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Application No. 13 726 295.2 - 1116	Ref. 10806 IB/mcd	Date 11.05.2018
Applicant Instituto de Medicina Molecular		

Communication under Rule 71(3) EPC

1. Intention to grant

You are informed that the examining division intends to grant a European patent on the basis of the above application, with the text and drawings and the related bibliographic data as indicated below.

A copy of the relevant documents is enclosed.

1.1 In the text for the Contracting States:

AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT
RO RS SE SI SK SM TR

Description, Pages

1-22 filed in electronic form on 19-03-2018

Claims, Numbers

1-3 filed in electronic form on 19-03-2018

Drawings, Sheets

1/6-6/6 as published

With the following amendments to the above-mentioned documents proposed by the division

Description, Pages 5

Comments

DESCRIPTION

Page 5: Sentence introduced to adapt the description to the claims.

1.2 Bibliographic data

The title of the invention in the three official languages of the European Patent Office, the international patent classification, the designated contracting states, the registered name(s) of the applicant(s) and the other bibliographic data are shown on **EPO Form 2056** (enclosed).

2. Invitation

You are invited, **within a non-extendable period of four months** of notification of this communication,

2.1 to EITHER approve the text communicated above and verify the bibliographic data (Rule 71(5) EPC)

(1) by filing a translation of the claim(s) in the other two official languages of the EPO

	Fee code	EUR
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(2a) by paying the fee for grant including the fee for publication:
minus any amount already paid (Rule 71a(5) EPC):

007	925.00 0.00
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Total amount:	925.00
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(3) by paying additional claims fees under Rule 71(4) EPC;
number of claims fees payable: 0
minus any amount already paid (Rule 71a(5) EPC):

016	0.00 0.00
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Total amount:	0.00
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Important: If the translations of the claims and fees have already been filed and paid respectively in reply to a previous communication under Rule 71(3) EPC, e.g. in the case of resumption of examination after approval (see Guidelines C-V, 6), **agreement as to the text to be granted** (Rule 71a(1) EPC) must be expressed within the same time limit (e.g. by approving the text and verifying the bibliographic data, by confirming that grant proceedings can go ahead with the documents on file and/or by stating which translations of the claims already on file are to be used).

Note 1: See "Notes concerning fee payments" below.

Note 2: Any overpaid "minus" amounts will be refunded when the decision to grant (EPO Form 2006A) has been issued.

Note 3: For the calculation of the grant fee under Article 2(2), No. 7, RFees (old fee structure), the number of pages is determined on the basis of a clean copy of the application documents, in which text deleted as a result of any amendments by the examining division is not shown. Such clean copy is made available via on-line file inspection only.

2.2 OR, in the case of disapproval, to request reasoned amendments or corrections to the text communicated above or keep to the latest text submitted by you (Rule 71(6) EPC).

In this case the translations of the claims and fee payments mentioned under point 2.1 above are NOT due.

The terms "amendment(s)" and "correction(s)" refer only to amendments or corrections of the application documents and not of other documents (e.g. bibliographic data, the designation of the inventor, etc.).

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the examining division requesting that you correct this deficiency (Rule 137(4) EPC).

2.3 Bibliographic data

Where you request a change or correction of bibliographic data in response to the Rule 71(3) communication, this will **not** cause the sending of a further communication under Rule 71(3) EPC. You will still have to pay the fees and file translations in reply to the Rule 71(3) communication in the case of 2.1 above, unless you also file a reasoned request for amendments or corrections in response to the Rule 71(3) communication (see case 2.2 above).

3. Loss of rights

If neither of the two possible actions above (see points 2.1 or 2.2) is performed in due time, the European patent application will be deemed to be withdrawn (Rule 71(7) EPC).

4. Further procedure

4.1 In the case of point 2.1 above

- 4.1.1 The decision to grant the European patent will be issued, and the **mention of the grant** of the patent will be published in the European Patent Bulletin, if the requirements concerning the translation of the claims and the payment of all fees are fulfilled and there is agreement as to the text to be granted (Rule 71a(1) EPC).

Note on payment of the renewal fee:

If a renewal fee becomes due before the next possible date for publication of the mention of the grant of the European patent, publication will be effected only after the renewal fee and any additional fee have been paid (Rule 71a(4) EPC).

Under Article 86(2) EPC, the obligation to pay renewal fees to the European Patent Office terminates with the payment of the renewal fee due in respect of the year in which the mention of the grant of the European patent is published.

Note on payment of the designation fee(s):

If the designation fee(s) become(s) due after the communication under Rule 71(3) EPC, the mention of the grant of the European patent will not be published until these fees have been paid (Rule 71a(3) EPC).

- 4.1.2 After publication, the **European patent specification** can be downloaded free of charge from the EPO publication server <https://data.epo.org/publication-server>.

4.1.3 Filing of translations in the contracting states

As regards translation requirements prescribed by the contracting states under Article 65(1) EPC, please consult the website of the European Patent Office

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In the case of a valid extension or validation

As regards translation requirements prescribed by the extension or validation states, please consult the website of the European Patent Office

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Failure to supply a prescribed translation in a contracting state, or in an extension or validation state may result in the patent being deemed to be void *ab initio* in the state concerned (Art. 65(3) EPC).

4.2 In the case of 2.2 above

If the present communication under Rule 71(3) EPC is based on an auxiliary request and, within the time limit, you maintain the main request or a higher ranking request which is not allowable, the application will be refused (Art. 97(2) EPC).

If the examining division gives its consent to the requested amendments or corrections, it will issue a new communication under Rule 71(3) EPC; otherwise, it shall resume the examination proceedings (Rule 71(6) EPC).

5. Filing of a divisional application

Any divisional application relating to this European patent application must be filed directly with the European Patent Office in Munich, The Hague or Berlin and will be in the language of the proceedings for the present application, or if the latter was not in an official language of the EPO, the divisional application may be filed in the language of the present application as filed (see Article 76(1) and Rule 36(2) EPC). Any such divisional application must be filed while the present application is still pending (Rule 36(1) EPC; Guidelines A-IV, 1.1.1).

6. Notes concerning fee payments

6.1 Making payments

For payments made via deposit account, please note that as from 1 December 2017 debit orders will only be carried out if filed in an electronically processable format (xml), using an accepted means of filing as laid down in the Arrangements for deposit accounts (ADA), published in the Supplementary publication in the Official Journal.

All relevant information related to the modes of payment of fees to the EPO can be retrieved from the EPO website at "**Making Payments**".

6.2 Information concerning fee amounts

Procedural fees are usually adjusted every two years, on even years, with effect from 1 April. Therefore, before making a payment, parties should verify the amounts actually due on the date of payment using the applicable version of the Schedule of fees and expenses, published as a Supplement to the Official Journal of the EPO, available on the EPO website (www.epo.org) at www.epo.org/schedule-of-fees. The "Schedule of fees" table allows the viewing, downloading and searching of individual fee amounts, both current and previous.

6.3 Note to users of the automatic debiting procedure

The fee for grant, including the fee for publication, and any additional claims fees due under Rule 71(4) EPC will be debited automatically on the date of filing of the translations of the claims, or on the last day of the period of this communication. However, if the designation fee(s) become(s) due as set out in Rule 71a(3) EPC and/or a renewal fee becomes due as set out in Rule 71a(4) EPC, these should be paid separately by another permitted way of payment in order not to delay the publication of the mention of the grant. The same applies in these circumstances to the payment of extension and validation fees. The same applies in these circumstances to the payment of extension and validation fees.

Note: If a waiver is expressed in response to a Rule 71(3) communication (see OJ EPO 2015, A52), the fee for grant, including the fee for publication/printing, and any additional claims fees will not be debited automatically. These fees must be paid separately by another means of payment allowed under the Rules relating to Fees.

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Enclosures: Text intended for grant
EPO Form 2056

Annex to EPO Form 2004, Communication pursuant to Rule 71(3) EPC

Bibliographical data of European patent application No. 13 726 295.2

For the intended grant of the European patent, the bibliographical data are set out below, for information:

Title of invention:

- NAGETIER-PLASMODIUMPARASITEN ALS PLATTFORMEN EINES MALARIA-IMPfstOFFES FÜR DEN GESAMTEN ORGANISMUS
- RODENT PLASMODIUM PARASITES AS PLATFORMS FOR A WHOLE-ORGANISM MALARIA VACCINE
- PARASITES DE PLASMODIUM DE RONGEUR UTILISÉS COMME PLATES-FORMES POUR UN VACCIN CONTRE LA MALARIA À BASE D'ORGANISMES ENTIERS

Classification: INV. A61P33/06

Date of filing: 17.04.2013

Priority claimed: PT / 17.04.2012 / PTA10626212

Contracting States*
for which fees have been paid:

AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

Extension States*
for which fees have been paid:

Validation States*
for which fees have been paid:

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- *) If the time limit for the payment of designation fees according to Rule 39(1) EPC has not yet expired and the applicant has not withdrawn any designation, **all Contracting States/Extension States/Validation States** are currently still deemed to be designated. See also Rule 71a(3) EPC and, if applicable, the above Note to users of the automatic debiting procedure.
- **) If two or more applicants have designated different Contracting States, this is indicated here.

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DESCRIPTION**"RODENT *PLASMODIUM* PARASITES AS PLATFORMS FOR A WHOLE-ORGANISM MALARIA VACCINE"****Field of the invention:**

This invention provides a method for inoculating a vertebrate host against malaria by administering to the host a live rodent *Plasmodium* organism and exploiting its cross-species protection potential. The invention further provides a vaccine composition comprising a rodent *Plasmodium* organism that is genetically engineered to express circumsporozoite protein (CS) of *P.falciparum*. The invention also provides for production of a vaccine composition, by suspending wild-type or genetically modified rodent *Plasmodium* organisms in a suitable pharmaceutically acceptable carrier solution.

Background of the invention:

The long-standing goal of an effective vaccine against malaria constitutes a crucial component of efforts to eradicate a disease that, according to recent estimates, kills over 1 million persons per year. Malaria vaccines can target sexual and mosquito stage parasite antigens, pre-erythrocytic vaccines that reduce asexual and sexual stage parasite burdens, asexual erythrocytic stage vaccines that reduce blood-stage parasite densities, and vaccines that disrupt parasite development in the vector. So far, vaccines against the early pre-erythrocytic stages have shown most success among current vaccine candidates [1], including the circumsporozoite (CS) protein-based leading vaccine candidate, RTS,S. However, recent results of a

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phase 3 trial of this subunit vaccine have revealed only modest efficacy of protection against severe malaria [2].

An alternative to subunit vaccine candidates is the use of a whole-organism approach. Such a strategy is based on the generation of immunity by attenuated sporozoites, the *Plasmodium* form that is injected by an infected mosquito into its vertebrate host. During a natural malaria infection, an asymptomatic parasite maturation and extensive replication phase inside hepatocytes leads to the generation of *Plasmodium* exoerythrocytic forms (EEFs) and precedes the release of erythrocyte-infectious merozoites that cause disease (reviewed in [3]). A few decades ago, it was shown that sterile protection of humans could be achieved through the injection *P. falciparum* radiation-attenuated sporozoites (RAS) [4]. More recently, it was shown that sporozoites deficient in certain genes, and which become impaired in complete *Plasmodium* development inside the liver hepatocyte (GAS), can confer long-lasting protection against malaria in rodents [5]. This has created renewed hopes for a whole-organism vaccine against malaria based on genetically attenuated *Plasmodium* sporozoites (GAS). Both RAS and GAS are able to invade hepatocytes but fail to complete their developmental process in the liver. Importantly, late liver stage-arresting parasites seem to trigger antimalarial immunity superior to early-arresting variants [6], although they might increase the risk of breakthrough infections.

The protective efficacy of RAS and GAS involves conserved mechanisms and seems to be mainly mediated through the activity of induced CD8⁺ T cells, although antibodies also contribute to protection. *Plasmodium* CS is the

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immunodominant protective antigen in both RAS and GAS [7] and previous studies have shown that protection could be achieved by immunization with CS alone. However, it is also clear that CS is not the sole immunogen at play in the immunity triggered by a whole-organism approach [8, 9].

EPSTEIN J E ET AL: "Live attenuated malaria vaccine designed to protect through hepatic CD8+Tcell Immunity", SCIENCE (NEW YORK, N.Y.) 28 OCT 2011 (2011-10-28), pages 475-480, ISSN: 1095-9203, reports the result of a clinical trial with a vaccine based on irradiation-attenuated *P.falciparum* sporozoite (PfSPZ). The vaccine was administered to volunteers by needle inoculation in the skin and was suboptimally immunogenic and protective. Animal studies suggest that a protective response might be obtained by changing the route of administration to intravenous.

VANBUSKIRK KELLEY M ET AL: "preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 106, no.31, August 2009 (2009-08), pages 13004-13009, ISSN: 0027-8424, describes a genetically attenuated *P.falciparum* which might be useful in a vaccine against malaria. The genetically attenuated parasites carry deletions in one or two sporozoite-expressed genes (P52 and P36) that cause development arrest during hepatocyte infection. The double gene deletion resulted in a stronger phenotype as assessed in a chimeric mouse model harboring human hepatocytes. The authors of this paper suggest that a *P.falciparum* parasite with the double P52/P36 deletion is a good candidate for a whole-organism vaccine against malaria.

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One major potential drawback of current pre-erythrocytic whole-organism malaria vaccination strategies is that they rely on the attenuation of *P. falciparum*, the deadliest human-infective parasite species. It has been shown that the radiation dose required to generate effective RAS must be finely tuned to meet minimal requirements. Indeed, sporozoites exposed to high radiation levels will not induce protection, while parasites exposed to low levels will induce breakthrough infection. Similarly to latter, breakthrough infections with different GAS have been reported [10]. Since a single sporozoite undergoing complete development in the liver can give rise to blood infection and malaria symptoms, a vaccination based on the attenuation of *P. falciparum* sporozoites poses safety concerns that cannot be ignored.

In this context, we hereby propose an alternative strategy for the development of a pre-erythrocytic, whole-organism vaccine against malaria, based on the use of rodent *Plasmodium* parasites as a non-pathogenic vector of human immunization. Here, we demonstrate that *P. berghei* is capable of infecting human hepatocytes, as required for optimal antigen presentation, while being unable to cause a blood-stage infection, thereby ensuring the enhanced safety of the proposed approach and we have demonstrated the potential for cross-species protection between rodent and human *Plasmodium* species.

We further propose that such cross-species protection can be enhanced by the genetic engineering of rodent *Plasmodium* organisms, to express antigens of their human-infective counterparts. We used a transgenic *P. berghei* parasite where the endogenous CS has been replaced by that of *P.*

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falciparum (PbCS_{Pf}) [11] to demonstrate that genetically engineered rodent *Plasmodium* organisms are able to elicit a specific immune response capable of binding and inhibit infection by *P. falciparum*. These results compound a new paradigm in malaria vaccination strategies with optimal immunogenic properties.

Summary of the invention

2 ■ The scope of the invention is defined by the appended claims. The content of this disclosure that extends beyond the scope of the claims is provided for information purposes only. ■ 2

The present invention proposes an alternative strategy for the development of a pre-erythrocytic, whole organism vaccine against malaria, based on the use of rodent parasites as primary immunizing vectors. Rodent *Plasmodium* organisms can be used to induce an immune response in human hosts capable of protecting against infection with human-infective *Plasmodium* spp., by exploiting the cross-species protection potential created by homologous molecules of the rodent parasite. Furthermore, the immunizing potential of these rodent *Plasmodium* organisms can be enhanced by introducing selected immunogenic antigens through genetic engineering. In this strategy, rodent *Plasmodium* organisms can carry antigens of human-infective *Plasmodium* spp. to elicit a specific protective immune response against human *Plasmodium* parasites, in addition to their natural cross-species protective capability, provided by evolutionarily conserved molecules.

The present invention describes a live rodent *Plasmodium* organism for use against human malaria.

A preferred embodiment of the present invention provides the live rodent *Plasmodium* organism genetically engineered to express circumsporozoite protein (CS) of *P.falciparum* for use as immunospecific single- or multi-stage

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immunoeffectors against human malaria caused by *P.falciparum* or *P.vivax*.

In another embodiment of the present invention, the previous live rodent *Plasmodium* organism is any member of the protozoan genus *Plasmodium* whose natural host is a rodent, including *P. berghei*, *P. yoeli*, *P. vinckei* and *P. chabaudi*.

A preferred embodiment of the present invention provides the use of the live rodent *Plasmodium* organism, for the manufacture of an admixture with a pharmaceutically acceptable carrier.

In another embodiment of the present invention, the live rodent *Plasmodium* organism is used for the manufacture of a vaccine against malaria.

Description of the drawings

The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of invention.

Figure 1. Rodent *Plasmodium* parasites infection and development within human hepatic cells.

A) *In vitro*. Representative pictures of Pb(WT) parasites developing 48 hours post infection in human hepatoma cell lines (Huh-7 and HepG2) and human immortalized hepatocytes (HC-04); **B)** *Ex vivo* development. Representative pictures of Pb(WT) parasites developing 48 hours post infection (upper panels) and 72 hours post infection (lower panels) in micro patterned co-cultures of human primary hepatocytes (scale indicates 10µm). **C)** Development of exo-erythrocytic forms

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of Pb(WT) parasites in Huh-7 and HepG2 hepatoma cell lines and HC-04 immortalized human hepatocytes 48 hours post infection as quantified by area measurement (line indicates mean of area and bars represent the 90 and 10 percentile); **D)** Number of exo-erythrocytic forms developing in Huh-7 and HepG2 hepatoma cells and HC-04 hepatocytes 48 hours post infection with 10000 sporozoites, presented as a % of the number of exo-erythrocytic forms after normalization for cell confluence (bars indicate standard deviation); **E)** Relative proportion of exo-erythrocytic forms from Pb(WT) parasites developing inside mouse (black bar) and human engrafted hepatocytes (grey bar) in the livers of FRG humanized mice 48 hours post infection; **F)** Development of exo-erythrocytic forms of Pb(WT) parasites inside mouse hepatocytes (black bars) or human hepatocytes (grey bars) in the FRG humanized mice at 48 hours post infection, as quantified by area measurement (line indicates mean of area and bars represent the 90 and 10 percentile); **G)** Pictures and projections of parasites developing 48 hours post-infection inside human hepatocytes of liver-humanized mice. Note that the *P. berghei* parasite (red) is clearly within a human, fumarylacetoacetate hydrolase-stained, hepatocyte (white). Scale bars correspond to 10 μ m.

Figure 2a and 2b. Rodent *Plasmodium* parasites infection of human erythrocytes *in vivo*.

A1, A2 and A3) Representative flow cytometry plots of measurements of parasitemia in the peripheral blood of chimeric and non-chimeric mice engrafted with human erythrocytes. Simultaneous staining with SYTO-16 for nucleic acids (x-axis) and TER-119 mAB for murine erythroid lineage (y axis) allows the distinction between human and mouse erythrocytes, as well as between infected and non-

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infected erythrocytes. Using this strategy, it is possible to identify human erythrocytes infected with *P. berghei* parasites as they are expected to fall in the same gate as a positive control of infected human erythrocytes with *P. falciparum*. (mE: murine erythrocytes; hE: human erythrocytes; imE: infected murine erythrocytes; ihE: infected human erythrocytes; bimE: background for infected murine erythrocytes; bihE: background for infected human erythrocytes, **A1**) Chimeric mice engrafted with human erythrocytes infected with *P. falciparum*; **A2**) non-chimeric mice infected with *P. berghei*; **A3**) Chimeric mice engrafted with human erythrocytes infected with *P. berghei*; **B1, B2 and B3**) Average percentage of infected erythrocytes from either human or murine origin, in chimeric and non-chimeric mice infected with Pb(WT) or *P. falciparum*, across time; **B1**) Average percentage of infected human erythrocytes (full line) as well as signal background obtained on the expected region for infected mouse erythrocytes (small dotted line) in chimeric mice engrafted with human erythrocytes infected with *P. falciparum*. **B2**) Average percentage of infected mouse erythrocytes (long dotted line) as well as signal background obtained on the expected region for infected human erythrocytes (small dotted line) in non-chimeric mice infected with *P. berghei*; **B3**) Average percentage of infected human erythrocytes (full line) as well as average percentage of infected mouse erythrocytes (long dotted line) in chimeric mice engrafted with human erythrocytes infected with *P. berghei*; **C1 and C2**) Erythrocyte populations obtained after magnetic separation of imE and ihE from chimeric mice engrafted with human erythrocytes infected with *P. berghei*. **C1**) Representative flow cytometry plot of magnetically separated *P. berghei* infected human erythrocytes present in the peripheral blood of chimeric

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mice engrafted with human erythrocytes. **C2)** Representative flow cytometry plot of magnetically separated *P. berghei* infected mouse erythrocytes present in the peripheral blood of chimeric mice engrafted with human erythrocytes; **D1 and D2)** Cultivation of erythrocyte populations obtained after magnetic separation of imE and ihE from chimeric mice engrafted with human erythrocytes infected with *P. berghei*. **D1)** Representative pictures of *P. berghei* parasites over-time in culture within human erythrocytes; **D2)** Representative pictures of *P. berghei* parasites over-time in culture within mouse erythrocytes; Note that *P. berghei* parasites in ihE obtained immediately after magnetic separation only show picnotic forms that eventually degenerate after 20 h in culture, while *P. berghei*-imE are already mature at the moment of separation and proceed towards complete trophozoite segmentation over time in culture. **E)** Representative pictures obtained by light and immunofluorescence microscopy observation of blood smears from infected chimeric mice showing multiple *P. berghei*-infected murine erythrocytes and abnormal, picnotic *P. berghei*-infected human erythrocytes in the same mice.

Figure 3. Rodent *Plasmodium* parasites interspecies cross-protection.

A1 and A2) CS-specific antibody titers in sera from human malaria patients (n=21); Black represent serum samples positive for *P. falciparum* CS; **A1)** *P. falciparum* CS-specific IgG titers; **A2)** *P. berghei* CS-specific IgG titers Black represent serum samples positive for *P. falciparum* CS; **B1 and B2)** Recognition and binding of sera from human malaria patients to *P. falciparum* and *P. berghei* sporozoites; **B1)** Proportion of human serum samples recognizing and binding to *P. falciparum* (right) and *P.*

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berghei sporozoites (left); Proportion binding to sporozoites (black) proportion not binding to sporozoites (grey); **B2)** Representative images of *P. falciparum* sporozoites binding assays (top) and of *P. berghei* sporozoites binding assays (bottom).

Figure 4. Genetically modified rodent *Plasmodium* parasites expressing human *Plasmodium* parasite antigens.

A) *Ex vivo*. Immunofluorescence microscopy of liver rodent *Plasmodium* parasite forms 48 hours after sporozoites infection of rodent primary hepatocytes; **B)** *In vivo*. Immunofluorescence microscopy 45 hours after *in vivo* sporozoites infection of C57BL/6 rodent malaria models. Note the presence of *P. falciparum* CS protein (green) in *Pb*(PfCS) parasites.

Figure 5. Immunogenicity of genetically modified rodent *Plasmodium* parasites.

A and B) Serum samples from mice infected with sporozoites from the different parasite lines were analyzed by ELISA to assay IgG and IgM responses induced against the circumsporozoite protein (CS) in mice challenged with either mock infected salivary-gland injected mice (SG) or mice infected with wild-type *P. berghei* (WT) or the genetically modified *Pb*CS_{Pf}. (PfCS); Titers are expressed in arbitrary units of fluorescence (AU) as the highest dilution of sera tested that gave a positive staining; **A)** IgG and IgM responses induced against *P. falciparum*-CS; **B)** IgG and IgM responses induced against *P. berghei*-CS; **C)** ELISPOT analysis of CS-specific T-cell responses in mice challenged with either mock infected salivary-gland injected mice (SG) or mice infected with wild-type *P. berghei* (WT) or the genetically modified *Pb*CS_{Pf}. (PfCS);

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the number of IFN- γ secreting cells in 10^6 splenocytes is presented; **D)** Binding affinity of immune sera from mice challenged with either mock infected salivary-gland injected mice (SG) or mice infected with wild-type *P. berghei* (WT) or the genetically modified PbCS_{Pf}. (PfCS) to wild-type *P. berghei* sporozoites, or genetically modified PbCS_{Pf} or *P. falciparum* sporozoites; **E)** Gliding motility inhibition assays of *P. falciparum* sporozoites in the presence of sera from mice challenged with either mock infected salivary-gland injected mice (SG) or mice infected with wild-type *P. berghei* (WT) or the genetically modified PbCS_{Pf}. (PfCS); **F)** Hepatic infectivity inhibition assays of *P. falciparum* in the presence of sera from mice challenged with either mock infected salivary-gland injected mice (SG) or mice infected with wild-type *P. berghei* (WT) or the genetically modified PbCS_{Pf} (PfCS).

Detailed description of the invention

The present invention relates to an alternative approach to malaria vaccination that combines unprecedented versatility with high efficacy, whilst ensuring complete safety. We have demonstrated that (i) *rodent malaria* sporozoites (spz) can be used as a whole-organism pre-erythrocytic vaccine capable of generating a strong immune response because they are able to successfully infect and develop in human liver cells; (ii) *rodent malaria parasites* are an extremely safe antigen delivery platform because they are completely unable to complete their life cycle inside human erythrocytes, which renders them unable to cause a disease-triggering human blood-stage infection; (iii) *rodent malaria parasites* can induce a high level of cross-species protection against human *Plasmodium* species because they possess conserved molecules that can be recognized by the

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human immune system iv) Genetically engineered rodent *Plasmodium* parasites are highly immunogenic, being able to trigger specific immune responses against an engineered human *Plasmodium* antigen capable of recognizing human *Plasmodium* parasites and inhibiting human *Plasmodium* infection. By rodent *Plasmodium* organism or rodent *Plasmodium* parasite is meant any member of the protozoan genus *Plasmodium* whose natural host is a rodent, including the four known species, *P. berghei*, *P. yoelii*, *P. vinckei* and *P. chabaudi*. By human *Plasmodium* organism or human *Plasmodium* parasite is meant any member of the protozoan genus *Plasmodium* known to cause human malaria, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.

(i) Rodent *Plasmodium* parasites infect human hepatocytes *in vivo*

We monitored in parallel the *in vitro* infection of one mouse and two human hepatoma cell lines (Hepa 1-6, HepG2 and Huh7), and one human immortalized hepatocyte line (HC04), and the *ex vivo* infection of mouse primary hepatocytes and human primary hepatocyte/fibroblast co-cultures by wild-type *P. berghei*, a rodent *Plasmodium* organism. Infection assessments by quantitative real-time PCR (qRT-PCR) and fluorescence microscopy showed that *P. berghei* traverses, invades and develops to similar extents in all *in vitro* systems studied and that it is able to invade and develop inside rodent and human primary hepatic cells *ex vivo* (Fig. 1A,B,C,D).

In order to ascertain whether *P. berghei* is able to infect human liver cells in an *in vivo* context, we injected sporozoites into liver-humanized mice (^{LH}mice) and

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monitored hepatic infection by confocal fluorescence microscopy. Our results show that *P. berghei* can effectively infect human hepatocytes engrafted in the livers of the chimeric ^{LH}mice. A comparison between infection of the human and mouse hepatocytes in the chimeric livers, reveals that the parasite is capable of similar development inside either type of cell (Fig. 1E-G).

Additionally, we employed a genetically engineered rodent *P. berghei* parasite where the endogenous CS has been replaced by that of *P. falciparum* (PbCS_{Pf}) [11]. We evaluated the hepatic infection behavior of this genetically modified parasite and showed that it retains the ability to infect human hepatic cells *in vitro*, as well as human primary hepatocytes as well as the livers of ^{LH}mice.

Overall, these results show that the rodent *P. berghei* parasite is fully competent to infect human hepatocytes, and that this competence is retained in the genetically engineered PbCS_{Pf} parasite, thereby fulfilling the immunity-generation condition for a malaria vaccine.

(ii) Rodent *Plasmodium* parasites are unable to cause a human blood-stage infection that leads to pathology

In order to be safe, a rodent *Plasmodium* organism-based malaria vaccine must ensure that the immunizing parasite is unable to cause disease in humans. This requires that rodent *Plasmodium* merozoites are unable to effectively invade and multiply inside human red blood cells (RBCs). In order to ascertain this, we employed a strategy based on the use of blood-humanized mice (^{BH}mice), engrafted with defined proportions of human erythrocytes, which have been

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developed as models to evaluate drug efficacy against *P. falciparum* infection [12]. This system can be coupled with the use of the nuclear SYTO-16 and the mouse-specific TER-119 dyes to distinguish infected from non-infected cells and human from rodent erythrocytes, respectively, and thereby monitor infection of either type of cell by flow cytometry (Fig. 2A1, 2A2, 2A3).

We started by infecting ^{BH}mice bearing different degrees of chimerism by transfusion of *P. berghei*-infected mouse blood and monitored the parasitemia in these mice at regular intervals, using non-chimeric mice as controls. Our results show that while the SYTO-16⁺/TER-116⁺ population increased to values ~5%, showing, as expected, infection of the mouse RBC (^mRBC) population, the SYTO-16⁺/TER-116⁻ population that would correspond to infected human RBCs (^hRBCs) never surpassed 0.2%, remaining within the range of the background levels observed for the non-humanized mice (Fig. 2A1, 2A2, 2A3, 2B1, 2B2, 2B3).

This result suggested that *P. berghei* is unable to infect ^hRBCs or that it may do so at very low levels. It is worth noting that this occurs in the context of a chimeric mouse, which contains a significant population of ^mRBCs that serve as an effective reservoir for the production of large numbers of merozoites. To further investigate the possibility of human RBC infection under these conditions, we analysed blood samples from these mice after staining with the nuclear dye DAPI and with TER-116. We found very rare instances of DAPI⁺/TER-116⁻ cells, which indicated that a small degree of invasion of ^hRBCs could indeed occur. However, we were unable to find any ^hRBC bearing more than a single parasite nucleus, suggesting that *P.*

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berghei is unable to replicate inside the few ^hRBCs that it may invade (Fig. 2E).

Crucially, *in vitro* cultivation of flow cytometry-isolated infected ^hRBCs showed that these parasites are indeed incapable of completing their intra-erythrocytic life cycle in ^hRBCs (Fig. 2C1, 2C2, 2D1, 2D2), rendering them safe for use in humans. Similar results were obtained when infection was carried out with the PbCS_{Pf} parasite.

Since in these experiments infection was initiated with second-generation merozoites, obtained by transfusion of infected RBCs, we decided to investigate the behavior of the first-generation merozoites that are produced in the liver. To do this, we infected ^{BH}mice with sporozoites collected from the salivary glands of *P. berghei*- or PbCS_{Pf}-infected mosquitoes and, using non-chimeric mice as controls, we carried out the same type of analysis as described above. Our results show that merozoites produced in the liver behave similarly to second-generation merozoites, showing that rodent *P. berghei* parasites are safe for use in humans and do not present the risks associated with inefficient attenuation of *P. falciparum* sporozoites.

(iii) Rodent *Plasmodium* parasites have a high cross-species protection potential against human *Plasmodium* parasites

We evaluated serum samples from African malaria-infected individuals from Cameroon and Tanzania for the presence of antibodies against the *P. berghei* and *P. falciparum* CS proteins and for their ability to recognize spz from both these species. Our results showed that while none of these samples contained antibodies against *P. berghei* CS (Fig.

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3A1, 3A2), 71% of them were able to recognize both *P. falciparum* and *P. berghei* spz (Fig. 3B1, 3B2). These data show that naturally acquired immunity against malaria includes an antibody response against conserved human *Plasmodium* parasites and rodent *plasmodium* parasites epitopes on spz, besides the CS protein. Overall, these results demonstrate that the use of rodent *Plasmodium* parasites as a vaccination platform has the potential to raise an immune response against currently unknown conserved antigens.

(iv) Immunization with genetically modified rodent *Plasmodium* parasites elicits specific highly effective protection against human *Plasmodium* infection

An additional advantage of our proposed vaccination method relies on the notion that we can enhance the intrinsic cross-species protection provided by rodent *Plasmodium* parasites by introducing antigens of human *Plasmodium* parasites through genetic modification, which will elicit highly effective specific immune responses.

To establish the proof-of-concept of our proposed strategy, we employed the rodent PbCS_{Pf} parasite. We used immunofluorescence microscopy to confirm that PbCS_{Pf} expresses *P. falciparum* CS in liver cells, either *ex vivo* (Fig. 4A) or *in vivo*. (Fig. 4B). We then evaluated the immunogenicity of these transgenic parasites in rodent models of infection and determined the specificity of this response for the engineered *P. falciparum* CS antigen. C57BL/6 mice were infected with PbCS_{Pf} sporozoites and subsequently treated with chloroquine to prevent the development of blood parasitemia and disease. Five days after the initiation of chloroquine treatment, the mice

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were sacrificed and immune serum was obtained from collected blood. Pre-immune serum from uninfected mice and serum from mice immunized with wild-type *P. berghei* were obtained and used as controls in these experiments. Antibodies against *P. falciparum* CS in the serum were quantified by ELISA (Fig. 5A, 5B). Our results show that mice immunized with the PbCS_{Pf} parasite produced significant amounts of this antibody, showing that immunization of rodents with PbCS_{Pf} elicited the generation of antibodies directed against *P. falciparum* CS, which are known to mediate protection against the human-infective parasite [13].

Moreover, we demonstrated a clear cellular immune response against *P. falciparum* CS epitopes (Fig. 5C). Finally, we showed that the serum of mice immunized with the genetically modified rodent PbCS_{Pf} can recognize and bind with high avidity to human *Plasmodium* sporozoites (Fig. 5D). Moreover, we showed that this immune serum is able to functionally inhibit the gliding motility (Fig. 5E) and hepatic cell invasion (Fig. 5F) of human *Plasmodium* parasites.

Overall, our data show that the genetic modification of rodent *Plasmodium* parasites can substantially increase the immunizing potential of these parasites against human *Plasmodium* parasites.

Conclusions

Whole-organism approaches such as those provided by radiation attenuated (RAS) and genetically attenuated (GAS) sporozoites appear as very attractive alternatives to subunit-based pre-erythrocytic vaccination strategies,

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despite considerable technological challenges in terms of manufacturing, formulation, and delivery of such attenuated sporozoite vaccines. However, both these approaches pose undeniable safety concerns that arise from the fact that they are based on the attenuation of *P. falciparum*, the most deadly human malaria parasite.

We propose an alternative strategy for the development of a pre-erythrocytic, whole-organism vaccine against malaria, based on the cross-species protection potential between rodent and human *Plasmodium* parasites. Such a vaccine may elicit cross-species protection by rodent antigens that may be immunogenic against their human *Plasmodium* spp. counterparts. Examples of cross-species protection induced by components other than CS following immunization with irradiated malaria sporozoites are available. This might be the case, for instance, of CelTOS, for cell-traversal protein for ookinetes and sporozoites, which has been recently identified as a target antigen for a pre-erythrocytic vaccine, based on its ability to induce protective immunity through humoral and cellular immune responses. CelTOS is highly conserved among the *Plasmodium* species and immunization with pre-erythrocytic antigen CelTOS from *P. falciparum* has been shown to elicit cross-species protection against a heterologous challenge with *P. berghei*, suggesting that the reverse effect might also take place.

Besides their cross-protective potential, the immunogenicity of rodent *Plasmodium* parasites can be enhanced by genetic engineering, effectively turning them into platforms for the delivery of immunogenic antigens of human-infective *Plasmodium* species, capable of eliciting

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highly efficient specific immune responses. Of course more than one antigen, already known or hitherto unknown, can be introduced, for either single or multiple stages of the *Plasmodium* life cycle, and for a single or multiple species of human *Plasmodium* organisms, because such redundancy may ensure an additional degree of protection against infection of parasitemia.

In this invention, we demonstrate that the rodent parasite *P. berghei* can infect human hepatocytes whilst being unable to cause a human blood stage infection. These constitute two of the essential premises for a rodent-based parasite to be evaluated as a vaccination strategy. The third one is that a transgenic rodent *Plasmodium* parasite may elicit a response against a challenge with a human-infective *Plasmodium* species. To demonstrate this assumption, we employed a *P. falciparum* CS-expressing mutant of *P. berghei* (PbCS_{Pf}) and showed that it retains the main features of its wild-type counterpart whilst eliciting a specific protective response against a *P. falciparum* challenge. This establishes the proof-of-principle of our proposed strategy and opens further avenues for the design and production of other vaccine candidates based on the same principle.

PbCSPf itself has the drawback that it produces relatively low numbers of salivary gland sporozoites and lower hepatic infectivity than wild-type *P. berghei*. This is most likely due to the absence of the endogenous CS, coupled with an inappropriate conformation of the *P. falciparum* protein in this parasite. Thus, several alternatives may be envisaged in order to improve the infection yields of PbCSPf, such as the heterologous expression of *P. falciparum* CS in a neutral locus, under the control of the endogenous CS

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promoter, in addition rather than as a replacement of the endogenous protein.

The use of rodent parasites as "piggy-backs" for human malaria genes can be extended to include antigens other than CS. Among the obvious candidates, to generate a pre-erythrocytic immune response, are the thrombospondin-related adhesion protein (TRAP) and liver stage antigen 1 (LSA-1), both of which are expected to present immunogenic potential.

In addition, sterile protective immunity against malaria is directed against a panel of novel *P. falciparum* antigens rather than one antigen in isolation. Or strategy also allows for the inclusion of blood-stage antigens. Apical membrane antigen I (AMA-I), erythrocyte-binding antigen-175 (EBA-175), and merozoite surface protein 1 (MSP-1) are prime candidates among these, given their established or proposed immunogenicity. When placed under the control of promoters, that enable their expression during the liver stages of infection, these antigens are expected to prime the host's immune system against *P. falciparum* blood-stages, creating an additional layer of protection. Moreover, this approach can be used to introduce a transmission blocking component in the pseudo-attenuated vaccine, by engineering gametocyte-specific genes, such as p48/45, into the rodent *Plasmodium* platform.

This strategy can be extended beyond the development of immunity against *P. falciparum*, as one may envisage the inclusion of *P. vivax* antigens, such as CS, orthologs of the *P. falciparum* genes listed above, Duffy-binding protein

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(DBP), or reticulocyte-binding proteins (RBPs), in the immunogenic platform.

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NEW CLAIMS

1.- A live rodent *Plasmodium* organism for use in a method of vaccination against human malaria caused by *P.falciparum* or *P.vivax*, characterized in that the live rodent *Plasmodium* organism is genetically engineered to express circumsporozoite protein (CS) of *P.falciparum*.

2.- A live rodent *Plasmodium* organism for use in a method of vaccination against human malaria according to the previous claim, wherein the rodent *Plasmodium* organism is any member of the protozoan genus *Plasmodium* whose natural host is a rodent, selected from the group consisting of *P.berghei*, *P. yoeli*, *P.vinckei* and *P.chabaudi*.

3.- Composition for use in a method of vaccination against human malaria comprising a live rodent *Plasmodium* organism according to any of the previous claims 1 - 2 in admixture with a pharmaceutically acceptable carrier.

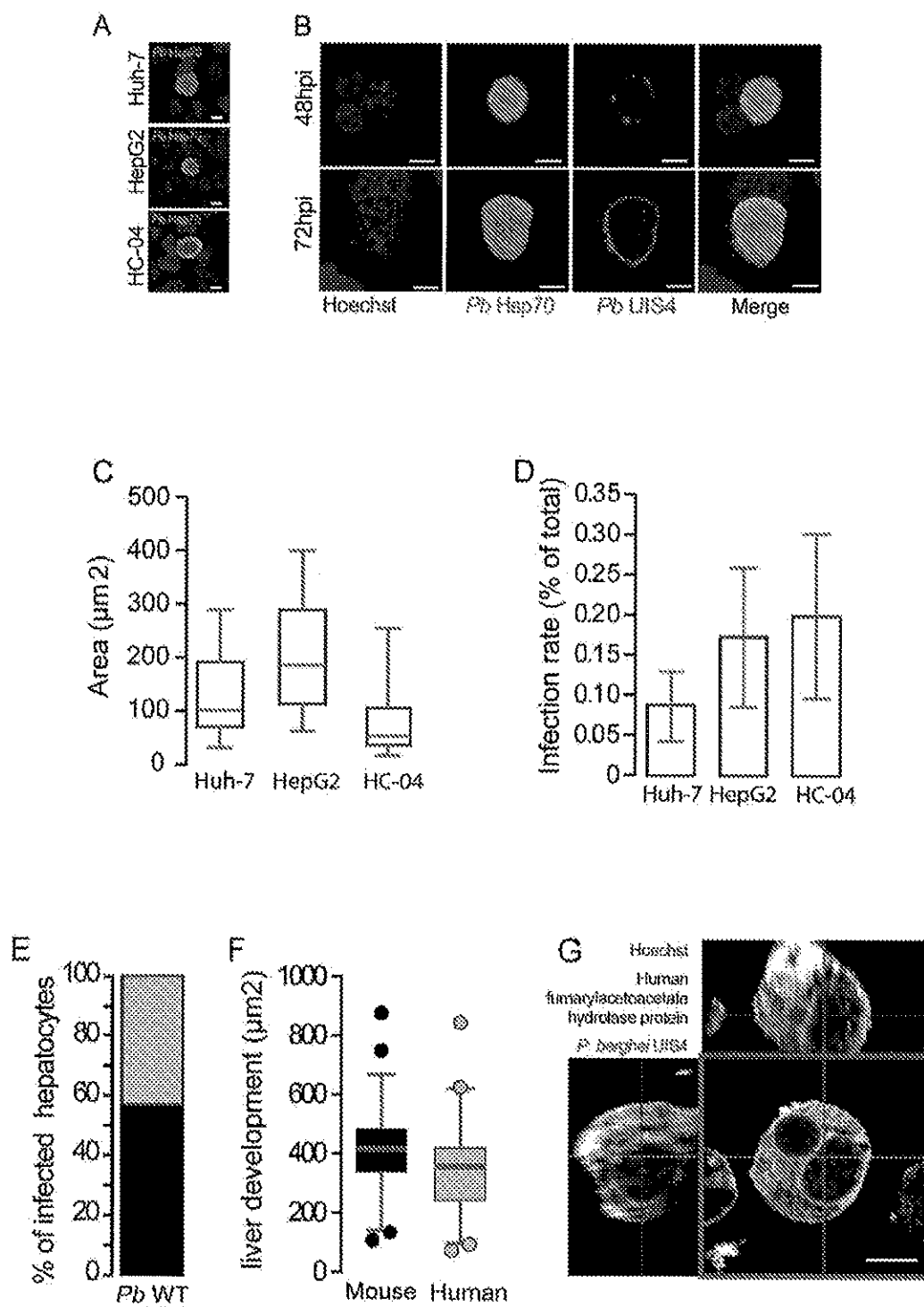


Figure 1

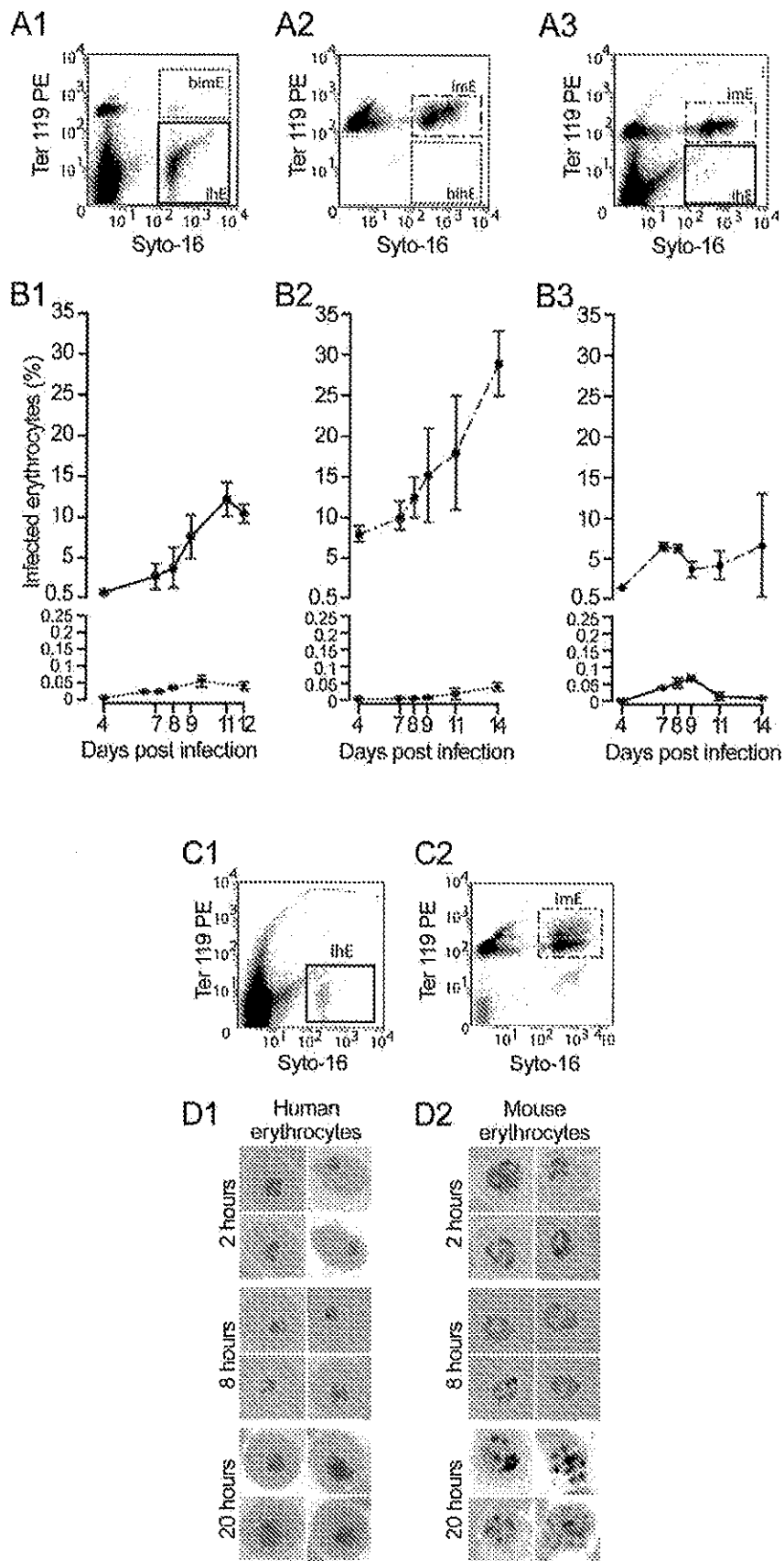


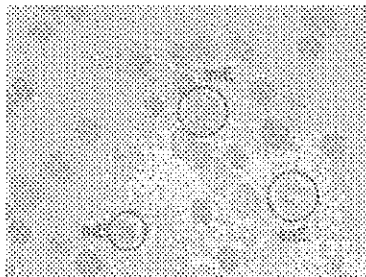
Figure 2a

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Light microscopy

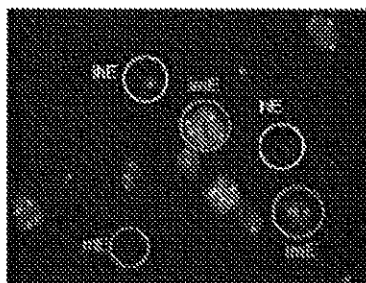
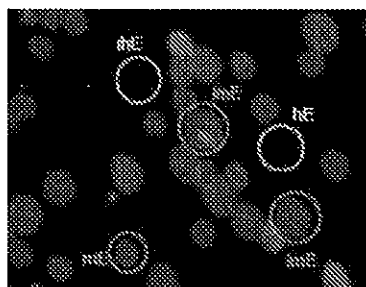
Immunofluorescence microscopy
(DAPI : nuclei)Immunofluorescence microscopy
(TER-119: rodent erythrocytes)

Figure 2b

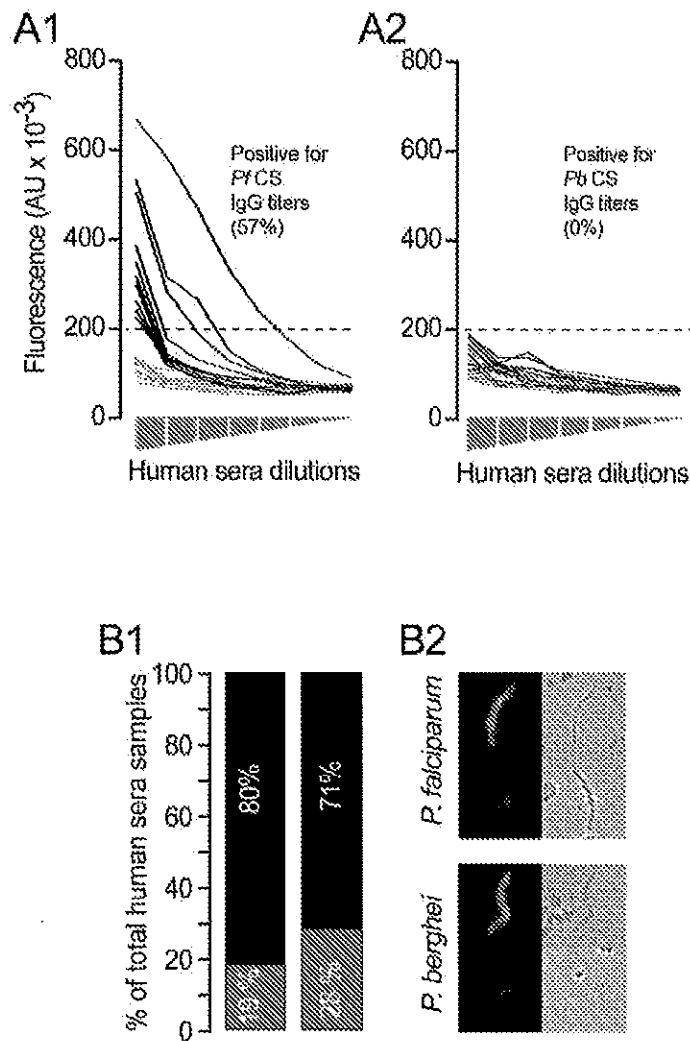


Figure 3

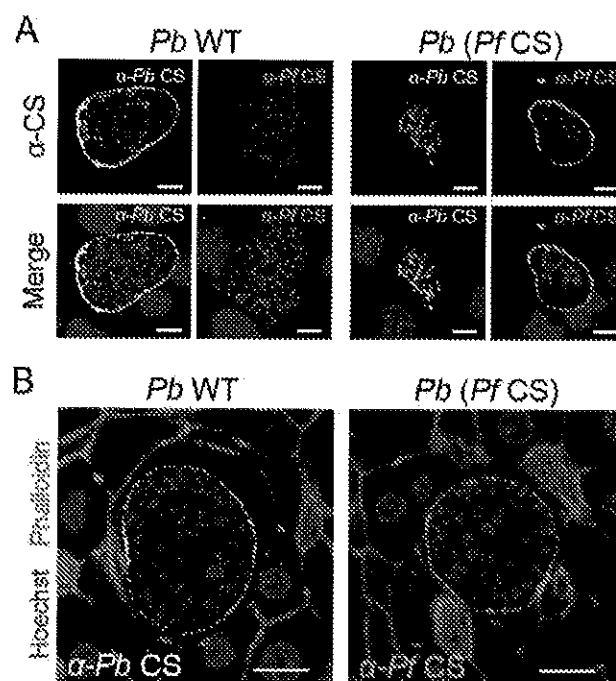


Figure 4

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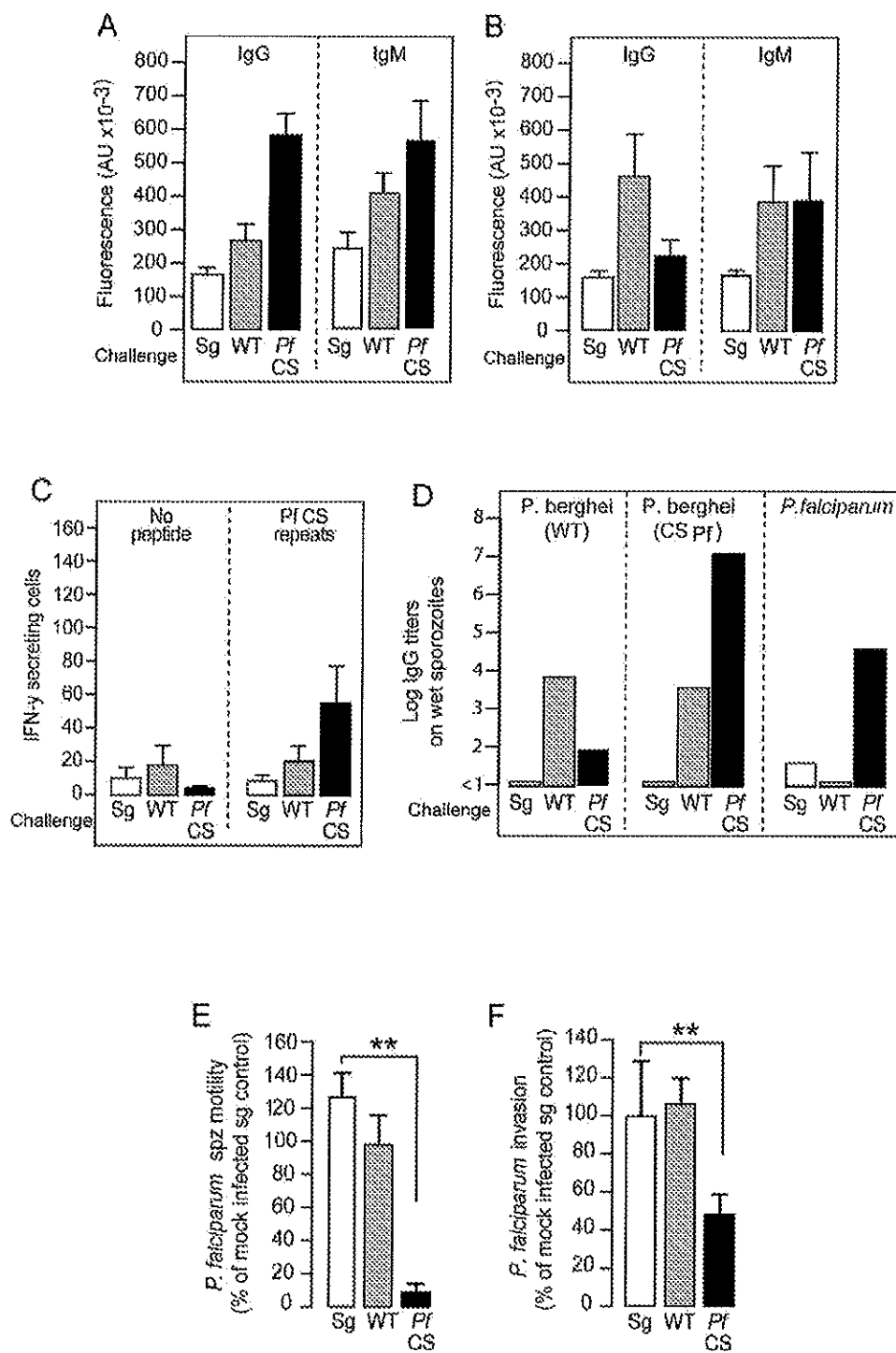


Figure 5