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Recombinant measles vaccine expressing malaria antigens induces long-term memory and protection in mice

Marie Mura1,2, Claude Ruffié1, Chantal Combredet1, Eduardo Aliprandini3, Pauline Formaglio2, Chetan E. Chitnis4, Rogerio Amino3 and Frédéric Tangy4

INTRODUCTION

Despite decades of malaria vaccine research, only RTS,S/AS01 vaccine candidate reached Phase III clinical trial to eventually show moderate protection of short duration.1 This led the World Health Organization to recommend additional pilot studies in three countries with enhanced pharmacovigilance.2 Nevertheless, these results are encouraging as they establish the feasibility of developing a malaria vaccine. Furthermore, the spread of artemisinin-resistant P. falciparum strains3 underlines the need for an effective vaccine for sustained protection against malaria. The rationale for malaria vaccine development relies on several observations. First, natural immunity is gradually acquired to severe, life-threatening malaria and then to clinical disease after several years of natural exposure.4 Nevertheless, this immunity is not sterile and quickly wanes if an individual leaves the endemic area. Continued exposure to parasites is, therefore, required to maintain immunological memory.5 Second, transfer of gamma-globulin fractions from semi-immune to naïve humans clears blood stage parasites and mitigates malaria disease.6 Finally, inoculation of irradiated attenuated sporozoites can protect humans against infectious challenge, but requires high and frequent doses, and immunity wanes after 6 months.7 Therefore, the induction of long-term memory is critical for sustained vaccine efficacy.

The RTS,S subunit vaccine is based on the Plasmodium falciparum (Pf) circumsporozoite protein (CS), which is expressed during the sporozoite and early liver stages, and is involved in adhesion and invasion of hepatocytes. CS is known as the lead antigen for inclusion in a pre-erythrocytic vaccine candidate. Based on data on efficacy elicited by CS in pre-clinical as well as human challenge models, the CS is considered a “gold standard” that can be used to evaluate different vaccine delivery platforms and prime-boost strategies.8–10 The CS is composed of a central and conserved Asparagine-Alanine-Asparagine-Proline (NANP) amino acid repeat sequence, known as the immunodominant B-cell epitope. Indeed, CS-specific antibodies and CD4+ T cell responses were associated with human protection during RTS,S-controlled human malaria infection trials (CHMI).11 However, RTS,S/AS01 did not induce CD8+ T cell response but prime-boost strategies with AdCh63 and MVA, which are non-replicative viral vectors, were disappointing.12,13

The measles virus (MV) vector-based vaccine platform offers new opportunities as a replicative but safe viral vector. It has not yet been used to deliver malaria antigens. The rationale for the use of MV is based on the following arguments: (i) MV is one of the safest and most effective human vaccines, eliciting life-long protective immunity against measles after a single injection; (ii) its production can be easily scaled up at low cost, which is important for developing countries where malaria is endemic; (iii) immunization with MV vector induces both humoral and cellular responses to the transgenes14–16; (iv) MV genome can integrate up to 6 kb in additional transcription units, allowing the expression of several malaria antigens; (v) phase I and phase II clinical trials with a
recombinant MV (rMV) vaccine expressing chikungunya virus-like particles showed that the vaccine was very immunogenic and, unlike non-replicative viral vector platforms, there was no impact of pre-existing immunity against measles vaccine\textsuperscript{(20,21); (vi)} in 2016, about 85% of the world’s children received one dose of measles vaccine by their first birthday through routine health services. A recombinant measles-malaria vaccine could therefore easily be integrated in vaccination schedules.

Based on previously obtained data in collaboration with R. and V. Nussensweig, we have generated rMV expressing the CS protein of Plasmodium berghei (Pb) and PF to establish proof of concept for the use of measles vector to express malaria antigens. In the CS\textsubscript{Pb} model, we demonstrate that rMV-CS\textsubscript{Pb} is able to induce sterile protection of mice or at least protect them from severe symptoms with reduced blood parasitemia. In the CS\textsubscript{PF} model, rMV-CS\textsubscript{PF} induced immunogenicity has a Th1 profile and is maintained from 3 weeks up to, at least, 4 months after the second immunization. Furthermore, we show the induction of CD4\textsuperscript{+} and CD8\textsuperscript{+} cellular responses. High antibody titers with long-term memory and induction of cellular response are keys for the development of a malaria vaccine with higher efficacy and long-term protection against P. falciparum malaria.

RESULTS

Production of rMVs expressing CS\textsubscript{P} and CS\textsubscript{F} proteins

We constructed an rMV expressing CS\textsubscript{P} protein (rMV-CS\textsubscript{P}) and an rMV expressing CS\textsubscript{F} protein (rMV-CS\textsubscript{F}) by inserting mammalian codon-optimized sequences of both proteins in additional transcription unit 2 (ATU2) of pTM-MV\textsubscript{Schw} plasmid, which encodes the antigenome of the Schwarz MV vaccine strain\textsuperscript{22} (Fig. 1a). The ATU2 allows high-level expression of the protein, as there is a decreasing gradient of gene expression generated by MV repressor “N” expression to low polymerase “L” expression. Both plasmids were transfected into HEK293T-helper cells for rescue and co-cultured with Vero cells for virus spread. The rescued rMV-CS\textsubscript{P} and rMV-CS\textsubscript{F} had slightly delayed growth curves, as compared to empty MV (Fig. 1b), but still reached high titers on Vero cells. Viral stocks were made from unique syncytia after rescue and are therefore considered as clonal. The expression of CS was assessed by Western blot, and found in the lysate and in the supernatant of infected Vero cells (Fig. 1c and Supplementary Fig. 1). The CS expression in infected cells forming syncytia was also demonstrated by immunofluorescence (Fig. 1d). For rMV-CS\textsubscript{F}, the stability of transgene expression was demonstrated after 10 passages of the recombinant virus on Vero cells by immunofluorescence, Western blot and sequencing. The stability of rMV-CS\textsubscript{P} was not tested as the mouse model was only used for proof of concept but the virus stock was successfully characterized by sequencing and CS\textsubscript{P} expression analysis.

Susceptibility of hCD46IFNAR mice to Pb ANKA challenge

Mice are naturally resistant to MV, which is restricted to human and non-human primates (NHPS). The usual mouse model to test rMV vaccine candidates is deficient for type-I IFN receptor (IFNAR) and expresses human receptor CD46 (hCD46).\textsuperscript{22} The genetic background of hCD46IFNAR mouse used here is 5v129, which has the same major histocompatibility complex haplotype as C57BL/6 mouse (H-2Db, H-2Kb, I-Ab). C57BL/6 mice infected with P. berghei ANKA (PbA) is a model for cerebral malaria, which leads to death. C57BL/6 mice are easily infected and highly susceptible, as compared to Balb/c mice.\textsuperscript{23,24} In order to validate the model of infection in hCD46IFNAR mice, we inoculated 5000 GFP-expressing PbA (GFP PbA) sporozoites in the footpad of six C57BL/6 and six hCD46IFNAR mice. We monitored the parasitemia and clinical symptoms from day 4 to day 6 post-inoculation. Although parasitemia was slightly higher in hCD46IFNAR group, there was no statistically significant difference between both groups of mice (Fig. 2a). So, we concluded that both mouse models were comparable for sporozoite challenge. These observations validated the use of hCD46IFNAR mouse for the rest of the study.

Immunogenicity and protective efficacy of rMV-CS\textsubscript{Pb} as a proof of concept

Six-week-old hCD46IFNAR mice (6 mice per group) received 10\textsuperscript{5} TCID\textsubscript{50} of rMV-CS\textsubscript{Pb}, or MV\textsubscript{Schw} as negative control, by intraperitoneal (i.p.) route at day 0 and at day 28. Sera were collected before the first immunization (control) and 3 weeks after each immunization. Antibodies to MV were induced at similar levels in all immunized mice (Fig. 2b). Antibodies to CS\textsubscript{Pb} were efficiently induced from the first immunization with limiting dilution titers of about 10\textsuperscript{4}, then boosted after the second immunization to reach 10\textsuperscript{5} (Fig. 2c). Mice were challenged 3 weeks after the second immunization with 5000 sporozoites of GFP-PbA injected in the footpad. In MV\textsubscript{Schw} immunized group (control), we sacrificed mice at day 6 post-challenge (Fig. 2d), due to start of cerebral symptoms, which were ethical endpoints of the study. In rMV-CS\textsubscript{Pb} immunized group, two mice (33%) achieved sterile protection (no detectable IRBC at day 10 post-challenge) and parasitemia was delayed for one mouse (Fig. 2e). The other mice showed a significant decreased parasitemia (Fig. 2f), with no observed severe symptoms. Moreover, at day 10 post-challenge, the parasitemia in rMV-CS\textsubscript{Pb} immunized mice was still <1%. So, immunization with rMV-CS\textsubscript{Pb} achieved sterile protection in 33% of hCD46IFNAR mice and completely protected mice from severe and lethal PbA-induced cerebral malaria.

Immunogenicity of rMV-CS\textsubscript{F}: Th1 IgG subtype profile and long-term memory

Six-week-old hCD46IFNAR mice (6 mice per group) received 10\textsuperscript{5} TCID\textsubscript{50} of rMV-CS\textsubscript{F}, or MV\textsubscript{Schw} as negative control, by i.p. route at day 0 and at day 28. Sera were collected before the first immunization (control), 3 weeks after each immunization, and 22 weeks after the first immunization for a group of 6 mice dedicated to long-term memory study. As for rMV-CS\textsubscript{Pb}, antibodies to MV were induced at similar levels in all immunized mice (Fig. 3a) and antibodies to CS\textsubscript{F} were efficiently induced from the first immunization with limiting dilution titers of about 10\textsuperscript{4}, then boosted after the second immunization to reach 10\textsuperscript{5} (Fig. 3b). Interestingly, this high antibody titer was maintained 22 weeks post-prime. The humoral response profile corresponded to Th1 polarization with high titers of IgG2a antibodies (Fig. 3c), as expected for a replicative viral vector. Mice were challenged 3 weeks after the second immunization (early challenge) or 22 weeks post-prime (late challenge) with 5000 sporozoites of recombinant GFP-Pb expressing CS\textsubscript{P} with CS\textsubscript{F} repeat sequence (rGFP-PbA-CS\textsubscript{F} repeat), microinjected in the mouse footpad. In MV\textsubscript{Schw} immunized group (control), all mice were sacrificed at day 6 post-challenge, due to start of cerebral symptoms. In rMV-CS\textsubscript{F} immunized group, there was no induction of sterile protection, but a decreased and delayed parasitemia, whether for early (Fig. 3d) or late challenge (Fig. 3e). Mice started to present symptoms of cerebral malaria at day 7 and were sacrificed to avoid unnecessary suffering. This decreased parasitemia was therefore less important than the one observed for rMV-CS\textsubscript{Pb}. For both studies, there was no correlation between protection and anti-CS antibody titers. We hypothesized that the observed difference was due to the challenge model with rGFP-PbA-CS\textsubscript{F} repeat that allow only to study protection relying on neutralizing antibodies directed against the repeat sequence. We therefore evaluated the cellular response in the Pf model.
Induction of specific cellular immune response

Cell-mediated immune response (CMI) elicited by immunization with rMV-CSF was assessed using IFNγ Elispot assay and intracellular cytokine staining (IFNγ and TNFα) on freshly extracted splenocytes collected 7 days after a single immunization with 1 x 10^5 TCID50 in 100 µl i.p. (Fig. 4 and Supplementary Fig. 2). Splenocytes were stimulated ex vivo with inactivated MV-Schwarz at an MOI of 1 or CSPI recombinant LPS-free protein at 50 µg/ml. A moderate but significant (p < 0.01, Mann–Whitney U test) number of MV-specific cells (up to 100/10^6 splenocytes) were detected by the ELISPOT assay (Fig. 4a), which corresponds to 5–10% of the number of MV-specific spots. The phenotype of MV-specific and CSPI-specific cells induced by rMV-CSF was analyzed by flow cytometry (Fig. 4b–e). The mean frequency of MV-specific T cells secreting IFNγ and TNFα in CD4+ cells (Fig. 4b) was, respectively, 1.5% and 0.2%. The mean frequency of MV-specific T cells secreting IFNγ and TNFα in CD8+ cells (Fig. 4c) was, respectively, 2.6% and 0.2%. The mean frequency of CSPI-specific T cells secreting IFNγ and TNFα in CD4+ cells (Fig. 4d) was, respectively, 0.16% and 0.14%. The mean frequency of CSPI-specific T cells secreting IFNγ and TNFα in CD8+ cells (Fig. 4e) was, respectively, 0.3% and 0.18%. An induction of CD4+ cells secreting IFNγ and CD8+ cells secreting IFNγ or TNFα was observed, as compared to control group but statistically not significant (p = 0.074, p = 0.057, and p = 0.088, respectively, Mann–Whitney U-test). Even if no CD8+ epitopes of CSPI were described in C57BL/6 mouse, we showed the induction of a moderate but significant CMI as early as 7 days after a single immunization with rMV-CSF, with CD4+ activated and CD8+ activated phenotype.

DISCUSSION

Following the moderate protection and short memory response induced by RTS,S vaccine candidate in phase III clinical trial, there is strong support for developing a second-generation malaria vaccine with higher efficacy and longer duration of protection. Because of its central place in infant vaccine schedules all over the world, measles provides a promising viral vector to deliver malaria antigens, either as a single delivery platform or in a prime boost strategy. We report here the first use of measles-based vaccine platform to deliver CS malaria antigen as a proof of concept of the feasibility and advantages of this vector, in a murine model. Importantly, we showed induction of cellular response and long-term memory with high antibody titers. These are the two main characteristics required for second-generation malaria vaccine candidates.

We first showed the possibility of stably expressing a malaria antigen using the MV as a delivery vector. CSPl and CSPI sequences were successfully inserted in MV-Schwarz genome and stably maintained after 10 passages in Vero cell culture, without any mutation. Nevertheless, we were unable to rescue a virus with CS native sequence and therefore mammalian codon-optimized sequence is required. The P. falciparum genome is AT rich and polyA/polyU probably disturbed measles polymerase, either for replication or transcription. As MV-Schwarz vector is able to insert sequences were successfully inserted in MV-Schwarz genome and therefore mammalian codon-optimized sequences were easily added to CS to improve vaccine efficacy.

Then we showed in the hCD46IFNAR mouse model the induction of high antibody titers that are maintained at least until 22 weeks post-prime in a two-immunization schedule with one-month interval. This maintenance of high antibody level was
Fig. 2 Immunogenicity and protective efficacy of rMV-CSPb. a Blood parasitemia of C57BL/6 and hCD46IFNAR mice after skin microinjection of 5000 sporozoites of *Plasmodium berghei* ANKA. Percentage of infected red blood cells (iRBCs) at day 4, 5, and 6 post-infection (p.i.) was log transformed for parasitemia normalization before statistical analysis. No statistically significant difference was observed between both groups. b, c Antibody response induced in hCD46IFNAR mice immunized with rMV-CSPb at day 0 and 4 weeks later. The data show the reciprocal endpoint dilution titers of specific antibodies to MV b and CSPb c. Percentage of asymptomatic d and non-infected e hCD46IFNAR mice (6 mice per group) immunized twice at one month of interval and challenged 3 weeks after with 5000 *Pb* ANKA sporozoites intradermally. f Log of parasitemia at day 4, 5 and 6 post-infection (p.i.) (lines represent means for each group). Asterisks (*) indicate significant mean differences (**p < 0.01; ***p < 0.001) measured by t-test after normalization. L.D. level of detection. N.I. threshold of parasitemia detection.
severe disease, with a reduced parasitemia, and no severe clinical symptoms. In the rGFP-PbA-CS Pf repeat challenge model, there was no sterile protection and reduction in parasitemia was less compared to the PbA model. This suggests that sterile protection was not induced by neutralizing antibodies directed against the repeat sequence of CS Pf, but may involve antibodies against C and N-terminal domains of CS and cell-mediated immune responses. In fact, phagocytic activity of antibodies induced by RTS,S/AS01 malaria vaccine has been correlated with full-length CS and C-terminal-specific antibody titer, but not to repeat region antibody titer.37 Accordingly, we showed a moderate but significant induction of cell-mediated immune response that appeared as early as 7 days after a single immunization, with an increase in CD8+ T cells secreting IFNγ or TNFα. As there is no described CD8+ epitope for CS Pf in H-2b mice, the increase observed, even if moderate, is of great interest. Indeed, protection against malaria has been correlated to CS Pf CD8+ T cell response in human immune system (HIS) mice harboring functional human CD8+ T cells.38 This major role for CD8+ T cells to induce protection was already shown by in vivo depletion of CD8+ T cells that abrogated sporozoite-induced protective immunity in mice.39 Thus, even if the protection resulting from rGFP-PbA-CS Pf repeated challenge model was not indicative of real protection, it brought indications of efficient immune mechanisms involved in protection.

To conclude, in this work we demonstrated the promising potential of using measles vaccine vector to deliver malaria antigens. We showed the induction of cellular immune responses and long-term memory with high antibody titers in mice, two critical desired characteristics for second-generation malaria vaccines. As expected, expression of CS alone was not able to induce sterile protection in all mice in this model. We rather used CS as a "gold standard" to validate the measles vector approach. Transposition to clinical trial with measles vector is facilitated by an excellent track record of safety and immunogenicity for other relevant antigens and pathogens, and a well-established production process that allows substantial acceleration in development timelines. Further recombinant measles-vectored malaria vaccine candidates expressing additional pre-erythrocytic and/or blood-stage antigens in combination with CS will be soon evaluated to yield synergistic effects and provide protection with higher efficacy for long duration. Such vectors should be rapidly introduced in clinical trials and easily tested in controlled malaria human challenge, thanks to the excellent safety of measles vaccine.

METHODS

Study design

This study aimed to evaluate the immunogenicity and efficacy of measles live attenuated vaccine as a vector to deliver malaria antigens. To this purpose, we designed, cloned, and rescued recombinant measles viruses (rMV) that expressed the CS of Pb ANKA (CS Pf ANKA full length sequence, mammalian codon optimized synthetic gene, Eurofins Genomics) or a truncated CS of Pf 3D7 (CS Pf, mammalian codon optimized synthetic gene,
aa 19–369 without GPI anchored signal at C-terminus and with the signal sequence from MV Fusion protein at N-terminus; Genscript, USA). Rescue of both recombinant viruses (rMV-CS\textsubscript{Pb} and rMV-CS\textsubscript{Pf}) was performed using the helper-cell-based rescue method described by Radecke et al.\textsuperscript{40,41} and modified by Parks et al.\textsuperscript{41} (supplementary methods). rMV-CS\textsubscript{Pb} and rMV-CS\textsubscript{Pf} were grown on Vero cells (ATCC, CCL-81). Then, groups of six 6-week-old mice deficient for type-I IFN receptor (IFNAR) and expressing human CD46 (hCD46),\textsuperscript{22} housed under pathogen-free conditions at the Institut Pasteur animal facility, were inoculated with 10\textsuperscript{5} TCID\textsubscript{50} of rMV-CS\textsubscript{Pb}, rMV-CS\textsubscript{Pf}, or MV-Schwarz as control, via the i.p. route. All the animal studies were repeated twice. To study cellular response, a single immunization was administered and spleens were extracted 7 days later. For humoral response and infectious challenge, two immunizations were administered within a 4 weeks interval. Sera were collected before the first immunization (day 0, negative control) and 4 weeks after each immunization, and 4 months after the second immunization to study long-term memory responses. Immunized mice were challenged with\textsuperscript{42} sporozoites expressing the green fluorescent protein (GFP) under the control of\textit{hsp70} promoter (GFP-Pb).\textsuperscript{43} Alternatively, mice immunized with rMV-CS\textsubscript{Pf} were challenged with\textit{P. berghei} NK65 sporozoites expressing the GFP under the control of the\textit{hsp70} promoter\textsuperscript{42} and a chimeric CSP\textsubscript{Pb} harboring the central repetitive region of\textit{CSPf} (rGFP-Pb-CSP\textsubscript{Pf} repeat).\textsuperscript{44} Sporozoites were freshly collected from the salivary gland of infected Anopheles stephensi in D-PBS and filtered using a 35 µm nylon mesh cell strainer snap cap (Corning Falcon). Infectious challenges were executed 4 weeks after the second immunization (early response), or 4 months after the second immunization (long-term memory response) by the microinjection of 5000 sporozoites in one microliter of D-PBS in the posterior footpad using a 35G microsyringe (World Precision Instruments). After challenge, parasitemia was monitored from day 3 to day 10. Blood samples (2 µl) were diluted in 500 µl of PBS and analyzed by flow cytometry (MacsQuant, Miltenyi Biotec). Doublets and clusters of red blood cells (RBCs) were excluded from counts. Single GFP\textsuperscript{+}RBCs (infected RBC, iRBCs) among total RBCs were estimated and data analyzed by the MACSQuantify™ Software. As no protection against blood stage parasites was expected, mice were sacrificed at day 10 post-challenge in the presence of iRBCs in order to avoid unnecessary suffering, or before in the presence of severe symptoms that were ethical endpoints (signs of cerebral malaria: motor troubles, ruffled fur, and sometimes convulsions). Non-parasitemic mice at day 10 were considered sterile protected.
Intracellular cytokine staining

Freshly extracted splenocytes from immunized mice were analyzed by flow cytometry for their capacity to secrete IFN-γ and TNF-α upon specific stimulation. Spleen cells were cultured for 16 h in U-bottom 96-well plates (1.0 × 10^5 cells/well) in a volume of 0.2 ml complete medium (MEM—10% FCS supplemented with non-essential amino-acids 1%, sodium pyruvate 1%, and β-mercaptoethanol). Cells were stimulated with PMA/ionomycin (#00-4970, ebioscience) as positive control, complete MEM as negative control, live attenuated MV-Schwarz virus at an MOI of 1, and CSPP LPS-free recombinant protein at 50 µg/ml. Brefeldin A (#B6542, Sigma) was then added at 10 µg/ml for 6 more hours of incubation. Stimulated cells were harvested, washed in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% w/w sodium azide (FACS buffer), incubated 10 min with Fc blocking Ab (CD16/32 clone 2.4G2, PharMingen) and surface stained in FACS buffer with Live/Dead fixable dead cell violet kit (#L34955, invitrogen), anti-mouse CD4-PECy7 mAb (#552775, BD Biosciences) and anti-mouse CD8-APCCH7 mAb (#560182, BD Biosciences) for 30 min at 4 °C in the dark. After washing, cells were fixed and permeabilised for intracellular cytokine staining using the Cytofix/ Cytoperm kit (#554922, BD Bioscience). Cells were then incubated in a mix of anti-mouse IFNy-APC mAb (#554413, BD Biosciences) and anti-mouse TNF-α-FITC mAb (#554418, BD Biosciences) diluted in permwass buffer (# 557885, BD Biosciences) for 30 min in the dark. After washing with permwass buffer and FACS buffer, cells were fixed with 1% formaldehyde in PBS. Data were acquired using a MacsQuant® Analyzer (Miltenyi Biotec), and analyzed using FlowJo™ 9.3.2 software and are presented as percentage of CD4^+ or CD8^+ cells expressing TNF-α or IFNy among total CD4 or CD8 populations.

Statistical analysis

Parasitemia was log transformed for normalization. Statistical analyses of normalized parasitemia were done using the t-test. Statistical analyses of cellular responses were done using the non-parametric Mann–Whitney U-test. Differences were considered statistically significant when p < 0.05.

Reporting summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the authors on reasonable request; see author contributions for specific data sets.

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AUTHOR CONTRIBUTIONS

M.M. and F.T. designed the study, in collaboration with C.E.C. and R.A. M.M. and C.C. performed the vaccine constructions and viral rescues. M.M. and P.F. and R.A. performed the mice experiments. R.A. and E.A. provided and maintained the transgenic parasites. M.M. performed the immunology experiments, analyzed the data and prepared the manuscript. All authors contributed to final manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper on the npj Vaccines website (https://doi.org/10.1038/s41551-019-0106-8).

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