parasite, especially a fragment which results from the deletion of a functional domain of the full-length (i.e., native) antigen, when said domain is not useful or is detrimental to the elicitation of an immune response in a host.

[029] In a particular embodiment of the invention, the lentiviral vector particles comprise in their genome, at least one recombinant polynucleotide which encodes a polypeptide(s) of an antigen from the circumsporozoite protein of a Plasmodium parasite, esp. of *Plasmodium falciparum* or *Plasmodium malariae, Plasmodium vivax, Plasmodium ovale* or *Plasmodium knowlesi*. It is especially a truncated version of the CSP and in particular a polypeptide devoid of the GPI anchoring motif of the CSP.

[030] In an embodiment of the invention, the lentiviral vector particles comprise in their genome a recombinant polynucleotide which encodes a polypeptide(s) of an antigen selected from the group of the sporozoite surface protein 2 (TRAP/SSP2), liver-stage antigen (LSA in

particular LSA3), Pf exported protein 1 (Pf Exp1)/Py hepatocyte erythrocyte protein (PyHEP17), and Pf antigen 2 (where Pf represents *Plasmodium falciparum* and Py represents *Plamsodium yoelii*), sporozoïte and liver stage antigen (SALSA), sporozoïte threonine and asparagines-rich (STARP) or other pre-erythrocytic antigen, possibly in addition to the polypeptide of an antigen of the CSP.

[031] In a particular embodiment of the invention, the polypeptide of the antigen of the malaria parasite is a fragment of the CSP protein and it is co-expressed by the vector genome with a polypeptide of another antigen of the malaria parasite, either an antigen from the pre-erythrocytic stage or an antigen of the erythrocytic stage. Antigens of the erythrocytic stage which may be used to design the polynucleotide encoding the polypeptide according to the invention are merozoïte surface protein 1 (MSP2), in particular Merozoïte surface protein 1 (MSP-1), Merozoïte surface protein 2 (MSP-2) merozoïte surface protein 3 (MSP-3), Merozoïte surface protein 4 (MSP-4), Merozoïte surface protein 6 (MSP-6), Ring-infected erythrocyte surface antigen (RESA), Rhoptry associated protein 1 (RAP-1), Apical membrane antigen 1 (AMA-1), Erythrocyte binding antigen (EBA-175), Erythrocyte membrane-associated giant protein or Antigen 332 (Ag332), dnaK-type molecular chaperone, Glutamate-rich protein (GLURP), in particular MSP3-GLURP fusion protein (WO 2004/043488; ref 28), Erythrocyte membrane protein 1 (EMP-1), Serine repeat antigen (SERA), Clustered-asparagine-rich protein (CARP), Cirumsporozoite protein-related antigen precursor (CRA), Cytoadherence-linked asexual protein (CLAG), Acid basic repeat antigen (ABRA) or 101 kDa malaria antigen, Rhoptry antigen protein (RAP-2), Knob-associated histidine-rich protein (KHRP), Rhoptry antigen protein (RAP), Cysteine protease, Hypothetical protein PFE1325w, Protective antigen (MAg-1), Fructose-bisphosphate aldolase, Ribosomal phosphoprotein P0, P-type ATPase, Glucose-regulated protein

(GRP78), Asparagine and aspartate-rich protein (AARP1), Interspersed repeat antigen or PFE0070w.}

[032] Antigens of the sexual stage which may be used to design the polynucleotide encoding the polypeptide according to the invention are Sexual stage and sporozoite surface antigen, Antigen Pfg27/25, Antigen QF122, 11-1 polypeptide, Gametocyte-specific surface protein (Pfs230) Ookinete surface protein (P25), Chitinase, Multidrug resistance protein (MRP).

[033] These antigens are disclosed by reference to *P. falciparum* and may have a counterpart in other *Plasmodium* species. They are reported in Vaughan K. et al (18).

[034] Vaughan K et al disclose in particular epitopes of said antigens that may be used by the skilled person as a basis to prepare the recombinant polynucleotide(s) used in the vector of the invention.

[035] The above-cited antigens of *Plasmodium* parasite have been disclosed in the prior art, including through their sequences which are available in data bases.

[036] The circumsporozoite protein (CSP) is one of the preferred antigens for the preparation of the lentiviral vector particles of the invention. It constitutes the sporozoite coat protein, which has been recognized in the past as the target of protective antibodies. Apart from its ability to elicit anti-CS antibodies, this antigen further contains T-epitopes including especially CD8+ T-cells epitopes and CD4+ T-cells epitopes. Particular CSP antigens are disclosed through their amino acid sequences as SEQ ID No 20, 23, 26, 27, 28, 29, 30, 31, or as SEQ ID No 32 for a consensus of these sequences. The sequence of *P. vivax* is given in GenBank as AY674050.1.

[037] In a particular embodiment of the invention, the lentiviral vector particles have in their genome a recombinant polynucleotide which encodes at least a polypeptide of the CSP-antigen of *Plasmodium yoelii* as illustrated in the examples or advantageously of *Plasmodium falciparum*,

e.g., a polypeptide corresponding to a fragment of said CSP-antigen devoid of the GPI-anchoring motif in *Plasmodium yoelii* is CSP DGPI having sequence SEQ ID No 21. Said GPI motif corresponds to the last 12 amino acid residues in the C-terminal part in the native amino acid sequence of the CSP antigen of *Plasmodium Yoelii* . The counterpart of said fragment of the CSP protein in *P. Falciparum* is disclosed in the figures and sequences (SEQ ID No 23 for the native protein, SEQ ID No 24 for the sequence devoid of the GPI motif, SEQ ID No 25 for the sequence truncated in the N-terminal end) and used to provide evidence in a suitable murine model, of the capacity of the polypeptide to elicit a protective immune response and even a sterilizing protection against malaria.

[038] In a particular embodiment of the invention, polynucleotide(s) of the lentiviral vector particles has(have) a mammalian codon optimized (CO) nucleotide sequence and optionally the lentiviral sequences of the genome of said particles has a mammalian codon optimized nucleotide sequence.

[039] 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.

[040] In a particular embodiment of the invention, the lentiviral vector particles disclosed herein further contain in their genome, a recombinant polynucleotide which encodes a polypeptide of an antigen of the blood stage of the cycle of the parasite as disclosed above and/or an antigen of the sexual stage.

[041] The polypeptide is either the native antigen or a modified version thereof, especially a fragment which comprises or consists in T-cell epitope(s) or B-cell epitope(s) or both.

[042] Examples of polypeptides expressed as a result of administering the lentiviral vector particles of the invention, are the polypeptides encoded by the vector plasmids (or sequence vectors) disclosed hereafter.

[043] The invention also relates especially to these vector plasmids, deposited at the CNCM (Paris, France) on April 20, 2010 and having the following features and accession number

pTRIP-|delta|**U3-CMV-MSP1$_{42}$-CO-WPRE**	CNCM I-4303
pTRIP-|delta|**U3-CMV-Hep17-CO-WPRE**	CNCM I-4304
pTRIP-|delta|**U3-CMV-Hep17**|delta|**SP-CO-WPRE**	CNCM I-4305
pTRIP-|delta|**U3-CMV-CSP-CO-WPRE**	CNCM I-4306
pTRIP-|delta|**U3-CMV-CSP**|delta|**SP-CO-WPRE**	CNCM I-4307
pTRIP-|delta|**U3-CMV-CSP**|delta|**GPI-CO-WPRE**	CNCM I-4308
pTRIP-|delta|**U3-CMV-CSP**|delta|**SP**|delta|**GPI-CO-WPRE**	CNCM I-4309

[044] These plasmids are described in the figures and sequences of the present application. The sequence of the transgene that they contain is from *P. yoelii*. Said transgene may be advantageously replaced by the appropriate sequence from *P. Falciparum.*

[045] The invention also concerns variants of these plasmids, where the polynucleotide encoding the polynucleotide of a *Plasmodium* antigen is modified to encode a functional immunogenic variant thereof or is substituted by a corresponding polynucleotide codon optimized from another *Plasmodium* strain especially from *Plasmodium falciparum* is modified to substitute the CMV promoter by one of the herein cited promoters.

[046] In the deposited plasmids the polynucleotide encoding the polypeptide of a *Palsmodium yoelii* antigen is codon optimized.

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

[048] As examples of typing envelope proteins for the preparation of the lentiviral vector particles, 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, VSV-G protein(s) of the New Jersey strain, VSV-G protein(s) of the Cocal strain, VSV-G protein of the Isfahan strain, VSV-G protein(s) of Chandipura strain, VSV-G protein(s) of Pyri strain or VSV-G protein(s) of the SVCV strain.

[049] 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 a N-terminal ectodomain, a transmembrane region and a C-terminal cytoplasmic tail. It is exported to the cell surface *via* the transGolgi network (endoplasmic reticulum and Golgi apparatus).

[050] 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.

[051] Alternatively, among VSV, Chandipura virus (CHPV), Cocal virus (COCV), Perinet virus (PERV), Piry virus (PIRYV), SVCV or Isfahan virus may be good candidates to design pseudotyping envelope proteins, and especially to prepare particles used for a boosting step of the immunization, accordingly providing second envelope protein(s) or third envelope protein(s), or further envelope protein(s) when the vector particles of the invention are used in a prime-boost administration regimen. When used accordingly, Cocal virus envelope protein(s) would be preferred for a late or last administration in a prime-boost regimen. However, Chandipura virus (CHPV) and Piry virus (PIRYV) may provide envelope proteins having low fusogenicity as a result of their lower affinity for their receptor, when comparing the vector titers obtained with particles prepared with different envelopes. Therefore in a first approach these envelopes may be excluded from the choice of envelopes in order to prepare particles with an efficient transduction capacity.

[052] The amino acid sequences and coding sequences of the VSV-G proteins referred to herein are disclosed in patent application WO 2009/019612. Particular examples of these amino acid sequences are also provided in the present application as SEQ ID No 77, 79, 82, 84, 86, 88, 90. Plasmids containing VSV-G encoding sequences are described in said application WO 2009/019612 which is incorporated by reference. The plasmids have been deposited at the CNCM (Paris, France). Nucleotide sequences encoding said envelope proteins are disclosed in the present application as SEQ ID No 76, 78, 81, 83, 85, 87, 89.

[053] In a particular embodiment of the invention, said first and second and if any said third or further, 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 relative good ability to fuse with DC would be preferred. Relative titers of transduction are illustrated in the examples.

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

[055] Polynucleotide encoding VSV envelope protein(s) (VSV-G) also targets splenocytes, in particular Antigen Presenting Cells (APC) or Dendritic Cells (DC), or liver cells including liver dendritic cells, hepatocytes or non parenchymal cells.

[056] The envelope protein(s), also designated sometimes as surface protein in particular viruses, are said to "originate" from a different organism, and especially from different RNA virus strains, meaning that in said protein(s), essential features of the corresponding protein(s) expressed in a determined RNA virus are maintained. Said essential features, relate to the structure or to the function of the protein and are those which enable especially the obtained protein(s) to be expressed at the surface of the vector particles for pseudotyping said vectors. The envelope proteins are then capable of being recognized and internalized in the target cells of the hosts when present on the vector particles.

[057] In a particular embodiment, protein(s) or glycoprotein(s), suitable for use in the design of pseudotyped lentiviral vectors of the kit of compounds are used as multimeric proteins, such as VSV-G protein which is trimeric.

[058] The 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).

[059] 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 or for transduction of cells suitable for the preparation of 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: 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 (19).

[060] These promoters may also be used in regulatory expression sequences involved in the expression of *gag-pol* derived proteins from the encapsidation plasmids.

[061] 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. (20). 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 20, 21) or a SODk packaging cell line, such as SODk0 derived cell lines, including SODk1 and SODk3 (ref 20, 22, 23, 24).

[062] The nucleotide sequence used for the expression of the envelope protein(s) required for pseudotyping the lentiviral vector particles may alternatively be modified, thus providing a variant with respect to the nucleic acid encoding the native envelope protein(s) used as reference. The modification may be carried out to improve the codons usage (codon optimization) in the cells for the preparation of the vector particles and/or in the transduced cells of the host. It may be modified to express a protein different from the native protein(s), especially one which has an improved pseudotyping capacity, an improved capacity in the level of production, or an improved capacity with respect to prevention of sero-neutralization (also designated as cross-reactive proteins) with other envelope protein(s) used in the kit of compounds.

[063] Such a modification of the polynucleotide encoding the envelope proteins(s) or modification of the envelope protein(s) (to generate variants of native envelopes) may affect and especially improve their level of production in a cell host or their ability to pseudotype the vector particles possibly by improving the density of envelope protein(s) associated with pseudovirions. Said modification may derive from a mutation in the amino acid sequence of said protein(s), for instance by addition, deletion or substitution of one or several nucleotides or nucleotidic fragments or may relate to post translational modifications and in particular to the glycosylation status of said envelope protein(s).

[064] The envelope protein(s) used to pseudotype the lentiviral vectors of the invention are indeed especially glycoproteins.

[065] It has already been shown that pseudotyping viral vectors with Vesicular Stomatitis Virus glycoprotein (VSV-G) enables the transduction of a large range of cell types from different species. This VSV-

G glycoprotein, in addition to its broad tropism, has an interesting stability when used for vector pseudotyping. Therefore, VSV-G have been used as a standard for evaluating the efficiency of other pseudotypes (Cronin J. et al, 2005).

[066] According to the invention, the lentiviral vector particles 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 a polypeptide of an antigen of a malaria parasite as disclosed herein under the control of regulatory expression sequences, and optionally comprising sequences for integration;
- an expression plasmid encoding a pseudotyping envelope derived from a 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 or a variant thereof 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.

[067] The invention thus also concerns lentiviral vector particles as described above, which are the product recovered from a stable cell line 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 truncated mammalian, especially human, codon-optimized sequence of the cs gene of a *Plasmodium* parasite, 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 or envelope proteins, wherein said polynucleotide is under the control of regulating expression sequences, in particular regulatory expression sequences comprising an inducible 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.

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

[069] The polynucleotide encodes at least one polypeptide of a malaria antigen according to any embodiment disclosed in the present application. In particular, it encodes a polypeptide which is a truncated mammalian, especially human, codon-optimized sequence of the cs gene of a *Plasmodium* parasite, especially of *Plasmodium falciparum*.

[070] In a particular embodiment, the polynucleotide encodes another polypeptide of a distinct antigen of the malaria parasite, or it encodes two or more polypeptides which originate and/or are derived from distinct antigens of said parasite as disclosed in the various embodiments. Accordingly, the vector plasmid may comprise several expression cassettes for the expression of the various polypeptides or may comprise bicistronic

or multicistronic expression cassettes where the polynucleotides encoding the various polypeptides are separated by an IRES sequence of viral origin (Internal Ribosome Entry Site), or it may encode fusion protein(s).

[071]The internal promoter contained the vector genome and controlling the expression of the polynucleotide encoding a polypeptide of an antigen of the malaria parasite (as a transgene or in an expression cassette) may be selected from the promoters of the following genes: 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 (19).

[072] 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.

[073]Alternatively, vector particles can be produced from co-transfection of the plasmids disclosed herein, in stable packaging cell lines which thus become capable of continuously secreting vector particles. Promoters used in the regulatory expression sequences involved for the expression of the envelope protein(s) are advantageously inducible promoters.

[074] The following particular embodiments may be carried out when preparing the lentiviral vector particles based on human lentivirus, and especially based on HIV virus.

[075] According to the invention, the genome of the lentiviral vector particles 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 transfecting human cells.

[076] 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.

[077] In a particular embodiment, the vector genome is defective for the expression of biologically functional GAG, and advantageously for biologically functional POL and ENV proteins. Accordingly, the vector genome is devoid of the sequence encoding these proteins.

[078]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. 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.

[079]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.

[080] 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 polypeptide of a malaria antigen, 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.

[081] The DNA flap (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).

[082]Sequences of DNA flaps have been disclosed in the prior art, especially in the above cited patent applications. These sequences are also disclosed as SEQ ID NO 69 to SEQ ID NO 75. 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) 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.

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

[084]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 178-base pair polymerase chain reaction (PCR) fragment encompassing the cPPT and CTS regions of the HIV-1 DNA (Zennou et al 2000).

[085]This PCR fragment may especially be derived from infective DNA clone of HIV-1 LAI especially pLAI3 of HIV1, as a fragment corresponding to the sequence from nucleotide 4793 to 4971. If appropriate, restriction sites are added to one or both extremities of the obtained fragment, for cloning. For example, Nar I restriction sites may be added to the 5' extremities of primers used to perform the PCR reaction.

[086]Therefore, the DNA flap used in the present invention, is deleted from the unnecessary 5' and 3' parts of the *pol* gene of the original lentiviral genome and is recombined with sequences of different origin.

[087]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.

[088]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.

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

[090]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.

[091]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.

[092]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.

[093]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).

[094]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, normally contained in *Pol*) to facilitate nuclear import.
5. Optionally post-transcriptional elements 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).

[095]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.

[096]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.

[097]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 (for HIV-1 lentiviral vectors).

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

[099] 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.

[0100] The envelope plasmid encodes the envelope protein(s) for pseudotyping which are disclosed herein, under the control of an internal promoter, as disclosed herein.

[0101] 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, 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 polypeptide of an antigen of the malaria parasite (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 contained in the described pTRIP or pThV plasmids of the invention illustrated in the examples .

[0102] In a 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.

[0103] 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 at least one polypeptide carrying epitope(s) of a pre-erythrocytic stage antigen of a *Plasmodium* parasite, in an immunogenic composition.

[0104] 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.

[0105] 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.

[0106] 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.

[0107] 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.

[0108] 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.

[0109] Another proposed substitution is the replacement of the amino acids residues RRK (positions 262 to 264) by the amino acids residues AAH.

[0110] 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 maximazing the efficiency of the immune response.

[0111] 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). The sequence of pTRIPΔU3.CMV-eGFP is provided as SEQ ID No 35 and is described in figure 11.

[0112] When the vector genome is derived from these particular plasmids, a sequence of a recombinant polynucleotide as disclosed in the present application is inserted therein, in addition or in replacement of the GFP coding fragment. The GFP coding sequence may also be substituted by a different marker. 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.

[0113] The WPRE sequence also contained in the particular deposited pTRIP vectors may optionally be deleted.

[0114] 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.

[0115] The concentration of particles produced can be determined by measuring the P24 (capsid protein for HIV-1) content of cell supernatants.

[0116] 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 retrotranscription 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 malaria parasite 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.

[0117] 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 at least one antigenic polypeptide, and particularly stimulating gene expression of the transgene and improving chromatin accessibility.

[0118] 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 polypeptide(s) of antigen(s) of malaria parasite, the at least one polypeptide epitope associated with the MHC molecule triggers a humoral or a cellular immune response.

[0119] 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.

[0120] 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;

(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);

wherein said first and second viral envelope pseudotyping protein(s) do not sero-neutralize with each other and are suitable for *in vivo* transduction of mammalian cells, especially of human cells.

[0121] The expression “combination of compounds” or alternatively “kit of compounds” means that the lentiviral vector particles constituting active ingredients of the kits or combinations, are provided as separate compounds in said kit or combination, and are intended for separate administration to a host, especially separate administration in time. Accordingly the invention enables to perform a prime-boost administration in a host in need thereof, where the first administration step elicits an immune, especially cellular, immune response and the later administration step(s) boost(s) the immune reaction including the cellular immune response. For each step of administration, it is preferred that the pseudotyping envelope protein(s) of the vector particles is different than the one used in the other step(s). Accordingly, the separate compounds of the

kit or combination of the invention have distinct particles at least due to the difference in their pseudotyping envelope proteins.

[0122] The compounds of the kit thus are provided separately in time to the host in need thereof, especially to a mammalian host, in particular a human host.

[0123] Accordingly, said lentiviral vectors can be provided in separate packages or can be presented in a common package for a separate use thereof.

[0124] Therefore, the notice included in the packages and comprising the directions for use, may indicate that said lentiviral vector particles which are pseudotyped with distinct pseudotyping envelope protein or pseudotyping envelope proteins are for separate administration in time, especially for priming and subsequently boosting an immune reaction in a host.

[0125] In accordance with the invention, in the combination of compounds it is provided lentiviral vector particles which are pseudotyped with a first determined heterologous viral pseudotyping envelope protein, or viral pseudotyping envelope proteins, and lentiviral viral vector particles which are pseudotyped with a second determined heterologous viral pseudotyping envelope protein or viral pseudotyping envelope proteins. Accordingly, said first and second heterologous viral envelope protein(s) are different and in particular are originating from different virus strains. Thus, the lentiviral vector particles contained in the separate compounds of the of the kit of compounds of the invention are distinct from each other, at least due to the particular pseudotyping envelope protein(s) used for pseudotyping the vector particles.

[0126] In a particular embodiment of the invention, the combination of compounds comprises a third or a further type of lentiviral vector particles wherein the pseudotyping envelope protein(s) of the third lentiviral

vector is different from said first and second pseudotyping envelope protein(s) and especially originates from a different virus strain.

[0127] When particles are successively administered which have different pseudotyping envelopes, the following order of administration with respect to said envelopes could be preferred: Indiana; New Jersey; Isfahan; SVCV/Cocal. Because Cocal pseudotyped lentiviral vectors seroneutralize several other envelopes, it is preferable, in the vaccination chronology, when Cocal envelopes are to be used in the preparation of particles, to administer particles pseudotyped with them as the last one in the administration regimen.

[0128] Apart from their pseudotyping envelope protein(s), the lentiviral vectors of the invention may be identical and especially may have identical vector genomes.

[0129] Alternatively, their vector genomes may be different, provided they carry the same recombinant determined polynucleotide (also designated as transgene), especially the same recombinant polynucleotide.

[0130] In another embodiment of the invention, the vector genomes of the lentiviral vectors are different by having at least one different recombinant polynucleotide, provided at least one of said different polynucleotides encodes polypeptide(s) having common antigenic determinant(s), or common epitope(s). Hence the different polynucleotides may be variants from each other that encode identical or variant polypeptides or may include sequences encoding different polypeptides.

[0131] A particular kit of compounds comprises lentiviral vectors wherein in at least one of the separate compounds, the vectors are pseudotyped with recombinant pseudotyping envelope protein(s) comprising combined domains or fragments originating from different envelope protein(s) of different viruses, especially of different genus of different species of VSV.

[0132] In a particular embodiment of the invention, at least one the first, second and if any third or further pseudotyping envelope protein(s) is (are) recombinant envelope protein(s) comprising the export determinant of the VSV-G of Indiana strain.

[0133] The export determinant of the VSV-G of the Indiana strain is a polypeptide encoded by the cytoplasmic fragment of the open reading frame of the envelope.

[0134] The export determinant of the VSV-G of the Indiana strain is a polypeptide comprising or having amino acid sequence YTDIE in the cytoplasmic tail (Nishimua N. et al. 2002).

[0135] Said recombinant envelope protein(s) may comprise the cytoplasmic tail of the VSV-G of an Indiana strain which is the intracellular portion of VSV-G delimited by a hydrophobic transmembrane domain.

[0136] A particular kit of compounds comprises lentiviral vectors wherein one or two or more of them is (are) pseudotyped with recombinant envelope protein(s) comprising the cytoplasmic domain of the indiana VSV and the ectodomain of a strain of a different VSV serotype. The transmembrane domain may also be the one of the Indiana VSV-G.

[0137] A particular kit of compounds comprises lentiviral vectors wherein one or both of them is (are) pseudotyped with recombinant envelope protein(s) comprising the transmembrane domain and the cytoplasmic domain of the indiana VSV and the ectodomain of the New-Jersey VSV.

[0138] Appropriate other modifications encompass mutations, especially point mutations, that improve pseudotyping. Such mutations for the VSV-G proteins may be carried out in the transmembrane domain by substituting or deleting one or several amino acid residues. Other examples of appropriate mutations are disclosed in Fredericksen B.L. et al (1995) or Nishimura N. et al (2003).

[0139] It is also especially possible to modify the glycosylation status of the VSV-G, in order to improve transduction efficiency of the lentiviral vector pseudotyped with these VSV-G proteins, when administered to a host.

[0140] VSV-G proteins from various strains of VSV are disclosed in the figures and their sequences can also be derived from databases, especially from GenBank. Especially the VSV-G proteins of Indiana and New-Jersey strains may be obtained by reference to the sequences disclosed as GenBank # AF170624 for New-Jersey VSV-G or GenBank # M11048 for Indiana strain.

[0141] Considering the glycoproteins of the New-Jersey and Indiana strains of VSV, it has been proposed that glycosylation at two asparagine residues (N180 and N336) favour the efficient pseudotyping of lentiviral vectors. This particular feature may be applied in the preparation of the lentiviral vectors of the invention.

[0142] The following constructs encoding VSV-G derived envelope proteins are particular examples of constructs for use in the preparation of the combination of lentiviral vector particles of the invention and are described in WO 2009/019612.

[0143] A VSV-G Indiana gene which is codon optimized as shown in SEQ ID No 76. A particular encapsidation plasmid is pThV-VSV.G (IND-CO) deposited at the CNCM (Paris, France) on October 10 2007, under number I-3842 or in an alternative version of the plasmid construct, on July 31, 2008, under number CNCM I-4056 is suitable for use in preparing pseudotyped particles with an envelope from VSV-G Indiana New-Jersey. Other constructs may be derived from this particular plasmid, especially by substituting the promoter for a promoter among those listed in the present application.

[0144] A VSV-G New-Jersey gene codon optimized is disclosed in SEQ ID No 78. A particular encapsidation plasmid is pThV-VSV.G (NJ-CO)

deposited at the CNCM (Paris, France) on October 10, 2007, under number I-3843 or in an alternative version of the plasmid construct, on July 31, 2008, under number CNCM I-4058 is suitable for use in preparing pseudotyped particles with an envelope from VSV-G Indiana New-Jersey. Other constructs may be derived from this particular plasmid, especially by substituting the promoter for a promoter among those listed in the present application.

[0145] Other envelope genes suitable to carry out the invention having codon optimized sequences are illustrated in WO 2009/019612and especially encompass VSV-G Chandipura gene and its expression product, VSV-G Cocal gene and its expression product, VSV-G Piry gene and its expression product, VSV-G Isfahan gene and its expression product, VSV-G Spring viremia carp virus gene and its expression product. A particular encapsidation plasmid, containing an envelope gene for VSV-G Cocal, is pThV-VSV.G (COCAL-CO) deposited at the CNCM (Paris, France) on July 31, 2008, under number CNCM I-4055. Another particular encapsidation plasmid, containing an envelope gene for VSV-G Isfahan, is pThV-VSV.G (ISFA-CO) deposited at the CNCM (Paris, France) on July 31, 2008, under number CNCM I-4057. Another particular encapsidation plasmid, containing an envelope gene for VSV-G Spring viremia carp virus, is pThV-VSV.G (SVCV-CO) deposited at the CNCM (Paris, France) on July 31, 2008, under number CNCM I-4059. These constructs are disclosed in patent application WO2009/019612.

[0146] Fusion envelope proteins, especially fusion proteins involving several different fragments of VSV-G proteins of different viruses and to the nucleic acid constructs encoding such proteins are used as alternative embodiments and are also disclosed in WO 2009/019612. A particular fusion envelope is the fusion between the ectodomain of the New-Jersey envelope protein and the transmembrane domain and

cytoplasmic domain of the Indiana envelope protein as illustrated in the herein provided sequences.

[0147] Another fusion envelope protein suitable to perform the invention comprises the ectodomain of one VSV-G protein selected among VSV-G Chandipura, VSV-G Cocal, VSV-G Pyri, VSV-G Isfahan, or VSV-G SVCV and the tranmembrane and cytoplasmic domains of VSV-G Indiana. A nucleic acid molecule encoding said fusion protein is advantageously a codon optimized nucleic acid. Nucleic acid encoding the fusion protein are also described as SEQ ID No 77, 79, 81, 83 85, 87, 89.

[0148] In a particular embodiment of the invention, a combination of compounds is provided, wherein the lentiviral particles of the separate compounds encode (i) a polypeptide of the CSP antigen or (ii) a polypeptide of the CSP antigen devoid of the GPI-anchoring motif (CSP deltaGPI) or a CSP protein truncated in the N-terminal end (CSP NTer or also CSP delta SP).

[0149] In a particular embodiment, these compounds or some of them further encode at least one additional polypeptide of an antigen of the malaria parasite chosen in the groups disclosed herein, the distinct polypeptides of said antigens being either expressed from the same lentiviral particles or from distinct lentiviral particles.

[0150] In another particular embodiment of the invention, these compounds or some of them further encode at least one additional polypeptide of an antigen of the malaria parasite chosen in the groups disclosed herein.

[0151] The invention concerns especially lentiviral vector particles or a combination of compounds as herein defined for the prophylactic immunization against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host.

[0152] Accordingly, the lentiviral vector particles, compositions comprising the same or the combination of compounds of the invention,

when administered to a host in needs thereof, especially to a mammalian in particular to a human host, elicit 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 malaria parasite. The immune response may either prevent the infection by the malaria parasite when such parasite is inoculated as sporozoite to the host or may prevent the onset or the development of a pathological state resulting from inoculation of malaria parasite in the form of sporozoite or prevent the onset or the development of the consequences of the generation of further forms of said parasite such a merozoite forms.

[0153] Accordingly, the lentiviral vector particles or the combination of compounds of the invention are suitable for prevention, control or inhibition of the onset of the pathology caused by inoculation of the parasite or by the induction of the exo-erythrocytic i.e., hepatic, stage of the cycle of the malaria parasite and in an advantageous embodiment are suitable to prevent, alleviate or inhibit the onset or development of the erythrocytic cycle of said parasite. Advantageously, it has been observed that the lentiviral vector particles of the invention used in a prime-boost regimen of administration enable the development of a protective immunity and especially enable a sterilizing protection against the malaria parasite-induced pathology. Such a sterilizing protection may result from controlling the consequences of the infection at the stage of liver infection, if not before, in cycle of the parasite.

[0154] In a particular embodiment of the invention, a composition of lentiviral vector particles is prepared wherein said lentiviral vector particles are formulated with a suitable administration vehicle for use for prophylactic immunization against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host.

[0155] 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 intramuscularly or, for children intranasally.

[0156] Accordingly, a combination of compounds can comprise separately provided compositions of lentiviral vector particles wherein each separate composition of the combination or kit of compounds comprises lentiviral vector particles, pseudotyped with a determined heterologous viral pseudotyping envelope protein or proteins, and wherein said pseudotyping envelope proteins do not cross-react with to sero-neutralize the pseudotyping envelope proteins of the lentiviral vector particles of another composition of the combination or kit of compounds.

[0157] Accordingly, such compositions or combination of compounds of said compositions are used for prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, said use involving an immunisation pattern comprising administering an effective amount of the lentiviral particles to prime the cellular immune response of the host and later in time administering an effective amount of lentiviral particles to boost the cellular immune response of the host, and optionally repeating (once or several times) said administration step for boosting, wherein the lentiviral particles administered in each of the priming or boosting steps are pseudotyped with distinct pseudotyping envelope protein(s) which do not cross-neutralise with each other, and wherein said priming and boosting steps are separated in time by at least 6 weeks, in particular by at least 8 weeks.

[0158] In the examples which follow where mice models have been treated according to the prime-boost regimen with lentiviral vector particles of the invention, it has been shown by the inventors that mice immunized according to such a regimen and challenged 6 months after the last

immunization step still exhibit a sterile protection for a significant proportion of the vaccinated mice (more than 40%) which illustrates that the lentiviral vector particles of the invention elicit a long-lasting sterile protection in a host, and would therefore constitute a suitable compound for immunization especially in a human host.

[0159] The invention relates, in a particular embodiment, to the lentiviral vector particles or combination of compounds as defined herein, for the prophylactic immunization against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, in a dosage regimen comprising separately provided doses of said lentiviral particles wherein the dose intended for priming and boosting the cellular immune response is a moderate dose and the dose intended for boosting the cellular immune response is higher than the dose for priming.

[0160] Accordingly, the dose intended for priming and boosting the cellular immune response which is used in the administration pattern, comprises from 10^7 TU to 10^9 TU of viral particles when integrative vectors are used ,the dose intended for children being in the range of 10^7TU and for adults in the range of 10^9TU . The dose intended for priming and boosting comprises from 10^8 to 10^{10} of lentiviral particles when integrative-incompetent vectors are used.

[0161] The lentiviral vector particles or the combination of compounds of the invention is especially used in a particular embodiment for the prophylactic immunization against malaria parasite infection or against parasite-induced pathology in mammalian, host, especially in a human host, in a dosage and administration regimen which is suitable to obtain at least one of the following effects in the host:

- eliciting sterile protection against malaria parasite infection, especially by *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, Plasmodium knowlesi* or *Plasmodium ovale* in a human host;

- inhibiting extracellular forms of malaria parasite;
- preventing hepatocytes infection by malaria parasite or inhibition of liver stage amplification of infection;
- eliciting a specific T-cell immune response against malaria parasite antigen(s), especially a CD8+ T-cell response and/or a specific CD4+ T-cell response;
- eliciting a B-cell response against parasite antigen(s);
- controlling parasitemia so as to reduce or alleviate the effects of infection by the malaria parasite;
- eliciting a protective cellular immunity against the infection by the parasite or against the parasite-induced pathology;
- eliciting memory T-cell immune response
- eliciting earlier and higher rebound of the CD4+ and CD8+ T-cell response during infection by the malaria parasite;
- eliciting earlier and strong CT (CD8+T) response by stimulating intra hepatic memory lymphocytes upon *Plasmodium* infection.
- preventing malaria parasite escape from immune response thereby allowing long-term control of the infection by the malaria parasite.

[0162] Among the above targeted effects, a cellular immune response (T-cell immune response), particularly a CD8-mediated cellular immune response or a CD4-mediated cellular immune response *i.e.,* an immune response which is mediated by activated cells harbouring CD8 or CD4 receptors, preferably Cytotoxic T lymphocytes (CTL) and memory T cell response are advantageously targeted when defining the immunization regimen of the lentiviral particles of the invention.

[0163] The immune response can also involve a humoral response *i.e.,* antibodies, elicited by said lentiviral vector particles, produced against said at least one polypeptide of the lentiviral vector. In a particular embodiment, said humoral response is a protective humoral response. The protective humoral response results mainly in maturated antibodies, having a high affinity for their antigen, such as IgG. In a particular aspect, the protective humoral response is T-cell dependent. In a particular embodiment, the protective humoral response induces the production of neutralizing antibodies.

[0164] In a particular embodiment of the invention, the lentiviral vector of the invention, even when used in a form which has defective integrase, is able to elicit an early immune response. The expression "*early immune response*" refers to a protective immune response (protection against the parasite or against the parasite-induced pathology) that is conferred within about one week after the administration of the composition.

[0165] In another particularly advantageous embodiment, the immune response conferred by the lentiviral particles of the invention 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, (as illustrated for mice in the examples a protection is still obtained after at least 6 months after the administration of the particles) which allows to consider that the protection may last in a human host over several years following the administration.

[0166] When the immune response includes a humoral response, the long-lasting response can be shown by the detection of specific antibodies, by any suitable methods such as ELISA, immunofluorescence (IFA), focus reduction neutralization tests (FRNT), immunoprecipitation, or Western blotting.

[0167] In a particular embodiment, said immune response, either humoral or cellular, early immune response and/or long-lasting immune response, is elicited with the non-integrative gene transfer vector, after a single administration of the composition of the invention.

[0168] The invention also concerns the use of the lentiviral vector particles or the use of a combination of compounds according to the definition given herein, for the manufacture of an immunogenic composition for prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host.

[0169] The invention also concerns a method of providing immunization in a mammalian host, especially in a human host, comprising the step of administering the lentiviral vectors of the invention to elicit the immune response, and optionally repeating the administration steps one or several times, to boost said response, in accordance with the present disclosure.

[0170] In a particular embodiment of the invention, the lentiviral vector particles or the combination of compounds 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.

[0171] The compositions quoted above can be injected in a host via different routes: subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.) or intravenous (i.v.) injection, oral administration and mucosal 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 expressed with respect to the content in

equivalent p24 antigen of vector particles (for HIV-1 lentiviral vectors) and can be determined.

[0172] 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.

Legend of the figures

[0173] **Figure 1. Nonintegrative lentiviral vector-based vaccination confers total inhibition of liver stage development. A.** Study design. Naive mice were primed at week 0 with 100 ng of TRIP.NI CS particles pseudotyped with the VSV-G Indiana (VSV-G Ind) envelope and then boosted at week 8 with 1500 ng of TRIP.NI CS particles pseudotyped with the VSV-G New Jersey (VSV-G NJ) envelope. One group of vaccinated mice were challenged with 80.000 sporozoites (spz) of *Plasmodium yoelii* (17XNL-gfp$^+$ strain) and protective efficacy was measured by quantifying the liver parasite load 40 hours later. A second group of vaccinated mice were challenged with 500 spz of *Plasmodium yoelii* (17XNL-gfp$^+$ strain) and protective efficacy was evaluated by monitoring blood stage parasitemia every other day from day 3 post injection until day 14 by Giemsa-stained blood smears. In the two cases, the challenges were performed one month after the last immunization. **B.** Results of the parasite loads quantified using real-time RT-PCR for *P.yoelii* 18S rRNA in the livers of challenged mice. Data are presented as the number of copies of plasmodium 18S rRNA detected in individual control mice (n=5) and vaccinated mice (n=4). Mean +/- SD of duplicate is shown. **C.** Results of the monitoring of blood stage parasitemia. 0 indicates absence of parasites, + indicates presence of parasites.

[0174] **Figure 2. A.** Study design. Mice primed with 100 ng of TRIP.NI CS particles pseudotyped with the VSV-G Indiana (VSV-G Ind) envelope and boosted 8 weeks later with 1500 ng of TRIP.NI CS particles pseudotyped with the VSV-G New Jersey (VSV-G NJ) envelope received a third immunization dose 5 months later with 1500 ng of TRIP.NI CS particles pseudotyped with the VSV-G Cocal. Vaccinated mice were challenged one month later with 500 spz of *Plasmodium yoelii* (17XNL strain) and protective efficacy was evaluated by monitoring blood stage parasitemia every other day from day 3 post injection until day 16 by Giemsa-stained blood smears. **B.** Percentage of mice completely protected against sporozoite challenge after a nonintegrative lentiviral vector-based regimen. **C.** Means of parasitemia of naive mice (CO-black curve), vaccinated mice completely protected (VAC-light grey curve) and vaccinated mice partially protected (VAC-grey curve) are depicted. **D.** Means of parasitemia from naive mice (CO- black), vaccinated mice partially protected (VAC-grey) and vaccinated mice completely protected (VAC-grey), 10 days after the challenge.

[0175] **Figure 3.** Gross morphology of spleens and livers from mice vaccinated (VAC) or not (CO) at the final killing (3 weeks post-challenge) with 500 sporozoites of *Plasmodium yoelii*.

[0176] **Figure 4.** CS protein-specific T cell responses from splenocytes of vaccinated mice 3 weeks after challenge. *Ex vivo* IFN□ ELISPOT was carried out using splenocytes from vaccinated mice harvested 3 weeks after the challenge with *Plasmodium yoelii*. Splenocytes were stimulated with synthetic peptides representing $CD8^+$ or $CD4^+$defined epitopes. Data are expressed as mean +/- SD of spot forming cells (sfc) of

duplicate wells. n=5 in the protected group and n=3 in the unprotected group. * : different from unprotected group p<0.05.

[0177] **Figure 5.** Optimized non integrative lentiviral vectors confer long term sterile protection against malaria (a) Vaccine schedule. Mice were primed with 100 ng of TRIP.NI CSP particles pseudotyped with the VSV-G Indiana envelope and boosted 8 weeks later with 1500 ng of TRIP.NI CSP particles pseudotyped with the VSV-G New Jersey envelope. Five months later, they received a third injection of TRIP.NI CS particles (1500 ng) pseudotyped with the VSV-G Cocal envelope. Animals were challenged six months later with 500 sporozoites of *Plasmodium yoelii* (17XNL strain) and protective efficacy was evaluated by monitoring blood stage parasitemia every other day from day 3 post injection until day 16. (**b**) Means of parasitemia from naive mice (CO- black), vaccinated mice partially protected (VAC-light grey (middle)) and vaccinated mice completely protected (VAC-grey (right)), 10 days after the challenge. (**c**) Tetramer analysis of the % of CSP-specific $CD8^+$ T cells from the spleen, the bone marrow and the liver of mice at the final killing (3 weeks post-challenge).Black bars indicate vaccinated mice partially protected and white bars indicate vaccinated mice completely protected. (d) INF-g ELISPOT quantification of CSP-specific CD8+ T cells in the spleen, the bone marrow and the liver of mice. * P< 0.05 (Student's t-test)

[0178] **Figure 6.** Hep17-specific T cell responses induced by nonintegrative lentiviral vectors. Naive mice (n=5/group) were immunized or not (-) i.p. with a single injection of various doses (100 or 600 ng) of nonintegrative lentiviral vectors coding for Hep17. At 11 days post-immunization, Hep17-specific cellular immune responses against the $CD8^+$

T cell epitopes (A) and the $CD4^+$ T cell epitopes (B) were assessed by IFN-γ ELISPOT. SFC, spot-forming cells.

[0179] **Figure 7.** Hep17-specific T cell responses induced by integrative lentiviral vectors. Naive mice (n=5/group) were immunized (or not: -) i.m. with a single injection of integrative lentiviral vectors ($1x10^7$ TU) coding for Hep17. At 11 days post-immunization, Hep17-specific cellular immune responses against the $CD8^+$ T cell epitopes (**A**) and the $CD4^+$ T cell epitopes (**B**) were assessed by IFN-γ ELISPOT. SFC, spot-forming cells.

[0180] **Figure 8.** CS- and Hep17 specific T cell responses elicited after coimmunizations with lentiviral particles. Naive mice (n=5/group) were immunized i.m. with a single injection of integrative lentiviral vectors ($1x10^7$ TU) coding for CS (named CSP in figure **A** and **B)** or Hep17 (named Hep17 in figure **C** and **D**). For coimmunization experiments, naive mice were injected into one quadriceps with TRIP.I CS and into the opposite quadriceps with TRIP.I Hep17 particles (named CSP+Hep in figure A,B,C,D). At 11 days post-immunization,CS-specific cellular immune responses (**A**) and Hep17-specific cellular immune responses (**C**) were assessed by IFN-γ ELISPOT. SFC, spot-forming cells. For *in vivo* cytotoxic assays, immunized mice were injected at day 11with target cells pulsed with CS peptides (**C**) or Hep17 peptides (**D**). Percentages of specific killing were determined 18 hours later, as described in Material and Methods section.

[0181] **Figure 9. A single dose of nonintegrative lentiviral vector coding for $MSP1_{42}$ elicits a strong and specific antibody**

response. A. Groups of adult mice (n=5) were immunized intraperitoneally with graded doses of TRIP.I $MSP1_{42}$. After 21days, pooled sera (5 mice per group) were assessed for the presence of MSP-1_{19}-specific antibodies. **B.** Mice were primed with 100 ng of TRIP.I $MSP1_{42}$ particles pseudotyped with the VSV-G Indiana envelope. 3 months later, mice were boosted with 1000 ng of TRIP.NI $MSP1_{42}$ particles pseudotyped with the VSV-G cocal envelope. Results are the mean titers of MSP-1_{19}-specific antibodies detected in the sera of mice 3 weeks after the last immunization.

[0182] **Figure 10. Alignments of *Plasmodium* CSP proteins and sequence of a consensus.**

[0183] **Figure 11. Restriction map of plasmid pTRIP-DeltaU3-CMV-eGFP.** SEQ ID No 33

[0184] **Figure 12. Restriction map of plasmid pTRIP-ΔU3-CMV-$MSP1_{42}$ CO-WPRE** (CNCM I-4303 or SEQ ID No 34).

[0185] **Figure 13. Restriction map of plasmid pTRIP-ΔU3-CMV-Hep17 CO-WPRE** (CNCM I-4304 or SEQ ID No 37).

[0186] **Figure 14. Restriction map of plasmid pTRIP-ΔU3-CMV-Hep17 ΔSP CO-WPRE** (CNCM I-4305 or SEQ ID No 40).

[0187] **Figure 15. Restriction map of plasmid pTRIP-ΔU3-CMV-CSP CO-WPRE** (CNCM I-4306 or SEQ ID No 43).

[0188] **Figure 16. Restriction map of plasmid pTRIP-ΔU3-CMV-CSP ΔSP CO-WPRE** (CNCM I-4307 or SEQ ID No 45).

[0189] **Figure 17. Restriction map of plasmid pTRIP-ΔU3-CMV-CSP ΔGPI CO-WPRE** (CNCM I-4308 or SEQ ID No 47).

[0190] Examples

[0191] With a view to assess whether lentiviral vectors may represent an alternative strategy, a nonintegrative lentiviral vector coding for a truncated form of the circumsporozoite (CS) protein of *Plasmodium yoelii* (TRIP.NI CS) was designed and assayed in an animal mice model relevant for malaria. The CS protein is distributed uniformely over the surface of sporozoites and is also detected in infected liver cells [4, 5]. Thus, the induction of humoral immune responses against the CS protein reduces the hepatocyte infectivity, whereas the cellular immune responses against this antigen kill parasite-infected hepatocytes. This concept was recently supported by an elegant study that demonstrated that the CS protein was the main target of protective immunity in the irradiated sporozoite immunization model [6]. Moreover, among all the vaccine candidates tested so far in clinical trials, only the CS protein-based vaccine RTS,S has been shown to reduce significantly malaria incidence and cases of severe malaria in endemic countries [7, 8].

[0192] In order to elicit optimal immune responses against the CS protein, we combined three strategies : 1) to increase the level of antigen expression in transduced cells, we inserted in the vector backone a mamalian codon-optimized sequence of the CS protein under the control of the strong cytomegalovirus promoter and we added downstream the transgene the woodchuck post-transcriptional regulatory element sequence to increase mRNA stabilization and export to the cytoplasm; 2) we deleted the GPI anchoring sequence located at the 3' end of the cs gene since deletion of GPI-anchoring motif has been shown to improve the

immunogenicity of the CS protein [9] 3) to increase the specific immune response, and in particular, to protect mice from infection by sporozoite challenge, mice received LV-based boosters. To circumvent the presence of neutralizing anti-envelope antibodies induced after the first immunization, lentiviral particles used for boost immunizations were pseudotyped with VSV-G envelopes from non-cross-reactive serotypes (VSV-G Indiana for the prime, VSV-G New Jersey and Cocal for the first and the second boost, respectively).

[0193] In a first series of experiment, mice were primed with a moderate dose of TRIP.NI CS and boosted 8 weeks later with a high dose of TRIP.NI CS (Figure 1a). To evaluate protection induced by this prime-boost regimen, BALB/c mice were challenged with 80.10^3 sporozoites of Plasmodium yoelii (17XNL gfp+ strain), the invasive form of the parasite present in the mosquitoe. The challenge was performed 4 weeks after the completion of immunization regimen. Forty hours after the challenge, the level of inhibition of liver stage development was determined by quantifying plasmodial 18S rRNA in the livers of mice. For this purpose, liver-extracted RNA was used for real-time PCR amplification of the plasmodial 18S rRNA sequences, using the EXPRESS One-Step SYBR® GreenER™ kit (invitrogen) and specific primers for the amplification of the 18S rRNA of Plasmodium yoelii. As shown in Figure 1b, the inhibition of liver stage development of the parasite was complete for all immunized mice, i.e., no parasite 18S rRNA could be detected by quantitative RT-PCR. In parallel experiments, protection was also assessed by examining blood smears of immunized mice, which were challenged with 500 Plasmodium yoelii sporozoites, for the occurrence of erythrocytic stages. Peripheral blood smears were obtained daily from day 3 to 14 post-challenge, stained with Giemsa and examined by microscopy, to determine whether immunized mice became parasitemic, i.e., failed to develop protection. As shown in Figure 1c, complete protection occured in 60% of immunized mice.

[0194] In a second series of experiments, we added a third injection of TRIP.NI CS pseudotyped with a VSV-G Cocal envelope which does not cross-react whith antbodies directed against the Indiana and New Jersey serotypes. One month after the last boost, immunized mice were challenged intravenously with 500 sporozoites ,(Figure 2a). Protective efficacy was evaluated by monitoring blood stage parasitemia every other day from day 3 post injection until day 21 by Giemsa-stained blood smears. After 5 days, all naive mice exhibited patent blood stage parasitemia. By contrast, 62,5% of immunized mice showed sterile immunity (as defined by the absence of parasitemia over the following 21 days) (Figure 2b). Moreover, as compared with naive mice, immunized mice that developped parasitemia displayed a significant delay in the course of erythrocyte invasion (Figure 2c). At day 10 post-challenge, immunized mice partially protected showed a twofold reduction in the level of parasitemia compared to naive mice, demonstrating that in this case, the vaccine afforded also an immune control, albeit partial, of the parasite (Figure 2d).

[0195] Hepatosplenomegaly is a prominent feature of malaria. We then performed a qualitative analysis of the organs from mice sacrified 3 weeks post-challenge. Naive mice infected with the parasite displayed dramatic splenomegaly (Figure 3). Moreover, spleens and livers showed dark pigmentation resulting from the accumulation of hemozoin produced by the parasite during digestion of red blood cell hemoglobin. By contrast, the capacity of 5 out of 8 vaccinated mice to mount a sterile immune response coincided with preservation of livers and spleens that displayed normal size and pigmentation.

[0196] In an attempt to understand why 1/3 of immunized mice did not shown sterile protection, we evaluated the CS protein-specific immune responses in vaccinated animals sacrified at 3 weeks post-challenge.

Challenged naive mice displayed no detectable CS protein-specific IFN-μ producing T cells (data not shown). By contrast, in the vaccinated group, mice fully protected exhibited five to eight fold greater CSP-specific T cell responses as compared with mice vaccinated but partially protected, emphasizing the critical importance of the strength of T cell responses for immune control (Figure 4).

[0197] Importantly, we also performed challenge experiments at 6 months after the last immunization. In this case, more than 40% of the vaccinated mice still failed to develop detectable parasitemia following challenge, illustrating the long-lasting sterile protection conferred by our vaccine strategy (Figure 5).

[0198] Taken together, these data demonstrated that a prime-boost regimen based on non integrative lentiviral vectors can confer a high degree of protection against challenging infectious agents such as plasmodium.

[0199] Based on these results, we are currently developing a multi-stage vaccine approach. The rationale of this strategy is to improve the protective efficiency conferred by our vaccine approach by inducing a multi-immune response directed against antigens expressed in the liver stage and targeted by T-cell responses, as well as antigens expressed in the blood-stage and targeted by antibody responses. To this end, we have selected two pre-erythrocytic stage antigens (CS protein and Hepatocyte Erythrocyte Protein 17 kDa – HEP17) and one erythrocytic stage antigen (The 42-kDa fragment of the Merozoite Surface Protein 1 - MSP-1_{42}). These antigens were selected because it has been shown that cytotoxic T cell responses specific of Hep17 are partially protective against sporozoite challenge and antibody responses specific of MSP-1_{42} can also protect mice against a lethal challenge with blood-stage parasites [10, 11]. Lentiviral vectors coding for Hep17 or MSP-1_{42}, were constructed as detailed in the Material and Methods part. To evaluate the immunogenicity of a single

injection of lentiviral particles expressing Hep17, groups of mice (n=5/group) were immunized with 100 ng (3,2 x 10^7 TU) or 600 ng (1,9 x 10^8 TU) of TRIP.NI Hep17 and specific immune responses were assayed by Elispot. As shown in Figure 6, relatively weak CD8 and CD4 responses could be detected in the spleens of immunized mice after stimulation with the 9-mer and 15-mer previously described [12].

[0200] We also tested the immunogenicity of TRIP.I Hep17 lentiviral particles. Groups of mice (n=5) were immunized im with 1x10^7 TRIP.I Hep17 particles. Hep-17-specific IFNg Elispot responses were evaluated 11 days later on splenocytes from immunized mice. As shown in Figure 7, the most robust responses were detected against the CD4+ T cell epitopes (KL14 and EK15) and against one CD8+ T cell epitope (LA9). We also evaluated the T-cell responses obtained after co-immunization of TRIP.I Hep17 particles with TRIP.I CS particles. Mice received two injections : one injection of 1x10^7 TRIP.I Hep17 particles in the left quadriceps and one injection of 1x10^7 TRIP.I CS particles in the right quadriceps. In parallel, groups of mice were immunized with TRIP.I CS particles alone (1x10^7 TU im) or TRIP.I Hep17 particles alone (1x10^7 TU im). At day 11, one part of immunized mice were sacrified for Elispot experiments. There was no huge difference between the frequency of CS-specific IFNg T cells in mice immunized with TRIP.CS particles alone or with TRIP.I CS and TRIP.I Hep17 particles (Figure 8A). To evaluate cytotoxic T cell response in immunized mice, we performed an in vivo cytotoxic assay (as described in Material and Methods). At day 11, groups of mice immunized with TRIP.I CS particles alone or coimmunized with TRIP.I CS and Hep17 particles were challenged by iv injection with target cells pulsed with CS peptides. As expected, mice immunized with TRIP.I CS particles lyzed efficiently target cells and we did not detect significant difference between group of mice immunized with TRIP.I CS particles alone and group of mice that received both TRIP.I CS and TRIP.I Hep17 particles

(Figure 8B). Taken together, these results demonstrated that TRIP.I Hep17 particles co-administered with TRIP.I CS particles did not significantly interfere with the CS-specific T cell response elicited by TRIP.I CS particles. We also evaluated the frequency of Hep17-specific IFNg T cells in mice immunized with TRIP.I Hep17 alone or co-administrated with TRIP.I CS particles. Frequencies of specific T cells responding to stimulation to the five 9-mer peptides (CD8+ T cell epitopes) were the same in the two groups, as well as those measured after stimulation with the KL14 epitope (CD4+ T cell epitope). Strikingly, the responses detected against the CD4+ T cell epitope EK15 were twice higher in mice co-immunized than in mice immunized with TRIP.I Hep17 alone (Figure 8C). As shown in figure 8D, the cytotoxic capacity of T cells against Hep17 peptides-pulsed targets were also greatly increased in mice co-immunized with TRIP.I Hep17 and TRIP.I CS particles. Collectively, these data demonstrate that CS-specific immune response enhances cytotoxic T cell responses specific for Hep17.

[0201] We next evaluated the ability of lentiviral vectors to initiate a B cell response against the blood stage malaria antigen merozoite surface protein-1 (MSP1). Mice (n=5) were immunized with various doses of integrative lentiviral vectors coding for the 42-kDa region of MSP1 from *Plasmodium yoelii* (TRIP.I $MSP1_{42}$) fused to at the N terminus to the secretory signal of the calreticuline. Three weeks after immunization, pooled sera collected from each group of immunized mice were tested for the presence of total anti-MSP1 antibodies directed against the protective C-terminal 19-kDa region ($MSP\text{-}1_{19}$) [13, 14] of the antigen. As shown in figure 9A, mice immunized with a dose as low as $1x10^6$ TU displayed detectable levels of anti- $MSP\text{-}1_{19}$ antibodies and immunizations with $1x10^7$ TU of this vector induced a strong secretion of anti- $MSP\text{-}1_{19}$ Ig with a mean titer reaching 2×10^3. To know whether anti-MSP1 response conferred by lentiviral vector immunization could be enhanced by a second immunization, mice immunized with 100 ng of TRIP.I $MSP1_{42}$ particles

pseudotyped with VSV-G Indiana envelope were boosted 3 months later with 1000 ng of TRIP.NI $MSP1_{42}$ particles pseudotyped with the VSV-G Cocal envelope (Figure 9B). 3 weeks after the last immunization, the levels of anti- $MSP\text{-}1_{19}$ antibodies in prime-boosted mice reached a mean value of 4×10^5 whereas the titer in the plasma of mice solely primed was 2×10^4. In conclusion, immunization with integrative lentiviral vectors can induce potent anti- $MSP\text{-}1_{19}$ Ig that have been shown to be protective against infection of red blood cells by parasites.

MATERIAL AND METHODS

[0202] **Animals and parasites**. Balb/c Ola Hsd (six-week-old female) were purchased from Harlan Laboratories (Gannat, France). All animal experiments were conducted in accordance with the guidelines of Animal Care at the Pasteur Institute. Infection experiments were performed with the *Plasmodium yoelii* (17XNL strain) wild-type or genetically modified to express the green fluorescent protein, allowing the detection of oocysts and sporozoites in living mosquitoes. *Plasmodium yoelii* was maintained by alternate cyclic passages in *Anopheles stephensi* and Balb/c mice. Mosquitoes were reared at the Center for Production and Infection of Anopheles (CEPIA) of the Pasteur Institute using standard procedures.

[0203] **Plasmid vectors construction.** The mammalian codon optimized form of the gene coding for the full-length of the Py CS protein (amino acids 1-367 ; GenBank Accession No. M58295) was synthesized by Geneart. Since deletion of the GPI-anchoring motif has been shown to improve the immunogenicity of the CS protein, we constructed a codon optimized form of the cs gene deleted of the sequence encoding the last 11 amino acids. This sequence was obtained by PCR amplification of a

fragment of the codon optimized cs gene using the following oligonucleotides (Sigma-Proligo) : (forward) 5'GGTACCGGATCCGCCACCATGAAGAAA TGCACC-3' (underlined is the BamHI site) ; (reverse) 5'-AGCTCGAGTCATCACAGGCTGTTGGACACGATGTTGAAGATGC-3' (underlined is the XhoI site). The resulting amplicon was cloned in a pCR 2.1-TOPO plasmid (Invitrogen) and sequenced (plasmid referred as pCR 2.1-TOPO CS). The pTRIP CS vector plasmid was generated by replacing the GFP sequence from pTRIP CMV-GFP-WPRE digested BamHI/XhoI by the truncated codon-optimized CS sequence obtained after a BamHI/xhoI digestion of the pCR 2.1-TOPO CS. For pTRIP Hep17, a mammalian codon-optimized sequence (Geneart) of the Py Hep17 gene (GenBank Accession No. U43539) including a kozak sequence and flanked of a BamH1 site in 5' and a XhoI site in 3' was cloned in pTRIP CMV-WPRE digested BamH1/XhoI. For MSP1 construct, a composite mammalian codon optimized sequence (Geneart) was designed to include : a sequence coding for the secretion signal of the calreticuline (MLLSVPLLLGLLGLAVA) fused to the codon optimized sequence of the Py $MSP1_{42}$ (GenBank Accession No. JO4668). The entire sequence digested BamH1/XhoI was cloned in pTRIP CMV-WPRE digested BamH1/XhoI.

[0204] Sequences of the pTRIP vectors are respectively designated as: SEQ ID NO 34, 37, 40, 43, 45 and 47.

[0205] **Lentiviral vector production.** Vector particles were produced by transient calcium phosphate co-transfection of 293T cells with the vector plasmid pTRIP CS, a VSV-G envelope expression plasmid (pHCMV-G) and the pD64V encapsidation GAG POL plasmid for the production of integration-deficient vectors (the D64V substitution in the catalytic domain of the integrase blocks the DNA cleaving and joining reactions of the integration step) as previously described [15] . Quantification of the p24 antigen content of concentrated vector particles was performed

with a commercial HIV-1 p24 enzyme-linked immunoabsorbent assay (ELISA) kit (Perkin Elmer Life Sciences). Vector titers of TRIP.I and TRIP.NI particles were determined by transducing HeLa cells treated with aphidicolin (SIGMA) and performing a quantitative PCR as previously described [15]. The titers of integrative and nonintegrative lentiviral vectors were similar according to p24 content and quantitative PCR measured in growth-arrested cells.

[0206] **Mice immunization and challenge.** Six-week-old BALB/c mice were intraperitoneally (i.p.) immunized with 100 ng of TRIP.NI CS vector particles pseudotyped with the VSV-G Indiana envelope, diluted in 0.1 ml Dulbecco's phosphate-buffered saline. Eight weeks later, mice were boosted i.p. with 1500 ng of TRIP.NI CS vector particles pseudotyped with the VSV-G New Jersey envelope. Challenge of the immunized and the control mice consisted of the injection of 80,000 sporozoites intravenously 4 or more weeks after the last immunization. The outcome of the challenge was determined by measuring the parasite burden in the liver of mice by using a quantitative real-time RT-PCR method, as detailed later. We also determined, in control and immunized groups of mice, whether or not mice developed parasitemia after i.v. inoculation of 500 sporozoites, by microscopic examination of Giemsa-stained thin blood smears obtained daily, from the third day after challenge up to day 14. Briefly, a small drop of blood from challenged mice was placed on a microscope slide. The drop was smeared by using a second slide, air-dried, and fixed in 100% methanol for 30 seconds. Fixed slides were stained for 30 minutes in a fresh solution of 10% Giemsa (Reactfs RAL) diluted in water (Volvic), rinsed with water and dried in air. The slides were observed with x100 oil immersion objective.

[0207] **Quantification of P. yoelii by Real-Time RT-PCR.** Quantification of the parasite loads in the liver of the challenged mice was performed as previously described [16] with some modifications. 40 hours after challenge, livers were harvested and RNA was extracted with the RNeasy mini kit (Qiagen). 2 µg of RNA was used for the quantification of parasite specific 18S rRNA. The reaction of real-time RT-PCR was carried out with the EXPRESS One-Step SYBR® GreenER™ kit (invitrogen) and specific primers for the amplification of the 18S rRNA of *P.yoelii*. The sequences of the primers (Sigma-Proligo) are : 5'-GGGGATTGGTTTTGACGTTTTTGCG-3' (forward primer) and 5'-AAGCATTAAATAAAGCGAATACATCCTTAT-3' (reverse primer). Experiments were performed with a LightCycler™ apparatus (Roche diagnostics). The quantity of parasite RNA copies was assessed by extrapolation of threshold fluorescence values onto an internal standard curve prepared from serial dilutions of a plasmid construct (pCR 2.1-TOPO plasmid-Invitrogen) containing the 18S cDNA PCR-amplified fragment of the parasite.

[0208] **Elispot Assay.** Nitrocellulose microplates (MAHA S4510, Millipore) were coated with capture antibody (Mouse IFNg Elispot pair, BD Pharmingen) and blocked with complete medium composed of RPMI 1640 Glutamax supplemented with 10 % FCS, antibiotic, Hepes, non-essential amino-acids, b-mercaptoethanol and sodium pyruvate. Splenocytes from vector-immunized mice were added to the plates in triplicates at $0{,}125x10^6$ cells/well. For quantification of CS-specific $CD8^+$ T cell reponses, splenocytes were incubated with 2 µg/ml of the peptides (PolyPeptide Laboratories France) SYVPSAEQI (Py $CS_{280-288}$) or IYNRNIVNRL (Py CS_{58-67}). To evaluate the CS-specific $CD4^+$ T cell responses, splenocytes were incubated with 2 µg/ml of the peptides SYVPSAEQILEFVKQI (Py $CS_{280-295}$). Twenty hours later, spots were revealed with the biotin-conjugated antibody (Mouse IFNg Elispot pair, BD Pharmingen) followed by streptavidin-AP

(Roche) and BCIP/NB substrate solution (Promega). Spots were counted using a Bioreader 2000 (Biosys, Karben, Germany) and results were expressed as IFNγ spot-forming cells (sfc) per million splenocytes. The same protocol was applied for quantification of Hep17-specific T cell responses. Peptides used for stimulation in Elispot and *in vivo* cytotoxic assay are summarized in Table 1.

[0209] **In vivo cytotoxic assay.** For target cell preparation, splenocytes from naive mice were labelled with various concentrations (high, 5 μM ; Low, 1 μM) of CFSE (carboxyfluorescein-diacetate succinimydyl ester, Vybrant CFDA-SE cell-tracer kit, Molecular Probes). Splenocytes labelled with high concentrations of CFSE were pulsed with combination of peptides at 5 μg/ml. The control population stained with low doses of CFSE was incubated in medium without peptides. Each mouse received 10^7 CFSE-labelled cells of a mix containing an equal number of cells from each fraction, through the retroorbital vein. After 15-18h, single-cell suspensions from spleen were analyzed by flow cytometry (Becton Dickinson, CellQuest software). The disappearance of peptide-pulsed cells was determined by comparing the ratio of pulsed (High CFSE fluorescence intensity) to unpulsed (Low CFSE fluorescence intensity) populations in immunized versus naive mice. The percentage of specific killing was established according to the following calculation : $(1-((CFSE_{low}$ naive$/CFSE_{high}$ naive$)/(CFSE_{low}$ immunized$/CFSE_{high}$ immunized$)))*100$.

Table 1. Sequences of CS and Hep17 synthetic peptides.

CSP

CD8+ T cell epitopes

SI9	Rs 280-88	**SYVPSAEQI**	Dominant
IL10	Rs 58-67	**IYNRNIVNRL**	Subdominant

CD4+ T helper cell epitopes with overlapping CD8+ T cell epitopes

SI16	Rs 280-95	**SYVPSAEQILEFVKQI**	Dominant
KD14	Rs 57-70	**KIYNRNIVNRLLGD**	Dominant (nested dom. CD8+ T cell epitope)
YK21	Rs 59-79	**YNRNIVNRLLGDALNGKPEEK**	Subdominant (nested subdom.CD8+Tcell epitope)

PyHEP17

CD8+ T cell epitopes (9-mer)

L9A	Rs 73-81	**LRKINVALA**	Subdominant
EN9	Rs 61-69	**EEIVKLTKN**	Subdominant
KV9	Rs 70-78	**KKSLRKINV**	Subdominant
IL9	Rs 76-84	**INVALATAL**	Subdominant
LL9	Rs 84-92	**LSVVSAILL**	Subdominant

CD4+ T cell epitope with nested CD8+ T cell epitopes (15-mer)

EK15	Rs 61-75	**EEIVKLTKNKKSLRK**	Dominant
KL14	Rs 71-84	**KSLRKINVALATAL**	Dominant

Ref

CSP:

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Py HEP17

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[0210] **Recombinant MSP1$_{19}$ protein.** *P.yoelii* YM MSP1$_{19}$ (aa 1649-1757) was amplified by PCR using the forward primer 5'-CGTGGATCCATGGACGGCATGGATCTGCTG-3' and the reverse primer 5'-GATGAATTCGGAGCTGCTGCTGCAGAACACG-3' from pTRIP MSP1$_{42}$ and cloned into the glutathione S-transferase (GST)-fusion protein expression vector pGEX-2T (Amersham Biosciences, Bucks, UK).

Escherichia coli BL21 star (Invitrogen) were transformed with pGEX-2T $MSP1_{19}$ and growth and induction were performed according to the manufacturer's instructions (pGEX vectors, GST gene fusion system, Amersham). After induction of the expression of the protein in BL21, cells were harvested and lysed using BugBuster reagent (Novagen). Recombinant protein was purified by GST bind resin chromatography using GST bind purification kit (Novagen) as per manufacturer's instructions.

[0211] **Measurement of serum antibody responses.** Sera were collected 3 weeks after the last immunization for the assessment of $MSP1_{19}$-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Recombinant GST- $MSP1_{19}$ fusion protein or GST control were adsorbed overnight at 4°C to 96 well Nunc-Immuno Maxisorp plates (Fischer Scientific, Wohlen, Germany) at 2 µg/ml in PBS. After three washes with 0.05%Tween 20 in PBS, wells were blocked by the addition of 100 µl of PBS containing 10% of foetal bovine serum (FBS) at room temperature for 1 hour. Plates were washed three times with 0.05%Tween 20 in PBS and 100 µl of tenfold serial dilutions of serum were added to the wells. After incubation for 2 hours at room temperature, the wells were washed and 100 µl of peroxydase goat anti-mouse immunoglobulin (H+L) (Jackson Immuno Research) diluted 1/4000 in PBS 10% FBS was added to each well. After incubation for 1 hour at room temperature, wells were washed and 100 µl of tetramethylbenzidine substrate reagent (BD Pharmingen) was added to each well. The plates were incubated at room temperature for 30 min, and 100 µl of 1N H_2SO_4 was added to stop the reaction. The plates were read for optical density at 450 nm. The endpoint titer was calculated as the reciprocal of the last dilution eliciting twice the optical density of sera from non immunized mice.

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CLAIMS

1. Lentiviral vector particle (i) which is pseudotyped with a determined heterologous viral envelope protein or viral envelope proteins originating from a RNA virus and (ii) which comprises in its genome at least one recombinant polynucleotide encoding at least one polypeptide(s) carrying epitope(s) of a pre-erythrocytic stage antigen of a *Plasmodium* parasite capable of infecting a mammalian host, wherein said epitope(s) encompass(es) T-epitope(s) and optionally B-epitope(s).

2. Lentiviral vector according to claim 1 which is a replication-incompetent HIV-based vector particle.

3. Lentiviral vector particle according to claim 1 wherein at least one recombinant polynucleotide comprises a nucleic acid sequence encoding a polypeptide(s) of an antigen from the circumsporozoite protein (CSP) of a *Plasmodium* parasite infecting humans, or encoding a polypeptide of an antigen selected from the group of the sporozoite surface protein 2 (TRAP/SSP2), liver-stage antigen (LSA), LSA3, Pf exported protein 1 (Pf Exp1), Pf antigen 2 sporozoïte and liver stage antigen (SALSA), sporozoïte threonine and asparagines-rich (STARP)..

4. Lentiviral vector particle according to any of the preceding claims which comprises in its genome a polynucleotide encoding a polypeptide chosen in the group of merozoïte surface protein 1 (MSP2), in particular Merozoïte surface protein 1 (MSP-1), Merozoïte surface protein 2 (MSP-2) merozoïte surface protein 3 (MSP-3), Merozoïte surface protein 4 (MSP-4), Merozoïte surface protein 6 (MSP-6), MSP3-GLURP fusion protein. Ring-infected erythrocyte surface antigen (RESA), Rhoptry associated protein 1 (RAP-1), Apical membrane antigen 1 (AMA-1), Erythrocyte binding antigen

(EBA-175), Erythrocyte membrane-associated giant protein or Antigen 332 (Ag332), dnaK-type molecular chaperone, Glutamate-rich protein (GLURP); MSP3-GLURP fusion protein, Erythrocyte membrane protein 1 (EMP-1), Serine repeat antigen (SERA), Clustered-asparagine-rich protein (CARP), Cirumsporozoite protein-related antigen precursor (CRA), Cytoadherence-linked asexual protein (CLAG), Acid basic repeat antigen (ABRA) or 101 kDa malaria antigen, Rhoptry antigen protein (RAP-2), Knob-associated histidine-rich protein (KHRP), Rhoptry antigen protein (RAP), Cysteine protease, Hypothetical protein PFE1325w, Protective antigen (MAg-1), Fructose-bisphosphate aldolase, Ribosomal phosphoprotein P0, P-type ATPase, Glucose-regulated protein (GRP78), Asparagine and aspartate-rich protein (AARP1), Interspersed repeat antigen or PFE0070w. and/or a polynucleotide encoding a polypeptide chosen in the group of Sexual stage and sporozoite surface antigen, Antigen Pfg27/25, Antigen QF122, 11-1 polypeptide, Gametocyte-specific surface protein (Pfs230) Ookinete surface protein (P25), Chitinase, Multidrug resistance protein (MRP).

5. Lentiviral vector particle according to any of the preceding claims wherein the recombinant polynucleotide(s) has(have) a mammalian codon optimized nucleotide sequence and optionally the HIV-based sequences of the genome have a mammalian codon optimized nucleotide sequence.

6. Lentiviral vector particle according to any of the preceding claims wherein a recombinant polynucleotide encodes at least a polypeptide of the CSP antigen, said polypeptide being devoid of the GPI-anchoring motif of said CSP.

7. Lentiviral vector particle according to any of the preceding claims, which is chosen among either integration-deficient vector particles or integration-competent vector particles.

8. Lentiviral vector particle according to any of the preceding claims, which is pseudotyped with viral transmembrane glycosylated (G) envelope protein(s) of a Vesicular Stomatitis Virus (VSV) chosen in the

group of VSV-G protein(s) of the Indiana strain, VSV-G protein(s) of the New Jersey strain, VSV-G protein(s) of the Cocal strain, VSV-G protein of the Isfahan strain, VSV-G protein(s) of Chandipura strain, VSV-G protein(s) of Pyri strain or VSV-G protein(s) of the SVCV strain.

9. Lentiviral vector particles according to any of the preceding claims which 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 HIV-1, DNA flap element and optionally comprising cis-active sequences necessary for integration, said vector plasmid further comprising (ii) a polynucleotide of a truncated mammalian, especially human, codon-optimized sequence of the cs gene of a *Plasmodium* parasite, 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 or envelope proteins, wherein said polynucleotide is under the control of regulatory expression sequences 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 *gag-pol* or modified *gag-pol* sequences are under the control of regulatory expression sequences.

10. Lentiviral vector particles according to any of the preceding claims which are the product recovered from a stable cell line 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 truncated mammalian, especially human, codon-optimized sequence of the cs gene of a *Plasmodium* parasite, 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 or envelope proteins, wherein said polynucleotide is under the control of regulating expression sequences, in particular regulatory expression sequences comprising an inducible 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.

11. Lentiviral particles according to any of the preceding claims, whose vector genome comprises lentiviral-based sequences, especially HIV-1-based sequences, which are devoid of functional lentiviral genes and comprise cis-active sequences necessary for packaging, reverse transcription, and transcription and further comprise a functional lentiviral, especially HIV-1, DNA flap element said vector genome being further characterized in that, in said cis-acting sequences at least one is a modified polynucleotide as follows:

a) the 3'LTR sequence from the lentiviral genome is truncated and devoid of the enhancer of the U3 region;

b) the 3' LTR sequence from the lentiviral genome is truncated and devoid of the U3 region or partly deleted in the U3 region;

c) the U3 region of the LTR5' is replaced by a non lentiviral U3 region or by a promoter suitable to drive tat-independent primary transcription.

12. A combination of compounds for separate administration to a mammalian host, which comprises at least :

(i) lentiviral vector particles as defined in any of the preceding claims which are pseudotyped with a first determined heterologous viral envelope protein or viral envelope proteins;

(ii) provided separately from lentiviral vector particles in (i), lentiviral vector particles as defined in any of the preceding claims which are pseudotyped with a second determined heterologous viral envelope protein or viral envelope proteins distinct from said first heterologous viral envelope protein(s);

wherein said first and second viral envelope protein(s) do not sero-neutralize with each other and are suitable for *in vivo* transduction of mammalian cells.

13. A combination of compounds according to the preceding claim, wherein said first and second viral envelope proteins are:

- VSV-G of Indiana strain and VSV-G of New Jersey strain respectively or *vice versa* or,
- wherein one or both of said first and second envelope proteins are modified versions of native VSV-G of Indiana strain or/and VSV-G of New Jersey strain or,

- wherein at least one of said first and second envelope proteins is a chimeric VSV-G protein wherein at least one of the following domains is from an Indiana strain: the export determinant YTDIE, the cytoplasmic tail, the transmembrane domain or the cytoplasmic domain or,
- the first viral envelope protein is either VSV-G of Indiana strain or VSV-G of New Jersey strain and the second viral envelope protein is selected in the group of VSV-G protein(s) of the Cocal strain, VSV-G protein of the Isfahan strain, VSV-G protein(s) of Chandipura strain, VSV-G protein(s) of Pyri strain and VSV-G protein(s) of the SVCV strain .

14. A combination of separately provided compounds according to claim 9 or 10, wherein the lentiviral particles encode distinct polypeptides including (i) a polypeptide of the CSP antigen or a polypeptide of the CSP antigen devoid of the GPI-anchoring motif and (ii) at least one additional polypeptide of an antigen of the malaria parasite chosen in the group of sporozoite surface protein 2 (TRAP/SSP2), liver-stage antigen (LSA), Pf exported protein 1 (Pf Exp1), Pf antigen 2, said distinct polypeptides of said antigens being either expressed from the same lentiviral particles or from distinct lentiviral particles.

15. A combination of separately provided compounds according to any of the preceding claims wherein lentiviral particles encode a polypeptide chosen in the group of a merozoite surface protein 1 (Msp-1) merozoite surface protein 2 (Msp-2), apical membrane antigen 1 (AMA-1), serine repeat antigen (SERA), GLURP antigen, *Pf* 155/RESA (Ring infected erythrocyte surface antigen) or a RHOPTRY-associated protein 1 (RAP-1) or RHOPTRY-associated protein 2 (RAP-2)

16. Lentiviral vector particles or combination of compounds according to any of the preceding claims, for the prophylactic immunisation

against malaria parasite infection or against parasite-induced pathology, in a mammalian host, especially a human host.

17. A composition of lentiviral vector particles or a combination of compounds as defined in any of the preceding claims, which is formulated with a suitable administration vehicle for use for prophylactic immunisation against malaria parasite infection or against parasite-induced pathology, in a mammalian host, especially in a human host, said use involving an immunisation pattern comprising administering an effective amount of lentiviral particles to prime the cellular immune response of the host and later in time administering an effective amount of lentiviral particles, to boost the cellular immune response of the host, and optionally repeating said administration step for boosting, wherein the lentiviral particles administered in each of the priming or boosting steps are pseudotyped with distinct envelope protein(s) which do not sero-neutralise with each other, and wherein said priming and boosting steps are separated in time by at least 6 weeks, in particular by at least 8 weeks.

18. Lentiviral vector particles or combination of compounds according to any of the preceding claims, which is used for the prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, in a dosage regimen comprising separately provided doses of said lentiviral particles wherein the dose intended for priming the cellular immune response is a moderate dose and the dose intended for boosting the cellular immune response is higher than the dose for priming.

19. Lentiviral vector particles or combination of compounds according to any of the preceding claims, which is used for the prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, in a dosage regimen comprising separately provided doses of said lentiviral particles wherein the dose intended for priming and for boosting the cellular immune

response comprises from 10^7 to 10^9 of lentiviral particles when integrative-competent vector particles are used and the dose intended for priming and for boosting the cellular immune response comprises from 10^8 to 10^{10} .of lentiviral particles when integrative-incompetent vector particles are used.

20. Lentiviral vector particles or combination of compounds according to any of the preceding claims, which is used for the prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, in a dosage and administration regime which is suitable to obtain at least one of the following effects in the host:

(I) eliciting sterile protection against malaria parasite infection, especially by *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium knowlesi* or *Plasmodium ovale* in a human host;

(II) inhibiting extracellular forms of malaria parasite;

(III) preventing hepatocytes infection by malaria parasite or inhibition of liver stage amplification of infection;

(IV) eliciting a specific T-cell immune response against malaria parasite antigen(s), especially a CD8+ T-cell response and/or a specific CD4+ T-cell response;

(V) eliciting a B-cell response against parasite antigen(s);

(VI) controlling parasitemia so as to reduce or alleviate the effects of infection by the malaria parasite;

(VII) eliciting a protective cellular immunity against the infection by the parasite or against the parasite-induced pathology;

(VIII) eliciting memory T-cell immune response

(IX) eliciting earlier and higher rebound of the CD4+ and CD8+ T-cell response during infection by the malaria parasite;

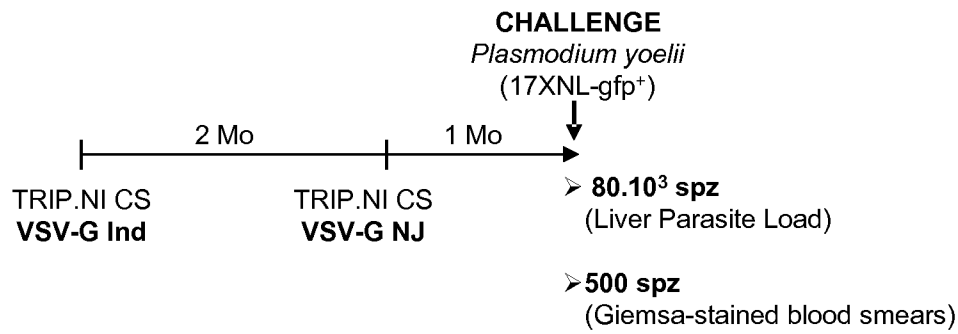
(X) eliciting earlier and strong CT (CD8+T) response by stimulating intra hepatic memory lymphocytes upon *Plasmodium* infection.

(XI) preventing malaria parasite escape from immune response thereby allowing long-term control of the infection by the malaria parasite.

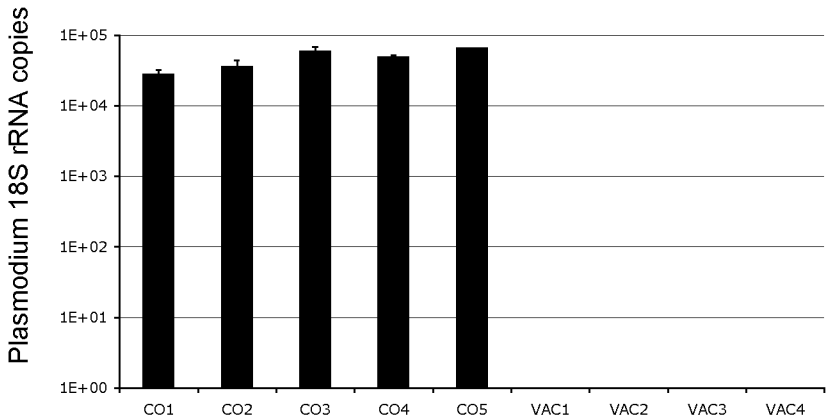
21. Use of lentiviral vector particles or of a combination of compounds as defined in any of the preceding claims, for the manufacture of an immunogenic composition for prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host.

22. Lentiviral vector particles or combination of compounds according to any of the preceding claims, which is used for the prophylactic immunisation against malaria parasite infection or against parasite-induced pathology in a mammalian host, especially in a human host, in association with an adjuvant compound and/or with an immunostimulant compound and an appropriate delivery vehicle.

A



B

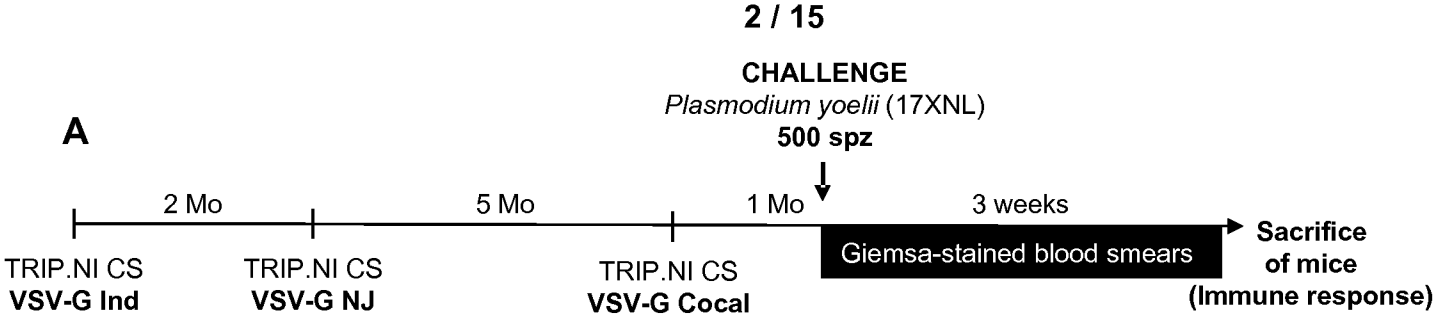


C

	3	4	5	6	7	8	9	10	11	12	13	14
CO1	0	0	0	0	+	+	+	+	+	+	+	+
CO2	0	0	0	0	+	+	+	+	+	+	+	+
CO3	0	0	0	0	+	+	+	+	+	+	+	+
CO4	0	0	0	0	0	0	0	0	0	0	0	0
CO5	0	0	0	0	+	+	+	+	+	+	+	+
VAC1	0	0	0	0	+	+	+	+	+	+	+	+
VAC2	0	0	0	0	+	+	+	+	+	+	+	+
VAC3	0	0	0	0	0	0	0	0	0	0	0	0
VAC4	0	0	0	0	0	0	0	0	0	0	0	0
VAC5	0	0	0	0	0	0	0	0	0	0	0	0

FIGURE 1

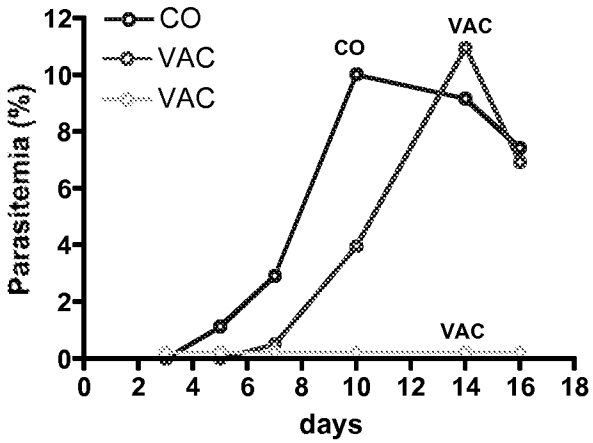
A



B

	Protected/ Challenged	% sterile immunity
CO	0/8	0
VAC	*5/8*	*62,5*

C



D

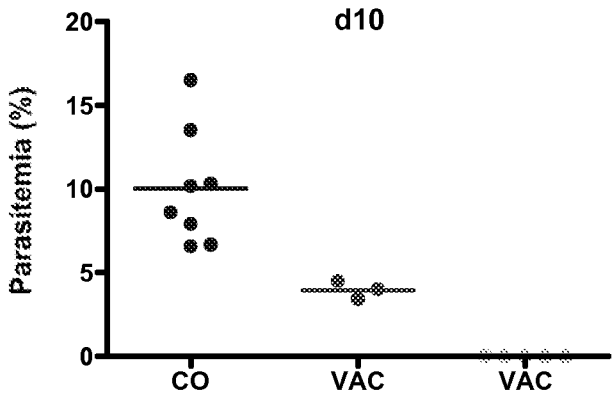


FIGURE 2

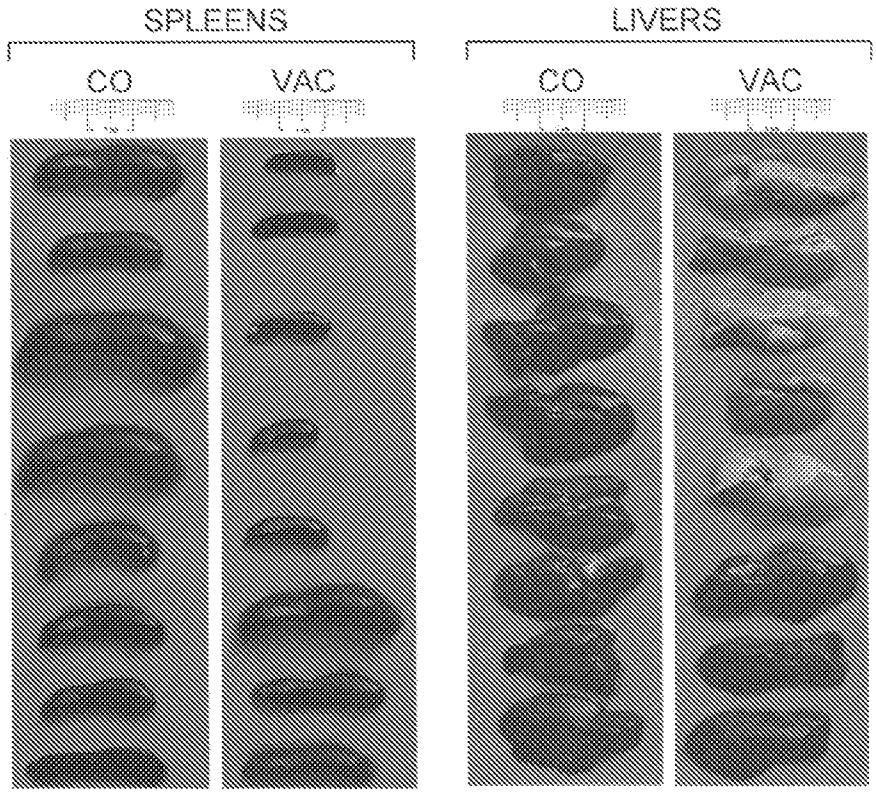


Figure 3

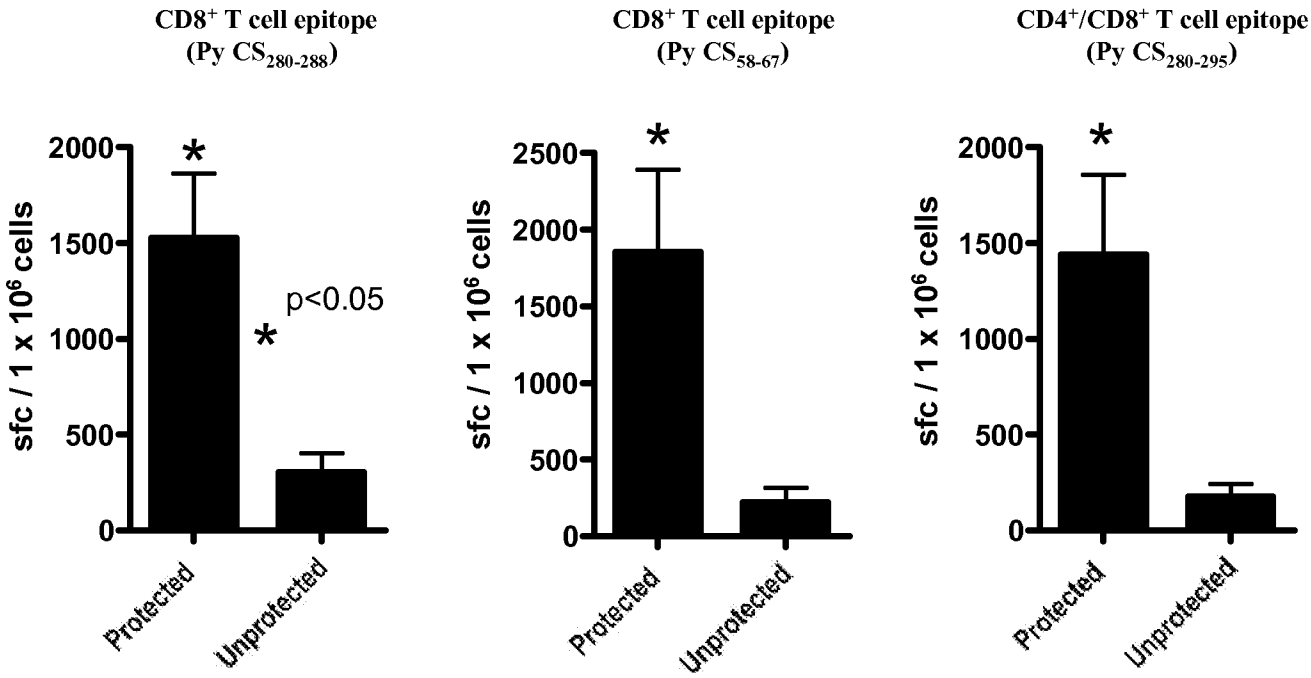

CD8+ T cell epitope
(Py CS280-288)
CD8+ T cell epitope
(Py CS58-67)
CD4+/CD8+ T cell epitope
(Py CS280-295)
sfc / 1 x 10^6 cells
2000
1500
1000
500
0
2500
*
p<0.05
Protected
Unprotected

FIGURE 4

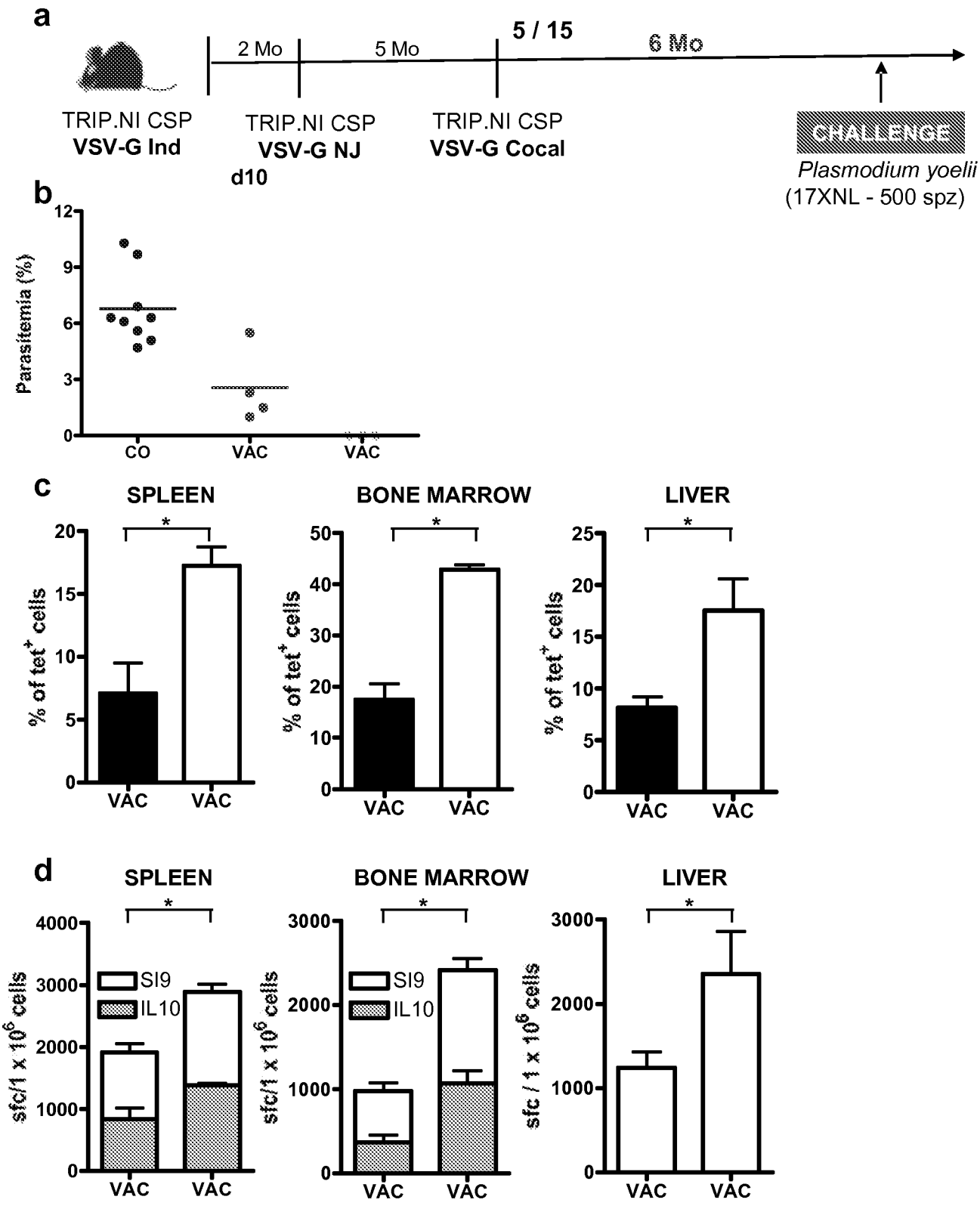
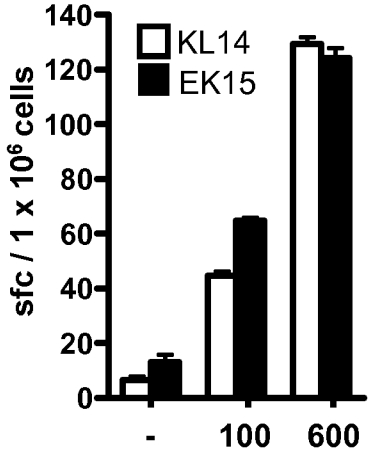

a
2 Mo
5 Mo
5 / 15
6 Mo
TRIP.NI CSP
VSV-G Ind
TRIP.NI CSP
VSV-G NJ
d10
TRIP.NI CSP
VSV-G Cocal
CHALLENGE
Plasmodium yoelii
(17XNL - 500 spz)
b
Parasitemia (%)
CO
VAC
VAC
c
SPLEEN
BONE MARROW
LIVER
% of tet+ cells
VAC
VAC
d
SPLEEN
BONE MARROW
LIVER
sfc/1 x 10^6 cells
SI9
IL10
VAC
VAC

FIGURE 5

A. Hep17-specific CD4+ T cell responses



B. Hep17-specific CD8+ T cell responses

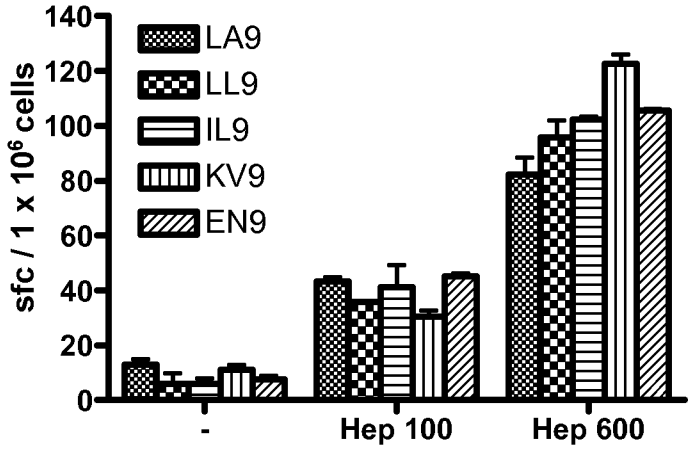
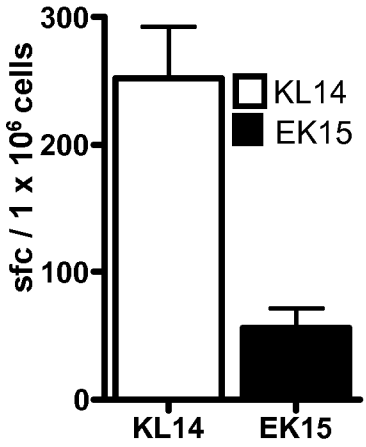


FIGURE 6

A. Hep17-specific CD4+ T cell responses



B. Hep17-specific CD8+ T cell responses

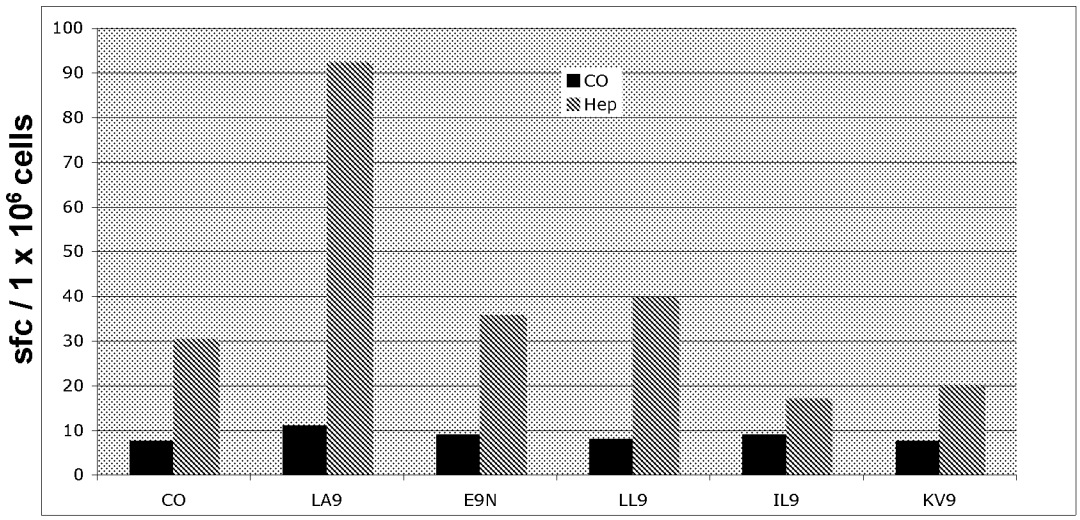
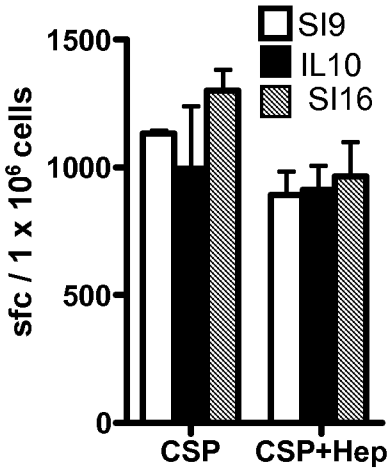


FIGURE 7

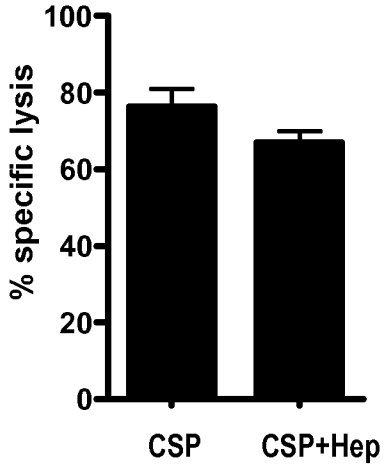
A ELISPOT

(CS-specific T cell responses)



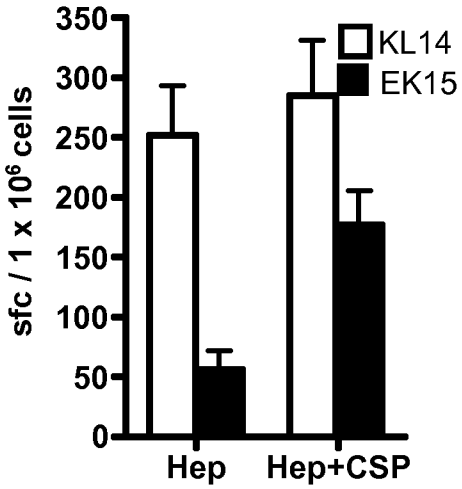
B *In vivo* cytotoxic assay

(CS-specific CTL response)



C ELISPOT

(Hep17-specific T cell responses)



D *In vivo* cytotoxic assay

(Hep17-specific CTL response)

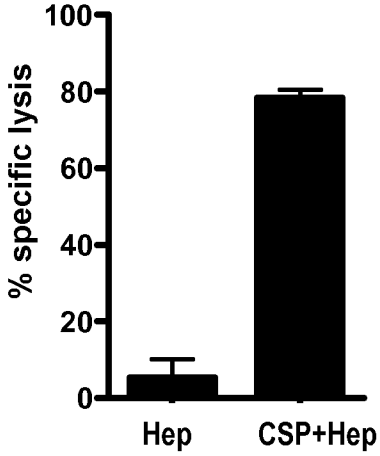


FIGURE 8

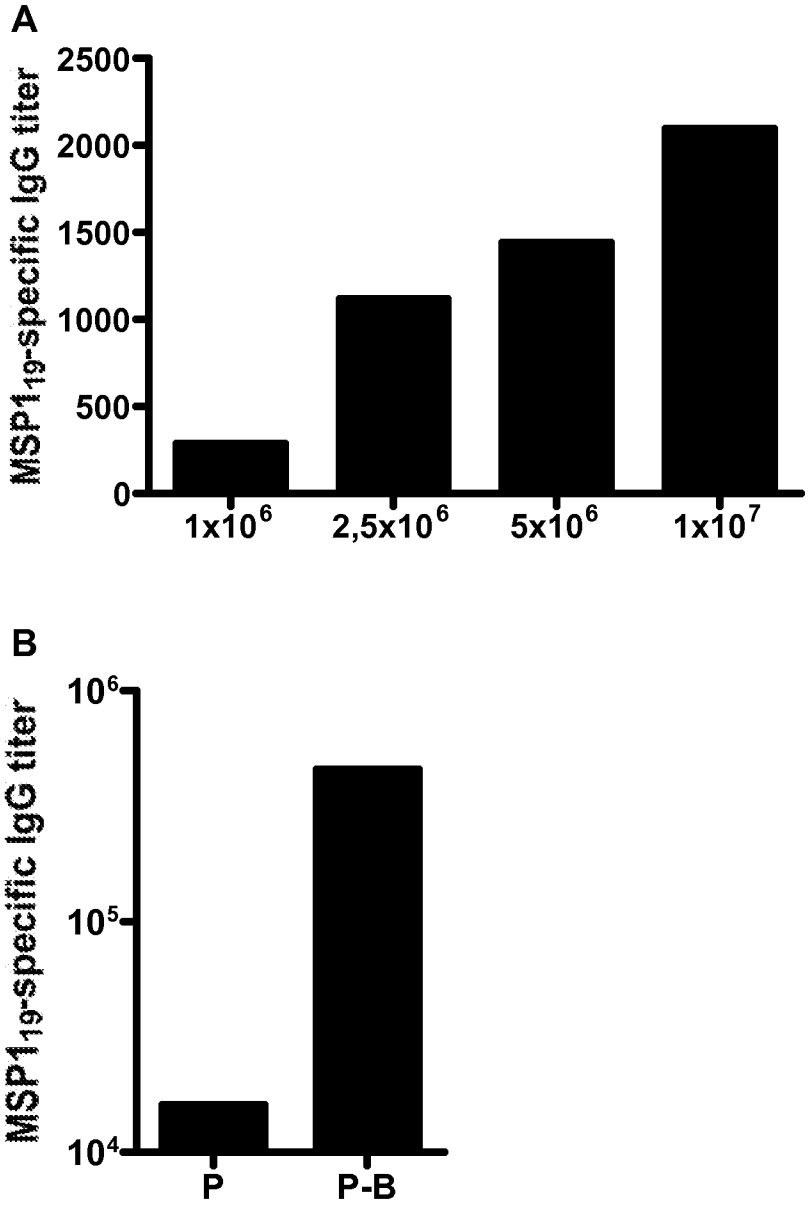

A
MSP1$_{19}$-specific IgG titer
2500
2000
1500
1000
500
0
1x10^6
2,5x10^6
5x10^6
1x10^7
B
MSP1$_{19}$-specific IgG titer
10^6
10^5
10^4
P
P-B

FIGURE 9

ALIGNEMENTS

```
                                1                                                  50
CSP Plasmodium falciparum     (1) ------------------------------------MMRKLAILSVSSFL
    CSP Plasmodium yoleii     (1) ------------------------------------MKKCTILVVASLL
   CSP Plasmodium berghei     (1) ------------------------------------MKKCTILVVASLL
  CSP Plasmodium malariae     (1) MKKLSVLAISSFLIVDFLFPGYHHNSNSTKSRNLSELCYNNVDTKLFNEL
  CSP Plasmodium coatneyi     (1) ------------------------------------MKNFILLAVSSIL
  CSP Plasmodium knowlesi     (1) ------------------------------------MRNFILLAVSSIL
CSP Plasmodium reichenowi     (1) ------------------------------------MMRKLAILSVSSFL
CSP Plasmodium gallinaceum    (1) ------------------------------------MKKLAILSASSFL
                Consensus     (1)                                      MKK  ILSVSSIL

                                51                                                 100
CSP Plasmodium falciparum    (15) FVEALFQEYQCYGSSSNTRVLNELN--YDNAGTNLYNELEMNYYGKQENW
    CSP Plasmodium yoleii    (14) LVDSLLPGYGQNKSVQAQRNLNELCY-------NEENDNKLYHVLNSKNG
   CSP Plasmodium berghei    (14) LVNSLLPGYGQNKIIQAQRNLNELCY-------NEGNDNKLYHVLNSKNG
  CSP Plasmodium malariae    (51) EVRYSTNQDHFYNYNKTIRLLNENNNEKDGNVTNERKKKPTKAVENKLKQ
  CSP Plasmodium coatneyi    (14) LVDLFPTHFGHNVDLSRAINLNGVS----------FNNVDTSLLGAAQVR
  CSP Plasmodium knowlesi    (14) LVDLFPTHFEHNVDLSRAINVNGVS----------FNNVDTSSLGAAQVR
CSP Plasmodium reichenowi    (15) FVEALFQEYQCYGSSSNTRVLNELNY--DNAGTNLYNELEMNYYGKQENW
CSP Plasmodium gallinaceum   (14) FADFLFQEYQHNGNYKNFRLLNEVCY--NNMNIQLYNELEMENYMSNTYF
                Consensus    (51) LVDAL Q Y  N  LS  RNLNEL Y       N YNELEM HVG A N

                               101                                                 150
CSP Plasmodium falciparum    (63) YSLKKNSRSLGENDDGNNEDNEKLRKP-----------KHKKLKQPADGN
    CSP Plasmodium yoleii    (57) KIYNRNIVNRLLGDALNGKPEEKKD---------------DPPKDGNKDD
   CSP Plasmodium berghei    (57) KIYNRNTVNRLLADAPEGKKNEKKN---------------EKIERNNKLK
  CSP Plasmodium malariae   (101) PPGDDDG---AGNDAGNDAGNDAGN-------------AAGNAAGNAAGN
  CSP Plasmodium coatneyi    (54) QSASRGRGLGEK--PKKKAEK-----------------KEEEPKKPNENK
  CSP Plasmodium knowlesi    (54) QSASRGRGLGEK--RKEGADKEKKKE-----------KEEEPKKPNENK
CSP Plasmodium reichenowi    (63) YSLKKNSRSLGENDDADNGDADNGDEGIDENRRHRNKEGKEKLKKPKHNK
CSP Plasmodium gallinaceum   (62) YNNKKTIRLLGENDNEANVNRANNNVAND---------NRANGNRGNVNR
                Consensus   (101)  S  RN    G ND    AD EK                  E  KKPN NK

                               151                                                 200
CSP Plasmodium falciparum   (102) PDPNANPNVDPNANPNVDPNANPNVDPNANPNANPNANPNANPNANPNAN
    CSP Plasmodium yoleii    (92) LPKEEKKDDLPKEEKKDDPPKDPKKDDPPK-----EAQNKLNQPVVADEN
   CSP Plasmodium berghei    (92) QPP-------PPPNPNDPPPPNPNDPPPP-------------------N
  CSP Plasmodium malariae   (135) AAGNAAGNAAGNAAGNAAGNAAGNAAGNDAGNAAGNAAGNAAGNAAGNAA
  CSP Plasmodium coatneyi    (85) LKQPVDGARDGPA-PAADGARDGPAPAADGA-------RDGPAPAADGAR
  CSP Plasmodium knowlesi    (90) LKQPDQAAPGAGG-EQPAPGAGGEQPAPGAG-------GERPAPGAGGE-
CSP Plasmodium reichenowi   (113) LKQPGNDNVDPNANPNVDPNANPNVDPNANP--------NVDPNANPNVD
CSP Plasmodium gallinaceum  (103) ANDRNIPYFRENVVNLNQPVGGNGGVQPAGGNGGVQPAGGNGGVQPAGGN
                Consensus   (151) L          PNA PN DP A PN    PAAG            A A A AN

                               201                                                 250
CSP Plasmodium falciparum   (152) PNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNVDPN
    CSP Plasmodium yoleii    (137) VDQGPGAPQGPGAPQGPGAPQGPGAPQGPGAPQGPGAPQGPGAPQGPGAP
   CSP Plasmodium berghei    (115) PNDPP--PPNPNDPAPPNANDPAPPNANDPAPPNANDPAPPNANDPAPPN
  CSP Plasmodium malariae    (185) GNDAGNAAGNAAGNAAGNAAGNAAGNAAGNAAGNAAGNAAGNAAGNDAGN
  CSP Plasmodium coatneyi    (127) DGPAPAADGARDGPAPAADGARDGPAPAADGARDGPAPAADGARDGPAPP
  CSP Plasmodium knowlesi    (131) -QPAPGAGGE--QPAPGAGGERPAPGAGGEQPAPGAGGEQPAPGAGGEQP
CSP Plasmodium reichenowi    (155) PNANPNVNPNANPNVDPNANPNVNPNANPNVNPNANPNVNPNANPNANPN
CSP Plasmodium gallinaceum   (153) GGVQPAGGNGGVQPAGGNGGVQPAGGNGGVQPAGGNGGAQPVAAGGGAQP
                Consensus   (201)  N APAA  NA  PA PNAG N APNAGG AP NANG ANPNA  GAAPP

                               251                                                 300
CSP Plasmodium falciparum   (202) ANPNANPNANPNANPNANPNANPN--ANPNANPNANPNANPNANPNANPN
    CSP Plasmodium yoleii    (187) QGPGAPQGPGAPQGPGAPQGPGA---PQGPGAPQGPGAPQGPGAPQEPPQ
   CSP Plasmodium berghei    (163) ANDPAPPNANDPAPPNANDPP--------PPNPNDPAPPQGNNNPQ--PQ
  CSP Plasmodium malariae    (235) AAGNAAGNAAGNAAGNAAGNAAGN--AAGNAAGNAAGNAAGNAAGNAAGN
  CSP Plasmodium coatneyi    (177) ADGARDGPAPPAADGARDGPAP----PAADGARDGPAPPAPQQGGN----
  CSP Plasmodium knowlesi    (178) APGAGGEQPAPGAGGERPAPGAGGERPAPGAGGEQPAPGAGGEQPA----
CSP Plasmodium reichenowi    (205) ANPNANPNANPNANPNANPNAN------PNANPNANPNANPNANPNANPN
CSP Plasmodium gallinaceum   (203) VVADGGVQPLRQEGDAEEDGGNGGAQPAGGNGGAQPAGGNGGAQPAGGNG
                Consensus   (251) A G A  NA PNAGPNA   A G   PAG AAPNAPA ANGNANPNA PN
```

FIGURE 10(A)

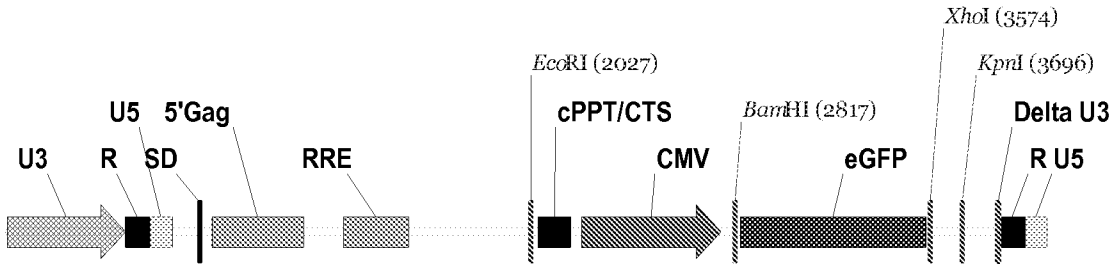
```
CSP Plasmodium falciparum  (250) ANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNRNVDENANA
     CSP Plasmodium yoleii  (234) QPPQQPPQQPPQQPPQQPPQQPPQQPPQQ------------------PRPQPDG
    CSP Plasmodium berghei  (203) PRPQPQPQPQPQPQPQPQPQPQPRPQ--------------------PQPQPGG
   CSP Plasmodium malariae  (283) AAGNAAGNAAGNAAGNAAGNAAGNAAGNAAGNAAG------NEKAKNKDNKVDA
   CSP Plasmodium coatneyi  (219) ---AAGQAQGGGNAGNKKAGDAAGN------------------------
   CSP Plasmodium knowlesi  (224) ---PAPRREQPAPGPGAGDGARGGN------------------------
 CSP Plasmodium reichenowi  (249) ANPNANPNANPNANPNRNNEANGQGHN--------------KPNDQNRNV
CSP Plasmodium gallinaceum  (253) GAQPAGGNGGAQPAGGNDAAKPDGG------------------NDDDKPE
                 Consensus  (301) A PNA PNANPN APN   NA GQ                    D N  A

                                  351                                          400
 CSP Plasmodium falciparum  (300) NSAVKNN---NNEE---PSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGIQ
     CSP Plasmodium yoleii  (266) NNNNNNNNGNNNEDSYVPSAEQILEFVKQISSQLTEEWSQCSVTCGSGVR
    CSP Plasmodium berghei  (234) NNNNKNNN---NDDSYIPSAEKILEFVKQIRDSITEEWSQCNVTCGSGIR
   CSP Plasmodium malariae  (327) NTNKKDNQEENNDSSNGPSEEHIKNYLESIRNSITEEWSPCSVTCGSGIR
   CSP Plasmodium coatneyi  (241) -AGAAKGQGQNNEGANVPNEKVVNDYLQKIRSTVTTEWTPCSVTCGNGVR
   CSP Plasmodium knowlesi  (246) -AGAGKGQGQNNQGANVPNEKVVNDYLHKIRSSVTTEWTPCSVTCGNGVR
 CSP Plasmodium reichenowi  (285) NENANANNAGRNNNNEEPSDKHIEEFLKQIQNNLSTEWSPCSVTCGNGIQ
CSP Plasmodium gallinaceum  (285) GGDEKSEEEKEDEPIPDPTQEEIDKYLKSILGNVTSEWTNCNVTCGKGIQ
                 Consensus  (351) N N K NNG NNE S VPSEK I EYLK IR SLTTEWSPCSVTCGNGIR

                                  401                                          450
 CSP Plasmodium falciparum  (344) VRIKPGSANKPKDELDYANDIEKKICKMEKCS-SVFNVVNSSIGLIMVLS
     CSP Plasmodium yoleii  (316) VRKRKNVNKQ--PENLTLEDIDTEICKMDKCS-SIFNIVSNSLGFVILLV
    CSP Plasmodium berghei  (281) VRKRKGSNKK--AEDLTLEDIDTEICKMDKCS-SIFNIVSNSLGFVILLV
   CSP Plasmodium malariae  (377) ARRKVGAKNK-KPAELVLSDLETEICSLDKCS-SIFNVVSNSLGIVLVLV
   CSP Plasmodium coatneyi  (290) LRRKAHAEKK-KPEDLTMDDLDVEVCAMDKCA-GIFNFVSNSLGLVILLV
   CSP Plasmodium knowlesi  (295) IRRRQNAGNK-KAEDLTMDDLEVEACVMDKCA-GIFNVVSNSLGLVILLV
 CSP Plasmodium reichenowi  (335) VRIKPGSAGKPKDQLDYENDLEKKICKMEKCS-SVFNVVNSSIGLIMVLS
CSP Plasmodium gallinaceum  (335) AKIKSTSANK-KREEITPNDVEVKICELERCSFSIFNVISNSLGLAIILT
                 Consensus  (401) VRRK GSANK K EDLTLDDLE EICKMDKCS SIFNVVSNSLGLVILLV

                                  451
 CSP Plasmodium falciparum  (393) FLFLN
     CSP Plasmodium yoleii  (363) LVFFN
    CSP Plasmodium berghei  (328) LVFFN
   CSP Plasmodium malariae  (425) LILFH
   CSP Plasmodium coatneyi  (338) LAFN-
   CSP Plasmodium knowlesi  (343) LALFN
 CSP Plasmodium reichenowi  (384) FLFLN
CSP Plasmodium gallinaceum  (384) FLFFY
                 Consensus  (451) LLFFN
```

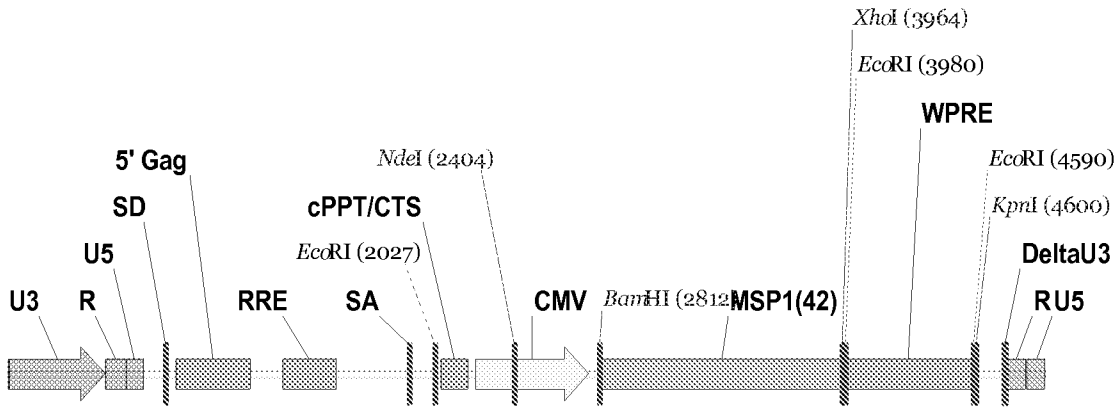
FIGURE 10(B)



pTRIP-DeltaU3-CMV-eGFP

4024 bp

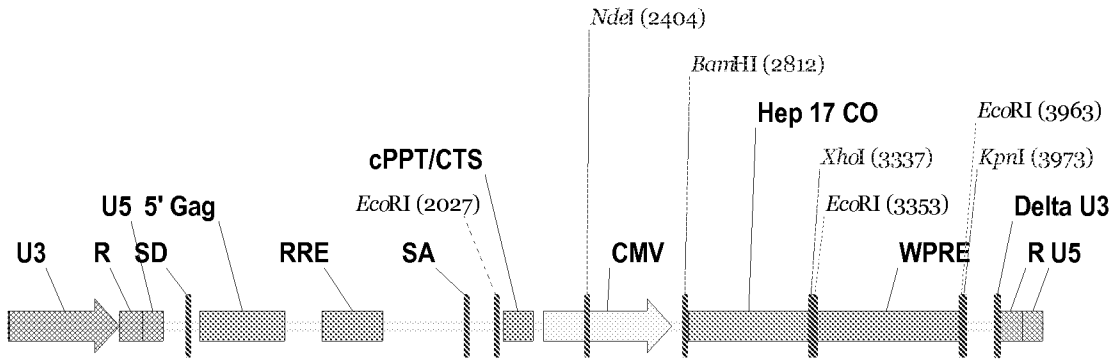
FIGURE 11



pTRIP-DeltaU3-CMV-MSP1(42)-CO-WPRE

4927 bp

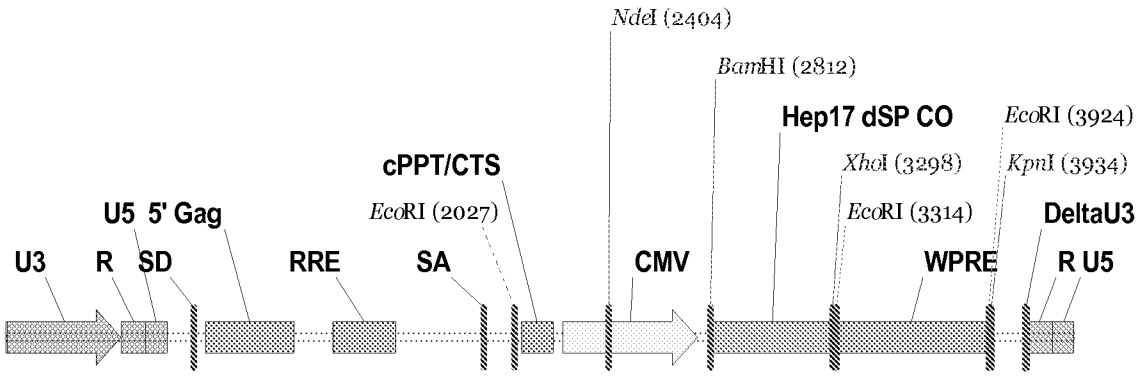
FIGURE 12



pTRIP-DeltaU3-CMV-HEP17 CO-WPRE

4300 bp

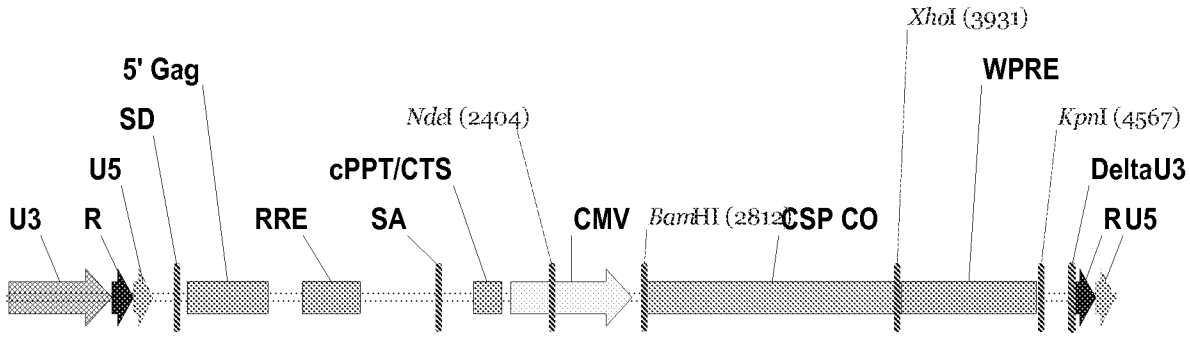
FIGURE 13



pTRIP-DeltaU3-CMV-Hep17 dSP CO WPRE

4261 bp

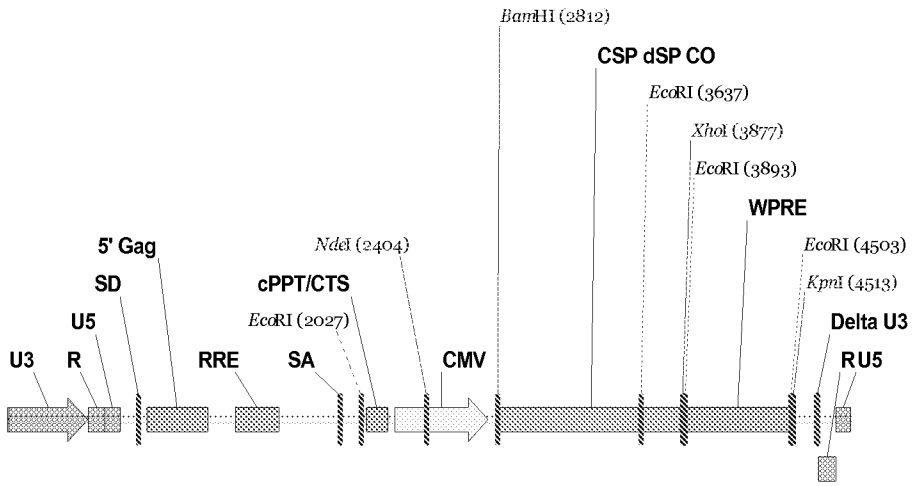
FIGURE 14



pTRIP-DeltaU3-CMV-CSP CO WPRE

4897 bp

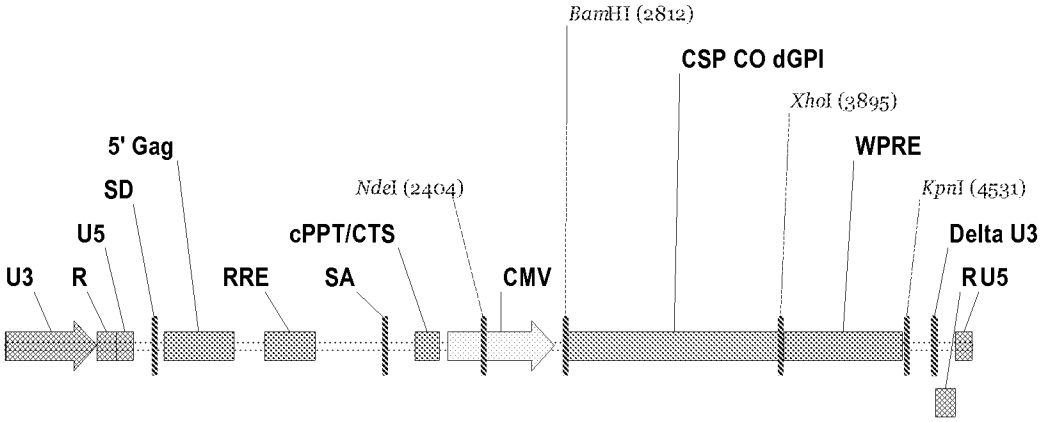
FIGURE 15



pTRIP-DeltaU3-CMV-CSP dSP CO WPRE

4840 bp

FIGURE 16



pTRIP-DeltaU3-CMV-CSP dGPI CO WPRE

4858 bp

FIGURE 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/056887

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/04 C12N15/869 A61K39/015 C12N15/867 C07K14/445
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2007/095201 A2 (UNIV CALIFORNIA [US]; SCHAFFER DAVID V [US]; YU JULIE H [US]) 23 August 2007 (2007-08-23) the whole document -----	1,2, 8-10,16, 21,22 1-5,7-22
Y	WO 2009/019612 A2 (PASTEUR INSTITUT [FR]; CHARNEAU PIERRE [FR]; BEIGNON ANNE-SOPHIE [FR];) 12 February 2009 (2009-02-12) cited in the application paragraph [0001] - paragraph [0174]; claims 1 2,5, 6,20; figures 1-3,6-20,24 ----- -/--	1-22

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 2011	15/07/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Chambonnet, F

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIMBACH KJ, RICHIE TL.: "Viral vectors in malaria vaccine development.", PARASITE IMMUNOL., vol. 31, no. 9, 26 June 2009 (2009-06-26), pages 501-519, XP002601705, cited in the application the whole document	1,3-5, 7-22
Y	TINE ET AL: "NYVAC-Pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for plasmodium falciparum malaria", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 9, no. 64, 1 September 1996 (1996-09-01), pages 3833-3844, XP002077342, ISSN: 0019-9567 the whole document	1,3,4
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Y	BRUNA-ROMERO O ET AL: "Enhanced protective immunity against malaria by vaccination with a recombinant adenovirus encoding the circumsporozoite protein of Plasmodium lacking the GPI-anchoring motif", VACCINE, ELSEVIER LTD, GB LNKD-DOI:10.1016/J.VACCINE.2004.03.050, vol. 22, no. 27-28, 9 September 2004 (2004-09-09), pages 3575-3584, XP004526937, ISSN: 0264-410X cited in the application the whole document	3,4,6, 13-16
Y	WO 2004/043488 A1 (STATENS SERUMINSTITUT [DK]; THEISEN MICHAEL [DK]; JEPSEN SOEREN [DK]) 27 May 2004 (2004-05-27) the whole document	1,4

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/056887

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SPEAKE C, DUFFY PE.: "Antigens for pre-erythrocytic malaria vaccines: building on success.", PARASITE IMMUNOL., vol. 31, no. 9, 1 September 2009 (2009-09-01), XP002601706, the whole document	1,3,4
A	DOBANO ET AL: "Identification of minimal CD8+ and CD4+ T cell epitopes in the Plasmodium yoelii hepatocyte erythrocyte protein 17kDa", MOLECULAR IMMUNOLOGY, PERGAMON, GB LNKD-DOI:10.1016/J.MOLIMM.2007.01.001, vol. 44, no. 11, 16 March 2007 (2007-03-16), pages 3037-3048, XP005938387, ISSN: 0161-5890 cited in the application the whole document	1
A	COUTANT F, FRENKIEL MP, DESPRES P, CHARNEAU P.: "Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine.", PLOS ONE, vol. 3, no. 12, E3973, 19 December 2008 (2008-12-19), pages 1-6, XP002602189, the whole document	1,2
A	BEIGNON AS, MOLLIER K, LIARD C, COUTANT F, MUNIER S, RIVIÈRE J, SOUQUE P, CHARNEAU P.: "Lentiviral vector-based prime/boost vaccination against AIDS: pilot study shows protection against Simian immunodeficiency virus SIVmac251 challenge in macaques.", VIROL., vol. 83, no. 21, 12 August 2009 (2009-08-12), pages 10963-10974, XP002602190, the whole document	1
A	BEIGNON A; MOLLIER K; LIARD C; MUNIER S; RIVIERE J; COUTANT F; BOULAY A; CALEECHURN L; SOUQUE P; BAUCHE C; CHARNEAU P: "Lentiviral vector-based vaccine against SIV infection and simian AIDS", RETROVIROLOGY, vol. 6, no. suppl. 3, 2009, page P302, XP002646859, the whole document	1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/056887

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	& AIDS Vaccine Meeting 2009; Paris, FRANCE; October 19 -22, 2009 -----	1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/056887

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   - [ ] on paper
   - [X] in electronic form

   b. (time)

   - [X] in the international application as filed
   - [ ] together with the international application in electronic form
   - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/056887

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007095201 A2	23-08-2007	US 2009220458 A1	03-09-2009
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