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The species specificity of immunity generated by live whole organism immunisation with erythrocytic and pre-erythrocytic stages of rodent malaria parasites and implications for vaccine development

Megumi Inoue [a,b,e,*], Jianxia Tang [c], Mana Miyakoda [b,d], Osamu Kaneko [a,b], Katsuyuki Yui [b,d], Richard Culleton [e,*]

[a] Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[b] Global COE Program, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[c] Key Laboratory on Technology for Parasitic Disease Prevention and Control, Ministry of Health, Jiangsu Institute of Parasitic Diseases (JIPD), Meiyuan Yangxiang 117, Wuxi, Jiangsu 214064, PR China
[d] Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Japan
[e] Malaria Unit, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

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ABSTRACT

A promising strategy for the development of a malaria vaccine involves the use of attenuated whole parasites, as these present a greater repertoire of antigens to the immune system than subunit vaccines. The complexity of the malaria parasite's life cycle offers multiple stages on which to base an attenuated whole organism vaccine. An important consideration in the design and employment of such vaccines is the diversity of the parasites that are infective to humans. The most valuable vaccine would be one that was effective against multiple species/strains of malaria parasite. Here we compare the species specificity of pre-erythrocytic and erythrocytic whole organism vaccination using live parasites with anti-malarial drug attenuation. The cross-stage protection afforded by each vaccination strategy, and the possibility that immunity against one stage may be abrogated by exposure to other stages of both homologous and heterologous parasites was also assessed. The rodent malaria parasites *Plasmodium yoelii yoelii* and *Plasmodium vinckei lentum* are to address these questions, as they offer the widest possible genetic distance between sub-species of malaria parasites infectious to rodents. It was found that both erythrocytic and pre-erythrocytic stage immunity generated by live, attenuated parasite vaccination have species-specific components, with pre-erythrocytic stage immunity offering a much broader pan-species protection. We show that the protection achieved following sporozoite inoculation with concurrent mefloquine treatment is almost entirely dependent of $CD8^+$ T-cells. Evidence is presented for cross-stage protection between erythrocytic and pre-erythrocytic stage vaccination. Finally, it is shown that, with these species, an erythrocytic stage infection of either a homologous or heterologous species following immunisation with pre-erythrocytic stages does not abrogate this immunity. This is the first direct comparison of the specificity and efficacy of erythrocytic and pre-erythrocytic stage whole organism vaccination strategies utilising the same parasite species pair.

1. Introduction

In 2010, 1.24 million deaths were attributed to malaria worldwide, 60% of which were children under 5 years of age (Murray et al., 2012). Despite reductions in incidence during the past 10 years, the disease remains a significant cause of morbidity and mortality of children in tropical regions, especially in sub-Saharan Africa.

Plasmodium infection is initiated by the bite of an infected mosquito, which can deposit up to 1,000 sporozoites into the skin (Medica and Sinnis, 2005). The majority of these sporozoites traverses the dermis and enter the blood circulation, eventually reaching the liver (Frevert, 2004). However, a small proportion of infected sporozoites remain in the dermis (Gueirard et al., 2010; Voza et al., 2012) or travel to lymph nodes (Chakravarty et al., 2007) where they develop and, presumably, stimulate the immune system (Good and Doolan, 2007). In the liver, sporozoites invade hepatocytes and undergo schizogony, producing thousands of

* Corresponding authors. Address: Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan (M. Inoue). Tel.: +81 95 8197903; fax: +81 95 8197805.
E-mail addresses: m.inoue@tm.nagasaki-u.ac.jp (M. Inoue), richard@nagasaki-u.ac.jp (R. Culleton).

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merozoites. These are then released into the bloodstream where they invade red blood cells (RBCs), initiating the erythrocytic cycle during which the clinical symptoms of malaria occur. This complex life cycle presents numerous possible targets for vaccine development.

The asymptomatic pre-erythrocytic stage of the *Plasmodium* life cycle offers one such attractive vaccine target. Laboratory investigations performed with rodent malaria parasites over 40 years ago first showed that immunisation with irradiation-attenuated sporozoites could invoke sterile immunity against the immunising strain (Nussenzweig et al., 1967). Since then, much research has followed, culminating in the recent successful vaccination of human volunteers with live sporozoites administered with contemporaneous anti-erythrocytic blood stage drugs (Roestenberg et al., 2009), and attempts to produce an effective human vaccine based on irradiation-attenuated sporozoites (Epstein et al., 2011).

Whole organism vaccines, attenuated by irradiation, drug treatment or genetic modification, are being developed against both the blood stages (Ting et al., 2008; Gerald et al., 2011) and pre-erythrocytic stages (Belnoue et al., 2004). The greater efficacy of whole organism compared with subunit vaccine strategies (Pinzon-Charry and Good, 2008; Good, 2011) is possibly due to their ability to induce immune responses against multiple parasite antigens, but the mechanisms underlying this remain to be elucidated. It is known, however, that parasite-specific hepatic $CD8^+$ T-cells play a major role in inducing protective immunity against the pre-erythrocytic stages (Romero et al., 1989; Rodrigues et al., 1991), whereas immunity against blood stage malaria parasite infection is thought to involve both humoral and cellular responses (McCarthy and Good, 2010).

In almost all malaria endemic areas, there exist more than one species of *Plasmodium* parasite capable of causing disease in humans (Guerra et al., 2006; Mueller et al., 2007). The interactions between these species may have practical implications for the development and deployment of an antimalarial vaccine. As the elimination of a single species from an endemic area may result in an increase in the incidence (and possible severity) of other species (Mayxay et al., 2004; Zimmerman et al., 2004), a vaccine that induces protection against multiple species is desirable. Furthermore, as blood stage malaria infections are known to modulate immunity (Mendis et al., 1990), the possibility exists that infection with one species may adversely affect the protection achieved, through vaccination, against another species. Specifically, it has been shown that a blood stage infection can abrogate the immunity achieved against the pre-erythrocytic stages of a homologous species (Orjih, 1985; Ocana-Morgner et al., 2003). Whether such an abrogating affect may occur following infection with a heterologous species is currently unknown.

It is possible that malaria parasites may induce an immunity that is protective against more than one species of *Plasmodium* (Sedegah et al., 2007; Purcell et al., 2008) with laboratory experiments with the rodent malaria parasites, *Plasmodium yoelii* and *Plasmodium berghei,* and field observations of human malaria (Maitland et al., 1997) suggesting that some cross-species immunity occurs.

Recently, it has been shown that immunisation using whole parasites derived from one particular stage in the *Plasmodium* life cycle may offer protection against other stages, in laboratory experiments with rodent malaria parasites (Kawabata et al., 2002; Belnoue et al., 2008). Furthermore, observations from moderately endemic areas have suggested that natural malaria infection confers protective immunity against a subsequent infection of a heterologous malaria parasite species (Gunewardena et al., 1994).

Here, using the rodent malaria parasites, *Plasmodium yoelii yoelii* and *Plasmodium vinckei lentum*, we investigated (i) the degree of cross-species protection following live, whole organism vaccination against either the pre-erythrocytic stages or the blood stages; (ii) the role of $CD8^+$ T cells in the induction of immunity through live, anti-malarial drug-attenuated sporozoite immunisations; (iii) the degree of cross-stage protection that results from immunisation with either the pre-erythrocytic stages or the blood stages; and (iv) the effect of both homologous and heterologous species blood stage infections on the immunity achieved against the pre-erythrocytic stages following live, whole organism vaccination.

Although previous work has examined the species specificity of blood stage immunity or pre-erythrocytic stage immunity individually, there are very few reports that directly compare blood stage immunity and pre-erythrocytic stage immunity using the same parasite species and similar vaccination methodology (in this case, exposure to live parasites followed by drug treatment). Furthermore, we believe that this is the first such report utilising *P. yoelii yoelii* and *P. vinckei lentum*, two of the most distantly related rodent malaria parasite subspecies.

2. Material and methods

2.1. Experimental animals and parasites

Female BALB/c mice, 7–8 weeks old (SLC Inc., Japan) at the time of the first immunisation were used for all experiments. The parasites used for immunisation and infection were *P. yoelii yoelii* 17XNL clone 1.1 (hereafter referred to as *P. yoelii*) originally obtained from Peter Preiser (Nanyang Technological University, Singapore) or *P. vinckei lentum* clone DS (hereafter referred to as *P. vinckei*), originally obtained from Richard Carter (University of Edinburgh, UK). All experiments were carried out in compliance with the British Home Office Animals (Scientific Procedures) Act 1986, and in accordance with the Animal Usage Committee of Nagasaki University, Japan.

2.2. Immunisation and infection

In order to generate pre-erythrocytic stage immunity, mice were inoculated twice (with a minimum of a 2-week interval) via the intradermal (i.d.) or i.v. route with 2,000–10,000 *P. yoelii* sporozoites (doses varied between experiments). Inoculations were carried out in a total volume of 100 μl, with i.d. inoculations administered in two inoculations of 50 μl to separate areas of the dorsal dermis following sterilisation of the area with 70% ethanol (Inoue and Culleton, 2011). Mice were concurrently treated with a 5 day regiment of mefloquine (MF) (20 mg/kg/per day, orally), beginning on the day of inoculation, to prevent the development of the erythrocytic cycle. Sporozoites were generated by allowing *Anopheles stephensi* mosquitoes to feed on mice inoculated i.v. with 1×10^6 *P. yoelii* or *P. vinckei* blood stage parasites 3 days previously. Fifteen to 21 days post-feeding, mosquito salivary glands were dissected in sterile PBS (Sigma–Aldrich Japan K.K.), crushed in a glass homogeniser, and sporozoite numbers determined using a haemocytometer. All mice were monitored by microscopy of Giemsa-stained thin blood smears prepared from tail vein blood to confirm the absence of blood stage parasites following immunisation from 48 h post sporozoite infection for 14 days and on the day following sporozoite immunisation or challenge infection.

Blood stage immunity was generated by i.v. inoculation of 1×10^6 parasitised RBCs (pRBCs) of *P. yoelii* or *P. vinckei* diluted in a total of 100 μl of 1:1 FCS and Ringer's solution. Infections were cured with a 5 day regimen of MF from day 4 or 5 p.i. Blood stage parasitaemia of all mice had reached between 25–35% for *P. yoelii* and 3–5% for *P. vinckei* infections on the day of MF cure.

All challenge infections with *P. yoelii* and *P. vinckei* sporozoites were performed by i.v. inoculation with sporozoites diluted in

100 μl of PBS. The number of sporozoites inoculated varied between experiments with 500–1,000 used for evaluating the development of blood stage parasitaemia and 1,500–3,000 administered for measuring liver parasite burdens.

Challenge infections of blood stage parasites of *P. yoelii* or *P. vinckei* were performed by i.p. or i.v. inoculation of 1×10^6 pRBCs diluted in 100 μl of 1:1 FCS and Ringer's solution. All challenge infections were carried out at least 14 days after the last MF dose was administered to allow blood plasma MF levels to reduce below a biologically active threshold. Control groups were subject to the same MF treatment regimens as the experimental groups. Before the challenge infections, Giemsa's solution-stained thin blood smears prepared from tail vein blood were observed to confirm the complete clearance of parasites from the blood. All challenge infected mice were followed up for 2 weeks post sporozoite infection.

2.3. RNA extraction and real time quantitative PCR (RT-qPCR)

Liver parasite burdens were assessed 42 h (*P. yoelii*) and 52 h (*P. vinckei*) post sporozoite inoculation using real-time quantitative PCR (RT-qPCR) measurement of parasite 18S rRNA gene copy number following reverse transcriptase PCR (RT-PCR) of total RNA extracted from whole mouse livers using a method modified from Kawabata et al. (2002). Mouse livers were dissected between 42–44 h p.i. with *P. yoelii* sporozoites and 50–52 h p.i. with *P. vinckei* sporozoites. Livers were homogenised in 5 ml of ISOGEN (Nippon Gene Co., Ltd., Japan) using a QIAgen TissueRuptor® (QIAGEN GmbH, Germany) and immediately stored at −80 °C. Extraction of total RNA was performed using the ISOGEN extraction system according to the manufacturer's instructions. One microgram of extracted total RNA was used to generate cDNA using the SuperScript® III RNase H RT system (Invitrogen, Germany), according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 in MilliQ water and 2 μl was used for RT-qPCR using the Power SYBR® Green PCR kit (Applied Biosystems, UK) on a 7500 Real Time PCR System (Applied Biosystems). Copy numbers of the parasite 18S rRNA gene were quantified with reference to a standard curve produced from known numbers of plasmids containing the same gene sequence. Inter-sample variation was controlled by the simultaneous quantification of the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene, against which the copy number of the parasite gene was calibrated. The *P. yoelii* 18S rRNA gene and *P. vinckei* 18S rRNA gene were amplified using the primers 18sF1 5′ GGAACGATGTGTGTCTAACACAAGGA 3′ and 18sR1 5′ CGCGTGCAGCCTAGTATATCTAAGGACA 3′, and the mouse G3PDH gene by the primers MmG3PDHF1 5′ CATCTGAGGGCCCACTGAAG 3′ and MmG3PDHR1 5′ TGCTCTTGAAGTCGCAGGAG 3′.

2.4. Monitoring of blood parasitaemia

For follow up of infections, Giemsa-stained thin blood smears prepared from tail vein blood were observed for the presence of parasites up to 2 weeks post challenge infection. Approximately 10,000 cells were observed per smear.

2.5. ELISA

To evaluate the cross-species reactivity of sera from immunised mice, ELISAs were performed using sera collected 2–3 days before challenge infections, with either *P. yoelii* or *P. vinckei* blood stage crude parasite extract as the plate-coating antigen. The plate-coating antigen was extracted by washing parasitised mouse blood with PBS and freeze–thawing three times at −80 °C. The freeze–thaw treated pellet was mixed for 1 min with 0.15% saponin in PBS solution, to lyse RBCs and the resulting parasite pellet collected after centrifuging at 3,500*g* for 5 min. The amount of protein in the parasite pellet was measured by NANODrop ND-1000 (NanoDrop Technologies, Inc. USA). Fifty microlitres of crude parasite extract was added to each well of the ELISA plate at 10 μg/ml (Sumitomo Bakelite Co., Japan) and incubated overnight at 4 °C. Plates were washed three times with 0.05% PBS/Tween (PBST) and blocked with a 30 min incubation at 37 °C with 200 μl per well of 5% skim milk (Difco™ Laboratories, USA) in PBST solution. Fifty microliters of mouse sera (1:100 dilution) were added to the coated wells and incubated at 37 °C for 2 h to allow the antibodies to bind to the parasite extract. Plates were then washed three times with PBST. Fifty microliters of horseradish peroxidase (HRP) conjugated anti-mouse IgG (1:3,000 dilution, R&D systems, USA) was added and incubated at 37 °C for 1.5 h. All wells were washed four times with PBST and 50 μl of ABTS Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., USA) was added and incubated at 37 °C for 10 min. Absorbance was measured on an iMARKTM microplate reader (Bio-Rad, USA) at 405 nm. Ninety-five percent cutoff measures (calculated by the mean of naïve + 3 × (mean S.D.)) were used to determine reactivity against each parasite extract.

2.6. IFA

IFAs against *P. yoelii yoelii* or *P. vinckei lentum* sporozoites were performed using the paraformaldehyde fixation method. Salivary gland sporozoites dissected in PBS were dropped onto glass slides, briefly treated with 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for fixation. Following blocking with 5% skim milk (Difco) in PBS, slides were incubated at 37 °C for 1 h with sera (one in 50 dilution) obtained from mice immunised with *P. yoelii* or *P. vinckei* sporozoites or with uninfected mosquito salivary glands (as controls). Sporozoite-bound antibodies were detected by incubating with Alexa 488 –α Mouse IgG (H+L) secondary antibody (one in 500 dilution, Molecular Probe, USA) at 37 °C for 30 min. Slides were also stained with DAPI (final concentration 1 μg/ml, Invitrogen) to aid sporozoite identification through nuclear staining.

2.7. In vivo $CD8^+$ T-cell depletion

Mice immunised by inoculation with *P. yoelii* sporozoites with contemporaneous MF treatment were given 0.36 mg anti-CD8 (clone 2.43) antibodies i.p. 2 days prior to sporozoite challenge infection. The dose required for $CD8^+$ T-cell depletion was confirmed previously by analysing the population of $CD8^+$ T-cells by FACS CANTO™ flow cytometry (Becton, Dickinson and Company, USA) in both peripheral blood and spleen lymphocytes. Non-$CD8^+$ T-cell depleted control mice were injected i.p. with PBS solution only.

2.8. Alignment of *P. vinckei lentumn* and *P. yoelii yoelii* circumsporozoite protein (CSP)

The major $CD8^+$ T-cell epitope for the *P. yoelii yoelii* circumsporozoite protein (CSP) is located at the C-terminus (Weiss et al., 1990) (amino acid sequence: SYVPSAEQI). To investigate whether there is a conserved peptide sequence corresponding to the *P. yoelii yoelii* CSP $CD8^+$ T-cell epitope, we aligned the peptide sequence of *P. yoelii* CSP (GenBank Accession Number AAA29558.1) and *P. vinckei lentum* CSP (GenBank Accession Number AAL36453.1) by CLUSTALW (http://www.genome.jp/tools/clustalw/).

2.9. Statistical analysis

Statistical analysis was performed using MINITAB® software v15 (LEAD Technologies, Inc., UK) and GraphPad Prism (GraphPad

Software, USA). The statistical significance of differences between treatment groups was assessed with two-tailed *t*-tests, for data that showed normal distribution according to Anderson–Darling tests for normality. The statistical significance of differences between groups that were not normally distributed was determined by non-parametric tests (i.e. Mann–Whitney test).

The statistical analysis of parasitaemia development in blood stage immunised mice challenge infected with homologous and heterologous blood stage parasites was performed by dividing the parasitaemia development into three phases. The three phases were designated: phase 1, the log-linear growth phase; phase 2, when the parasitaemia reaches a maximum; and phase 3, the time from peak parasitaemia to parasite clearance. Phase 1 corresponded to days 1–3 and days 1–5, phase 2 to days 3–5 and days 5–7, and phase 3 to days 5–14 and days 7–11 in *P. yoelii* or *P. vinckei* blood stage-infected groups, respectively. For the comparison of peak parasitaemia, we compared day 4 parasitaemia in both immunised and naïve *P. yoelii*-infected groups, and day 5 for immunised and day 6 for naïve *P. vinckei*-infected groups, respectively. To compare the difference in parasite growth (phase 1) or clearance (phase 3) between immunised and non-immunised groups, the gradient of the fitted line describing the increase/decrease (formula: $y = ax + b$, value a serving as the gradient) in parasitaemia during that period was analysed. *P*-values below 0.05 were considered significant.

3. Results

3.1. Species specificity of immunity generated by live malaria parasite vaccination with anti-malarial drug attenuation

3.1.1. Immunisation with live P. yoelii sporozoites inoculated contemporaneously with MF induces sterile immunity against challenge with P. yoelii, but not P. vinckei, sporozoites

Female Balb/c mice were twice immunised by i.v. inoculation of 1×10^4 *P. yoelii* sporozoites with concurrent MF treatment, with a period of 14 days between inoculations. Non-immunised control mice were treated with MF in the same way. Two weeks following the last MF dose, all mice were challenged i.v. with 1,000 sporozoites of either *P. yoelii* or *P. vinckei* for the parasitaemia evaluation groups and 1,500 or 3,000 sporozoites for groups that were sacrificed for liver parasite burden analysis. Livers were dissected from mice which had been given 1,500 or 3,000 sporozoites i.v., 42 h (*P. yoelii* challenged) or 52 h (*P. vinckei* challenged) p.i. to measure the parasite liver burden. Blood parasitaemia development was measured in mice which had received an i.v. inoculation of 1,000 sporozoites.

Liver parasite burdens measured by RT-qPCR in mice immunised against *P. yoelii* sporozoites and challenged with *P. yoelii* sporozoites were significantly reduced (by 99%) compared with non-immunised mice (Student's two-tailed *t*-test, $t = 7.72$, $P < 0.001$, Degrees of Freedom (df) = 8, Fig. 1A). Liver parasite burdens measured by RT-qPCR of mice immunised against *P. yoelii* sporozoites and challenge infected with *P. vinckei* were significantly reduced by 80% compared with non-immunised mice challenge infected with *P. vinckei* sporozoites (Student's two-tailed *t*-test, $t = 4.41$, $P = 0.002$, df = 8, Fig. 1B). Parasitaemia development was monitored by Giemsa-stained thin blood smears up to 15 days p.i. Mice immunised with *P. yoelii* sporozoites and challenged with *P. yoelii* sporozoites did not develop parasitaemia during the 15 days of observation (Fig. 1C). In contrast, mice immunised with *P. yoelii* sporozoites and challenged with *P. vinckei* sporozoites all developed parasitaemia (Fig. 1D). However, the time to patency (i.e. the first day at which parasites were observed in the blood using light microscopy) of the mice in the latter group was extended by 2 days (first appearance was day 6 post-challenge) compared with naïve mice infected with *P. vinckei* sporozoites (day 4). In addition, peak parasitaemia was reduced by 92% and parasites were cleared from the blood 2 days earlier than in the *P. yoelii* immunised group compared with the naïve group.

3.1.2. Sporozoite immunisation generates species-specific antibodies detectable by IFA

Protective immunity generated by the inoculation of sporozoites is mediated both by $CD8^+$ T-cells and antibodies (Schofield et al., 1987; Kumar et al., 2009). Antibodies may play a role by inhibiting the motility of sporozoites (Stewart et al., 1986). However, whether this antibody-mediated inhibition of sporozoites is species specific is unknown. To determine whether antibodies generated against sporozoites react in a species-specific manner, sera drawn from mice 4 weeks following immunisation with either *P. yoelii* or *P. vinckei* sporozoites were tested by IFA for reactivity with *P. yoelii* or *P. vinckei* mosquito salivary gland sporozoites. *P yoelii* sporozoites reacted with *P. yoelii* sporozoite immune sera but not with *P. vinckei* sporozoite immune mouse sera or naïve sera (Fig. 1E). In the reciprocal experiment, *P. vinckei* sporozoites reacted with *P. vinckei* sporozoite immune sera but not with *P. yoelii* sporozoite immune sera or naïve sera (Fig. 1F).

3.1.3. $CD8^+$ T-cells are involved in pre-erythocytic stage immunity generated by live sporozoite inoculation with MF treatment

$CD8^+$ T-cells are of crucial importance in conferring protective immunity against the liver stages following immunisation with irradiated (Weiss et al., 1988), genetically attenuated (Jobe et al., 2007), or with contemporaneous chloroquine administered with live sporozoites (Belnoue et al., 2004). To evaluate the role of $CD8^+$ T-cells in the protection generated by immunisation with live sporozoites and concurrent MF treatment, two groups of mice were i.d. immunised twice (with a 2 week interval between immunisations) with 2,000–2,500 *P. yoelii* sporozoites with concurrent MF treatment. Two days prior to challenge infection, one group of immunised mice was depleted of $CD8^+$ T-cells. Six weeks after the last immunisation, mice were challenged with 1,500 i.v. *P. yoelii* sporozoites. Livers of all challenged mice were dissected 42–44 h p.i. and liver parasite burdens were determined by RT-qPCR using, as a template, cDNA generated from total RNA extracted from infected mouse livers. Liver parasite burdens of immunised mice were significantly lower than in non-immunised mice (Mann Whitney test, $U < 0.01$, $P = 0.0079$, Fig. 2). The mean liver parasite burden of $CD8^+$ T-cell depleted immunised mice was not significantly different to that of the non-immunised mice (Mann Whitney test, $U = 7.0$, $P = 0.3095$, Fig. 2). The liver parasite burden of immunised mice was significantly lower than that of the $CD8^+$ T-cell depleted immunised mice (Mann Whitney test, $U = 2.00$, $P = 0.0317$). Finally, $CD8^+$ T-cell depletion did not affect the liver parasite burden of non-immunised mice (Man-Whitney test, $U = 6.00$, $P = 0.222$, Fig. 2).

3.1.4. Alignment of P. vinckei lentumn and P. yoelii yoelii CSP

The CSP is thought to be a major antigen for immunity against the pre-erythrocytic stages of malaria parasites. It is dominantly expressed on the sporozoite surface (Romero et al., 1989). A major H2-Kd-binding $CD8^+$ T-cell epitope was previously identified in the C-terminal region of *P. yoelii yoelii* CSP (Weiss et al., 1990) with the amino acid sequence: SYVPSAEQI. To evaluate whether this epitope is conserved in *P. vinckei lentum* CSP, we aligned the two CSP sequences (Supplementary Fig. S1) and demonstrated that the *P. yoelii yoelii* $CD8^+$ T cell epitope sequence is not conserved in *P. vinckei lentum* CSP.

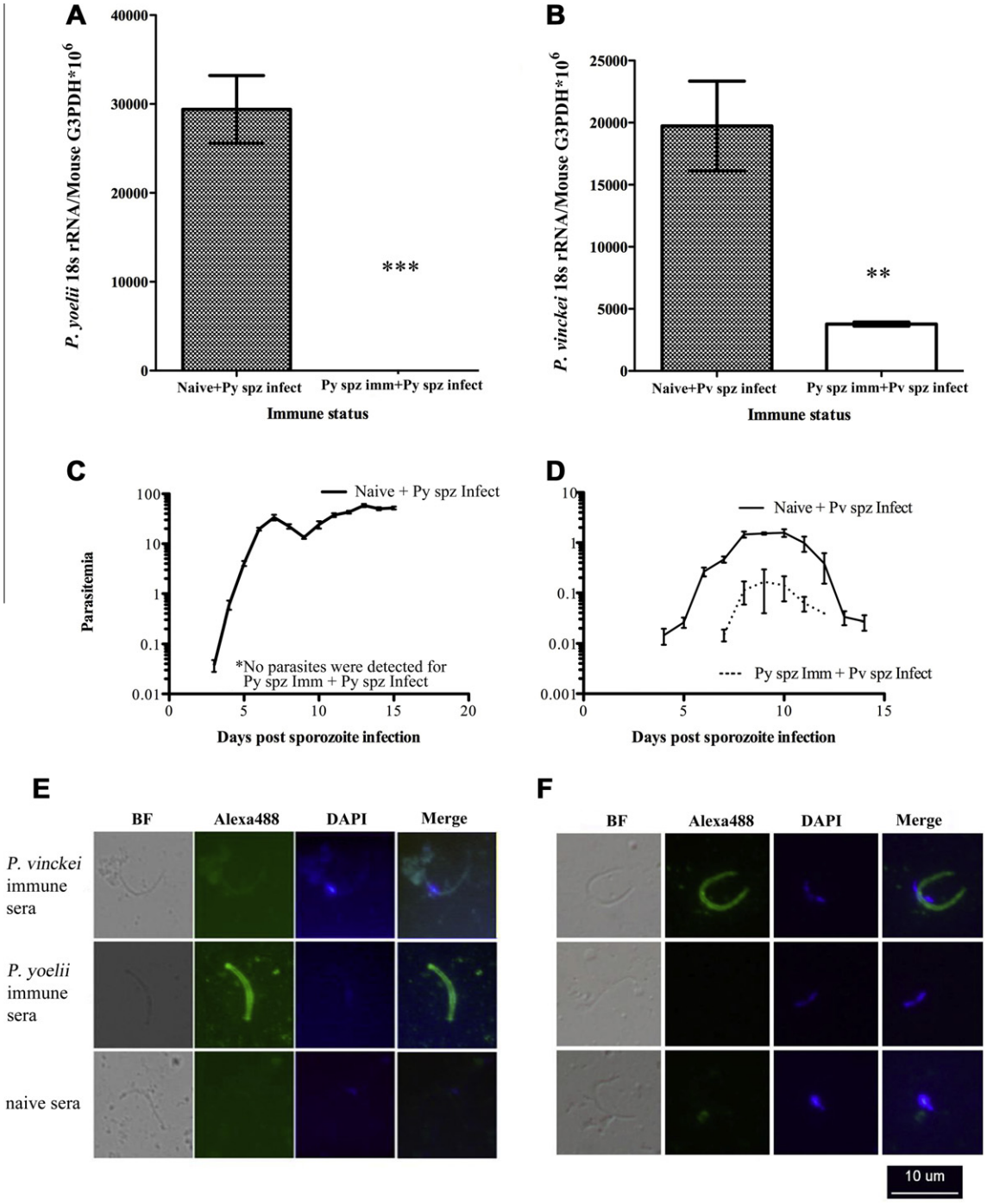

Fig. 1. The immunity generated by immunisation with live sporozoites of *Plasmodium yoelii yoelii* is species-specific. (A, B) Immunisation with live sporozoites with contemporaneous mefloquine (MF) treatment inhibits homologous and heterologous parasite development in the liver. (A) Female Balb/c mice ($n = 5$ per group) were i.v. immunised twice with 10,000 live *P. yoelii* sporozoites (Py spz Imm) and treated for 5 days with MF, beginning on the day of inoculation. Control (naïve) groups were treated with MF only. Both groups were challenged by i.v. infection with 1,500 *P. yoelii* sporozoites 2 weeks after the cessation of MF treatment (Py spz Infect). (B) Balb/c mice ($n = 5$ per group) were i.v. immunised twice with 10,000 live *P. yoelii* sporozoites and treated with MF as above. Control groups also received MF treatment. Both groups were challenged by i.v. infection with 1,500 *Plasmodium vinckei lentum* sporozoites 2 weeks after the cessation of MF treatment (Pv spz Infect). Bars show the mean liver parasite burden (parasite 18S rRNA copy number/mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) copy number $\times 10^6$). Error bars show the S.E.M. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared with naïve groups by a student's two-tailed *t*-test. These data are representative of the results of two independent experiments. (C, D) Blood stage parasitaemia of *P. yoelii* sporozoites immunised and challenge infected with *P. yoelii* sporozoites (Py spz Imm + Py spz Infect), and non-immunised Balb/c mice challenge infected with *P. yoelii* sporozoites (Naïve + Py spz Infect) (C) or *P. vinckei* sporozoites (Pv spz Infect) (D). *Plasmodium yoelii* sporozoite immunised mice and non-immunised mice were challenge infected i.v. with 1,000 *P. yoelii* or *P. vinckei* sporozoites. Each group contained five mice and parasitaemia development was monitored from day 2 until day 15 p.i. These data are representative of the results of two independent experiments. (E, F) Sera from mice immunised with *P. yoelii* or *P. vinckei* sporozoites react against sporozoites in a species-specific manner. Sera collected from mice immunised against *P. yoelii* or *P. vinckei* sporozoites collected 2 weeks after immunisation were assayed with *P. yoelii* (E) or *P. vinckei* (F) salivary-gland sporozoites. BF, bright field; Alexa 488, Alexa 488 –α Mouse IgG (H+L) antibody; Merge, BF, Alexa 488 and DAPI merged.

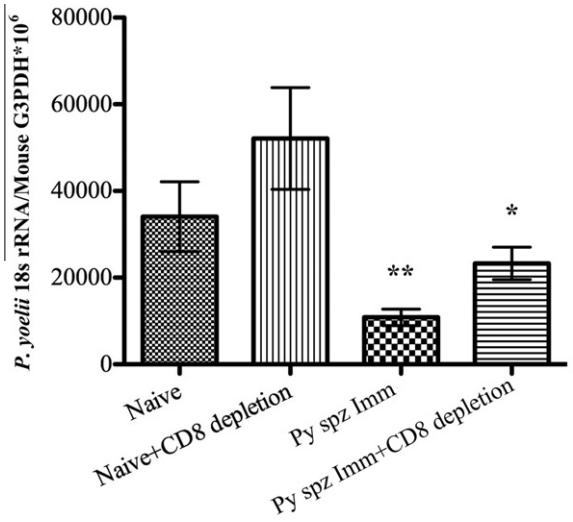

Fig. 2. CD8$^+$ T-cell involvement in immunity generated by inoculation of live *Plasmodium yoelii yoelii* sporozoites with concurrent mefloquine (MF) treatment. Mice were intradermally (i.d.) immunised twice with 2,000–2,500 *P. yoelii* sporozoites and treated with MF on days 0–5 (Py spz Imm). Control (naïve) mice were treated with MF only, in the same way. Anti-CD8$^+$ T-cell antibodies (clone 2.43) were i.p. inoculated into mice 2 days prior to challenge infection (CD8 depletion). All mice were infected i.v. with 1,500 *P. yoelii yoelii* sporozoites and liver parasite burdens were measured 42 h p.i. Bars show the mean liver parasite burden of five mice per group, with error bars indicating the S.E.M. Significance was tested by Mann Whitney tests between Naïve versus Py spz Imm ($P = 0.0079$), Naïve versus Py spz Imm+CD8 depletion ($P = 0.3095$), Naïve versus Naïve+CD8 depletion ($P = 0.222$) and Py spz Imm versus Py spz Imm+CD8 depletion ($P = 0.0317$). $^*P < 0.05$ (Py spz Imm versus Py spz Imm+CD8 depletion); $^{**}P < 0.01$ (Naïve versus Py spz Imm).

3.1.5. The immunity generated by MF cured blood stage infection of live parasites is species specific

Immunisation with live blood stage parasites was performed by inoculating mice with 1×10^6 pRBCs and drug treating the resulting infections with a 5 day regimen of MF 5 days p.i. Six weeks after MF treatment, mice were challenge infected with 1×10^6 pRBCs of *P. yoelii* or *P. vinckei*. Immunisation with *P. yoelii* pRBCs completely protected against infection with the homologous species (Fig. 3A), but did not protect against infection with the heterologous (*P. vinckei*) species. However, some cross-protection was observed, as immunisation with *P. yoelii* pRBCs significantly reduced the phase 2 peak parasitaemia of *P. vinckei* infection compared with parasite growth in naïve mice, and the clearance of parasites in the blood was faster by 1 day (Student's two-tailed *t*-test, $t = 16.90$, $P < 0.001$, df = 8, Fig. 3B). Similarly, immunisation with *P. vinckei* pRBCs completely protected against infection with the homologous species (Fig. 3B) but did not protect against *P. yoelii* challenge. However, when challenged with *P. yoelii* parasites, the phase 2 peak parasitaemia of *P. vinckei* immunised mice was significantly reduced compared with the naïve mice (Student's two-tailed *t*-test, $t = 3.64$, $P = 0.007$, df = 8, Fig. 3A). Comparison of the gradient of the linear fitted curve for phase 1 (log linear growth) and for phase 3 (parasite clearance) showed no significant difference between heterologous immunised groups and naïve groups (Fig. 3A and B).

3.1.6. Immunisation with pRBCs induces antibodies that react with both homologous and heterologous species crude parasite antigen by ELISA

Sera drawn from mice 4 weeks after immunisation were tested by ELISA for reactivity against *P. yoelii* or *P. vinckei* blood stage crude parasite antigen. Both *P. vinckei* and *P. yoelii* immune sera contained significant levels of antibodies specific to the homologous parasite crude extract (Student's two-tailed *t*-test, $t = 26.8$, $P < 0.0001$, df = 18, Fig. 3C and $t = 15.6$, $P < 0.0001$, df = 18, Fig. 3D). Furthermore, although much lower compared with reactions against homologous parasite crude extract, significant levels of antibodies against the heterologous species were measured (Student's two-tailed *t*-test, $t = 4.31$, $P = 0.0004$, df = 18, Fig. 3C and $t = 8.067$, $P < 0.0001$, df = 18, Fig. 3D).

3.2. Cross species and cross stage protection by immunisation with live malaria parasites

3.2.1. Immunisation with live sporozoites of P. yoelii induces an immunity that is active against the erythrocytic stages of P. yoelii and P. vinckei

Mice were twice immunised with 5,000 *P. yoelii* sporozoites i.v with concurrent oral MF treatment as described in Section 3.1.1. Two weeks after the last administration of MF, all mice were challenged i.v. with 1×10^6 *P. yoelii* or *P. vinckei* pRBCs. Parasitaemia development was monitored by Giemsa-stained thin blood smears (Fig. 4A and B). Mice immunised against *P. yoelii* sporozoites and infected with *P. yoelii* pRBCs developed significantly lower parasitaemia from the first day of monitoring compared with non-immunised mice and lower parasitaemias throughout the period of monitoring (Mann Whitney test, U < 0.01, $P = 0.0018$, Fig. 4A). However, mice immunised against *P. yoelii* sporozoites and infected with *P. vinckei* pRBCs showed no significant difference in parasitaemia on the first or second day of monitoring, but there were significant differences on days 3 and 4 p.i. (Mann Whitney test, U < 0.01, $P = 0.0002$ Fig. 4B). In order to determine whether the route of administration of parasites during immunisations and challenges were of importance (Inoue and Culleton, 2011), these experiments were repeated by immunising i.d., and challenging i.p. Comparable results were recorded (Supplementary Fig. S2).

3.2.2. Immunisation with live blood stage parasites does not generate significant immunity against liver stage parasites, but does affect the subsequent growth of blood stage parasites of both the homologous and heterologous species

In order to determine whether immunisation with live blood stages generates cross stage immunity, mice were immunised with *P. yoelii* or *P. vinckei* blood stages by inoculating 1×10^6 pRBCs of each species and curing with a 5 day oral MF treatment from day 4 or 5 post-inoculation. The average parasitaemia ranged between 28–37% in *P. yoelii* immunised mice and 5–7% in *P. vinckei* immunised mice on the day of MF treatment. Three weeks after the immunisation, immunised and naïve mice were challenged i.v. with 2,500 *P. yoelii* sporozoites by inoculation. Forty-two hours p.i., livers were dissected from half of the mice in each group and liver parasite burdens were evaluated. The rest of the mice were left, to follow the parasitaemia development by Giemsa-stained thin blood smears. Liver parasite burdens of mice immunised with both homologous and heterologous blood stage parasites were comparable with the naïve mice (Student's two-tailed *t*-test, $t = 1.737$, $P = 0.428$, for naïve versus homologous immunised and $t = 0.579$, $P = 0.230$, for naïve versus heterologous immunised, Fig. 5A). Mice immunised with *P. yoelii* pRBCs were completely protected against the development of microscopy-detectable blood stage parasites following challenge with *P. yoelii* sporozoites. However mice immunised with *P. vinckei* pRBCs developed detectable parasitaemia when challenged with *P. yoelii* sporozoites. The initial parasitaemia measured 48 h post sporozoite infection in mice immunised with *P. vinckei* pRBC was significantly lower than non-immunised mice following challenge with *P. yoelii* sporozoites (Mann Whitney test, U = 0.5, $P = 0.0295$, Fig. 5B).

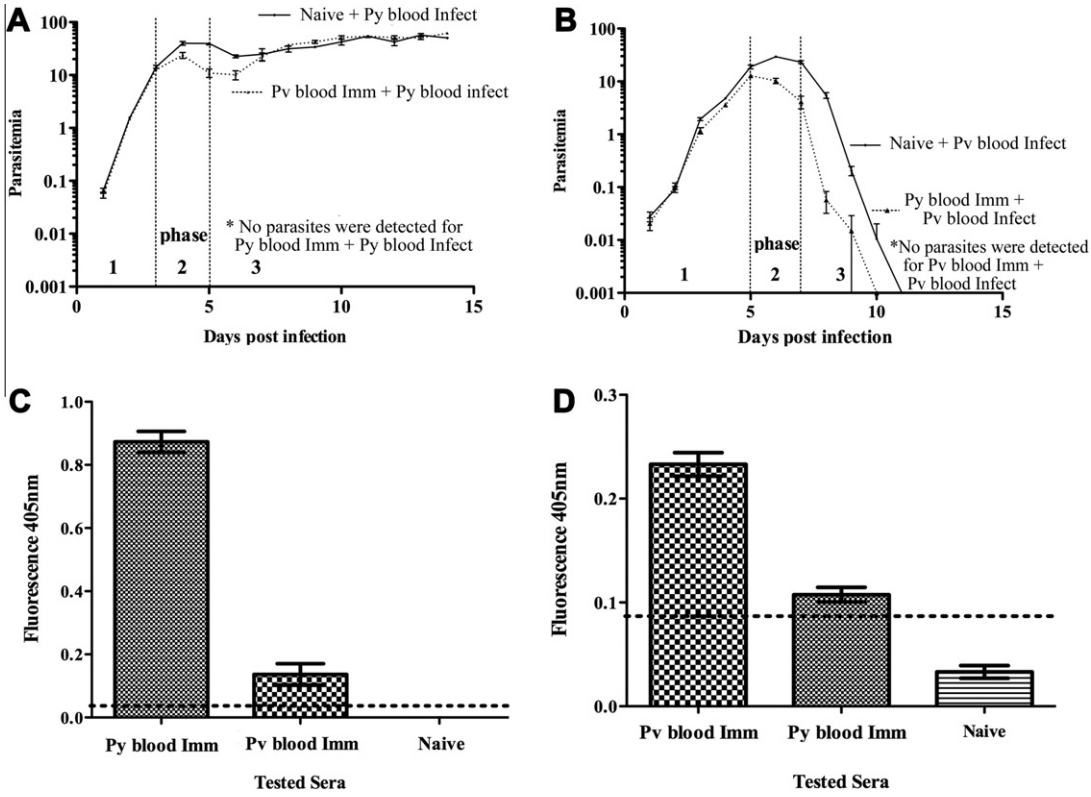

Fig. 3. The immunity generated by infection with blood stages of *Plasmodium yoelii yoelii* and *Plasmodium vinckei lentum* is species-specific. Vaccination with live blood stage parasites of *P. yoelii* or *P. vinckei*, followed by 5 day mefloquine (MF) treatment beginning on day 5 p.i., induces species-specific immunity. Groups of five mice were immunised i.v. with 1×10^6 *P. yoelii* or *P. vinckei* blood stage parasites and treated with MF from day 5 p.i. for 5 days (Py blood Imm/Pv blood Imm). Control (naïve) mice were treated with MF. All mice were challenge infected 6 weeks after the last MF treatment with 1×10^6 of *P. yoelii* (Py blood Infect; A) or *P. vinckei* (Pv blood Infect; B) blood stage parasites. The peak parasitaemia was compared between naïve and heterologous immunised mice by a student's two tailed *t*-test, $P = 0.007$ for Fig. 2A and $P < 0.001$ for Fig. 2B. Bold lines indicate parasitaemia curves of naïve mice and dotted lines of heterologous immunised mice. Error bars indicate the S.E.M. ($n = 5$ per group). (C, D) *Plasmodium yoelii* and *P. vinckei* blood stage parasite immune sera reacts against blood stage parasite antigen in a species-specific manner. Sera from mice infected with 1×10^6 *P. yoelii* or *P. vinckei* blood stage parasites and cured with MF were collected 5 weeks p.i. (Py blood Imm/Pv blood Imm). Sera from control mice treated with MF alone were collected at the same time. Sera were incubated with crude *P. yoelii* blood stage extract (C), or crude *P. vinckei* blood stage extract (D). Results indicate the mean fluorescence of 10 mice per group, with error bars marking the S.E.M. The dotted line indicates the 95% cutoff for positive responses (mean of naïve + 3 × (mean S.D.)). Significance was tested by a Student's two-tailed *t*-test between Py blood Imm versus Naïve ($P < 0.0001$) and Pv blood Imm versus Naïve ($P < 0.0004$), respectively, in C and Pv blood Imm versus Naïve ($P < 0.0001$) and Py blood Imm versus Naïve ($P < 0.0001$), respectively, in D.

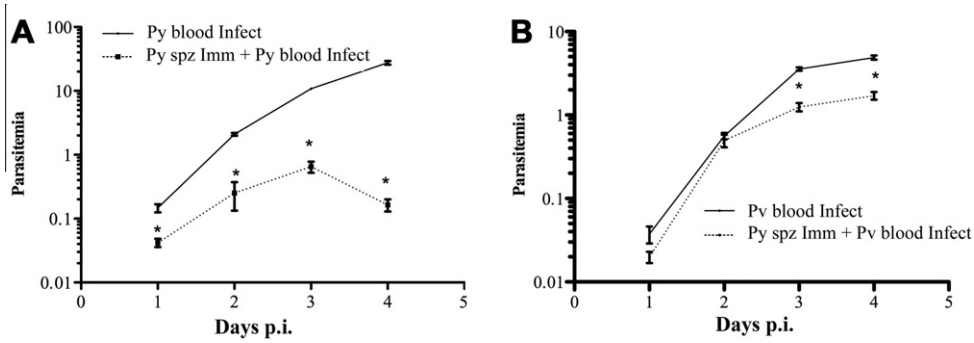

Fig. 4. Immunisation with live *Plasmodium yoelii yoelii* sporozoites induces cross stage and cross species immunity. (A, B) Immunisation with live *P. yoelii* sporozoites and mefloquine (MF) treatment induces immunity that is active against both *P. yoelii* and *Plasmodium vinckei lentum* blood stage infection. Groups of 10 mice were i.v. immunised twice with 5,000 *P. yoelii* sporozoites and treated with MF (Py spz Imm). Control (naïve) groups were composed of six mice and were also MF treated. All groups were challenged with 1×10^6 *P. yoelii* (A) or *P. vinckei* (B) blood stage parasites (Py blood Infect/Pv blood Infect). Mann Whitney tests comparing the significance of the differences in parasitaemia between immunised and non-immunised groups indicated that for *P. yoelii* challenged animals (A) differences were significant (*) on all days tested, whereas parasitaemias were significantly different between the *P. vinckei* challenged groups (B) on days 3 and 4. The data represent repeats of four independent experiments.

3.2.3. Blood stage infections of either homologous or heterologous species do not abrogate the immunity achieved against the pre-erythrocytic stages via live sporozoite immunisation

The immunity generated by immunisation with live *P. yoelii* sporozoites with concurrent MF treatment is protective against a homologous species sporozoite infection but does not inhibit the growth of a heterologous species sporozoite infection. To evaluate the possibility of blood stage malaria infection abrogating the pre-erythrocytic stage immunity generated by live sporozoite infection, we immunised mice twice with i.v. 5,000–10,000 *P. yoelii*

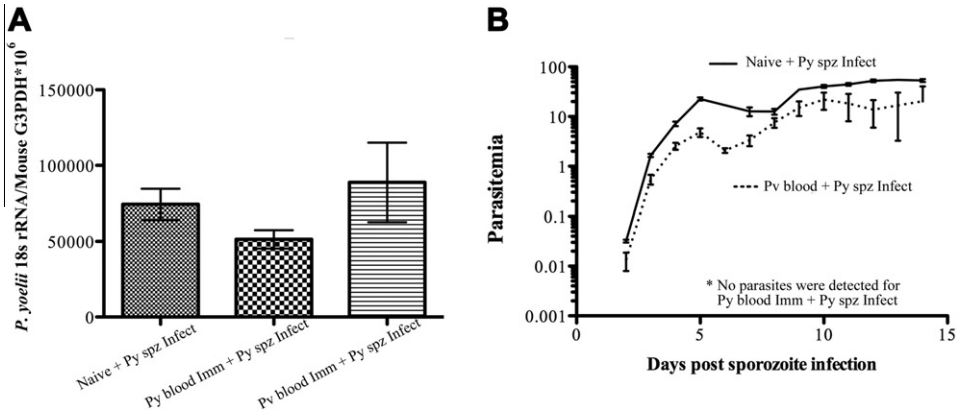

Fig. 5. Immunisation with live blood stage parasites does not affect liver parasite burden but reduces the initial parasitaemia in immunised mice. (A, B) Immunisation with live *Plasmodium yoelii yoelii* or *Plasmodium vinckei lentum* blood stage parasites confers immunity that is active against the parasite's development in the blood but not in the liver when infected with *P. yoelii* sporozoites. Groups of six mice were infected with 1×10^6 *P. yoelii* or *P. vinckei* blood stage parasites and treated for 5 days with mefloquine (MF) from day 4 p.i (Py blood Imm/Pv blood Imm). Control (naïve) mice were subjected to MF treatment alone. Three weeks post MF treatment, all mice were challenge infected i.v. with *P. yoelii* sporozoites (Py spz Infect). Forty-two hours post sporozoite infection, liver parasite burdens were measured in half of the mice in each group (A) and parasitaemia was monitored in the remaining mice (B). There was no significant difference in the liver parasite burden after sporozoite infection challenge between non-immunised and Py blood Imm or Pv blood Imm mice (A) (Student's two-tailed *t*-test, $P = 0.428$, for naïve versus homologous immunised (Py) and $P = 0.230$, for naïve versus heterologous immunised (Pv)). Mann Whitney tests showed that the difference in the initial parasitaemia of non-immunised versus *P. vinckei* immunised groups was significant ($P = 0.0295$, (B)). The data represent repeats of two independent experiments. G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

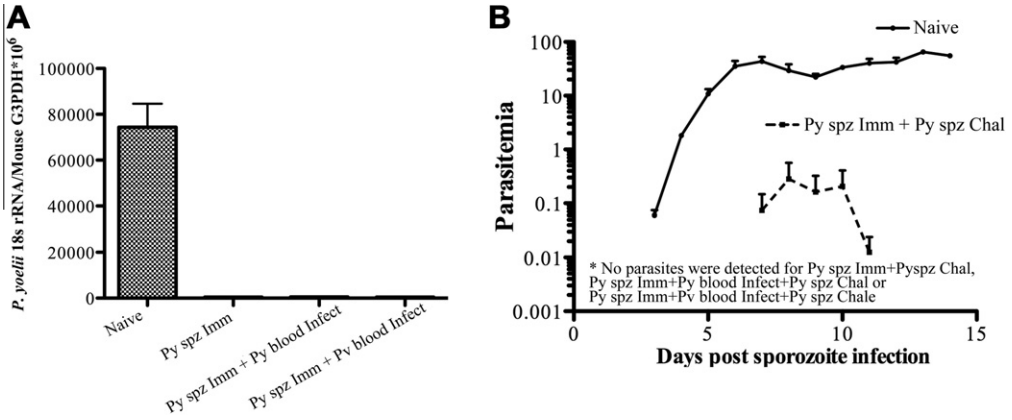

Fig. 6. Blood stage infection following immunisation with live *Plasmodium yoelii yoelii* sporozoites does not abrogate the immunity acquired by sporozoite immunisation. (A, B) Three groups of five mice each were immunised i.v. twice with 10,000 *P. yoelii* sporozoites with concurrent MF treatment (Py spz Imm). Two weeks after the second immunisation, one group was infected i.v. with 1×10^6 *P. yoelii* blood stage parasites (Pv blood Infect) and another group was infected with 1×10^6 *Plasmodium vinckei lentum* blood stage parasites (Pv blood Infect). Blood stage parasite-infected groups were treated with MF from day 4 p.i. for 5 days. Control (naïve) groups also received MF treatment. All mice were challenge infected with 1,000 *P. yoelii* sporozoites (Py spz Chal) 4 weeks after the final MF was administered. Liver parasite burden was measured by real time quantitative PCR (A). Bars indicate the mean liver parasite burden (parasite 18S rRNA copy number/Mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) copy number $\times 10^6$), error bars indicating the S.E.M., of five mice per group. Differences in liver parasite burdens between immunised (Py spz Imm), immunised and homologous blood stage infected (Py spz Imm + Py blood Infect + Py spz Chal), immunised and heterologous blood stage infected groups (Py spz Imm + Pv blood Infect + Py spz Chal) compared with the naïve group (naïve + Py spz Chal) were all significant (Mann Whitney test, $P = 0.0159$, (A). Parasitaemia development was monitored from day 2 p.i. for 14 days (B). All naïve mice developed parasitaemia by day 3 p.i. whereas one out of four immunised mice developed a low transitory parasitaemia. No parasites were detected in groups that were immunised and exposed to homologous or heterologous blood stage parasites. $n = 5$ mice per group, error bars indicate the S.E.M. The data represent repeats of two independent experiments.

Table 1
Summary of the results of two independent experiments designed to test the abrogating effect of blood stage infection on immunity against the pre-erythrocytic stages of *Plasmodium yoelii yoelii* (Py) and *Plasmodium vinckei lentum* (Pv).

Experiment	Immunization route and dose	Blood stage parasitaemia intervention	Challenge infection dose	Number of immunised mice protected	Number abrogated by blood stage parasitaemia intervention
A	i.v., 5,000 × 2	Py pRBC, 1×10^6 i.v. Pv pRBC, 1×10^6 i.v.	500 Py i.v.	3/5 (60%)	0/5 (0%) 0/5 (0%)
B	i.v. 10,000 × 2	Py pRBC, 1×10^6 i.v. Pv pRBC, 1×10^6 i.v.	1,000 Py i.v.	3/4 (75%)	0/4 (0%) 0/4 (0%)

pRBC, parasitised red blood cells.

sporozoites with concurrent MF treatment. Two weeks after the last immunisation, mice were infected with blood stage parasites of *P. yoelii* or *P. vinckei* and cured on day 4 p.i. with a 5 day oral MF treatment. Four weeks after the blood stage infection was cured, all mice were challenged with *P. yoelii* sporozoites and liver parasite burden and subsequent development of parasitaemia was assessed.

Immunised mice had significantly lower liver parasite burdens than non-immunised mice, irrespective of whether or not they received a blood stage infection of either *P. yoelii* or *P. vinckei*, following immunisation (Mann Whitney test, $U = 0.00$, $P = 0.0159$, Fig. 6A).

Following challenge with *P. yoelii* sporozoites, parasitaemia developed in all naïve mice, but no parasites were observed in immunised mice that had a subsequent exposure to homologous or heterologous blood stage parasite infection (Table 1). One mouse in the immunised group that did not receive a blood stage infection developed a very low transitory "break-out" infection (Fig. 6B). These results indicate that blood stage infection of either homologous or heterologous parasite species does not abrogate the immunity generated by the inoculation of *P. yoelii* sporozoites with concurrent MF treatment.

4. Discussion

Malaria eradication has recently returned to the world health agenda. Although control strategies targeting the mosquito vector and the use of anti-parasitic drugs are effective, it is difficult to envisage the goal of malaria eradication being reached without the development of an effective vaccine. One promising approach to the development of such a vaccine is the use of attenuated whole parasites. As the malaria parasite has a multi-stage life cycle, the question of which stage would offer the best candidate for an attenuated whole organism vaccine arises. Vaccination with attenuated sporozoites has been shown to confer sterile protection against homologous species in experiments conducted with non-human malaria parasites (Nussenzweig et al., 1967; Belnoue et al., 2004; Engwerda and Good, 2005) as well as in vaccination trials with human parasites (Hoffman et al., 2002; Roestenberg et al., 2009). Furthermore, low dose blood stage parasitic infections treated with drugs also confer protection in humans (Pombo et al., 2002). In this study we investigated the comparative cross-species protection afforded by vaccination with both blood stage and pre-erythrocytic stage whole parasites.

We found that immunisation with live *P. yoelii yoelii* sporozoites inoculated with contemporaneous MF treatment induced an immunity that drastically reduced the numbers of homologous species and heterologous species parasites present in the liver 42 h post-sporozoite inoculation, and completely stopped the development of a patent blood stage infection of homologous species parasites. We found that this protection was dependent on the presence of $CD8^+$ T-cells, as has been previously shown for a similar vaccination strategy using chloroquine as the anti-blood stage drug (Belnoue et al., 2004) and in work utilising irradiated sporozoites (Schofield et al., 1987; Weiss et al., 1988), and genetically attenuated parasites (Douradinha et al., 2011). When mice immunised in the same way were challenged with sporozoites of a heterologous species, in this case *P. vinckei lentum*, there was also a significant reduction in liver parasite burden, but to a lesser degree than that observed upon homologous challenge. Most importantly, all immunised mice challenged with *P. vinckei* sporozoites developed patent blood stage parasite infections, although the severity of these infections was dramatically reduced compared with those that developed in non-immunised mice. Given that the immunity generated against the pre-erythrocytic stages is dependent on $CD8^+$ T-cells, then we can assume that the cross-protection observed between *P. yoelii* and *P. vinckei* in the liver must involve conserved antigens that contain $CD8^+$ T-cell epitopes. The CSP is often cited as the major antigen involved in eliciting $CD8^+$ T-cell immunity against liver stage malaria parasites. Alignment of the *P. yoelii* and *P. vinckei* CSP protein sequence demonstrated that the previously defined $CD8^+$ T-cell epitope of *P. yoelii* (Weiss et al., 1990) is not found in the *P. vinckei* CSP. This is indicative of pre-erythrocytic stage immunity targeting an antigen other than CSP. Furthermore, experiments using late liver-stage arresting genetically attenuated sporozoites have demonstrated that protective liver stage immunity targets an antigen expressed in the late liver stages (Butler et al., 2011). Combined with the previous reports, our results support the recent observations that question the importance of the role played by CSP in inducing pre-erythrocytic stage immunity (Khusmith et al., 1991; Doolan et al., 1996; Gruner et al., 2007; Mauduit et al., 2009, 2010).

Supporting the role of cellular rather than humoral responses in eliciting cross-species protection at the pre-erythrocytic stage, IFA showed that antibodies generated by live sporozoite inoculation with contemporaneous MF treatment react in a species-specific manner. These results are in agreement with the experiments performed with simian *Plasmodium* parasites (Nussenzweig and Chen, 1974) which showed that antisera against sporozoites reacted against homologous species but not with heterologous species.

There was a significantly large reduction in the parasitaemia of *P. vinckei* sporozoite inoculated *P. yoelii* sporozoite-immunised mice compared with naïve mice infected with *P. vinckei* sporozoites. There are multiple possibilities that may account for this. Firstly, the large reduction in *P. vinckei* liver-stage parasites in these mice would lead to vastly fewer exo-erythrocytic merozoites being released into the blood stream. As lower initiating doses of blood stage rodent malaria parasites are known to result in reduced parasitaemia (Timms et al., 2001), this might explain the reduced parasitaemia in our cross-challenge experiment. Secondly, it is possible that the immunity generated against the pre-erythrocytic forms of *P. yoelii* is not only cross-species protective, but also cross-stage protective, so that antigens are conserved between *P. yoelii* and *P. vinckei*, and antibodies raised against them are protective against the blood stages as well as the pre-erythrocytic stages. Related to this is the possibility that our vaccination protocol, which allows the release of exo-erythrocytic merozoites into the blood, and their invasion of red blood cells before they are killed at the trophozoite stage by the MF treatment, may elicit protection purely against the blood stage forms that is cross-species protective. However, given that mice immunised against blood stage *P. yoelii* only, and challenged with blood stages of *P. vinckei,* did not show a great deal of cross protection, we consider this last scenario unlikely.

Despite the large degree of cross-species protection against parasites developing in the liver, there was no cross-species protection from the development of blood stage infections following sporozoite challenge. These results differ from previous observations in which cross-species protection was observed between *P. berghei* and *P. vinckei* (Nussenzweig et al., 1969) and between *P. yoelii* and *P. berghei* following sporozoite immunisation (Sedegah et al., 2007; Purcell et al., 2008). In the latter case, this is possibly due to the larger antigenic diversity between *P. yoelii* and *P. vinckei* compared with that of *P. yoelii* and *P. berghei* (Perkins et al., 2007). It is also possible that differences in immunisation protocol (live sporozoite and contemporaneous MF treatment were used in our experiments, whereas irradiated or genetically attenuated sporozoites were used in previous works) and numbers of sporozoites for challenge infection play a role in inducing immunity that varies in its specificity (Inoue and Culleton, 2011).

Inoculation with live blood stage parasites and clearance of the infection with anti-malarial drugs induces immunity that confers

sterile protection against homologous species blood stage challenge, but not against heterologous species infection. These results are in accordance with those achieved in evaluating the degree of cross species protection induced by the inoculation of non-viable whole blood stage parasites (McColm and Dalton, 1983).

Data from challenge infections with heterologous species parasites show a reduction in peak parasitaemia but not in phase 1 parasitaemia growth or phase 3 parasitaemia clearance. These results, together with the cross-species reactivity of immune sera, indicate that an antibody-mediated immune response probably plays a role in generating cross-species protection against the blood stages. These findings are in accordance with several earlier reports which show a correlation between blood stage-specific antibodies and protection against malaria infection (Hirunpetcharat et al., 1997; Soe et al., 2004).

Studies with rodent malaria parasites have previously demonstrated cross-stage reactivity of the immune responses against between sporozoites and blood stage parasites (Golenser et al., 1976; Kawabata et al., 2002). Our results also demonstrate stage-transcending protection by both sporozoite and blood stage immunisation. Immunisation with live sporozoites reduced the subsequent parasite growth of both homologous and heterologous blood stage parasites, with a larger reduction in parasitaemia of homologous blood stage challenges. These results are in disagreement with experiments conducted with *P. berghei* sporozoite-immunised mice that were challenged with *P. berghei* or *P. vinckei* blood stage parasites, where no significant degree of protection was seen (Nussenzweig et al., 1969). These differences could be due to variations in immunisation protocols involving different administration routes and parasite attenuation methods, and a combination of different parasites and mouse backgrounds (Weiss, 1990; Inoue and Culleton, 2011). The stage-transcending protective effect is possibly due to the nature of a live sporozoite plus anti-blood stage drug immunisation regimen, which allows the release of liver stage merozoites, and their invasion into erythrocytes, as discussed above. That protection can be elicited by such a short exposure to small numbers of blood stage parasites by cell mediated immunity involving both $CD4^+$ T-cells and $CD8^+$ T-cells has previously been demonstrated in humans (Pombo et al., 2002). An alternative explanation for the phenomenon of sporozoite induced cross protection against blood stage parasites is the presence of shared antigens between stages. Proteomic analysis has demonstrated that close to 90% of the proteins expressed in late liver stage schizonts are present in blood stage parasites (Tarun et al., 2008).

We also evaluated whether the immunity generated by the administration of blood stage parasites could protect against the growth of parasites in the liver. We found no significant differences in the liver parasite burden of mice immunised with homologous (*P. yoelii*) or heterologous (*P. vinckei*) blood stage parasites and non-immunised mice challenged with sporozoites of *P. yoelii*. However immunisation with *P. yoelii* blood stage parasites completely protected against the development of blood-stage parasitaemia following homologous sporozoite challenge, whereas immunisation with *P. vinckei* blood stage parasites did not stop the development of parasitaemia when challenged with *P. yoelii* sporozoites. There was, however, a significant reduction in the initial parasitaemia (measured 48 h after sporozoite challenge) in heterologous blood stage immunised mice. It is known that blood stage immunity is induced by both $CD4^+$ T-cells and antibodies whereas pre-erythrocytic stage immunity is mediated by intrahepatic $CD8^+$ T cells and in part by antibodies and $CD4^+$ T cells (Wipasa et al., 2002; Schwenk and Richie, 2011). The different immune mechanisms between blood stage immunity and pre-erythrocytic stage immunity could be the reason that no significant difference was seen in parasite liver burdens of blood stage immunised and naïve mice. The significant reduction in initial parasitaemia in heterologous blood stage immunised mice could be due to species and stage transcending antibodies generated by blood stage immunisation. This combined with the liver parasite burden showing no significant differences between immunised and naïve mice suggest that the cross-species protection acts only on the blood stage parasites.

There is evidence that malaria parasites suppress the immune responses of their hosts. For example, reduced vaccine efficiency was observed in children vaccinated during malaria infection (Williamson and Greenwood, 1978) and diminished antibody responses were recorded in children with acute malaria infection (Greenwood et al., 1972). Experiments with rodent malaria parasites have also demonstrated the immune-suppressive effect of blood stage infection (Ahvazi et al., 1995; Ocana-Morgner et al., 2003). It has been shown that blood stage malaria infections can abrogate the immunity achieved against the pre-erythrocytic stages. Orjih (1985) showed that mice immunised with *P. berghei* live sporozoites achieved complete protection against re-challenge with sporozoites if the immunising inoculation was performed in the presence of chloroquine, preventing the establishment of a blood stage infection. However, if chloroquine was not administered until after the blood stage cycle had commenced, mice were not protected against challenge with sporozoites. In our experiments we have shown that the immunity generated against the pre-erythrocytic stages is species specific, to the degree that immunisation with one species allows the development of blood stages of a heterologous species. These phenomena raise the possibility that immunisation against the pre-erythrocytic stages of one species of malaria parasite might allow the establishment of a blood stage infection of a second species, and that this may abrogate the immunity induced against the immunising species. Given this, we conducted experiments to evaluate whether a blood stage infection of either a homologous or heterologous species could negatively impact on the immunity generated against the pre-erythrocytic stages. After repeated experiments, we found no effect of a subsequent blood stage infection of either a homologous or a heterologous species on the immunity achieved against the pre-erythrocytic stages. This suggests that blood stage malaria parasite infection – following clearance – does not abrogate immunity to pre-erythrocytic stages.

In summary, we have compared the specificity of pre-erythrocytic stage and blood stage immunity using *P. yoelii yoelii* and *P. vinckei lentum*, two distantly related rodent malaria parasite species. Our results show that, despite a degree of species specificity in the immunity generated by both pre-erythrocytic stages and blood stages, pre-erythrocytic stages conferred a far greater species transcending protection to the growth of parasites in the liver and to the subsequent development of parasites in the blood compared with immunisation with blood stage parasites.

Furthermore, we found no abrogative effect of blood stage infection on pre-erythrocytic stage immunity. Based on these observations we conclude that vaccines targeting pre-erythrocytic stages would offer a wider degree of protection against genetically polymorphic malaria parasites than those targeting the blood stages. These findings are of importance when considering the design of whole-organism based vaccines for deployment in areas where multiple malaria parasite species are prevalent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.07.001.

References

Ahvazi, B.C., Jacobs, P., Stevenson, M.M., 1995. Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria. J. Leukoc. Biol. 58, 23–31.

Belnoue, E., Costa, F., Frankenberg, T., Vigário, A., Voza, T., Leroy, N., Rodrigues, M., Landau, I., Snounou, G., Rénia, L., 2004. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J. Immunol. 172, 2487–2582.

Belnoue, E., Voza, T., Costa, F.T., Gruner, A.C., Mauduit, M., Rosa, D.S., Depinay, N., Kayibanda, M., Vigario, A.M., Mazier, D., Snounou, G., Sinnis, P., Renia, L., 2008. Vaccination with live *Plasmodium yoelii* blood stage parasites under chloroquine cover induces cross-stage immunity against malaria liver stage. J. Immunol. 181, 8552–8558.

Butler, N., Schmidt, N., Vaughan, A., Aly, A., Kappe, S., Harty, J., 2011. Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. Cell Host Microbe 9, 451–513.

Chakravarty, S., Cockburn, I.A., Kuk, S., Overstreet, M.G., Sacci, J.B., Zavala, F., 2007. CD8$^+$ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. Nat. Med. 13, 1035–1041.

Doolan, D.L., Sedegah, M., Hedstrom, R.C., Hobart, P., Charoenvit, Y., Hoffman, S.L., 1996. Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. J. Exp. Med. 183, 1739–1746.

Douradinha, B., van Dijk, M., van Gemert, G.J., Khan, S.M., Janse, C.J., Waters, A.P., Sauerwein, R.W., Luty, A.J., Silva-Santos, B., Mota, M.M., Epiphanio, S., 2011. Immunization with genetically attenuated P52-deficient *Plasmodium berghei* sporozoites induces a long-lasting effector memory CD8$^+$ T cell response in the liver. J. Immune Based Ther. Vaccines 9, 6.

Engwerda, C.R., Good, M.F., 2005. Interactions between malaria parasites and the host immune system. Curr. Opin. Immunol. 17, 381–387.

Epstein, J., Tewari, K., Lyke, K., Sim, B., Billingsley, P., Laurens, M., Gunasekera, A., Chakravarty, S., James, E., Sedegah, M., Richman, A., Velmurugan, S., Reyes, S., Li, M., Tucker, K., Ahumada, A., Ruben, A., Li, T., Stafford, R., Eappen, A., Tamminga, C., Bennett, J., Ockenhouse, C., Murphy, J., Komisar, J., Thomas, N., Loyevsky, M., Birkett, A., Plowe, C., Loucq, C., Edelman, R., Richie, T., Seder, R., Hoffman, S., 2011. Live attenuated malaria vaccine designed to protect through hepatic CD8$^+$ T cell immunity. Science 334, 475–555.

Frevert, U., 2004. Sneaking in through the back entrance: the biology of malaria liver stages. Trends Parasitol. 20, 417–441.

Gerald, N., Majam, V., Mahajan, B., Kozakai, Y., Kumar, S., 2011. Protection from experimental cerebral malaria with a single dose of radiation-attenuated, blood-stage *Plasmodium berghei* parasites. PLoS ONE 6.

Golenser, J., Spira, D.T., Verhave, J.P., Meuwissen, J.H., 1976. Specific lymphocyte transformation in murine malaria. Z. Parasitenkd. 50, 95–98.

Good, M., 2011. A whole parasite vaccine to control the blood stages of Plasmodium - the case for lateral thinking. Trends Parasitol. 27, 335–340.

Good, M., Doolan, D., 2007. Malaria's journey through the lymph node. Nat. Med. 13, 1023–1027.

Greenwood, B.M., Bradley-Moore, A.M., Bryceson, A.D., Palit, A., 1972. Immunosuppression in children with malaria. Lancet 1, 169–172.

Gruner, A.C., Mauduit, M., Tewari, R., Romero, J.F., Depinay, N., Kayibanda, M., Lallemand, E., Chavatte, J.M., Crisanti, A., Sinnis, P., Mazier, D., Corradin, G., Snounou, G., Renia, L., 2007. Sterile protection against malaria is independent of immune responses to the circumsporozoite protein. PLoS ONE 2, e1371.

Gueirard, P., Tavares, J., Thiberge, S., Bernex, F., Ishino, T., Milon, G., Franke-Fayard, B., Janse, C., Ménard, R., Amino, R., 2010. Development of the malaria parasite in the skin of the mammalian host. Proc. Natl. Acad. Sci. USA 107, 18640–18645.

Guerra, C., Snow, R., Hay, S., 2006. Mapping the global extent of malaria in 2005. Trends Parasitol. 22, 353–361.

Gunewardena, D., Carter, R., Mendis, K., 1994. Patterns of acquired anti-malarial immunity in Sri Lanka. Memórias do Instituto Oswaldo Cruz 89 (Suppl. 2), 63–68.

Hirunpetcharat, C., Tian, J.H., Kaslow, D.C., van Rooijen, N., Kumar, S., Berzofsky, J.A., Miller, L.H., Good, M.F., 1997. Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of *Plasmodium yoelii* expressed in *Saccharomyces cerevisiae*: correlation of protection with antigen-specific antibody titer, but not with effector CD4$^+$ T cells. J. Immunol. 159, 3400–3411.

Hoffman, S.L., Goh, L.M., Luke, T.C., Schneider, I., Le, T.P., Doolan, D.L., Sacci, J., de la Vega, P., Dowler, M., Paul, C., Gordon, D.M., Stoute, J.A., Church, L.W., Sedegah, M., Heppner, D.G., Ballou, W.R., Richie, T.L., 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J. Infect. Dis. 185, 1155–1164.

Inoue, M., Culleton, R.L., 2011. The intradermal route for inoculation of sporozoites of rodent malaria parasites for immunological studies. Parasite Immunol. 33, 137–142.

Jobe, O., Lumsden, J., Mueller, A.K., Williams, J., Silva-Rivera, H., Kappe, S.H., Schwenk, R.J., Matuschewski, K., Krzych, U., 2007. Genetically attenuated *Plasmodium berghei* liver stages induce sterile protracted protection that is mediated by major histocompatibility complex Class I-dependent interferon-gamma-producing CD8$^+$ T cells. J. Infect. Dis. 196, 599–607.

Kawabata, Y., Udono, H., Honma, K., Ueda, M., Mukae, H., Kadota, J., Kohno, S., Yui, K., 2002. Merozoite surface protein 1-specific immune response is protective against exoerythrocytic forms of *Plasmodium yoelii*. Infect. Immun. 70, 6075–6082.

Khusmith, S., Charoenvit, Y., Kumar, S., Sedegah, M., Beaudoin, R.L., Hoffman, S.L., 1991. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. Science 252, 715–718.

Kumar, K.A., Baxter, P., Tarun, A.S., Kappe, S.H., Nussenzweig, V., 2009. Conserved protective mechanisms in radiation and genetically attenuated uis3(-) and uis4(-) *Plasmodium* sporozoites. PLoS ONE 4, e4480.

Maitland, K., Williams, T., Newbold, C., 1997. *Plasmodium vivax* and *P. falciparum*: biological interactions and the possibility of cross-species immunity. Parasitol. Today 13, 227–258.

Mauduit, M., Gruner, A.C., Tewari, R., Depinay, N., Kayibanda, M., Chavatte, J.M., Franetich, J.F., Crisanti, A., Mazier, D., Snounou, G., Renia, L., 2009. A role for immune responses against non-CS components in the cross-species protection induced by immunization with irradiated malaria sporozoites. PLoS ONE 4, e7717.

Mauduit, M., Tewari, R., Depinay, N., Kayibanda, M., Lallemand, E., Chavatte, J.M., Snounou, G., Renia, L., Gruner, A.C., 2010. Minimal role for the circumsporozoite protein in the induction of sterile immunity by vaccination with live rodent malaria sporozoites. Infect. Immun. 78, 2182–2188.

Mayxay, M., Pukrittayakamee, S., Newton, P.N., White, N.J., 2004. Mixed-species malaria infections in humans. Trends Parasitol. 20, 233–240.

McCarthy, J.S., Good, M.F., 2010. Whole parasite blood stage malaria vaccines: a convergence of evidence. Hum. Vaccin 6, 114–123.

McColm, A.A., Dalton, L., 1983. Heterologous immunity in rodent malaria: comparison of the degree of cross-immunity generated by vaccination with that produced by exposure to live infection. Ann. Trop. Med. Parasitol. 77, 355–377.

Medica, D.L., Sinnis, P., 2005. Quantitative dynamics of *Plasmodium yoelii* sporozoite transmission by infected anopheline mosquitoes. Infect. Immun. 73, 4363–4369.

Mendis, K., David, P., Carter, R., 1990. Human immune responses against sexual stages of malaria parasites: considerations for malaria vaccines. Int. J. Parasitol. 20, 497–502.

Mueller, I., Zimmerman, P., Reeder, J., 2007. *Plasmodium malariae* and *Plasmodium ovale*—the "bashful" malaria parasites. Trends Parasitol. 23, 278–361.

Murray, C., Rosenfeld, L., Lim, S., Andrews, K., Foreman, K., Haring, D., Fullman, N., Naghavi, M., Lozano, R., Lopez, A., 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 379, 413–444.

Nussenzweig, R., Vanderberg, J., Most, H., Orton, C., 1967. Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. Nature 216, 160–162.

Nussenzweig, R.S., Vanderberg, J.P., Most, H., Orton, C., 1969. Specificity of protective immunity produced by x-irradiated *Plasmodium berghei* sporozoites. Nature 222, 488–489.

Nussenzweig, R.S., Chen, D., 1974. The antibody response to sporozoites of simian and human malaria parasites: its stage and species specificity and strain cross-reactivity. Bull. World Health Organ. 50, 293–297.

Ocana-Morgner, C., Mota, M.M., Rodriguez, A., 2003. Malaria blood stage suppression of liver stage immunity by dendritic cells. J. Exp. Med. 197, 143–151.

Orjih, A.U., 1985. Acute malaria prolongs susceptibility of mice to *Plasmodium berghei* sporozoite infection. Clin. Exp. Immunol. 61, 67–71.

Perkins, S.L., Sarkar, I.N., Carter, R., 2007. The phylogeny of rodent malaria parasites: simultaneous analysis across three genomes. Infect. Genet. Evol. 7, 74–83.

Pinzon-Charry, A., Good, M.F., 2008. Malaria vaccines: the case for a whole-organism approach. Expert Opin. Biol. Ther. 8, 441–448.

Pombo, D.J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K., Mahakunkijcharoen, Y., Martin, L.B., Wilson, D., Elliott, S., Eisen, D.P., Weinberg, J.B., Saul, A., Good, M.F., 2002. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. Lancet 360, 610–617.

Purcell, L.A., Wong, K.A., Yanow, S.K., Lee, M., Spithill, T.W., Rodriguez, A., 2008. Chemically attenuated *Plasmodium* sporozoites induce specific immune responses, sterile immunity and cross-protection against heterologous challenge. Vaccine 26, 4880–4884.

Rodrigues, M., Cordey, A., Arreaza, G., Corradin, G., Romero, P., Maryanski, J., Nussenzweig, R., Zavala, F., 1991. CD8$^+$ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. Int. Immunol. 3, 579–664.

Roestenberg, M., McCall, M., Hopman, J., Wiersma, J., Luty, A.J., van Gemert, G.J., van de Vegte-Bolmer, M., van Schaijk, B., Teelen, K., Arens, T., Spaarman, L., de Mast, Q., Roeffen, W., Snounou, G., Renia, L., van der Ven, A., Hermsen, C.C., Sauerwein, R., 2009. Protection against a malaria challenge by sporozoite inoculation. N. Engl. J. Med. 361, 468–477.

Romero, P., Maryanski, J.L., Corradin, G., Nussenzweig, R.S., Nussenzweig, V., Zavala, F., 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature 341, 323–326.

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R., Nussenzweig, V., 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature 330, 664–666.

Schwenk, R., Richie, T., 2011. Protective immunity to pre-erythrocytic stage malaria. Trends Parasitol. 27, 306–320.

Sedegah, M., Weiss, W.W., Hoffman, S.L., 2007. Cross-protection between attenuated *Plasmodium berghei* and *P. yoelii* sporozoites. Parasite Immunol. 29, 559–565.

Soe, S., Theisen, M., Roussilhon, C., Aye, K.S., Druilhe, P., 2004. Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. Infect. Immun. 72, 247–252.

Stewart, M.J., Nawrot, R.J., Schulman, S., Vanderberg, J.P., 1986. *Plasmodium berghei* sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies. Infect. Immun. 51, 859–864.

Tarun, A., Peng, X., Dumpit, R., Ogata, Y., Silva-Rivera, H., Camargo, N., Daly, T., Bergman, L., Kappe, S., 2008. A combined transcriptome and proteome survey of malaria parasite liver stages. Proc. Natl. Acad. Sci. USA 105, 305–315.

Timms, R., Colegrave, N., Chan, B.H., Read, A.F., 2001. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitology 123, 1–11.

Ting, L.-M., Gissot, M., Coppi, A., Sinnis, P., Kim, K., 2008. Attenuated *Plasmodium yoelii* lacking purine nucleoside phosphorylase confer protective immunity. Nat. Med. 14, 954–962.

Voza, T., Miller, J.L., Kappe, S.H., Sinnis, P., 2012. Extrahepatic exoerythrocytic forms of rodent malaria parasites at the site of inoculation: clearance after immunization, susceptibility to primaquine and contribution to blood stage infection. Infect. Immun. 80, 2158–2164.

Weiss, W.R., Sedegah, M., Beaudoin, R.L., Miller, L.H., Good, M.F., 1988. $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. Proc. Natl. Acad. Sci. USA 85, 573–576.

Weiss, W.R., 1990. Host-parasite interactions and immunity to irradiated sporozoites. Immunol. Lett. 25, 39–42.

Weiss, W.R., Houghten, R.A., Good, M.F., Berzofsky, J.A., Miller, L.H., Hoffman, S.L., 1990. A CTL epitope on the circumsporozoite protein of *P. yoelii*. Bull. World Health Organ. 68 (Suppl. 2), 99–103.

Williamson, W.A., Greenwood, B.M., 1978. Impairment of the immune response to vaccination after acute malaria. Lancet 1, 1328–1329.

Wipasa, J., Elliott, S., Xu, H., Good, M.F., 2002. Immunity to asexual blood stage malaria and vaccine approaches. Immunol. Cell Biol. 80, 401–414.

Zimmerman, P.A., Mehlotra, R.K., Kasehagen, L.J., Kazura, J.W., 2004. Why do we need to know more about mixed *Plasmodium* species infections in humans? Trends Parasitol. 20, 440–447.