COMBINED MEASLES-MALARIA VACCINE

FIELD OF THE INVENTION

The present invention relates to a combined measles-malaria vaccine containing different attenuated recombinant measles-malaria vectors comprising a heterologous nucleic acid encoding several *Plasmodium falciparum* antigens. Preferably, it relates to viral vectors that comprise nucleic acids encoding the circumsporozoite (CS) protein of *P. falciparum*, the merozoite surface protein 1 (MSP-1) of *P. falciparum*, and its derivatives (p-42; p-83-30-38) in its glycosylated and secreted forms, and apical membrane antigen1 (AMA1) of *P. falciparum*, in its anchored or secreted form. The viral vector stems from an attenuated measles virus, based on a strain that is used as a vaccine and is efficient in delivering the gene of interest and that binds to and infects the relevant immune cells efficiently. In a preferred embodiment, the CS, the MSP1 and the AMA1 proteins are generated from the virus such that they will give rise to a potent immune response in mammals, preferably humans; the expression of the proteins is elevated due to human codon optimisation. Furthermore, the invention relates to the use of the recombinant vaccine in the prophylactic treatment of malaria.

BACKGROUND INFORMATION

Measles Virus

The invention relates to a vaccine containing recombinant attenuated measles viruses expressing antigens of *Plasmodium falciparum* (*Pf*) and to their use for the preparation of recombinant measles-malaria vaccine which will confer immunity against both Measles and Malaria antigens.

Measles virus (MV) is a member of the order Mononegavirales, i.e. viruses with a non-segmented negative-strand RNA genome. The non segmented genome of MV has an antimessage polarity; thus, the genomic RNA is not translated either *in vivo* or *in vitro*. Furthermore, it is biologically active only when it is very specifically associated with three viral proteins in the form of a ribonucleoprotein (RNP) complex (see below). Transcription and replication of non-segmented (-) strand RNA viruses and their assembly as virus particles have been reviewed extensively (1). Transcription and replication of measles virus do not involve the nucleus of the infected cells but rather take place in the cytoplasm of infected cells. The genome of the measles virus comprises genes encoding six major structural proteins from the six genes (designated N, P, M, F, H and L) and additionally two-non structural proteins derived from the P gene, C and V, involved in counteracting the constitutive immune responses and in regulation of transcription/replication. The gene order is 3' N, P (including C and V), M, F, H, and L 5'. In addition, from the 3'-terminal region a short leader RNA of about 50 nucleotides is transcribed. The cited genes respectively encode the proteins of the ribonucleocapsid (RNP) of the virus, i.e., the nucleoprotein (N), the phosphoprotein (P),

and the large polymerase/replicase protein (L), which very tightly associate with the genome RNA, forming the RNP. The other genes encode the proteins of the viral envelope including the hemagglutinin (H), the fusion (F) and the matrix (M) proteins. The transcription of the MV genes follows a decreasing gradient: when the polymerase operates on the genomic template it synthesizes more RNA made from upstream genes than from downstream genes. In this discontinuous transcription mode the mRNAs are capped and polyadenylated. Conversely, in the replication mode, the L protein produces full length antigenomic and genomic RNA which are immediately covered with N, P and L proteins to form infectious progeny RNPs.

The measles virus has been isolated in 1954: Enders and Peebles inoculated primary human kidney cells with the blood of David Edmoston, a child affected by measles, and the resulting Edmoston strain of MV (2) was subsequently adapted to growth in a variety of cell lines. Adaptation to chicken embryos, chick embryo fibroblasts (CEF), and/or dog kidney cells and human diploid cells produced the attenuated Edmonston A and B (3), Zagreb (EZ) and AIK-C seeds. Edmonston B was licensed in 1963 as the first MV vaccine. Further passages of Edmonston A and B on CEF produced the more attenuated Schwarz and Moraten viruses (3) whose sequences have recently been shown to be identical (4; 5). Because Edmonston B vaccine was reactogenic, it was abandoned in 1975 and replaced by the Schwarz/Moraten vaccine. Several other vaccine strains are also used: AIK-C, Schwarz F88, CAM70, TD97 in Japan, Leningrad-16 in Russia, and Edmonston Zagreb. The CAM70 and TD97 Chinese strains were not derived from Edmonston. Schwarz/Moraten and AIK-C vaccines are produced on CEF. Zagreb vaccine is produced on human diploid cells (WI-38). Today, the Schwarz/Moraten, AIK-C and EZ vaccines are commonly used (6), but in principle, any one of these attenuated vaccine strains, which are all of the one unique MV serotype, proven to be safe and to induce long-lasting immune responses, can be used for the purposes of the invention.

MV vaccines induce life-long immunity after a single or two low-dose injections. Protection against measles is mediated both by antibodies and by CD4 and CD8 T cells. Persistence of MV-specific antibodies and CD8 cells has been shown for as long as 25 years after vaccination (7).

MV vaccine is easy to produce on a large scale in most countries and can be distributed at low cost. Because the attenuation of MV genome results from an advantageous combination of numerous mutations, the vaccine is very stable and reversion to pathogenicity has never been observed (6).

Regarding safety, MV replicates exclusively in the cytoplasm, ruling out the possibility of integration into host DNA. These characteristics make live attenuated MV vaccine an attractive candidate to be used as a multivalent vaccination vector. Such a vaccine may prove as efficient in eliciting long-lasting immune protection against other pathogenic agents as against the vector virus itself.

Martin Billeter and colleagues cloned cDNA corresponding to the antigenome of Edmonston MV, and established an original and efficient reverse genetics procedure to rescue the virus (8), as described in International Patent Application WO 97/06270. The recombinant measles virus is recovered from the helper cell line 293-3-46, stably transfected and expressing MV N an P proteins as well as bacteriophage T7 RNA polymerase. For rescue of any variant or recombinant MV the helper cell line is then transiently transfected with an expression plasmid encoding L protein, and most importantly with any antigenomic plasmid appropriately constructed to yield any mutated or recombinant antigenomic RNA compatible to give rise to progeny MV. The transient transfection step leads first to the transcription, preferably by the resident T7 RNA polymerase. The resulting antigenomic RNA is immediately (*in statu nascendi)* covered by the viral N, P and L proteins, to yield antigenomic RNP from which genomic RNP is produced. Second, the genomic RNP is transcribed by the attached L, to yield all viral mRNAs and the respective proteins. Finally, both genomic and antigenomic RNPs are amplified by replication.

In a slight variation of this procedure, rather than using stably transfected 293-3-46 helper cells, commercially available 293T cells have been transiently transfected, using simultaneously all 5 plasmids detailed in the original patent description, those encoding N, P and T7 polymerase (previously used to create the helper cell line) as well as the plasmid encoding L and the antigenomic plasmid. Note that in the "fully transient transfection" procedure it is possible to use also variant expression plasmids and to avoid the use of T7 RNA polymerase altogether, utilizing instead the resident RNA polymerase II to express also the L protein and the antigenome (9).

To rescue individual recombinant MVs the antigenomic plasmids utilized comprise the cDNA encoding the full length antigenomic (+)RNA of the measles virus recombined with nucleotide sequences encoding the heterologous antigen of interest (heterologous nucleotide sequence), flanked by MV-specific transcription start and termination sequences, thus forming additional transcription units (ATUs). This MV Edmonston strain vector has been developed by the original MV rescue inventors for the expression of foreign genes (10), demonstrating its large capacity of insertion (as much as 5 kb) and the high stability in the expression of transgenes (11; 12), such as Hepatitis B virus surface antigen, simian or human immunodeficiency viruses (SIV or HIV), mumps virus, and human IL-12. In particular, early on, recombinant measles virus expressing Hepatitis B virus surface and core antigens either individually or in combination have been produced and shown to induce humoral immune responses in genetically modified mice.

From the observation that the properties of the measles virus and especially its ability to elicit high titers of neutralizing antibodies *in vivo* and its property to be a potent inducer of long lasting cellular immune response, the inventors have proposed

that it may be a good candidate for the production of recombinant viruses expressing antigens from *P. falciparum*, to induce neutralizing antibodies against said Malaria parasite which preferably could be suitable to achieve at least some degree of protection in animals and more preferably in human hosts.

Especially, MV strains and in particular vaccine strains have been elected in the present invention as candidate vectors to induce immunity against both measles virus and *P. falciparum* parasite whose constituent is expressed in the designed recombinant MV, in exposed infant populations because they are having no MV immunity.

Adult populations, even already MV immunized individuals, may however also benefit from MV recombinant immunization because re-administering MV virus under the recombinant form of the present invention results in a boost of anti-MV antibodies (13).

The invention relates in particular to the preparation of recombinant measles viruses bearing heterologous genes from *P.falciparum* parasites.

The advantageous immunological properties of the recombinant measles viruses according to the invention can be shown in an animal model which is chosen among animals susceptible to measles viruses, and wherein the humoral and/or cellular immune response against the heterologous antigen and/or against the measles virus is determined. Among such animals suitable to be used as model for the characterization of the immune response, the skilled person can especially use transgenic mice expressing CD46, one of the specific receptors for MV. The most promising recombinants can then be tested in monkeys.

The recombinant measles virus nucleotide sequence must comprise a total number of nucleotides which is a mutiple of six. Adherence to this so-called "rule of six" is an absolute requirement not only for MV, but for all viruses belonging to the subfamily *Paramyxovirinae*. Apparently, the N protein molecules, each of which contacts six nucleotides, must cover the genomic and antigenomic RNAs precisely from the 5' to the 3' end.

It is of note that the location of the ATUs can vary along the antigenomic cDNA. Thus, taking advantage of the natural expression gradient of the mRNAs of MV mentioned above, the level of expression of inserted ATUs can be varied to appropriate levels. Preferred locations of ATUs are upstream of the L-gene, upstream from the M gene and upstream of the N gene, resulting in low, medium and strong expression, respectively, of heterologous proteins.

Malaria parasite

Malaria currently represents one of the most prevalent infectious diseases in the world, especially in tropical and subtropical areas. Per year, malaria infections lead to severe illnesses in hundreds of million individuals worldwide, killing between 1 and 3 million, primarily young infants in developing and emerging countries. The

widespread occurrence and elevated incidence of malaria are a consequence of the widespread ban of DDT and the increasing numbers of drug-resistant parasites as well as insecticide-resistant parasite vectors. Other factors include environmental and climatic changes, civil disturbances, and increased mobility of populations.

Malaria is caused by the mosquito-borne hematoprotozoan parasites belonging to the genus *Plasmodium* from the phylum Apicomplexa. Four species of *Plasmodium* genus infect humans: *P. malariae*, responsible for Malaria quartana, *P. vivax* and *P. ovale*, both of which cause Malaria tertiana, and *P. falciparum*, the pathogen of Malaria tropica and responsible for almost all fatal infections. Many others cause disease in animals, such as *P. yoelii* and *P. berghei* in mice.

Malaria parasites have a life cycle consisting of several stages. Each stage is able to induce specific immune responses directed against the corresponding occurring stage-specific antigens. Malaria parasites are transmitted to man by several species of female Anopheles mosquitoes. Infected mosquitoes inject the "sporozoite" form of the malaria parasite into the mammalian bloodstream. Sporozoites remain for a few minutes in the circulation before invading hepatocytes. At this stage, the parasite is located in the extra-cellular environment and is exposed to antibody attack, mainly directed to the "circumsporozoite" (CS) protein, a major component of the sporozoite surface. Once in the liver, the parasites replicate and develop into so-called "schizonts." These schizonts occur in a ratio of up to 20,000 per infected cell. During this intra-cellular stage of the parasite, main players of the host immune response are T-lymphocytes, especially CD8+ T-lymphocytes. After about one week of liver infection, thousands of so-called "merozoites" are released into the bloodstream. Apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) are both present on merozoites that emerge from infected liver cells: they are essential components of the asexual blood-stage merozoite, responsible for invasion of erythrocytes. Once they enter red blood cells, they become targets of antibody-mediated immune response and T-cell secreted cytokines. After invading erythrocytes, the merozoites undergo several stages of replication, giving rise to so-called "trophozoites" and to schizonts and merozoites, which can infect new red blood cells. A limited amount of trophozoites may evolve into "gametocytes," which constitute the parasite's sexual stage. When susceptible mosquitoes ingest erythrocytes, gametocytes are released from the erythrocytes, resulting in several male gametocytes and one female gametocyte. The fertilization of these gametes leads to zygote formation and subsequent transformation into ookinetes, then into oocysts, and finally into salivary gland sporozoites. Targeting antibodies against gametocyte stage-specific surface antigens can block this cycle within the mosquito mid gut. Such antibodies will not protect the mammalian host but will reduce malaria transmission by decreasing the number of infected mosquitoes and their parasite load.

The MSP-1 is synthesised as 190-200 kDa (d-190) precursor which is proteolytically processed into fragments of 83, 30, 38 and 42 kDa (d-42) during schizogony (14). At the time of erythrocytic invasion the 42-kDa is further cleaved to yield a 33 kDa fragment which is shed with the rest of the complex, and a 19 kDa fragment, which contains two epidermal growth factor (EGF)-like domains, that remains associated with the merozoite membrane during invasion. This secondary cleavage is a pre-requisite for successfully erythrocyte invasion (15).

MSP-1 is an essentially dimorphic protein exhibiting high conservation within the dimorphic alleles characterised by the K1 and MAD20 prototypes.

AMA-1 (16) is a structurally conserved type I integral membrane protein, comprising 622 aa in *P. falciparum* (*Pf*AMA-1), organised in a cytosolic region (50 aa), a transmembrane region, and an ectodomain, which folds as an a N-terminal pro-sequence and three domains (DI, DII, DIII) Expression of the protein is maximal in late schizogony: the precursor of AMA-1 (83 kDa) is processed proteolytically, to cleave away the pro-sequence, converting the protein into a 66 kDa form, which allows the merozoite relocalisation. Antibodies recognise mainly DI and DII, and appear to react equally well with several allelic variants. Antibody responses to DIII are generally low, levels increasing in adults (17, 18).

*Pf*AMA-1 contains 64 polymorphic positions (9 in the pro-sequence, 52 in the ectodomain, 3 in the cytosolic region), most of them are dimorphic, which are important epitopes for host immune responses. To develop *Pf*AMA-1-based vaccines it should be important to cover the polymorphisms: Diversity Covering (DiCo 1, 2 and 3) *Pf*AMA-1 are artificial sequences representing, to the greatest extent possible, the naturally occurring polymorphism of the *Pf*AMA1 ectodomain. It has been shown that they induce immune responses which are functional against a range of parasites carrying diverse *Pf*AMA1 alleles. This approach may offer a means by which vaccines targeting *Pf*AMA1 can be produced such that a strong and a functional protection against the broad range of naturally occurring *Pf*AMA1 alleles can be induced. (19).

The CS protein (CSP) has about 420 aa and a molecular weight of 58 kDa. It represents the major surface protein of sporozoites: its function is fundamental for the maturation of sporozoites from oocystis and for the invasion of hepatocytes, which is mediated from a conserved motif of positively charged aminoacids. CSP is organised into two non-repetitive regions at 5' and 3' ends, and a variable species-specific central region, consisting of multiple repeats of four-residues-long motifs, which represents the main epitope within the CSP. Since CSP continues to be detectable for at least the first 3 days of schizogony, it is considered an attractive vaccine target for both antibody-mediated immuno response, directed against extracellular sporozoites, and cell-mediated immuno responses, directed against schizonts (20).

Current approaches to malaria vaccine development can be classified according to the different stages in which the parasite can exist, as described above.

Three types of possible vaccines can be distinguished: i) pre-erythrocytic vaccines, which are directed against sporozoites and/or schizont-infected cells. These types of vaccines are primarily CS-based, and should ideally confer sterile immunity, mediated by humoral and cellular immune responses, preventing malaria infection; ii) asexual blood-stage vaccines, which are directed against merozoites- infected cells: MSP1 and AMA1 are leading malaria vaccine candidates, designed to minimize clinical severity. These vaccines should reduce morbidity and mortality and are meant to prevent the parasite from entering and/or developing in the erythrocytes; iii) transmission-blocking vaccines, which are designed to hamper the parasite development in the mosquito host. This type of vaccine should favour the reduction of population-wide malaria infection rates. Next to these vaccines, the feasibility of developing malaria vaccines that target multiple stages of the parasite life cycle is being pursued in so-called multi-component and/or multi-stage vaccines.

Today's global malaria vaccine portfolio looks promising with 47 new vaccine candidates, 31 in preclinical development, narrowing down to 16 in clinical trials. One of these, the RTS,S vaccine, being developed by GSK Biologicals and PATH-MVI, should enter final phase III clinical trials in 2008 (21). Other interesting vaccine candidates are those based on live recombinant viruses used as vector, such as Modified Vaccinia Ankara (MVA), as described in International Patent Application US2006127413, poxvirus (US6214353, AU7060294, AU1668197, WO9428930, and US5756101), adenovirus (US2007071726, US2005265974, US2007088156 and CA2507915), cold-adapted attenuated influenza virus, or based on yeasts, such as *Pichia pastoris* and *Saccharomyces* spp., or on bacterial expression systems, such as *Salmonella* spp. (US5112749) and *Escherichia coli* (EB0191748) (22).

Currently, no commercially available vaccine against malaria is available, although the development of vaccines against malaria has already been initiated more than 30 years ago. Many factors make malaria vaccine development difficult and challenging. First, the size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system. Understanding which of these can be a useful target for vaccine development has been complicated, and to date at least 40 different promising antigens have been identified. Second, the parasite changes through several life stages even while in the human host, presenting, at each stage of the life cycle, a different subset of molecules to the immune system. Third, the parasite has evolved a series of strategies that allow it to confuse, hide, and misdirect the human immune system. Finally, it is possible to have multiple malaria infections of not only different species but also of different strains at the same time.

Hence the present invention fulfil the long felt need of prior art by providing combined measles-malaria vaccine containing different attenuated recombinant measles-

malaria vectors comprising a heterologous nucleic acid encoding several *Plasmodium falciparum* antigens.

SUMMARY OF THE INVENTION

In one embodiment of the present invention provides a combined measles-malaria vaccine comprises a recombinant measles vaccine virus which express malaria antigens capable of eliciting immune response and protection both against measles and malaria.

In another embodiment, the present invention provides the recombinant measles vaccine virus having nucleotide sequence which expresses MSP1 malaria antigen. In preferred embodiment, recombinant measles vaccine virus having nucleotide sequence which expresses malaria antigen d190 or d83-30-38 or d42 in both anchored and secreted forms from 3D7 strain and the FCB1 strain.

In yet another embodiment, the present invention provides the recombinant measles vaccine virus having nucleotide sequence which expresses Diversity Covering (DiCo) AMA1 malaria antigen.

In yet another embodiment, the present invention provides the recombinant measles vaccine virus having nucleotide sequence which expresses CS malaria antigen.

DESCRIPTION OF THE FIGURES

Fig. 1: Schematic representation of the antigenomic DNA p(+)MV-EZ of measles virus. p(+)MV-EZ is a plasmid derived from pBluescript containing the complete sequence of the measles virus (Edmoston Zagreb), under the control of the T7 RNA polymerase promoter (T7), containing three ATU respectively in position 1 (before the N gene of the measles virus), 2 (between the P and the M genes of the measles virus) and 3 (between the H and the L genes of the measles virus), and exactly terminated by the hepatitis delta ribozyme and T7 RNA polymerase terminator (δ T7t). The size of the plasmid is 18941 bp.

Fig. 2: Representation of the MSP-1 synthetic gene (d-190) from 3D7 strain. The coding nucleotides on the flanking regions of the d-190 gene fragments (d-83-30-38 and d-42) and the corresponding amminoacids are shown. Unique restriction sites added for cloning procedures are in colours; SP: signal peptide; GPI: glycosyl-phosphatidil-inositol sequence coded for membrane-anchored region.

Fig. 3: Representation of the MSP-1 synthetic gene (d-190) from FCB1 strain. The coding nucleotides on the flanking regions of the d-190 gene fragments (d-83-30-38 and d-42) and the corresponding amminoacids are shown. Unique restriction sites added for cloning procedures are in colours; SP: signal peptide; GPI: glycosyl-phosphatidil-inositol sequence coded for membrane-anchored region. SP and GPI regions are from 3D7 strain.

Fig. 4. Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d190-3D7. It is a plasmid derived from p(+)MV-EZ containing d-190 malaria gene (3D7 strain), 5253 bp, coding the GPI-anchored form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 24323 bp.

Fig. 5: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d190*-3D7. It is a plasmid derived from p(+)MV-EZ containing d-190* malaria gene (3D7 strain), 5160 bp, coding the secreted form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 24227 bp.

Fig. 6: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-d190-3D7 or p(+)MV_3-EZ-d190*-3D7. It is a plasmid derived from p(+)MV-EZ containing the d-190 malaria gene (3D7 strain), 5253 bp, coding the GPI-anchored form of the protein, or the d-190* malaria gene (3D7 strain), 5160 bp, coding the secreted form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The recombinant plasmid p(+)MV_3-EZ-d190 is 24323 bp, and p(+)MV_3-EZ-d190* is 24227 bp

Fig. 7: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d83-30-8-3D7. It is a plasmid derived from p(+)MV-EZ containing d-83-30-38 malaria gene (3D7 strain), 4122 bp, coding the GPI-anchored form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 23195 bp.

Fig. 8: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d83-30-38*-3D7. It is a plasmid derived from p(+)MV-EZ containing d-83-30-38* malaria gene (3D7 strain), 4029 bp, coding the secreted form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 23105 bp.

Fig. 9: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-d83-30-38-3D7 or p(+)MV_3-EZ-d83-30-38*-3D7. It is a plasmid derived from p(+)MV-EZ containing d-83-30-38 malaria gene (3D7 strain), 4122 bp, coding the GPI-anchored form of the protein, or the d-83-30-38* gene (3D7 strain), 4029 bp, coding the secreted form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The recombinant plasmid p(+)MV_3-EZ-d83-30-38 is 23195 bp, p(+)MV_3-EZ-d83-30-38* is 23105 bp.

Fig. 10: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d42-3D7. It is a plasmid derived from p(+)MV-EZ containing d-42 malaria gene (3D7 strain), 1347 bp, coding the GPI-anchored form of the protein, cloned in

position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20417 bp.

Fig. 11: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d42*-3D7. It is a plasmid derived from p(+)MV-EZ containing d-42* malaria gene (3D7 strain), 1254 bp, coding the secreted form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20345 bp.

Fig. 12: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-d42-3D7 or p(+)MV_3-EZ-d42*-3D7. It is a plasmid derived from p(+)MV-EZ containing d-42 malaria gene (3D7 strain), 1347 bp, coding the GPI-anchored form of the protein, or the d-42* malaria gene (3D7 strain), 1254 bp, coding the secreted form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The recombinant p(+)MV_3-EZ-d42 is 20417 bp, the p(+)MV_3-EZ-d42* is 20345 bp.

Fig.13: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-d190-FCB1. It is a plasmid derived from p(+)MV-EZ containing d-190 malaria gene (FCB1 strain), 5013 bp, coding the GPI-anchored form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 24083 bp.

Fig. 14: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-d190-FCB1. It is a plasmid derived from p(+)MV-EZ containing the d-190 malaria gene (FCB1 strain), 5013 bp, coding the GPI-anchored form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The recombinant plasmid p(+)MV_3-EZ-d190 is 24083 bp.

Fig. 15: Representation of the CS synthetic gene. The coding nucleotides on the flanking regions of the CS gene and the corresponding amminoacids are shown. Unique restriction sites added for cloning procedures are in colours.

Fig. 16: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-CS. It is a plasmid derived from p(+)MV-EZ containing CS gene, 1119 bp, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20219 bp.

Fig. 17: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-CS. It is a plasmid derived from p(+)MV-EZ containing CS gene, 1119 bp, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20219 bp.

Fig. 18: Representation of the DiCo-1 complete synthetic gene. The coding nucleotides on the flanking regions of the DiCo1 complete gene domains (ecto and trans-cyto) and the corresponding amminoacids are shown. Unique restriction sites

added for cloning procedures are in colours; SP: signal peptide human codon optimised.

Fig. 19: Representation of the DiCo-1 ecto synthetic gene. The coding nucleotides on the flanking regions of the DiCo1 ecto domain and the corresponding amminoacids are shown. Unique restriction sites added for cloning procedures are in colours; SP: signal peptide (human codon optimised.

Fig. 20: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-DiCo1-complete. It is a plasmid derived from p(+)MV-EZ containing DiCo1 complete gene, 1689 bp, coding the transmembrane form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20753 bp.

Fig. 21: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-DiCo1-complete. It is a plasmid derived from p(+)MV-EZ containing DiCo1 complete gene, 1689 bp, coding the transmembrane form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20753 bp.

Fig. 22: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_2-EZ-DiCo1-ecto. It is a plasmid derived from p(+)MV-EZ containing DiCo1 ecto gene, 1458 bp, coding the secreted form of the protein, cloned in position two of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20525 bp.

Fig. 23: Schematic representation of the recombinant measles-malaria plasmid, p(+)MV_3-EZ-DiCo1-ecto. It is a plasmid derived from p(+)MV-EZ containing DiCo1 ecto gene, 1458 bp, coding the secreted form of the protein, cloned in position three of the measles genome by *Sgr*AI-*Bss*HII digestion. The size of the recombinant plasmid is 20525 bp.

Fig. 24: Complete nucleotide sequence of p(+)MV_2EZ-GFP. The sequence can be described as follows with reference to the position of the nucleotides:

- 592-608 T7 promoter
- 609-17354 MV Edmoston Zagreb antigenome
- 4049-4054 *Mlu*I restriction site
- 4060-4067 *Sgr*AI restriction site
- 4079-4084 *Bss*HII restriction site
- 4085-4801 Green Fluorescent Protein (GFP) ORF
- 4805-4810 *Bss*HII restriction site
- 4817-4822 *Aat*II restriction site
- 17355-17580 HDV ribozyme and T7 terminator

Fig. 25: Complete nucleotide sequence of p(+)MV$_3$EZ-GFP. The sequence can be described as follows with reference to the position of the nucleotides:

- 592-608 T7 promoter
- 609-17359 MV Edmoston Zagreb antigenome
- 9851-9856 *Mlu*I restriction site
- 9862-9869 *Sgr*AI restriction site
- 9886-9891 *BssH*II restriction site
- 9892-10608 Green Fluorescent Protein (GFP) ORF
- 10612-10617 *BssH*II restriction site
- 10624-10629 *Aat*II restriction site
- 17360-17585 HDV ribozyme and T7 terminator

Fig. 26: AN101TE: this is the MSP1 d-190 3D7 sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-1903D7 signal peptide
- 100-105 *BamH*I restriction site
- 4014-4020 *BstE*II restriction site
- 5152-5157 *Acl*I restriction site
- 5158-5250 GPI sequence
- 5251-5253 STOP codon

Fig. 27: AN102TE: this is the MSP1 d-190* 3D7sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-190*3D7 signal peptide
- 100-105 *BamH*I restriction site
- 4014-4020 *BstE*II restriction site
- 5152-5157 *Acl*I restriction site
- 5158-5160 STOP codon

Fig. 28: AN103TE: this is the MSP1 d-83-30-38 3D7 sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-83-30-38 3D7 signal peptide
- 100-105 *BamH*I restriction site
- 4014-4020 *BstE*II restriction site

- 4021-4026 *AclI* restriction site
- 4027-4119 GPI sequence
- 4120-4122 STOP codon

Fig. 29: AN104TE: this is the MSP1 d-83-30-38* 3D7 sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-83-30-38* 3D7signal peptide
- 100-105 *BamHI* restriction site
- 4014-4020 *BstEII* restriction site
- 4027-4029 STOP codon

Fig. 30: AN105TE: this is the MSP1 d-42 3D7sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-42 3D7 signal peptide
- 100-105 *BamHI* restriction site
- 108-114 *BstEII* restriction site
- 1246-1251 *AclI* restriction sites
- 1252-1344 GPI sequence
- 1345-1347 STOP codon

Fig. 31: AN106TE: this is the MSP1 d-42* 3D7 sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-42* 3D7 signal peptide
- 100-105 *BamHI* restriction site
- 108-114 *BstEII* restriction site
- 1246-1251 *AclI* restriction sites
- 1252-1254 STOP codon

Fig. 32: AN107TE: this is the MSP1 d-190 FCB1 sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 d-190 FCB1 signal peptide
- 100-105 *BamHI* restriction site

- 146-151 *HindIII* restriction site
- 3825-3831 *BstEII* restriction site
- 4912-4917 *AclI* restriction sites
- 4918-5010 GPI sequence
- 5011-5013 STOP codon

Fig. 33: AN108TE: this is the CS sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-1116 CS sequence
- 1117-1119 STOP codon

Fig. 34: AN109TE: this is the DiCo 1 complete sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 DiCo1 complete signal peptide
- 100-105 *BamHI* restriction site
- 106-1686 DiCo 1 complete sequence ORF
- 1687-1689 STOP codon

Fig. 35: AN110TE: this is the DiCo 1 ecto sequence ORF cloned by the inventors. The sequence can be described as follows with reference to the position of the nucleotides:

- 1-3 Start codon
- 4-99 DiCo1 ecto signal peptide
- 100-105 *BamHI* restriction site
- 106-1455 DiCo 1 ecto sequence ORF
- 1456-1458 STOP codon

Fig. 36: Comparable cytopathic effects produced on Vero cells after infection with the recombinant Measles-p-42 Malaria virus MV virus vaccine.

Fig. 37: Expression of the d-42 3D7 transgene inserted into position three of the Measles vector (MV_3EZ-d-42 *Sgr*AI). Cell lysates from passage 1, 5 and 10 analysed by Western Blot against empty Measles vector (MVEZ) and a negative control (MV_3L1, a recombinant MV-Papilloma virus).

Fig. 38: Expression of the d-42 3D7 transgene inserted into position three of the Measles vector (MV_3EZ-d-42 *Sgr*AI) analysed by immunofluorescence, compared with empty Measles vector (MVEZ) and a negative control (MV2EZL1, a recombinant MV-

Papilloma virus). Arrows point to the same syncythia as they looked using an optical microscope before and after immunostaining.

Fig. 39: Growth kinetics curve of the recombinant Measles-p-42 Malaria virus compared with that of the MV virus vaccine.

Fig. 40: Expression of the d-190 FCB1 transgene inserted into position two and three of the Measles vector ($MV_{2\text{-}3}$EZ-d-190 *Sgr*AI FCB1). Cell lysates analysed by Western Blot against empty Measles vector (MVEZ) and a negative control (MV2EZL1, a recombinant MV-Papilloma virus).

Fig. 41: Growth kinetics curve of the recombinant Measles-p-190-FCB1 Malaria virus compared with that of the MV virus vaccine.

DETAILED DESCRIPTION OF THE INVENTION

The object of the invention is the production of a combined measles-malaria vaccine from a recombinant Measles vectors capable of containing stably integrated DNA sequences which code for CS, MSP-1 or partial sections of it and AMA-1 or partial sections, in the secreted or surface anchored forms, of *P. falciparum*.

The invention shall also include the rescue of recombinant MV-Malaria viruses which are capable of infection, replication and expression of *Pf*CS, *Pf*MSP-1 and *Pf*AMA-1 antigens in susceptible transgenic mice, monkeys and human host.

Furthermore, the invention intends to include the construction of multivalent recombinant measles-malaria vectors, in which two different antigens are simultaneously cloned and expressed in the same vector, conferring immunity against both of them.

Moreover, the invention relates to the combination of three different recombinant measles-malaria viruses, each carrying a different gene and expressing different antigens, in a manner to elicit immuno response in the host, directed against the different stages of the parasite's life-cycle.

In addition, the invention includes a process to produce recombinant measles-malaria viruses which are avoided of defective interfering particles (DIs). The DIs are known to significantly inhibit the growth of virus in any production system and to successfully suppress immune response in human individuals.

Furthermore, the invention comprises a method to produce a vaccine containing such recombinant viruses.

The examples below describe the preferred mode of carrying out the invention. It should be understood that these examples are provided for illustration and should not be construed as limiting the scope of the invention in any way.

Example 1: construction of recombinant MV-PfMSP-1 plasmids

All cloning procedures were done as per the techniques described in Sambrook *et al.* (1989).

All the restriction enzymes were from New England BioLabs; the oligonucleotides PCR primers and DNA polylinkers were from Invitrogen.

*Pf*MSP1 and its fragments (d-83-30-38 and d-42) either in the secreted and anchored form, have been chemically synthesized and human codon optimised. They have been cloned into the pZE21MV intermediate vector and have been slightly modified by adding *Sgr*AI cloning site at the 5' end followed by an optimised Kozak sequence (TCATCA). These modifications have been checked by sequencing at MWG Biotech.

List of the recombinant MV-*Pf*MSP-1 plasmids, GPI-anchored and secreted (*) forms, from 3D7 strain, which belongs to the MAD20 prototype, and from FCB1 strain, which belongs to the K1 prototype:

p(+)MV_2EZ-d-190-*Sgr*AI (3D7)
p(+)MV_3EZ-d-190-*Sgr*AI (3D7)
p(+)MV_2EZ-d-83-30-38-*Sgr*AI (3D7)
p(+)MV_3EZ-d-83-30-38-*Sgr*AI (3D7)
p(+)MV_2EZ-d-42-*Sgr*AI (3D7)
p(+)MV_3EZ-d-42-*Sgr*AI (3D7)
p(+)MV_2EZ-d-190*-*Sgr*AI (3D7)
p(+)MV_3EZ-d-190*-*Sgr*AI (3D7)
p(+)MV_2EZ-d-83-30-38*-*Sgr*AI (3D7)
p(+)MV_3EZ-d-83-30-38*-*Sgr*AI (3D7)
p(+)MV_2EZ-d-42*-*Sgr*AI (3D7)
p(+)MV_3EZ-d-42*-*Sgr*AI (3D7)
p(+)MV_2EZ-d-190-*Sgr*AI (FCB1)
p(+)MV_3EZ-d-190-*Sgr*AI (FCB1)

1a) Construction of p(+)MV_2EZ-d-190-*Sgr*AI (3D7, 24323 bp) and p(+)MV_3EZ-d-190-*Sgr*AI (3D7, 24323 bp).

1µg of MV plasmid DNA containing the green fluorescent protein (GFP) (p(+)MV_{2-3}EZ-GFP Berna strain, 19774 bp: figure 24 and 25) was digested with one unit of both *Sgr*AI and *Bss*HII restriction enzymes, for two hours at their optimal temperature, in 50µl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (19048 bp) was excised from the gel, purified by QIAEX gel purification and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1 µg/ml.

1μg of d-190 gene, inserted into an intermediate plasmid (pZE21MV-d-190 *Sgr*AI, 7564 bp,) was taken out by *Sgr*AI-*Bss*HII digestion (one unit of each enzyme), for two hours at their optimal temperature, in 50μl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (5275 bp) was excised from the gel, purified by QIAEX gel purification kit and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1 μg/ml.

Thus, the vector (MV DNA: figure 1) and the insert (d-190 DNA: figure 2), were ligated in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase and its own reaction buffer in 10□μl final volume.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-190-3D7 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 9335) is represented in figure 4 and its Open Reading Frame (ORF) is listed in figure 26.

The d-190- 3D7 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 15137) is represented in figure 6.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 21884) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-190-3D7 viruses.

1b) Construction of p(+)MV_2EZ-d-83-30-38-*Sgr*AI (3D7, 23195 bp) and p(+)MV_3EZ-d-83-30-38-*Sgr*AI (3D7, 23195 bp).

The measles vectors were prepared as detailed described in example 3a.

The pZE21MV-d-190 *Sgr*AI was digested *Bst*EII-*Acl*I to cut out the d-42 fragment; a polylinker, with cohesive *Bst*EII and *Acl*I ends, had been ligated to obtain the intermediate plasmid pZE21MV-d-83-30-38-*Sgr*AI (6436 bp).
The sequence of the polylinker was: 5'-GTCACCAGCGGCCGCAA-3'.
1μg of pZE21MV-d-83-30-38 SgrAI was digested *Sgr*AI-*Bss*HII (one unit of each enzyme), for two hours at their optimal temperature, in 50μl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (4147bp) was excised from the gel, purified by QIAEX gel purification kit and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1□μg/ml.

Thus, the vector (MV DNA: figure 1) and the insert (d-83-30-38 DNA: figure 2), were ligated in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase and its own reaction buffer in 10□µl final volume.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences were then aligned with the assumed ones using a DNA Strider software.

The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-83-30-38-3D7 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 8207) is represented in figure 7 and its Open Reading Frame (ORF) is listed in figure 28.

The d-83-30-38-3D7 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 14006) is represented in figure 9.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 20756) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-83-30-38-3D7 viruses.

1c) Construction of p(+)MV_2EZ-d-42-*Sgr*AI (3D7, 20417bp) and p(+)MV_3EZ-d-42-*Sgr*AI (3D7, 20417 bp).

The measles vectors were prepared as detailed described in example 3a.

1µg of d-42gene, inserted into an intermediate plasmid (pZE21MV-d-42 *Sgr*AI, 3658 bp) was taken out by *Sgr*AI-*Bss*HII digestion (one unit of each enzyme), for two hours at their optimal temperature, in 50µl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (1369 bp) was excised from the gel, purified by QIAEX gel purification kit and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1□µg/ml.

Thus, the vector (MV DNA: figure 1) and the insert (d-42 DNA: figure 2), were ligated in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase and its own reaction buffer in 10□µl final volume.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes

digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-42-3D7 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 5429) is represented in figure 10 and its Open Reading Frame (ORF) is listed in figure 30.

The d-42-3D7 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 11231) is represented in figure 12.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 17978) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-42-3D7 viruses.

The recombinant Measles-p-42 Malaria viruses and MV vaccine induced similar cytopathic effect (figure 36). The transgene is rather stably expressed: its expression was completely maintained in all analysed progeny clones derived from single original rescued clones after ten serial virus passages in human diploid cell MRC5 (figure 37-38).

The growth curves of recombinant MV-Malaria virus and MV vaccine showed the same kinetics (figure 39).

1d) Construction of p(+)MV_2EZ-d-190*-*Sgr*AI (3D7, 24227 bp) and p(+)MV_3EZ-d-190*-*Sgr*AI (3D7, 24227 bp).

The measles vectors were prepared as detailed described in example 3a.

Using the intermediate vector pZE21MVd-190-*Sgr*AI as template, a PCR reaction has been performed to delete the GPI anchor region, which is located between *Acl*I (pos. 5434) and *Cla*I (pos. 5536) sites.

PCR amplifications were carried out using the proofreading *Pfu* DNA polymerase (Stratagene). DNA sequences of the synthetic oligonucleotides primers are given in lower case for the MV nucleotides and in upper case for non MV nucleotides; sequences of relevant restriction endonucleases recognition sites are underlined.

The following oligonucleotides primers have been used: For-*Cla*I, 5'-CCAATAAACGTTTAAT AGatcgattacgcgcgctctagc -3', and Rev-*Avr*II, 5'-gcctttgagtgagctgatacc–3'.

For-*Cla*I is homologous to the template at the level of the *Cla*I and *Bss*HII sites and contains an overhang (in upper case) with two stop codons (TAATAG), the *Acl*I site (AACGTT), and a 6 bp long-protection site for *Acl*I (CCAATA). In the so-called PCR-GPI and in the final construct d-190*, *Acl*I will become close to *Cla*I.

Rev-AvrII is homologous to the template (from pos. 5704 to 5724).

PCR product was 207 bp-long: its digestion with *Acl*I+*Avr*II and ligation with the pre-digested *Acl*I+*Avr*II intermediate vector pZE21MVd-190-*Sgr*AI has produced pZE21MVd-190*-*Sgr*AI.

In detail, the digestion of the vector with *AclI+AvrII* has produced two bands of 7318 bp and 246 bp (containing the GPI region to delete): the 7.3 kb-fragment was purified from agarose gel by using QIAEX II purification kit (Qiagen) and was ligated to the digested *AclI-AvrII* PCR (insert) to obtain pZE21MVd-190*-*SgrAI*.

To screen for positive clones, *NcoI* digestion has be done, producing a single band of 7 kb from the d-190* intermediate vector, and two bands of 1.3 and 5.7 kb from the original GPI-anchor construct.

To construct the definitive recombinant p(+)MeV$_2$EZ-d190* and p(+)MeV$_3$EZ-d190* (figure 5 and figure 6), according to the "rule of six", MeV vectors and intermediate plasmid were digested with *SgrAI+BssHII* and afterwards ligated each other.

In detail, pZE21MVd-190*-*SgrAI* digested *SgrAI+BssHII* has produced three bands, 5.2 kb + 1.3 kb + 900 bp. D-190* sequence was contained in the 5.2 kb fragment, that has been cut, purified and ligated with MeV$_2$EZ and MeV$_3$EZ vectors *SgrAI+BssHII* digested (19 Kb in length), in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-190*- 3D7 gene, inserted into position 2 of the MV vector (*SgrAI*, pos. 4060, and *BssHII*, pos. 9239) is represented in figure 5 and its Open Reading Frame (ORF) is listed in figure 27.

The d-190*- 3D7 gene, inserted into position 3 of the MV vector (*SgrAI*, pos. 9862, and *BssHII*, pos. 15041) is represented in figure 6.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 21788) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant MV$_{2\text{-}3}$-d-190*-3D7 viruses.

1e) Construction of p(+)MV$_2$EZ-d-83-30-38*-*SgrAI* (3D7, 23105 bp) and p(+)MV$_3$EZ-d-83-30-38*-*SgrAI* (3D7, 23105 bp).

The measles vectors were prepared as detailed described in example 3a.

The intermediate vector pZE21MVd-190-*SgrAI* was digested *BstEII-ClaI* to cut out the d-42 fragment and the GPI region, which is located between *AclI* (pos. 5434) and *ClaI* (pos. 5536) sites; a polylinker, with cohesive *BstEII* and *ClaI* ends, had been ligated to obtain the intermediate plasmid pZE21MV-d-83-30-38*-*SgrAI* (6346 bp).

The sequence of the polylinker was: 5'-GTCACCGGGGAATAATAGCGCAT-3'.

DNA sequence of the synthetic oligonucleotide polylinker is given in upper case for non MV nucleotides; sequences of relevant restriction endonucleases recognition sites are underlined.

Polylinker contains the *Bst*EII (GTCACC) and *Cla*I (AT) sticky ends, two stop codons (TAATAG), and a triplet (GCG) to keep the rule of six.

1µg of pZE21MV-d-83-30-38* SgrAI was digested *Sgr*AI-*Bss*HII (one unit of each enzyme), for two hours at their optimal temperature, in 50µl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (4057bp) was excised from the gel, purified by QIAEX gel purification kit and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1□µg/ml.

Thus, the vector (MV DNA: fig. 1) and the insert (d-83-30-38* DNA: fig. 2), were ligated in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase and its own reaction buffer in 10□µl final volume.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences were then aligned with the assumed ones using a DNA Strider software. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-83-30-38*- 3D7 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 8117) is represented in figure 8 and its Open Reading Frame (ORF) is listed in figure 29.

The d-83-30-38*- 3D7 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 13919) is represented in figure 9.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 20666) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-83-30-38*-3D7 viruses.

1f) Construction of p(+)MV_2EZ-d-42*-*Sgr*AI (3D7, 20345bp) and p(+)MV_3EZ-d-42*-*Sgr*AI (3D7, 20345 bp).

The measles vectors were prepared as detailed described in example 3a.

Using the intermediate vector pZE21MVd-42-*Sgr*AI (3658 bp) as template, a PCR reaction has been performed to delete the GPI anchor region, which is located between *Acl*I (pos. 1528) and *Cla*I (pos. 1630) sites.

PCR amplifications were carried out using the proofreading *Pfu* DNA polymerase (Stratagene). DNA sequences of the synthetic oligonucleotides primers are given in lower case for the MV nucleotides and in upper case for non MV nucleotides; sequences of relevant restriction endonucleases recognition sites are underlined.

The following oligonucleotides primers have been used: For-*Cla*I, 5'-CCAATAAACGTTTAAT AGatcgattacgcgcgctctagc -3', and Rev-*Avr*II, 5'-gcctttgagtgagctgatacc–3'.

For-*Cla*I is homologous to the template at the level of the *Cla*I (pos. 1630) and *Bss*HII (pos. 1639) sites and contains an overhang (in upper case) with two stop codons (TAATAG), the *Acl* site (AACGTT), and a 6 bp long-protection site for *Acl* (CCAATA). In the so-called PCR-GPI and in the final construct d-42*, *Acl* will become close to *Cla*I. Rev-AvrII is homologous to the template (from pos. 1798 to 1818).

PCR product was 207 bp-long: its digestion with *Acl*+*Avr*II and ligation with the pre-digested *Acl*+*Avr*II intermediate vector pZE21MVd-42-*Sgr*AI has produced pZE21MVd-42*-*Sgr*AI.

In detail, the digestion of the vector with *Acl*+*Avr*II has produced two bands of 3412 bp and 246 bp (containing the GPI region to delete): the 3.4 kb-fragment was purified from agarose gel by using QIAEX II purification kit (Qiagen) and was ligated to the digested *Acl*-*Avr*II PCR (insert) to obtain pZE21MVd-42*-*Sgr*AI.

To screen for positive clones, *Nco*I digestion has be done, producing a single band of 3.4 kb from the d-42* intermediate vector, and two bands of 1.3 and 2.3 kb from the original GPI-anchor construct.

To construct the definitive recombinant p(+)MeV$_2$EZ-d42* and p(+)MeV$_3$EZ-d42*, according to the "rule of six", MeV vectors and intermediate plasmid were digested with *Sgr*AI+*Bss*HII and afterwards ligated each other.

In detail, pZE21MVd-42*-*Sgr*AI digested *Sgr*AI+*Bss*HII+*Spe*I has produced four bands, 1.3 kb + 936 bp + 800 bp + 400 bp. D-42* sequence was contained in the 1.3 kb fragment, that has been cut, purified and ligated with MeV$_2$EZ and MeV$_3$EZ vectors *Sgr*AI+*Bss*HII digested (19 Kb in length), in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-42*-3D7 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 5357) is represented in figure 11 and its Open Reading Frame (ORF) is listed in figure 31.

The d-42*-3D7 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 11159) is represented in figure 12.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 17906) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-42*-3D7 viruses.

1g) Construction of p(+)MV_2EZ-d-190-*Sgr*AI (FCB1, 24083 bp) and p(+)MV_3EZ-d-190-*Sgr*AI (FCB1, 24083 bp).

First of all, the cloning of the synthetic gene for MSP-1 of the FCB1 strain into the intermediate plasmid pZE21MV-*Sgr*AI has been performed, keeping the signal peptide and the GPI-anchor region from MSP-1 of 3D7 strain. D-190 gene (FCB1) was obtained stepwise from an intermediate vector, called pZE23f-GX-190H, as follow:

i). 1 µg of the plasmid pZE21MV-d-190-SgrAI (3D7) was digested with *Hind*III + *Acl*I restriction enzymes, for two hours at their optimal temperature, in 50µl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (2558 bp), corresponding to the vector, was excised from the gel, purified by QIAEX gel purification and the DNA concentration was calculated by absorbance at 260 nm.

ii). a PCR reaction was performed, using the pZE23f-GX-190H as template, in order to amplify and recover the d-42 portion of the MSP-1/FCB1. PCR amplification was carried out using the proofreading *Pfu* DNA polymerase (Stratagene). DNA sequences of the synthetic oligonucleotides primers are given in lower case for the MV nucleotides and in upper case for non MV nucleotides; sequences of relevant restriction endonucleases recognition sites are underlined.
The following oligonucleotides primers have been used, designed on the pZE23f-GX-190H sequence: For-1 FCB1, 5'-CCCAAGCTTccaggtggtcaccggAgagctgtcactcc -3', and Rev-1 FCB1, 5'- GCCTGCaacgttGCTagagctggagcaGaaGatcccgtcg -3'.
For-1 FCB1 is homologous to the template from pos. 4509 to pos. 4538, comprising the *Bst*EII site (ggtcacc). The A (in upper case) was a t in the template, and it has been modified to eliminate a *Sgr*AI site. It contains an overhang (in upper case) with the *Hind*III site (AAGCTT), after its 3 bp long-protection site (CCC).
Rev-1 FCB1 contains an *Acl*I site (aacgtt), preceded by a 6-bp protection site (GCCTGC). It was introduced a triplet GCT, coding for a serine, to keep the rule of six; two a have been modified in G to avoid a poly(A) site.
The obtained PCR-*Hind*III-*Acl*I (1.1 kb) has been digested *Hind*III + *Acl*I and ligated, overnight at 16°C in an equimolar ratio, to the pre-digested pZE21MV-d-190-*Sgr*AI with *Hind*III + *Acl*I (step i), obtaining the pZE21MV-d-42-*Sgr*AI-FCB1 (3657 bp). XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and

selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and by restriction enzymes digestion with *Hind*III + *Acl*I (expected fragments 2558 bp + 1099 bp).

iii). the pZE21MV-d-42-*Sgr*AI–FCB1, obtained as described in step ii, has been digested *Hind*III + *Bst*EII (*Hind*III, pos. 428, and *Bst*EII, pos. 440), and the proper band (3645 bp), corresponding to the opened vector, was loaded on a 1% agarose gel, excised from the gel, purified by QIAEX gel purification and the DNA concentration was calculated by absorbance at 260 nm.

iv). The pZE23f-GX-190H was digested *Hind*III + *Bst*EII and the proper band of 3679 bp (insert), corresponding to the d-83-30-38/FCB1 fragment, was purified from the gel, as previously described.

v). the *Hind*III + *Bst*EII digested fragment of 3657 bp (vector), obtained from pZE21MV-d-42-*Sgr*AI–FCB1, has been ligated to the *Hind*III + *Bst*EII fragment of 3679 bp (insert), containing the d-83-30-38/FCB1 and obtained by digestion from pZE23f-GX-190H. Ligation was done in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase, obtaining the pZE21MV-d-190-*Sgr*AI–FCB1 (7324 bp). Afterwards, XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion.

To construct the p(+)MV$_2$EZ-d-190-*Sgr*AI-FCB1 and p(+)MV$_3$EZ-d-190-*Sgr*AI-FCB1, the measles vectors were prepared as detailed described in example 3a.

1µg of d-190/FCB1 gene, inserted into an intermediate plasmid (pZE21MV-d-190 *Sgr*AI-FCB1, 7324 bp), was taken out by *Sgr*AI-*Bss*HII digestion (one unit of each enzyme), for two hours at their optimal temperature, in 50µl final volume. All the digested DNA was loaded onto a 1% agarose gel, run at 80 Volt for about 2 hours. Then, the proper band (5035 bp) was excised from the gel, purified by QIAEX gel purification kit and the DNA concentration was calculated by absorbance at 260 nm and adjusted to 1□µg/ml.

Thus, the vector (MV DNA: figure 1) and the insert (d-190/FCB1 DNA: figure 3), were ligated in an equimolar ratio overnight at 16°C, using one unit of T4 DNA Ligase and its own reaction buffer in 10□µl final volume.

XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

The d-190- FCB1 gene, inserted into position 2 of the MV vector (*Sgr*AI, pos. 4060, and *Bss*HII, pos. 9095) is represented in figure 13 and its Open Reading Frame (ORF) is listed in figure 32.

The d-190- FCB1 gene, inserted into position 3 of the MV vector (*Sgr*AI, pos. 9862, and *Bss*HII, pos. 14897) is represented in figure 14.

The genome's length (starting at ACC, pos. 609, to GGT, pos. 21884) of the recombinant Measles-Malaria plasmids was a multiple of six, allowing the rescue of the recombinant $MV_{2\text{-}3}$-d-190-FCB1 viruses.

The transgene is rather stably expressed: its expression was completely maintained in all analysed progeny clones derived from single original rescued clones after ten serial virus passages in human diploid cell MRC5 (figure 40).

The growth curves of recombinant MV-Malaria virus and MV vaccine showed the same kinetics (figure 41).

Example 2: designing of DiCo1 nucleic acid sequence.

Starting from the aminoacidic DiCo1 sequence (ecto, trans and cytoplasmic domains: aa 97-622) and using the DNA Strider software, a correspondent nucleic acid sequence has been designed comparing the DiCo1 DNA degenerate sequence to a selected *Pf*AMA1 gene (accession number AAG141.1), which represents the most similar sequence to the DiCo1 after BLAST alignment.

At the 5' end suitable unique restriction sites has been added (*MluI* and *Sgr*AI) as cloning sites, followed by an optimal KOZAC sequence and a human optimised Signal Peptide (SP). At the 3' end, two stop codons and a *Bss*HII cloning site have been added. Following this scheme, we designed two nucleotides sequences (respecting the "rule of six" for the further expression into the measles vector), encoding the anchored and the secreted forms of the DiCo1 protein: the first gene comprises the ectoplasmasmic, the transmembrane and cytoplasmic domains (Figure 18), while the second one corresponds to the ectodomain alone (Figure 19). The two sequences has been human codon optimised by GENEART, to reduce AT% content, to avoid poly(A) sequence and RNA instability motif.

DiCo1 complete ORF and DiCo1 ectodomain ORF are listed respectively in figure 34 and 35.

Example 3: construction of recombinant MV-*Pf*AMA-1 plasmids

All cloning procedures were done as per techniques described in Sambrook *et al.* (1989).

*Pf*AMA1, and in particular Diversity Covering sequences 1 (DiCo1) either in the secreted and anchored form, have been chemically synthesized and human codon optimised.

The codon optimised DiCo1 secreted and anchored forms were digested *Sgr*AI+*Bss*HII and ligated, overnight at 16°C in an equimolar ratio, to the pre-digested MeV_2EZ and MeV_3EZ vectors (19 Kb in length), using one unit of T4 DNA Ligase, obtaining the following recombinant MV- *Pf*AMA-1 plasmids: p(+)MV_2EZ-DiCo1-complete (Figure 20), p(+)MV_3EZ-DiCo1-complete (Figure 21), p(+)MV_2EZ-DiCo1-ecto (Figure 22), and p(+)MV_3EZ-DiCo1-ecto (Figure 23).

Example 4: construction of recombinant MV-*Pf*CS plasmids

Construction of p(+)MV_2EZ-CS-*Sgr*AI (20219 bp) and p(+)MV_3EZ- CS-*Sgr*AI (20219 bp)

All cloning procedures were basically as described in Sambrook *et al.* (1989).

*Pf*CS1, cloned into an intermediate vector pAdApt35Bsu.CS.Pfalc.aa-sub.gcc, has been amplified by PCR, and directly cloned into the definitive MV vectors, obtaining two recombinant MV-*Pf*CS plasmids: p(+)MV_2EZ-CS and p(+)MV_3EZ-CS.

In detail, a PCR reaction was performed, using the pAdApt35Bsu.CS.Pfalc.aa-sub.gcc as template, in order to amplify and recover the CS gene (figure 15). PCR amplification was carried out using the proofreading *Pfu* DNA polymerase (Stratagene). DNA sequences of the synthetic oligonucleotides primers are given in lower case for the MV nucleotides and in upper case for non MV nucleotides; sequences of relevant restriction endonucleases recognition sites are underlined.

The following oligonucleotides primers have been used, designed on the pAdApt35Bsu.CS.Pfalc.aa-sub.gcc sequence: For-*Sgr*AI, 5'-ACTTCTCACCGGTGTggaagcttgccac
catgat -3', and Rev-*Bss*HII-CS 5'- TAGCGCGCtctagaggatccttatcagc -3'.
For-*Sgr*AI is homologous to the template from pos. 1356 to pos. 1375, comprising the *Hind*III site (aagctt). It contains an overhang (in upper case) with *Sgr*AI restriction site (CACCGGTG), after 6-bp long-protection site (ACTTCT).
Rev-*Bss*HII-CS contains an overhang (in upper case) with *Bss*HII restriction site (GCGCGC), which will be close to *Xba*I (tctaga) in the PCR-CS (1187 bp).
The obtained PCR-CS has been digested *Sgr*AI + *Bss*HII and ligated, overnight at 16°C in an equimolar ratio, to the pre-digested MeV_2EZ and MeV_3EZ vectors *Sgr*AI+*Bss*HII (19 Kb in length), using one unit of T4 DNA Ligase, obtaining, respectively, p(+)MV_2EZ-CS-*Sgr*AI (20219 bp, figure 16) and p(+)MV_3EZ- CS-*Sgr*AI (20219 bp, figure 17). The CS ORF is listed in figure 33.
XL10 Gold chemical competent cell were then transformed with all ligation volume, following a standard transformation protocol (Sambrook *et al.* 1989), plated and selected on LB-Agar plates for ampicillin resistance. Colonies were screened by DNA plasmid preparation (QIAGEN, mini- midi and maxi kit) and restriction enzymes digestion. The right clones were sent to MWG for sequencing: the sequences, aligned with the assumed ones using a DNA Strider software, showed 100% identity.

Example 5: Cells and viruses

Cells were maintained as monolayers in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 5% Foetal Calf Serum (FCS) for Vero cells (African green monkey kidney) and with 10% FCS and 1% penicillin/streptomycin (P/S) for 293T cells (human embryonic kidney); DMEM supplemented with Glutamax (F12) and 10% FCS for MRC-5 (human foetal fibroblast); DMEM supplemented with 10% FCS and 1.2 mg/ml of G 418 for 293-3-46.

To grow MV virus stocks reaching titers of about 10^7 pfu/ml, recombinant viruses and the vaccine strain Edmoston Zagreb were propagated in MRC-5 cells: plaque purification was carried out by transferring a syncythium to 35 mm MRC-5 cell culture which was expanded first to a 10 cm dish, and afterwards to a 175 cm flask. Virus stocks were made from 175cm^2 cultures when syncythia formation was about 90% pronounced. Medium corresponding to the so-called "free-cell virus fraction" was collected, freeze and thawed three times and spun down to avoid cell debris. The medium was then stored at –80°C. Cells, which correspond to the so-called "cell-associated virus fraction", were scraped into 3 ml of OPTIMEM (Gibco BRL) followed by three rounds freezing and thawing, spun down and the cleared surnatant stored at –80°C.

Example 6: Transfection of plasmids and rescue of MV viruses

293T cells were seeded into a 35mm well to reach ~ 50-70% confluence when being transfected. 4 h before transfection, the medium was replaced with 3 ml DMEM containing 10% FCS. All recombinant plasmids were prepared according to the QIAGEN plasmid preparation kit. The kit for the Ca^{2+}phosphate coprecipitation of DNA was from Invitrogen.

Cells were co-transfected with the plasmids in the follows final concentration: pCA-L 0.5 µg, pCA-N 0.5 µg, pCA-P 0.1 µg, pCA T7 1 µg and the recombinant Measles-Malaria plasmid 4 µg. All five plasmids, diluted in H_2O, were added in a Eppendorf tube containing 2M $CaCl_2$, the mix was added to another Eppendorf tube containing HEPES buffer under shaking conditions, and was incubated 30 min at room temperature (RT). Thus, the co-precipitates were added dropwise to the culture and the transfection was carried out at 37°C and 5% CO_2 for about 18h. Then, the transfection medium was replaced with 3ml of DMEM containing 10% FCS.

Another way to obtain recombinant measles-malaria vaccine viruses is described hereafter, using the 293-3-46 helper cell (human embryonic kidney cells), stably expressing the measles N and P proteins as well as the T7 RNA polymerase. The viral RNA polymerase (large protein, L) was expressed by co-transfecting the cells with 15 ng of the plasmid peMCLa. To improve transfection efficiency 300 ng of pSC6-T7 Neo were added. Calcium-phosphate method was used for transfection.

First syncytia appeared 3-4 days after transfection when the cells were still subconfluent. To allow syncytia formation to progress more easily, almost confluent cell monolayer of each 35mm well were then transferred to a 10 cm dish. Each syncytium was taken up in 300 µl of transfection medium and put in a sterile Eppendorf tube containing 700 µl of OPTIMEM, freeze and thaw for three rounds, and stored at –80°C.

Example 7: virus titration by plaque assay

Serial 10-times dilutions of virus preparations were carried out using OPTIMEM to a final volume of 0.5 ml. Each dilution was added on 35 mm Vero cell cultures. After 1 h of virus adsorption, the inoculum was removed and the infected cells were overlaid with 2ml of DMEM containing 5% FCS and 1% low melting point agarose (LMP agarose). After 5 days of incubation at 37°C and 5% CO_2, cultures were fixed with 1ml of 10% TCA for 1 h, then UV cross-linked for 30 min. After removal of the agarose overlay, cell monolayers were stained with crystal violet dissolved in 4% ethanol, washed with water and the plaques were counted under the inverted microscope.

Example 8: MRC-5 virus serial passages of recombinant viruses

Rescued viruses were serially passaged 10-times on MRC5 cells, seeded into 10 cm diameter plates, that were infected with the standard and the recombinant MV viruses at MOI of 0.01 PFU/cells. After monolayer was full infected, 1% surnatant of each culture was used to infect the subsequent MRC5 cells monolayer. To test transgene expression and stability, viruses from passage 1, 5, and 10 were used for further characterisation of expression by Western blot and immunofluorescence.

Example 9: Western blot, immunofluorescence

To analyse the expression either MV and Malaria, Western blot and immunofluorescence were carried out.

For Western blot, Vero cells seeded on 35 mm dish (1-5x10^5) were monitored the next day for 90% confluence and infected with cleared virus suspension from cell-associated virus fraction, using 0.1 MOI (Multiplicity Of Infection), including MVEZ as control. When about 80% syncythia formation was observed, cells were first washed with PBS and then scraped in 1 ml PBS and collected in an Eppendorf tube, and centrifuge at 2000 RPM/4 min. Cells were then lysated 5 min/RT with 70 µl of lysis buffer (1% NP-40, 50 mM Tris pH 8, 150 mM NaCl) supplemented with protease inhibitor cocktail (Complete Mini, Roche, 1 836 153). Surnatants were cleared by centrifuge at 13000 RPM/5 min, and transferred into a new tube: 30 µl of 4x loading buffer (Invitrogen) were added; samples were mixed and boiled at 95°C/2 min, spun down and stored at –20°C.

An SDS-PAGE migration was performed, running a NuPAGE 12% Bis-acrylamide gel in reducing conditions, using 1x Running Buffer, for 50 min at 200V (start 100-125 mA, end 60-80 mA).

Then, semi-dry method was used to transfer separated cell-proteins to Nitrocellulose Membrane, at 14V/1h30.

As first antibodies, rabbit polyclonal against MSP1-p-83, diluted in PBST at least 1:30000, and against MSP1-p-42, diluted at least1:50000, were used. The second antibody was a swine anti-rabbit antibody coupled to horse-radish peroxidase allowing the visualization of the bands by the enhanced chemiluminescence kit (ECL™, Amersham LifeScience).

For immunofluorescence, Vero cells were seeded on a 24mm x 24mm glass cover slips in 35 mm wells, cultured overnight and infected with rescued recombinant virus. 3 days after infection cells on coverslips were fixed with 3,7% paraformaldehyde in PBS, and permeabilized with 0.1% TX-100, washed with blocking solution (PBS containing 1% BSA) for 1h, and stained with the specific antibodies. Mouse hybridoma supernatant mAb 5.2, which recognises a EGF-like domain in the p-19 portion of p-42, was used in a dilution 1:100 followed by FITCH conjugated goat anti-mouse serum, diluted 1:250.

Example 10: growth kinetics curve

MRC5 cells seeded on 35 mm dish (1-5x10^5) were monitored for 90% confluence and infected with cleared virus suspension from cell-associated virus fraction, using 0.1 MOI, including MVEZ as control. Samples, corresponding to the so-called "free-cell virus fraction" and to the so-called "cell-associated virus fraction", were collected daily for one week and titrated.

Example 11: Mice immunisation

The immunogenic power of the rescued recombinant MV-Malaria viruses described was proven by immunisation tests performed on transgenic mice IFNAR/CD46, susceptible to MV infections. The animals were kept under optimal hygienic conditions and were immunized at 6-8 weeks of age. Below is provided an example of mice immunization with two recombinant Measles-Malaria virus: the MeV2EZ-d-p42-SgrAI (the GPI anchored form) and the MeV2EZ-d-p42* (the secreted form). Immunisation was performed intra-muscularly using 10^5 PFU of each recombinant MV-Malaria in three injections at 0, 4 and 8 weeks. Mice immunized with recombinant-empty Measles (rMVEZ13- Empty cloned) served as negative control. UV inactivated rMV was used as a control to determine the effect of virus replication on activation of immune responses. The immune response of the MV vectored antigen was tested compared to the purified d-42 protein (0.5mg/ml): mice

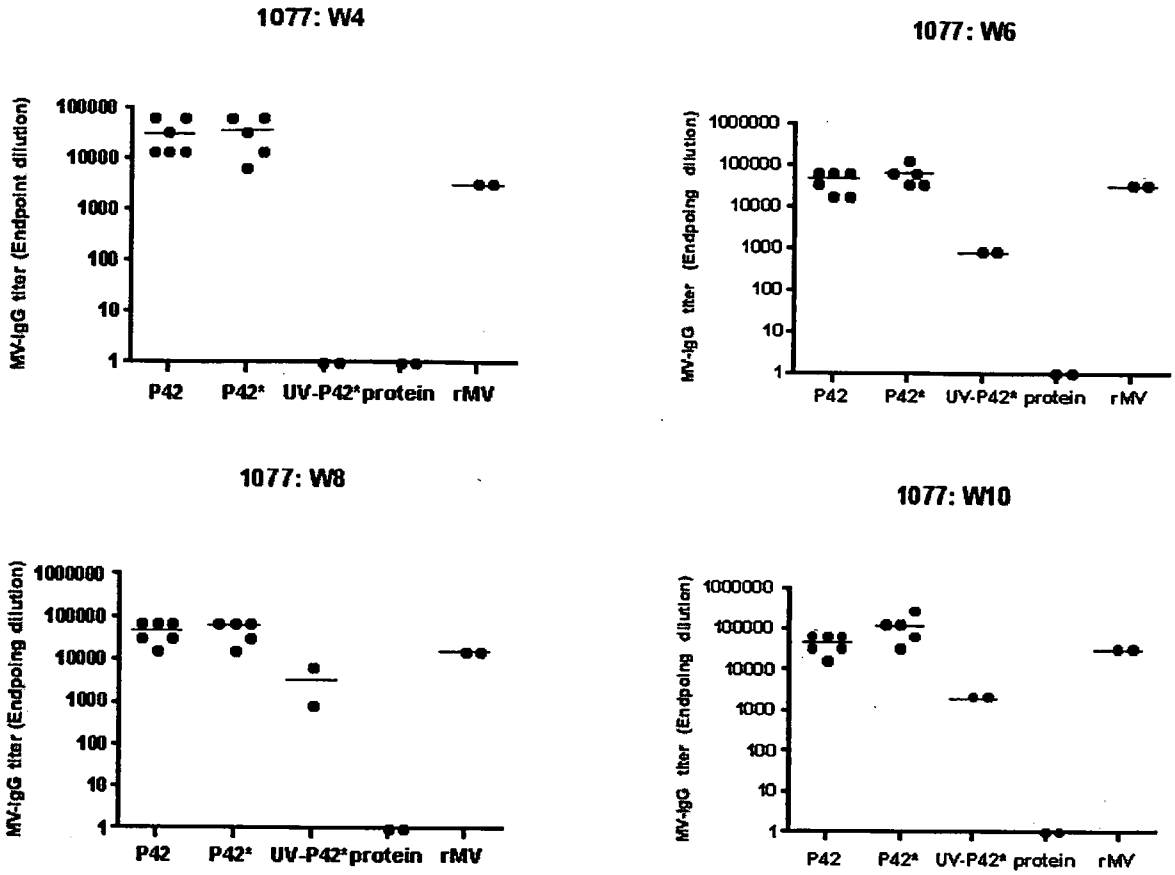
were immunized sub cutaneously with 20 µg of protein in Incomplete Freund's Adjuvant.

The presence of MV-specific antibodies in the sera from the immunised IFNAR/CD46 mice (6 per test group and 3 for control group) was determined by ELISA using 96-microwell plates, coated with Measles virus EIA bulk (ATCC VR-24), for IgG antibody detection. Protein was diluted 0.6 µg/ml with 0.05 M carbonate buffer (pH 9.4), and 100 µl per well was added to 96-well-microtiter plates. The plates were incubated overnight at 4°C, washed with PBS/0.05% Tween 20 (PT) (ph 7.4), incubated with PT (0.1 ml/well)-10% BSA for 60 min at 37°C, and washed again with PT. Serial 2-folds dilutions of the tested sera were added (100 µl/well), and the plates were incubated for 60 min at 37°C. The plates were washed with PT and were incubated with 100 µl of goat anti-mouse IgG HRP diluted 1:2000 in PT for 30 min at 37°C. The plates were washed with PT and incubated with 100 µl OPD (o-Phenylendiamin, Fluka 78411). The reaction was stopped after 3-4 min. Plates were read on a MicroElisa Reader at a wave length of 490 nm. Readings higher than three-folds negative controls were scored as positive reaction.

The presence of MV-Malaria-specific antibodies in the sera of immunised CD46 mice (at least 10 per test group) was determined by ELISA assay. Briefly, 96-microwell plates were coated 50 ng/ well MSP-1-d42 3D7 strains, diluted with carbonate buffer pH 9.4. The plates were incubated overnight at 4°C, washed with PBS/0.05% Tween 20 (PT). Subsequently, unspecific interaction were blocked with 10% defatted milk dissolved in PT for 1hour at 37°C and wells were washed again with PT. The plates were consecutively incubated with various dilutions of mouse sera (starting at 1:200, followed by serial two-fold dilutions), peroxidase-conjugate goat anti-mouse IgG and with OPD substrate. Optical density values were measured at 490 nm. Values above the cut-off background level (mean value of sera from MV immunised mice multiplied by a factor of 2.1) were considered positive. Titres were depicted as reciprocal end-dilutions.

The humoral immune responses against Measles are shown in Figure A. The humoral immune responses against Malaria p42 are shown in Figure B.

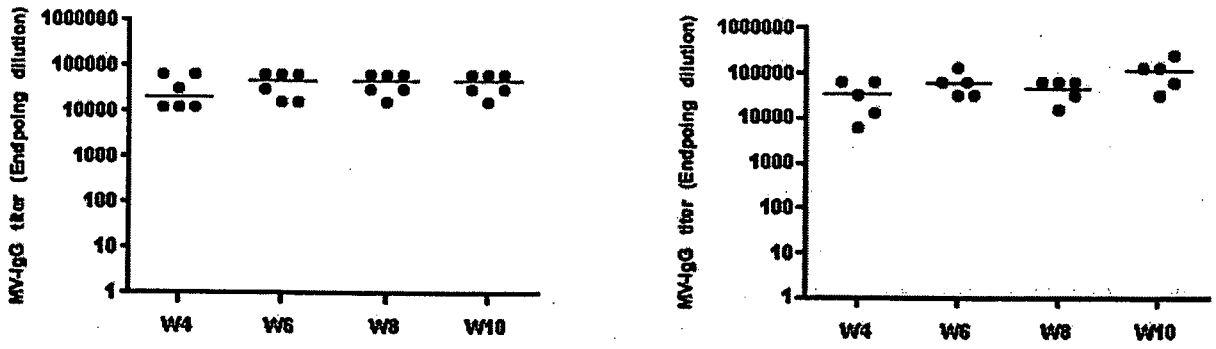
Figure A: Humoral response against Measles



rMeV2EZ-d-p42-SgrAI

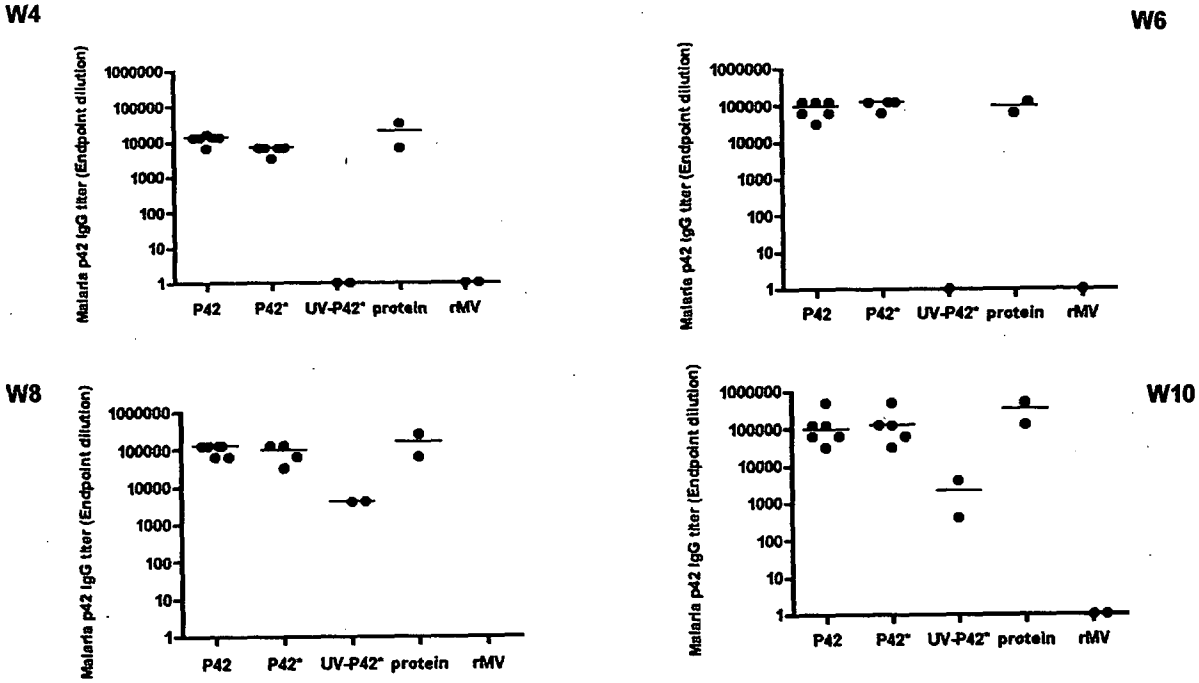
rMeV2EZ-d-p42*-

5 SgrAI



Blood was taken regularly and tested for Measles IgG Titers.

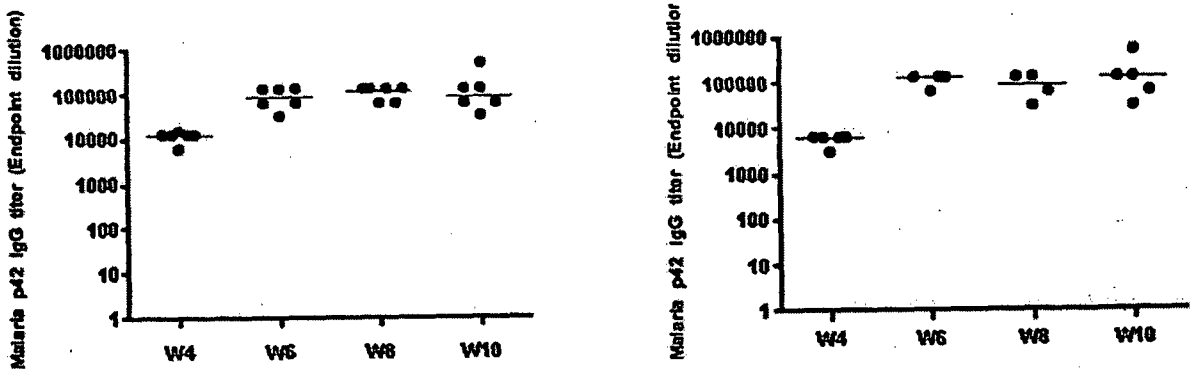
Figure B: Humoral response against Malaria



5

rMeV2EZ-d-p42-SgrAI

rMeV2EZ-d-p42*-SgrAI



10

Blood was taken regularly and tested for Malaria IgG Titers

Example 12: Purification of recombinant measles virus expressing malaria antigens from Defecting Interfering Particles (DIs) by plaque purification.

It is known from literature that after a certain number of passages with *Paramyxoviruses*, and in particular with measles virus, an accumulation of defective interfering particles (DIs) will occur (23, 24). It has been described that these DIs develop various defects: negative impact on vaccine safety, negative influence on virus yields in production, genome instability and suppression of immune reaction after vaccination. In order to avoid such DIs with our new recombinant viruses, we have applied the method of plaque purification as described in example 6 with the exception that we use MRC5 cell instead of 293T cells. After the formation of clear, well defined syncytia we aspirated under the microscope with a micropipette such material for further passaging in a fresh MRC5 tissue culture.

Example 13: Purification of recombinant measles virus expressing malaria antigens from Defecting Interfering Particles (DIs) by end point dilution.

The end point dilution technique was applied in microplates: in all wells a fresh monolayer of MRC5 cells had just developed. The virus suspension containing recombinant measles-malaria viruses was prepared in two fold dilutions. From the well of the latest monolayer where a syncytia was detected the supernatant was aspirated with a pipette. The supernatant was mixed with a suspension containing MRC5 cells. This mixture was incubated at 4°C for 1 hour. Finally, it was transferred in a small Costar flask and incubated at 35°C/5%CO_2 and harvested for purify recombinant measles-malaria virus after ten days.

Example 14: Production of a combined measles-malaria vaccine

The working seed of the described recombinant measles-malaria virus has been incubated on MRC5 cell monolayer in 1750cm^2 roller bottles at 35 °C for ten days. The cells have been monitored every day for status of health and confluence. On day ten at highest level of syncytia formation, the supernatant was pumped in a steel cylinder for storage in liquid nitrogen. The same procedure was repeated two days later. After performing of all the tests (virus titer, genome stability, virus safety, cell safety, chemical analysis, sterility and others), the harvests have been thawed up and mixed with stabilizer containing gelatine, sorbitol, amminoacids and other sugars to final dilution of 10^5. With a automated filling machine small lyo bottles (F3) have been inoculated with 0.5ml each. A specially calculated lyophilisation program was used to guarantee maximal survival of the product during the freeze-drying process.

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We claim:

1. A combined measles-malaria vaccine comprising a recombinant measles vaccine virus which express malaria antigens capable of eliciting immune response and protection both against measles and malaria.
2. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses single or different malaria antigens.
3. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses MSP1 malaria antigen.
4. The combined measles-malaria vaccine as claimed in claim1 wherein MSP1 malaria antigen is 190 to 200KDa (d190).
5. The combined measles-malaria vaccine as claimed in claim1 wherein MSP1 malaria antigen is d83-30-38.
6. The combined measles-malaria vaccine as claimed in claim1 wherein MSP1 malaria antigen is d42.
7. The combined measles-malaria vaccine as claimed in any preceding claims wherein the recombinant measles vaccine virus expresses MSP1 malaria antigen in both anchored and secreted forms.
8. The combined measles-malaria vaccine as claimed in any preceding claims wherein the recombinant measles vaccine virus expresses MSP1 malaria antigen in both anchored and secreted forms 3D7 strain and MAD 20 preferably the FCB1 strain.
9. The combined measles-malaria vaccine as claimed in any preceding claims wherein the recombinant measles vaccine virus expresses MSP1 malaria antigen in both anchored and secreted forms FCB1 strain.
10. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses Diversity Covering (DiCo) AMA1 malaria antigen.
11. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses DiCo-1 of AMA1 malaria antigen
12. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses DiCo-2 of AMA1 malaria antigen
13. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses DiCo-3 of AMA1 malaria antigen
14. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses DiCo-1, DiCo-2 and DiCo-3 of AMA1 malaria antigen

15. The combined measles-malaria vaccine as claimed in claim10 to claim 14 wherein the recombinant measles vaccine virus expresses Diversity Covering (DiCo) of AMA1 malaria antigen in trans membrane and secreted forms.

16. The combined measles-malaria vaccine as claimed in claim1 wherein the recombinant measles vaccine virus expresses CS malaria antigen

17. The combined measles-malaria vaccine as claimed in any preceding claims wherein the malaria antigen is cloned between P and M or H and L protein of recombinant measles vaccine virus.

18. A measles vaccine virus vector comprising the nucleotide sequence of antigen of malaria.

19. The vector as claimed in claim 18 wherein the nucleotide sequence is selected from figure 26 to figure 35.

20. The vector as claimed in claim 18 and claim 19 wherein the measles vaccine virus vector further comprises nucleotide sequence selected from figure 24 to figure 25.

21. The vector as claimed in claim 18 wherein the nucleotide sequence encodes malaria antigens selected from d83-30-38 and d42 and d190 fragments of MSP1 or Diversity Covering (DiCo) AMA1 or CS protein.

22. A host comprising the vector of claim 18.

23. The host as claimed in claim 22 is selected from E.coli or mammalian cell line.

24. The combined measles-malaria vaccine as claimed in any preceding claims wherein the recombinant measles virus originating from a vaccine strain derived from Edmoston Zagreb.

25. The combined measles-malaria vaccine as claimed in any preceding claims wherein recombinant measles vaccine virus which expresses at least one malaria antigens selected from d83-30-38 and d42 and d190 fragments of MSP1 or Diversity Covering (DiCo) AMA1 or CS protein.

26. The combined measles-malaria vaccine as claimed in any preceding claims wherein recombinant measles vaccine virus which expresses two or more malaria antigens selected from d83-30-38 and d42 and d190 fragments of MSP1 or Diversity Covering (DiCo) AMA1 or CS protein or combination thereof.

27. The combined measles-malaria vaccine as claimed in any preceding claims wherein recombinant measles vaccine virus comprises the following sequences.

MSP-1 d-190-3D7	AN101TE
MSP-1 d-190*-3D7	AN102TE
MSP-1 d-83-30-38-3D7	AN103TE

MSP-1 d-83-30-38*-3D7	AN104TE
MSP-1 d-42-3D7	AN105TE
MSP-1 d-42*-3D7	AN106TE
MSP-1 d-190-FCB1	AN107TE
CS	AN108TE
DiCo1-complete	AN109TE
DiCo1-ecto	AN110TE

28. The combined measles-malaria vaccine as claimed in any preceding claims wherein recombinant measles vaccine virus encods in addition to the malaria antigens protein with adjuvantic properties.
29. The vaccine as claimed in any preceding claims comprising recombinant measles viruses encoding in addition to the malaria antigens an interleukin, preferably interleukin 2.
30. The vaccine as claimed in any preceding claims comprises one of the described recombinant measles malaria viruses or a mixture of two to several such viruses.
31. The vaccine as claimed in any preceding claims wherein the described recombinant measles malaria viruses or a mixture of two to several such viruses devoid of defective interfering particles (DIs)
32. The vaccine as claimed in any preceding claims wherein the adventitiously arisen DI particles have been eliminated by plaque purification.
33. The vaccine as claimed in any preceding claims wherein the adventitiously arisen DI particles have been eliminated by end point dilution.
34. The vaccine as claimed in any preceding claims wherein the adventitiously arisen DI particles have been eliminated by physical methods such as differential centrifugation.
35. The vaccine as claimed in any preceding claims being a component of a combined vaccine where the other components are rubella, mumps, varicella or another life attenuated vaccine virus, naturally attenuated or recombinant, alone or in combination.
36. The vaccine as claimed in any preceding claims comprises suitable stabilizer, such as gelatin and/or human serum albumin and sorbitol as main components, ideal for parenteral application.
37. The vaccine as claimed in any preceding claims comprises with a suitable stabilizer and/or adjuvant ideal for intranasal application.
38. The vaccine as claimed in any preceding claims comprises suitable stabilizer and/or adjuvant ideal for inhalation application.
39. The vaccine as claimed in any preceding claims comprises suitable stabilizer and/or adjuvant ideal for oral application.

40. The vaccine as claimed in any preceding claims comprises suitable stabilizer and/or adjuvant ideal for trans dermal application.

41. The vaccine as claimed in any preceding claims comprises suitable stabilizer and/or adjuvant ideal for any suppository formulation.

42. A composition of combined measles-malaria vaccine comprising stabilizer and/or adjuvant.