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ORIGINAL ARTICLE

Induction of immunity following vaccination with a chemically attenuated malaria vaccine correlates with persistent antigenic stimulation

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Abstract

Objectives. Blood stage malaria parasites attenuated with seco-cyclopropyl pyrrolo indole (CPI) analogues induce robust immunity in mice to homologous and heterologous malaria parasites and are being considered for the development of a human vaccine. However, it is not understood how attenuated parasites induce immunity. We showed that following vaccination, parasite DNA persisted in blood for several months, raising the possibility that ongoing immune stimulation may be critical. However, parasites were not seen microscopically beyond 24 h postvaccination. We aimed to provide a mechanistic understanding of immune induction. **Methods.** Mice were vaccinated with chemically attenuated *Plasmodium chabaudi* parasites. PCR and adoptive transfer studies were used to determine the presence of parasites and antigen *in vivo*. In other experiments, *Plasmodium falciparum* parasitised red blood cells were attenuated *in vitro* and RNA and antigen expression studied. **Results.** We show that blood transferred from vaccinated mice into naïve mice activates T cells and induces complete protective immunity in the recipient mice strongly suggesting that there is persistence of parasite antigen postvaccination. This is supported by the presence of parasite RNA in vaccinated mice and both RNA and antigen expression in *P. falciparum* cultures treated with CPI drugs *in vitro*. In addition, drugs that block parasite growth also prevent the induction of immunity in vaccinated mice, indicating that some growth of attenuated parasites is required for immune induction. **Conclusions.** Attenuated parasites persist at submicroscopic levels in the blood of mice postvaccination with the ability to activate T cells and induce ongoing protective immune responses.

Keywords: cellular immunity, chemical attenuation, malaria, vaccines.

INTRODUCTION

Malaria vaccine development has a renewed focus on whole-parasite approaches. This arose because of antigenic polymorphism and poor immunogenicity of subunit vaccine candidates. Even the recently licensed vaccine, RTS,S, can provide only modest and short-term protection,[1–3] which correlates with a lack of antigenic boosting following sporozoite exposure and antigenic polymorphism of the vaccine.[4] In 2011 and 2013, the first clinical trials of a whole-parasite malaria sporozoite vaccine were reported.[5,6] Six of six volunteers were protected in one arm of the study following challenge with parasites of a homologous strain. More recently, heterologous immunity following deliberate challenge[7] and protection of adults from natural infection[8] were reported, although the degree of protection was significantly less than that reported following homologous challenge and remains unexplained. Little is known about the factors that regulate immunity to whole-parasite vaccines.

One factor that may be critical to efficacy is parasite dose. It is well known that antigen dose can influence the type of immune response induced. We published data showing that humans and mice exposed to very low doses of blood-stage parasites were able to mount vigorous cellular immune responses ($CD4^+$ T cells and $CD8^+$ T cells) to parasitised red blood cells (pRBC) without demonstrable antibody responses.[9,10] Conversely, a high dose, or patent infection, led to apoptosis of effector T cells. In the murine study, protection was assessed and demonstrated against homologous and heterologous parasite strains.

Recently, we developed a chemically attenuated blood-stage vaccine by treating pRBCs from the rodent parasites *Plasmodium chabaudi* and *Plasmodium yoelii* with various seco-cyclopropyl pyrrolo indole (CPI) analogues, including centanamycin and tafuramycin A (TFA).[11–13] These drugs alkylate parasite DNA. We observed that vaccination induced substantial immune responses and protection. The immunity was long-lasting, dependent on $CD4^+$ T cells and required that the membranes of the attenuated pRBCs were intact. Following immunisation, we could not detect parasites in the blood of mice; however, we did show that parasite DNA could be detected for $>$ 110 days.[11] We hypothesised that persisting submicroscopic attenuated parasites were critical for immunity.

There are little data that document persisting DNA and antigen in malaria immunity and protection following vaccination. One study with *P. berghei ANKA* irradiated sporozoites found parasite DNA persisting in hepatocytes of rats and mice for up to 6 months after immunisation.[14] DNA was detected by *in situ* hybridisation of liver sections. Treatment of immunised rats with primaquine to eliminate the exoerythrocytic stages impacted protective immunity. Animals that received primaquine 7 days after inoculation with irradiated sporozoites and then challenged with sporozoites were fully protected (100%), but protection waned when rats were challenged on day 30 (58% protection) or day 90 (16% protected). However, treating immunised rats with primaquine 1 month after immunisation did not abrogate protection (for up to 90 days).

Additional studies in mice utilising irradiated sporozoites genetically attenuated sporozoites, or sporozoites administered under chloroquine cover found similar results for antigen persistence. Cockburn *et al.*[15] demonstrated that the circumsporozoite protein (CSP) present on the surface of sporozoites persists for >8 weeks after immunisation with irradiated sporozoites. This was demonstrated by adoptive transfer of CSP-specific $CD8^+$ T cells into immunised mice where the T cells proliferated, produced cytokines and expressed cell surface markers consistent with engaging CSP in the recipient mice. Similar results were observed by Butler *et al.*[16] In all studies, there was no significant reduction in CSP persistence as measured by T-cell activation when assessed up to 45 days postvaccination. Administration of live sporozoites under chloroquine treatment (which allows liver stage development and suppresses blood-stage infection) protects mice.[17] Concurrent treatment with primaquine abrogates liver stage development and protection. These data are consistent and relevant to liver stage immunity, but the need for antigenic persistence in blood-stage immunity has not been studied.

We investigated persistence of parasite nucleic acids (RNA and DNA) as well as antigen using a variety of methods for chemically attenuated blood stages of rodent (*Plasmodium chabaudi AS*) and human (*Plasmodium falciparum*) malaria species. We examined the role of antigen persistence in protection and observed that although only very low levels of antigen are required to induce immunity, vaccine efficacy is lost if parasites are eliminated by drug treatment early after exposure. These results are critical to the development of vaccines for human malaria parasites.

RESULTS

Low levels of persisting nucleic acids postvaccination

We initially confirmed previous data showing the persistence of parasite DNA in the blood of mice postvaccination.[11] Similar to what was described previously, parasite DNA was detectable intermittently in the blood during this period (Table 1). The blood of all mice that received a WT infection (no chemical attenuation) remained positive for DNA and RNA at all time points, although these mice succumbed to infection prior to the day-13 analyses (data not shown). We also detected DNA in the liver of at least two mice that had received attenuated parasites from each time point up to day 13.

To ascertain whether parasites were metabolically active, we used real-time PCR to detect transcripts of the 18S rRNA gene from the same mice as above (Table 1, Supplementary table 1). RBCs were positive in all four mice at 1 h and at day 1. However, the RBCs of all mice were negative on days 2, 5 and 13. However, PCR from liver tissue was positive in two of three mice at day 13 and splenic tissue was positive in the same two mice. Mice that had detectable RNA by PCR all had DNA detectable by PCR. These data suggested the presence, at low level, of metabolically active parasites able to synthesise new RNA transcripts up to 2 weeks postattenuation. Because mice were sacrificed at each time point, we were unable to follow DNA or RNA longitudinally in individual mice; however, we previously showed that DNA levels in blood fluctuated near the level of detection in the same mice over a 6-month period,[11] consistent with an immune response suppressing, but not eliminating, an active infection.

We then studied RNA expression in attenuated *P. falciparum* pRBC cultured *in vitro* using fluorescence *in situ* hybridisation (FISH). *P. falciparum* cultures were either treated with 2 μM TFA or DMSO for 30 min, washed and returned to culture. Fluorescence from a peptide nucleic acid (PNA) probe specific for the 18S rRNA gene was detected in parasites 48 h after treatment with DMSO (Figure 1a) or 2 μM TFA (Figure 1b). Fluorescence was not observed in

Table 1. Persistence of DNA and RNA to the 18S rRNA gene as determined by qPCR

		1 h		1 day		2 days		5 days		13 days	
Mouse	Sample	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
1	RBC	+	+	+	+	–	–	–	–	–	–
	Spleen	+	+	+	–	–	–	–	–	–	–
	Liver	+	+	+	–	–	–	+	–	+	–
2	RBC	+	+	+	+	+	–	–	–	–	–
	Spleen	+	+	+	–	–	–	–	–	+	+
	Liver	+	+	+	–	–	–	+	–	+	+
3	RBC	+	+	+	+	–	–	–	–	+	–
	Spleen	+	+	+	–	–	–	–	–	+	+
	Liver	+	+	+	–	+	–	+	–	+	+
4	RBC	+	+	+	+	–	–	–	–		
	Spleen	+	+	+	–	–	–	–	–		
	Liver	+	+	+	–	+	–	+	–		

pRBCs, parasitised red blood cells; TFA, tafuramycin A.

BALB/c mice were immunised with a single dose 10^6 *P. chabaudi* AS pRBCs attenuated with 2 μM TFA i.v. At the times indicated, 3 or 4 mice were sacrificed and blood, spleens and livers processed. RNA and DNA were amplified to the 18S ribosomal RNA gene. (+) indicates two or three of triplicate samples had $C_t < 40$. (–) indicates no amplification or at least two of the triplicates showed a C_t value > 40. C_t values for the RNA studies are given in Supplementary table 1.

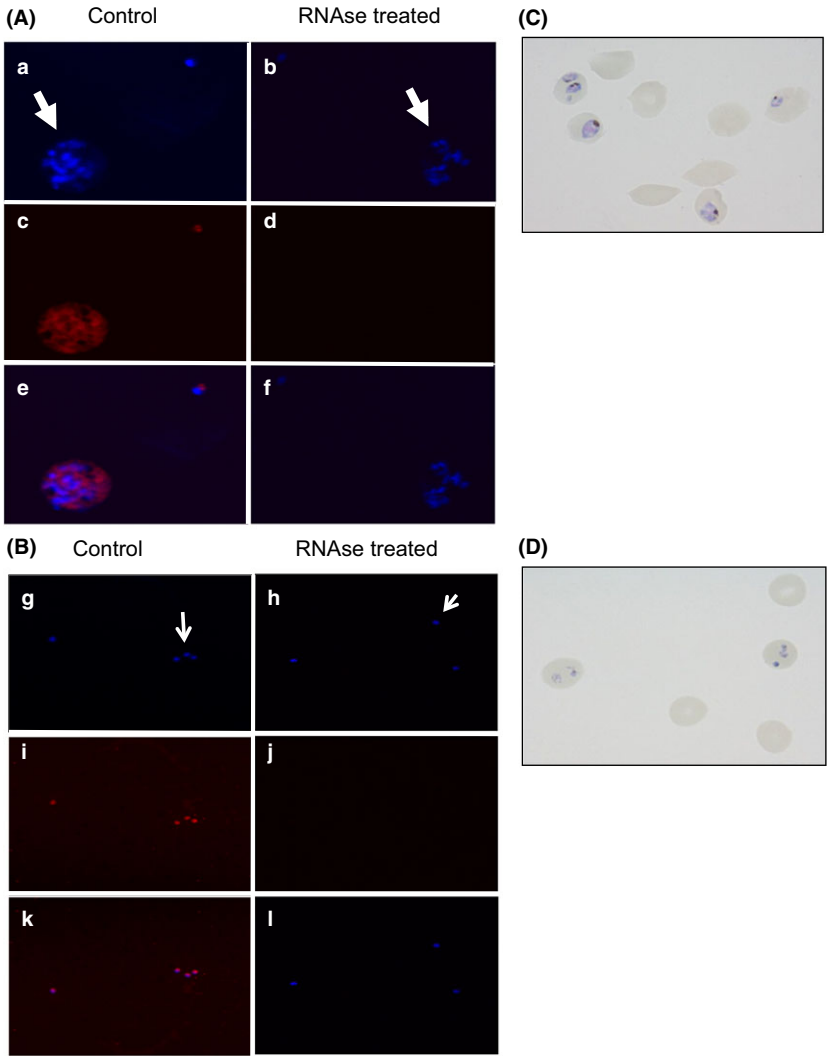

Figure 1. Fluorescence *in situ* hybridisation analysis of effect of tafuramycin (TFA) treatment on *Plasmodium falciparum* RNA transcription at 48 h post-treatment. *P. falciparum*-infected erythrocytes were treated with either DMSO **(a)** or 2 μM of TFA **(b)** for 30 min, washed and returned to culture. Parasite RNA transcription status was assessed at 48 h by treating the slides with either 1 mg mL^{-1} of RNAse A (b, d, f, h, j, l) or a control buffer (a, c, e, g, i, k). Blue indicates DAPI intercalation into DNA of the nucleus of the parasite. Red indicates Texas red conjugated to the 18S ribosomal RNA probe. Magenta in the merged images (e, f, k, l) indicates colocalisation of 18S rRNA and DNA. Giemsa staining of parasites from DMSO **(c)** and TFA **(d)** treated cultures. Arrows, rings; large arrow, schizonts. DAPI, 4′,6-diamidino-2-phenylindole.

slides treated with RNAse A (Figure 1), demonstrating that the probe primarily detected parasite RNA. The RNA probe colocalised to the 4′,6-diamidino-2-phenylindole (DAPI) staining of the parasite DNA in the nucleus demonstrating that RNA transcripts were present 48 h post-TFA attenuation of *P. falciparum* cultures. As the average half-life of RNA is between 9.5 and 65.4 min (from ring to late schizont),[18] this suggested that new RNA transcripts were synthesised during this time period even in TFA-attenuated parasites.

Attenuated parasites express antigen and induce immunity

We then analysed the morphology of specific antigens and structural proteins in *P. falciparum* cultures following treatment. Due to unavailability of TFA at that time, these experiments were performed using the closely related seco-CPI analogue, centanamycin (CM). Both TFA and CM attenuate parasites to a comparable degree, and rodent parasites attenuated with either drug induce comparable

protection as determined by significantly reduced parasite burden and clinical scores following challenge infection.[11] Asynchronous *P. falciparum* 3D7 cultures (or synchronised as rings or schizonts) were treated with CM. Smears were prepared 48 h postdrug treatment and immunofluorescence staining performed to detect parasite DNA and different antigens. Smears indicated the presence of parasites in culture for up to 7 days (Supplementary figure 1). However, from 48 h, unhealthy and dying parasites were visible in thin smears of attenuated parasites and absent in control cultures (Supplementary figure 1b). Cultures were stained with an antibody to EBA-175, a microneme marker that emerges in late-stage schizonts[19] and appears as a ring around each nucleus in control parasites (Figure 2). In CM-treated parasites, EBA-175 staining was either absent or weak and diffuse. These results suggested that the production and localisation of EBA-175 antigen in late schizonts are disrupted within 48 h of CM treatment. Next, we examined an antigen expressed earlier in the life cycle, RAP-1, which is localised to vesicles

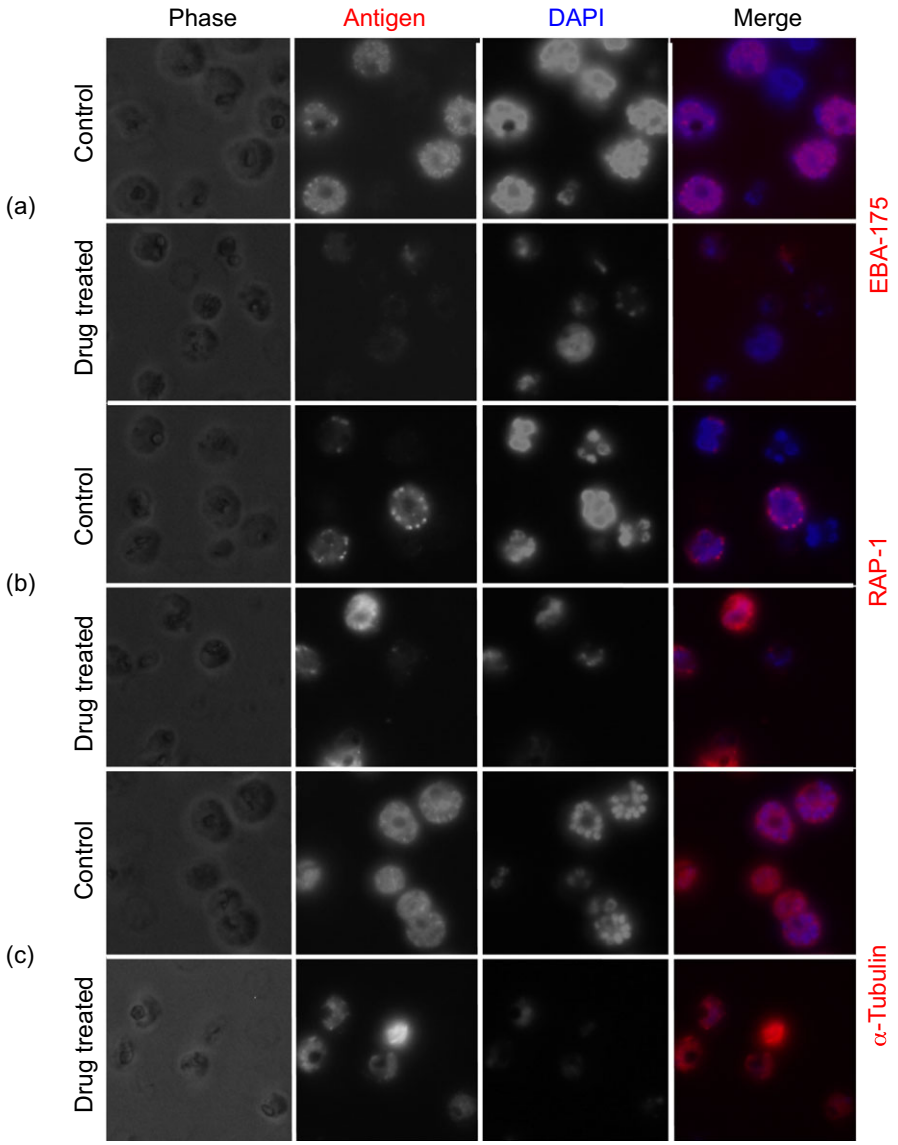

Figure 2. Expression of antigens in CM-attenuated *Plasmodium falciparum* at 48 h post-treatment. **(a)** EBA-175 staining of control CM-attenuated *P. falciparum* cultures. EBA-175 (red), microneme marker, DAPI DNA stain (blue). **(b)** RAP-1 staining of control and CM-attenuated *P. falciparum* cultures. RAP-1 (red), rhoptry marker, DAPI DNA stain (blue). **(c)** α-Tubulin staining of control and CM-attenuated *P. falciparum* cultures. α-Tubulin (red) microtubules, DAPI DNA stain (blue). DAPI, 4′,6-diamidino-2-phenylindole; CM, centanamycin.

within rhoptries during the third nuclear division of schizogony and remains visible as rhoptries mature.[20,21] RAP-1 vesicles are arranged in a regular pattern around the nucleus in control cells but are clumped together with diffuse staining in CM-treated parasites (Figure 2). RAP-1 antigen expression was present in attenuated parasites; however, the localisation was disrupted. Additionally, staining was performed for parasite-specific α-tubulin (which forms a heterodimer with β-tubulin to create microtubules), as this is critical for parasite development including mitosis.[22] In control parasite cultures, the distribution of α-tubulin is observed within the cytoplasmic microtubules of the nascent daughter parasites (Figure 2) while diffuse and disorganised within the attenuated parasites. Additionally, DAPI staining of the parasite nuclei was diffuse at 48 h post-CM treatment, likely from arrested parasite growth and DNA damage (Figure 2). These results demonstrate that parasite structural components are present post-treatment, but their expression pattern is atypical.

Having found evidence to support *P. chabaudi* and *P. falciparum* persistence postchemical attenuation, and in particular the ability of *P. falciparum* antigens to persist albeit in a dysregulated structure *in vitro*, we hypothesised that antigen would persist *in vivo.* We reasoned that if antigen did persist *in vivo*, then the blood of previously vaccinated mice would be able to induce a parasite-specific immune response when transferred to naïve mice.

One hundred microlitres of blood was transferred from mice that were immunised 7 days prior with 10^6 TFA-attenuated normal RBCs (nRBC controls) or 10^6 TFA-attenuated *P. chabaudi*-infected RBCs (parasite vaccine) into ten naïve mice. This procedure was repeated twice such that the recipient mice received three inoculations, each 2 weeks apart. To exclude transfer of activated immune cells, which may have mediated protection, the blood was irradiated at 18 Gy prior to transfer. This dose is sufficient to prevent lymphocyte proliferation[23] but over 50 times lower than the dose to adequately kill blood-stage parasites.[24] Other mice received the attenuated parasite vaccine at the same time, and controls received nRBC treated with TFA. The procedure is outlined in Figure 3a. One week after each blood transfer, mice were bled for qPCR (parasite DNA) and flow cytometry to assess T-cell activation. Modest activation of $CD4^+$ T cells was observed after the primary transfer (Figure 3b), and $CD8^+$ T cells were activated after the first and second blood transfers (Figure 3c). Even though $CD4^+$ T cells, and not $CD8^+$ T cells, are known to be critical for protection following vaccination with chemically attenuated pRBC,[11,13] significant activation of both $CD4^+$ and $CD8^+$ T cells is observed in the peripheral blood following administration of vaccine. The amount of parasite DNA detected by qPCR from pRBC-vaccinated mice increased with additional transfers of blood; no DNA was detected after primary transfer but was detected in some mice after boosts (Figure 3d). In keeping with the evidence of immunological activation, we observed that mice that received either the attenuated pRBC vaccine or blood from the cohort of vaccinated mice were protected against parasitaemia and lethal challenge (Figure 3e) while controls developed high parasitaemia and succumbed. Although a small number of immune cells would have been transferred during the blood transfers, these cells were irradiated and the number of cells would have been significantly less than what is expected for nonirradiated cells to transfer protection.[25] Furthermore, we tested the ability of irradiated spleen cells to proliferate and secrete cytokines in response to parasite stimulation. Whereas nonirradiated splenocytes from TFA-treated pRBC-vaccinated mice proliferated vigorously to parasite stimulation *in vitro* and secreted interferon-γ and IL-2, irradiated splenocytes neither proliferated nor secreted cytokines in response to parasite (data not shown). It is most unlikely that transferred serum antibodies would have contributed to protection since transfer of 1.5 mL of serum from mice vaccinated thrice with chemically attenuated parasites did not alter parasite burden over the course of infection in challenged mice compared with mice that received control serum although mice that received control serum did succumb to malaria earlier than those that received 1.5 mL of serum from immune mice (Supplementary figure 2).

Malaria chemotherapy prevents immune induction following vaccination

To confirm the need for persisting parasite for induction of immunity, we next vaccinated mice but treated them with two separate chemotherapy regimens to destroy infected RBCs. Mice were immunised with three doses of TFA-

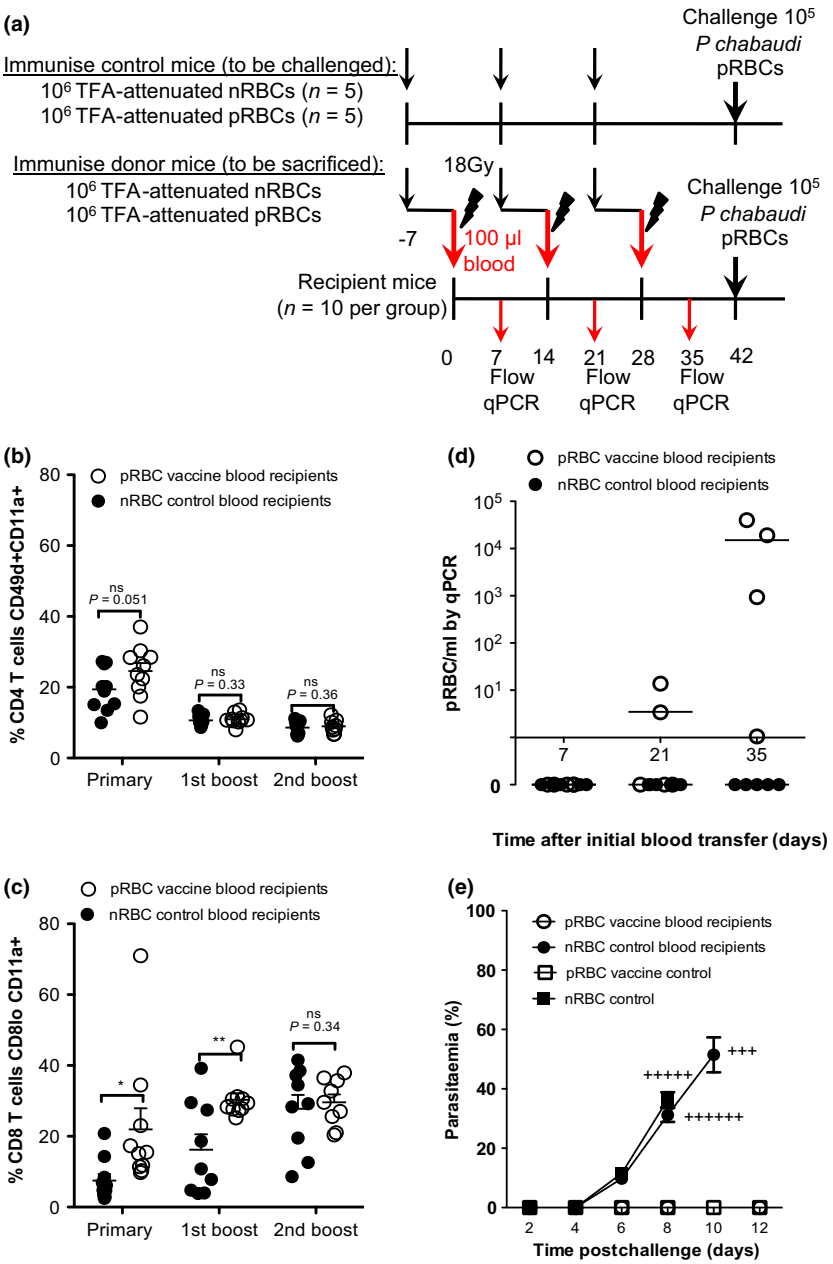

Figure 3. Adoptive transfer of irradiated blood from TFA-attenuated nRBCs and pRBCs. **(a)** Experimental timeline and schematic. Proportion of antigen-experienced CD4 **(b)** and CD8 **(c)** T cells in blood of recipient mice on day 7 after each adoptive transfer (*$P < 0.05$; **$P < 0.01$). **(d)** qPCR to detect parasite DNA in recipient mice day 7 after each adoptive transfer. **(e)** Parasitaemia after challenge of recipient and control mice with 10^5 *P. chabaudi* AS pRBCs i.v. As shown in this experiment, there were 10 donor mice and 20 recipient mice. TFA, tafuramycin A; pRBCs, parasitised red blood cells; nRBCs, normal RBCs.

attenuated pRBCs (vaccine) or TFA-treated nRBCs as a control, or infected with wild-type *P. chabaudi AS* and treated with malaria chemotherapy at 24 h postinoculation as depicted in Figure 4a. Mice received five consecutive doses of Malarone™ (containing atovaquone, a parasite electron transport chain inhibitor, and proguanil that via its metabolite cycloguanil acts as a dihydrofolate reductase inhibitor) or seven consecutive doses of doxycycline (an antimicrobial that inhibits parasite protein synthesis and destroys the apicoplasts) commencing 24 h postvaccination. Mice that received the vaccine (no chemotherapy) controlled parasite growth

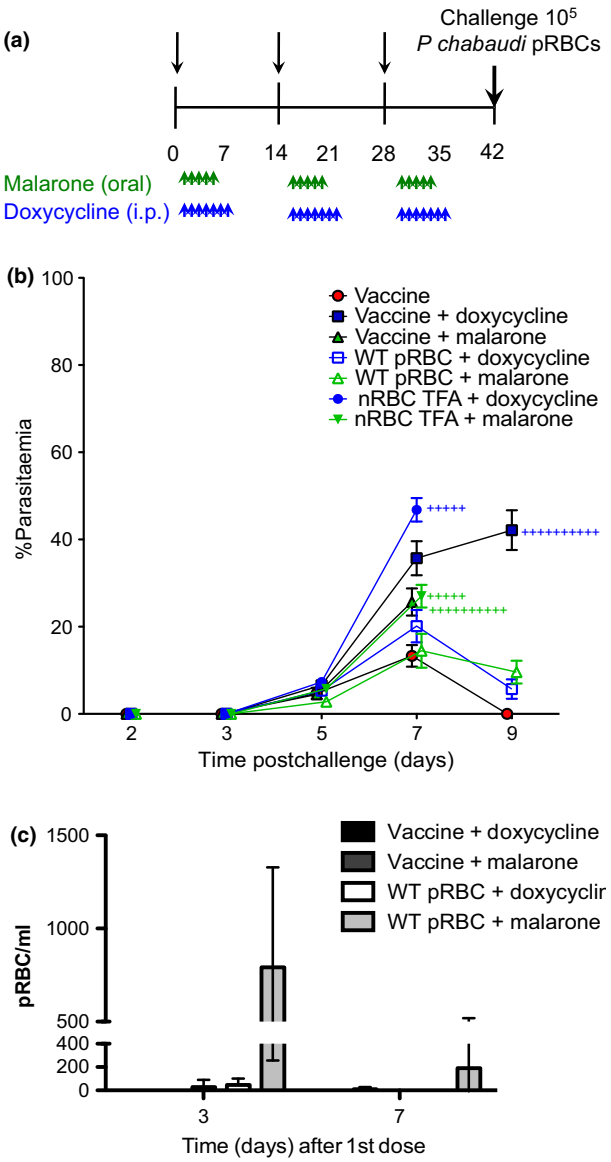

Figure 4. Malaria chemotherapy after vaccination abrogates protection. **(a)** Experimental timeline and schematic. **(b)** Parasitaemia and survival after challenge of recipient and control mice with 10^5 *P. chabaudi* AS pRBCs i.v. + = mouse died. **(c)** Detection of parasite DNA by qPCR after first dose vaccine or wild-type infection. In this experiment, 10 mice per group were used for the vaccine groups and five mice per group were used for the nRBC control groups. pRBCs, parasitised red blood cells; nRBC, normal RBC.

and survived similar to the wild-type infection and drug-cure groups (Figure 4b). All control mice that received TFA-attenuated nRBCs succumbed to the lethal infection. Similarly, the vaccinated mice that received doxycycline or Malarone™ at 24 h postinoculation all developed high parasitaemia and died. DNA was not detected in mice on days 3 or 7 after vaccination in mice that received Malarone™ and only at the limit of detection ($C_t > 40$) in the vaccinated mice treated with doxycycline (on day 3 postvaccination) (Figure 4c). We could not detect parasite DNA by qPCR in the vaccinated and treated mice prior to challenge (data not shown). Thus, killing of the vaccine parasites with chemotherapy blocked the generation of protective immunity normally observed by vaccination. These results strongly suggest that parasite persistence is required for longer than 24 h and that this, as measured by DNA and RNA, is likely to be crucial in mediating the protection observed after blood transfer 7 days postimmunisation.

DISCUSSION

In this study, we provide strong evidence that chemically attenuated parasites persist in vaccinated animals at low levels and that treatment of vaccinated mice with antimalaria chemotherapy prevents the acquisition of immunity. These data, together with evidence of persisting DNA and RNA, and evidence strongly suggesting the persistence of antigen as well, provide an understanding of the mechanism of induction of protective immune responses following vaccination with chemically attenuated parasites.

The primary evidence comes from studies whereby blood from vaccinated mice, taken 1-week postvaccination, was able to adoptively immunise naïve mice. Parasite DNA was detectable in the blood of some of the recipient mice after second transfer and third transfer; however, it seems very unlikely that transferred DNA, *per se*, was responsible for inducing protection in recipient mice. We previously showed that large numbers of killed parasites (without adjuvant) are unable to induce immunity.[26] It thus seems most likely that persisting antigen in the blood of mice, and still within RBCs,[11] has induced both a cellular immune response and protection.

This, together with evidence of T-cell activation post-transfer, provides strong evidence that transferred antigen was responsible for inducing protection. Furthermore, the ability of two separate antimalaria chemotherapy regimens (doxycycline or Malarone™) to prevent induction of immunity provides further evidence that not only was the transferred antigen present in the form of parasitised RBCs but also that the parasites responsible for induction of immunity were replicating. Doxycycline causes loss of

apicoplasts essential for parasite growth,[27] and Malarone™ interferes with pathways involved in the biosynthesis of folate cofactors required for DNA synthesis as well as collapsing mitochondrial membrane potential.[28] To our knowledge, these drugs are not known to have any effect on parasites that are already dead. While we cannot prove that there was subpatent parasite growth post-transfer of blood from vaccinated mice, we note that the number of parasites detected by qPCR in peripheral blood post-transfer did increase over time.

We studied attenuated *P. falciparum* parasites to investigate the expression and localisation of parasite-specific antigens and demonstrated that antigens were expressed but mislocalised. Additionally, many parasites were unhealthy and began to die at 48 h post-treatment and were not able to maintain sustained detectable growth in culture. However, at 48 h, attenuated parasites still had detectable RNA, suggesting that they maintained some functional and metabolic activity. We could not quantify how many parasites were killed *in vitro*. However, by 48 h all RNA present within the parasites at the time of attenuation would have been metabolised; thus, attenuated parasites appear able to synthesise new RNA transcripts. Together, these results suggested that although attenuated parasites have greatly reduced viability and growth, they are able to maintain some functionality at least during the first 48 h of culture. The aberrant expression of parasite antigens observed in attenuated *P. falciparum* parasites 48 h post-treatment does not suggest that these antigens have lost their immunogenicity. Clearly, they are still recognised by specific antibodies and there is no reason to suspect that their ability to stimulate T cells would have been diminished as T cells recognise epitopes from processed antigens. However, we are not suggesting that the *P. chabaudi* homologues of the antigens that we have studied are specifically responsible for induction of immunity. Rather, it is likely that many antigens remain and are able to stimulate protective T-cell responses.

The question as to why parasites may persist in immune animals has not been answered. One possibility is that immune mice are able to control but not eliminate residual parasites. This would explain why we have been able to detect DNA for over 100 days.[11] The lifespan of an uninfected mouse red blood cell is approximately 40 days, suggesting that parasite DNA detected beyond that time is due to parasite replication or persistence of DNA in some other form. We have previously shown that plasmacytoid dendritic cells can harbour infectious parasites, but their lifespan is even shorter than that of RBCs.[29] Live *P. berghei* parasites have been shown to persist for an extended period of time even in the presence of immunity.[30] Our PCR data showed that in most mice that had received attenuated parasites, DNA could be detected in liver tissue throughout the study period, suggesting that low-level parasite replication was occurring in that organ. Of interest, we observed previously that dendritic cells in the liver contained parasitised RBCs for at least 1-week postvaccination.[11] We do not know whether the attenuated parasites are replicating within RBCs residing within the liver or in an alternative drug-induced form in the liver. A plausible explanation for vaccine-induced immunity is that sustained parasite replication is occurring in the liver and that dendritic cells in that location initiate protective responses.

Whole parasite vaccines for malaria are now being assessed in human trials, but their interaction with the immune system and mode of action are poorly understood. The data presented here strongly suggest that attenuated parasites persist at levels undetectable by standard diagnostic methodologies. Antigens from these persistent parasites stimulate immune responses critical to induction of protective immunity. As such, these results are of great significance to the development of whole-parasite vaccines for malaria.

METHODS

Mice

Four- to 6-week-old female A/J mice (H-2^a) and BALB/c (H-2^d) were purchased from the Animal Resource Centre (Willetton, Western Australia).

Parasites

Cloned lines of *P. chabaudi* AS parasites were obtained from Queensland Institute of Medical Research from stock provided by Richard Carter (University of Edinburgh, United Kingdom). Challenge infections were performed by i.v. injection of 10^5 pRBCs. Studies with *P. falciparum* were performed with strain 3D7.

PCR

Isolation of parasite DNA and qPCR was as described[11] detecting the 18S ribosomal RNA gene of *P. chabaudi* AS.

Attenuation

Chemical attenuation of *P. chabaudi* AS parasites was performed as described[11] using 2 μM of the CPI analogues, TFA or CM. *P. falciparum* parasites were grown in 96-well plates as described.[20] For attenuation, the cultures were treated with CM or TFA for 30 min at 37°C. For controls, cultures were treated with DMSO for 30 min at 37°C. All *P. falciparum* parasite cultures were washed twice with complete RPMI to remove CM, TFA or DMSO and then returned to culture. At different time points postattenuation (e.g. 48 h), the effect of CM or TFA was examined by antigen expression and FISH. For CM-attenuated cultures, parasite survival and growth were monitored by thin blood film microscopy performed daily, on days 1–7 and then continued on days 14, 21 and 28.

Antigen expression studies

Plasmodium falciparum blood-stage parasites that were magnetically enriched to prepare the mature schizonts were fixed in 3% paraformaldehyde, 10 mM piperazine-N,N′-bis (2-ethanesulfonic acid) buffer (PIPES) pH 6.4, PBS by incubation for 30 min at room temperature on 22 mm^2 poly-L-lysine-coated coverslips and then permeabilised for 10 min with 0.25% Triton X-100, PBS. Coverslips with parasites were washed in PBS, and blocked for 1 h in 3% BSA in PBS and then incubated for 1 h at room temperature with the following antibodies: mouse monoclonal antibodies directed against EBA 175 (1:50 dilution), RAP1 (1:100 dilution) and tubulin (1:500 dilution) diluted in blocking buffer. After three washings in PBS, the coverslips were incubated for 30 min with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:500) (Invitrogen) and then washed three times in PBS. The samples were fixed for 15 min in 3% paraformaldehyde, washed twice in PBS and incubated for 10 min in 2 μg mL^{-1} DAPI. After two washings in PBS, the coverslips were briefly rinsed in dH_2O, mounted onto slides with Vectashield antifade medium and sealed with nail polish. Widefield epifluorescence images were acquired by using a 100 × 1.3NA oil objective on an Olympus microscope equipped with a DP71 CCD camera.

Fluorescence *in situ* hybridisation

Giemsa-destained thin blood smears were washed with 1× PBS twice for 2 min. Excess buffer was removed, and the slides were treated either with 1 mg mL^{-1} of RNAse A or 1× PBS containing 5 mM of $CaCl_2$ and 5 mM $MgCl_2$ for 30 min at 37°C. After three washes with 1× PBS, the slides were placed for 3 min in a protease solution consisting of 25 μL of 100 mg mL^{-1} pepsin stock in 35 mL of 10 mM HCl. Slides were thoroughly rinsed with 1× PBS, followed by increasing concentrations of ethanol solution (75%, 80% and 100%) for 2 min, and then air-dried. Ten microlitres of Texas red-labelled specific PNA probe [75 μM of PNA in 70% formamide, 10 mM Tris, pH 7.5, 1% Human Cot-1 DNA (Abbott Molecular Inc)] was applied to the sample, which was coverslipped, and denaturation was performed by incubation for 3 min at 90°C. Slides were then moved to a dark, closed container for hybridisation at 37°C for 2 h. Coverslips were carefully removed, and the slides were washed sequentially in 0.2× SSC buffer at 42°C for 20 min and 2× SSC buffer containing 0.2% of Tween 20, followed by a 1-min wash in 1× PBS. At this step, slides were mounted with Vectashield (Vector Labs, CA, USA) antifade mounting medium containing DAPI and stored at 4°C until used. The PNA probe complementary to *P. falciparum* 18S ribosomal RNA gene (PNA Bio Inc, CA, USA) has the sequence 5′-TCATCTTTCGAGGTGAC-3′ with an N-terminal covalently linked Texas red fluorescent dye. The probe stains both DNA and RNA. Staining is primarily detected from RNA, as the DNA concentration is low relative to RNA concentration.

Confocal microscopy

Images were taken using a Leica SP5 laser scanning confocal microscope equipped with 100X/1.44 N.A. oil-immersion objective lens. A 405-nm laser was used for excitation of DAPI and a 543-nm laser for Texas red. The microscope hardware was controlled, and data were captured using the LAS AF software (version 3.30). All images were captured using identical zoom and pinhole settings, while images for each

fluorophore were captured using identical laser power settings, photomultiplier gain and offset.

Persisting nucleic acid detection by PCR

BALB/c mice were immunised with a single dose 10^6 *P. chabaudi* AS pRBCs attenuated with 2 μM TFA i.v. At 1 h and then at days 1, 2, 5 and 13 postvaccination, mice were sacrificed (4 mice per time point except 3 mice for day 13) and blood, spleens and livers harvested. RNA was isolated from 50 μL of RBCs, spleen cells and liver tissue (50 mg). Samples were lysed with TRIzol and purified using RNA Mini Kit (Life Technologies). RNA was quantified using Nanodrop, and 500 ng RNA was treated with DNase I to remove genomic DNA. cDNA was made using random hexamers and PCR targeting the 18S rRNA gene was performed. For DNA estimation, a positive (+) response was recorded if the cycle threshold (C_t) values for two or three of the triplicate samples were < 40. For RNA estimation, a positive response was recorded if the C_t values for two or three samples in the presence of reverse transcriptase were < 40. In all tests, the negative reverse transcriptase controls gave either no amplification or at least two of the triplicates showed a C_t value > 40, indicating the absence of any significant amounts of genomic DNA.

Adoptive transfer studies

AJ mice were immunised as outlined below with 10^6 pRBCs or 10^6 naïve nRBCs, both attenuated with 2 μM TFA and administered by i.v. injection. Seven days after vaccination, donor mice were sacrificed, cardiac punctures performed and blood irradiated with 18 Gy. Subsequently, 100 μL of irradiated blood was transferred into recipient mice. Blood from recipient mice was taken via submandibular bleeds at days 7, 21 and 35 postinitial blood transfer for flow cytometry (day 7 after each blood transfer and boost) and qPCR performed. qPCR was done as previously described.[11]

Flow cytometry

To assess T-cell activation in blood, flow cytometry was done as previously described.[11] Briefly, RBCs were lysed with ACK and Fc-receptors blocked with supernatants from cell line 2.4G2 (ATCC) for 10 min on ice. Samples were stained on ice for 20 min with the following antibodies: anti-CD4V500 (Clone RM 4-5), anti-CD8 PerCP-Cy5.5 (Clone 53-6.7), anti-CD11a FITC (Clone 2D7), anti-CD49d PE (Clone R1-2). After washing, cells were re-suspended in 1% paraformaldehyde in PBS, kept at 4°C protected from light until run on a BD LSRFortessa cytometer. Data were analysed with FlowJo software.

Malaria chemotherapy

Twenty-four hours after vaccination with 10^6 pRBCs or 10^6 naïve nRBCs both attenuated with 2 μM TFA or inoculation with 10^6 wild-type (nonattenuated) *P. chabaudi* AS parasites some mice received 1 mg doxycycline (i.p.) for 7 days, or Malarone™ orally (208 μg atovaquone, 83 μg proguanil) for 5 days as outlined below. The numbers of mice per group were TFA-attenuated *P. chabaudi* AS vaccine ($n = 5$), TFA-attenuated *P. chabaudi* AS vaccine plus Malarone ($n = 10$), TFA-attenuated *P. chabaudi* AS vaccine plus doxycycline ($n = 10$), TFA-attenuated nRBCs ($n = 5$), TFA-attenuated nRBCs plus Malarone ($n = 5$), TFA-attenuated nRBCs plus doxycycline ($n = 5$), infection with wild-type *P. chabaudi* AS parasites plus Malarone ($n = 5$), wild-type *P. chabaudi* AS parasites plus doxycycline ($n = 5$). Mice were inoculated on days 0, 14, 28 and were challenged with 10^5 *P. chabaudi* AS pRBCs i.v. 2 weeks after the final inoculums of vaccines, infection or TFA-attenuated nRBCs.

Statistical analysis

All statistical analysis was conducted using GraphPad Prism software version 6. An unpaired, one-tailed *t*-test was used when comparing two experimental groups. Data represent mean ± SE.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.