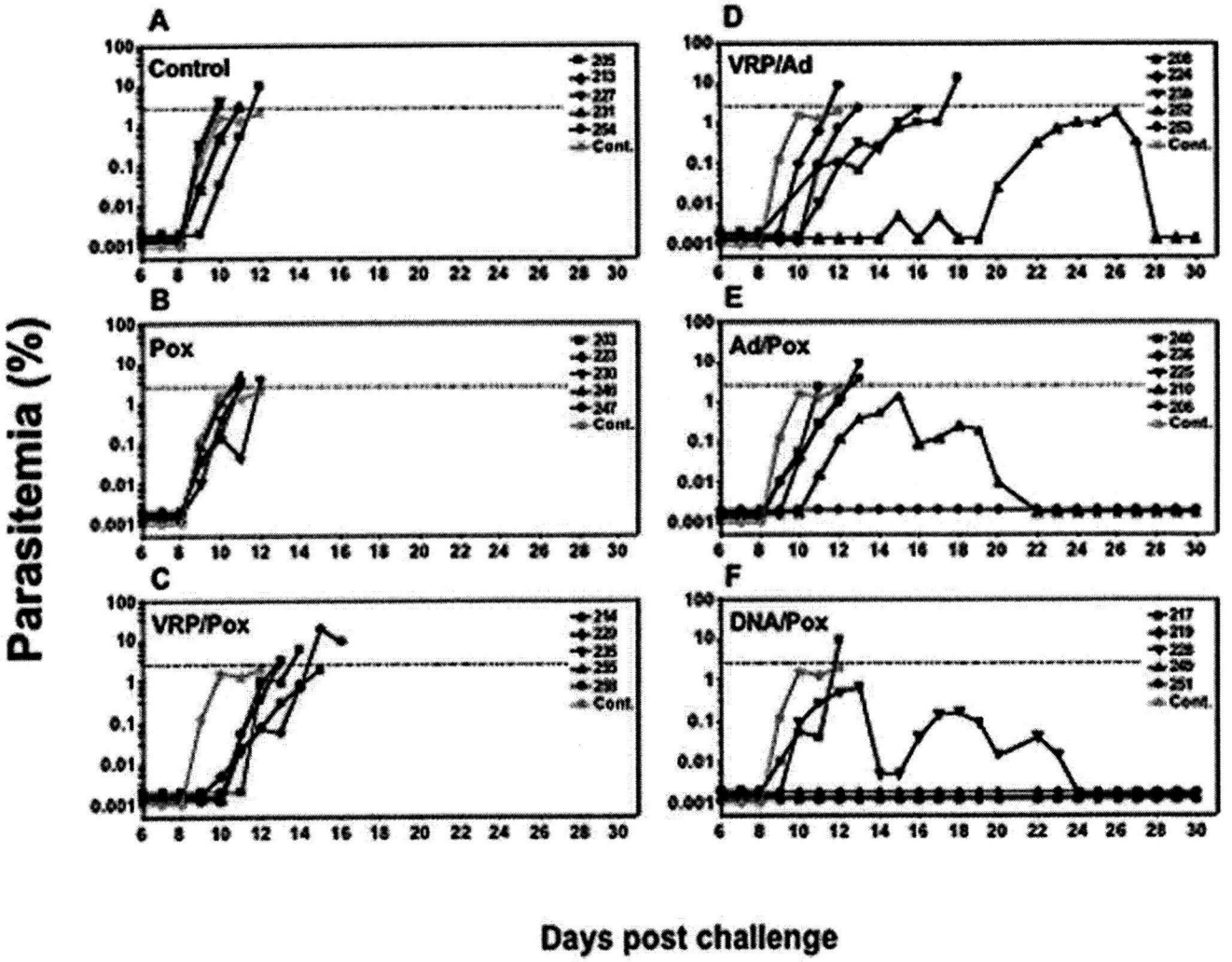


Figure 1. Daily parasitemias from individual monkeys after sporozoite challenge. Panel A, Control group: average parasitemia levels of 5 individual animals was presented as a thick grey line (Cont.) and is included in all 6 panels for comparison; B, Pox group; C, VRP/Pox group; D,VRP/Ad group; E, Ad/Pox group; F, DNA/Pox group; The dotted line in each panel shows the parasitemia level 2% at which we treated animals with anti-malaria drug. One monkey (206) in Panel E, and 3 monkeys (219, 249 and 251) in Panel F that had no detectable parasitemia are shown as horizontal lines.
doi:10.1371/journal.pone.0006559.g001

9.5 days). One of the two infected animals controlled its initial parasitemia at 1% and then self-cured, while the other exceeded 2% parasitemia on day 12. We have never previously sterilely protected such a high proportion of animals given any Pk4 vaccine. We discuss possible reasons for this high level of protection below.

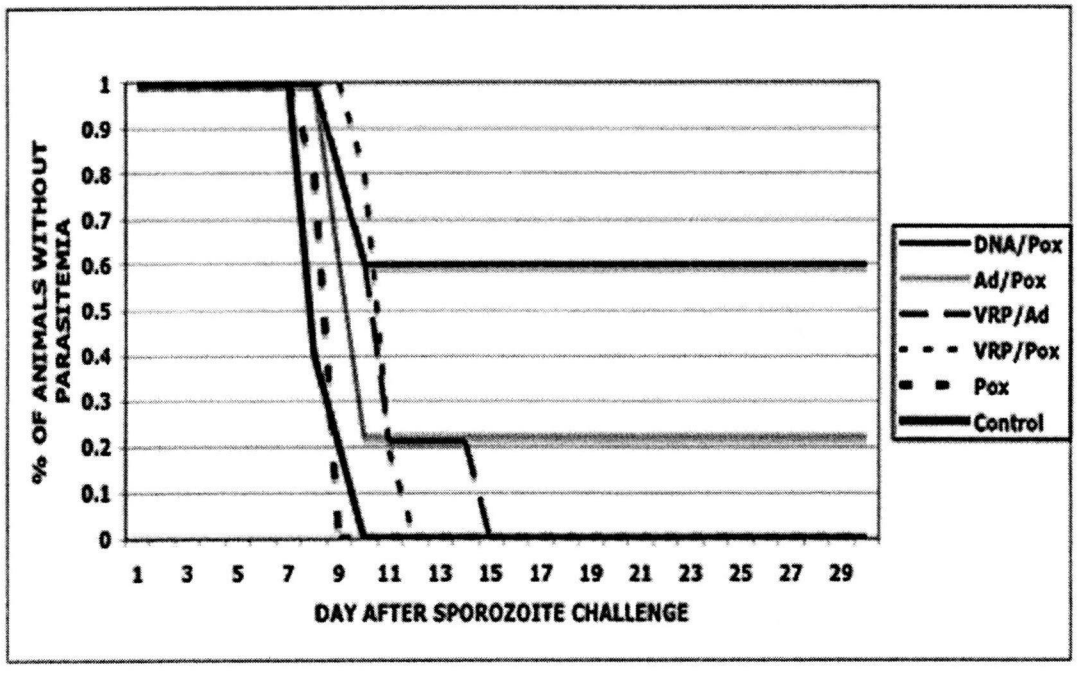
Fig. 2a shows Kaplan-Meyer curves of the percentage of animals in each group having parasites detected in the blood by day after challenge. Figure 2b is a similar graph showing the percentage of each group exceeding 2% parasitemia by day after challenge. The DNA/Pox group is the only vaccine group that had any endpoints statistically different from the Control group ($p = 0.06$ and 0.02 for day of first parasitemia and>2% parasitemia respectively, Log-rank Test). The other vaccine groups appear less protective than the DNA/Pox vaccine but differences do not reach statistical significance.

Pk4 vaccine effects against specific stages of the malaria lifecycle.

Because the Pk4 vaccine contains antigens that are expressed in sporozoites, liver stages, and blood stages of malaria, it is difficult to assign protective roles to particular vaccine components. CSP and SSP2 are found on sporozoites and in early hepatic stages. AMA1 and MSP1, which are expressed during late hepatic stages and merozoites, could contribute to protection at both the hepatic and blood stages of infection. In addition, there is evidence that AMA1 is present in sporozoite[31]. The time to first detection of parasites in the blood could be increased by vaccine effects at several points in the life cycle: by inhibition of sporozoite invasion of liver cells, by killing of infected hepatocytes or prolongation of hepatic parasite maturation, or by inhibition of parasite replication in red cells. Because a slowing of parasite growth provides more time for induction of immune responses to the blood stages of the parasite, delays in the early phases of infection could also affect peak parasitemias. Thus prolongation of either endpoint (time to first parasite detected in the blood or time to reach>2% parasitemia) may indicate a mixture of stage-specific and antigen-specific effects.

However, two outcomes have clear relationships to protection against specific stages of the parasite life cycle. The first is sterile protection. On the assumption that the release of any parasites

A



B

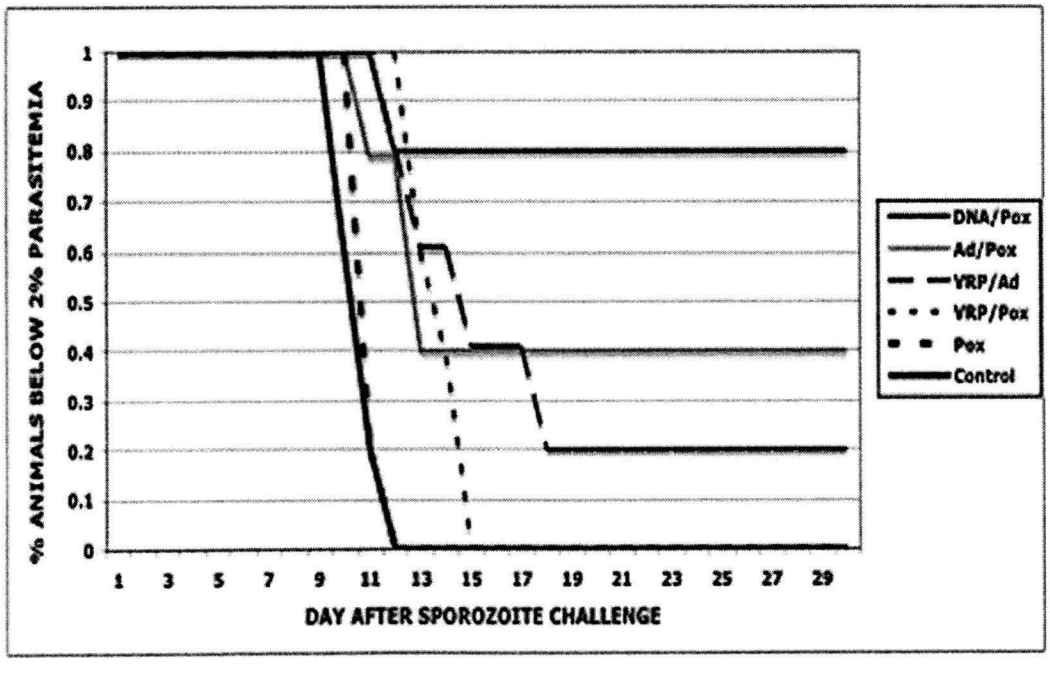


Figure 2. Kaplan-Meyer curves showing parasitemia endpoints for the six experimental groups. Panel A. shows the percentage of animals in each vaccine group without parasites detected in blood. Panel B. shows the percentage of animals with parasitemia below 2%. X axis shows day since sporozoite challenge. In each panel the DNA/Pox group shows the highest level of protection.
doi:10.1371/journal.pone.0006559.g002

from the liver will eventually lead to a patent infection (such as animal 252, Panel D, Figure 1), we believe that the four animals that never had parasitemia provide evidence for complete protection against sporozoites and liver stages of the parasite. The second outcome with a straightforward interpretation is self-cure. Three animals became infected but limited their parasitemia without need for drug treatment, indicating an effective immune response against the blood stages of the parasite. Even prior to their decline in parasite counts, the self-cure animals showed a slower rate of growth than Controls (Figure 3). Between days 1–2 and 2–3 of parasitemia, the mean rate of increase for the 5 Control animals was 0.03% and 0.37% per day, a nearly exponential progression. For the 3 self-cure monkeys, the mean rate of increase between days 1–2, 2–3, and 3–4 was 0.07%, 0.11%, and 0.15%, a more constant rate of growth. We believe that immune responses to blood stage antigens must have caused these slower growth rates.

Immunogenicity of different vaccine regimens

We measured circulating antibody and T cell responses to each of the four P. knowlesi antigens in the vaccine. Antibody measures included ELISA against each of the four vaccine antigens, and IFAT against whole fixed sporozoites and infected red cells. ELISA data is shown in Figures 4a. IFAT data is similar to ELISA data, with high titers to CSP or SSP2 giving high IFAT titers against sporozoites, and high ELISA titers to AMA1 or MSP1 giving high IFAT titers against infected red cells (data not shown). T cell responses were measured by IFN-γ ELISPOT assay and flow cytometric analysis of intracellular IFN-γ and IL-2 production. Data from the on IFN-γ ELISPOT on PBMCs are shown in 4b. Data from the flow cytometric studies were comparable to the IFN-γ ELISPOT (data not shown).

No significant immune responses were detected in samples from pre-vaccination samples. Three weeks after the last of three vaccinations with either Pk4 VRPs or Pk4 DNA plasmids, we detected no statistically significant immune responses to any of the four antigens by ELISA or ELISPOT. In contrast, three weeks after a single dose of Pk4 Ad5 (Figure 4a), there were significant increases in antibody responses to three of four antigens CSP, SSP2, and MSP1 $p<0.05$). The Ad5 vaccine also induced measurable ELISPOT responses to each of the four antigens in some animals(Figure 4b), although the group differences were not statistically significant.

All monkeys received a viral 'booster' vaccination at week 60 with either a control poxvirus (Control group), the Pk4 Ad5 viruses (VRP/Ad group), or the Pk4 poxviruses (four remaining groups). Blood was taken seven days later for measurement of immune responses and sporozoite challenge occurred 5 days after this blood sampling.

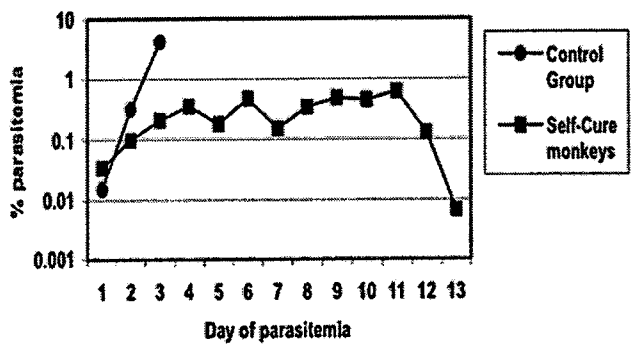


Figure 3. Mean parasitemias of the 5 Control monkeys compared to the 3 monkeys from vaccine groups which contained their parasitemia below 2%. The X axis is normalized so day 1 is the first day parasites were detected in the blood for each animal. Monkeys which controlled their parasitemias had slower growth rates.
doi:10.1371/journal.pone.0006559.g003

In the samples taken 7 days after the final vaccination, there were significant differences between the experimental groups in ELISA and ELISPOT responses to each of the four vaccine antigens (analysis by ANOVA, results not shown). We then compared immune responses with the Control group by T test using Tukey's Adjustment for multiple comparisons (Table 2). The group vaccinated with control Pox or Pk4 Pox (unprimed) seven days previously had no immune responses significantly different from Controls. In contrast, all prime/boost vaccine groups developed immune responses to some or all vaccine antigens that were statistically different from Controls, but there were no statistically significant differences between any prime/boost vaccine groups. Of note, the Ad/Pox and DNA/Pox vaccines were the only ones which induced significant ELISPOT responses to CSP, and these two vaccine groups were the only two which contained sterilely protected animals. The DNA/Pox group with 3/5 animals sterilely protected was the only vaccine group which produced statistically significant antibody responses to CSP.

Association between immune responses and protection against sporozoite challenge

We were interested to know if the magnitude of any immune response was associated with protection against malaria independent of which vaccine the animal received. We approached this question in two ways. First we analyzed immune responses of all 30 monkeys with respect to the two protective endpoints, 'day of first parasitemia' or 'day>2% parasitemia'. As discussed previously, we believe that immune responses to both pre-erythrocytic and erythrocytic stage antigens could contribute to any protective effect identified by these two endpoints. In the second approach, we focused on the four sterilely protected monkeys (sterile protection reflecting immune responses targeting pre-erythrocytic stages) and the three monkeys that self-cured their parasites (self-cure reflecting immune responses targeting blood stages), comparing immune responses in these protected animals to the other monkeys in the same vaccine groups.

To analyze the relationship between immune responses of all 30 monkeys and protection we used Cox Proportional Hazard analysis. Table 3 shows that considered one at a time many immune responses to vaccine antigens were significantly associated with protection. All ELISPOT responses except for CSP had important effects on both day to first parasite and day>2% parasitemia. We have found this same lack of correlation of ELISPOT responses of PBMC to CSP in previous P. knowlesi vaccine studies [19]. For the ELISA data, both SSP2 and MSP1 responses had a significant effect on both protective endpoints. However, when we fit models using the immune responses to the four vaccine antigens simultaneously, neither ELISA nor ELISPOT responses to any one vaccine antigen were significantly correlated with either protective endpoint (data not shown).

In a second set of analyses, we focused on the immune responses in the sterilely protected and self-cure animals. We compared the immune responses to all four vaccine antigens of these protected animals with those of the other non-protected monkeys in the same vaccine groups. Comparing responses of the four sterilely protected monkeys with the six unprotected animals in the DNA/Pox and Ad/Pox groups, only the MSP1 ELISA and ELISPOT showed a trend toward higher values in the sterilely protected monkeys but this was not statistically significant (data not shown). There were also no differences in immune responses between the

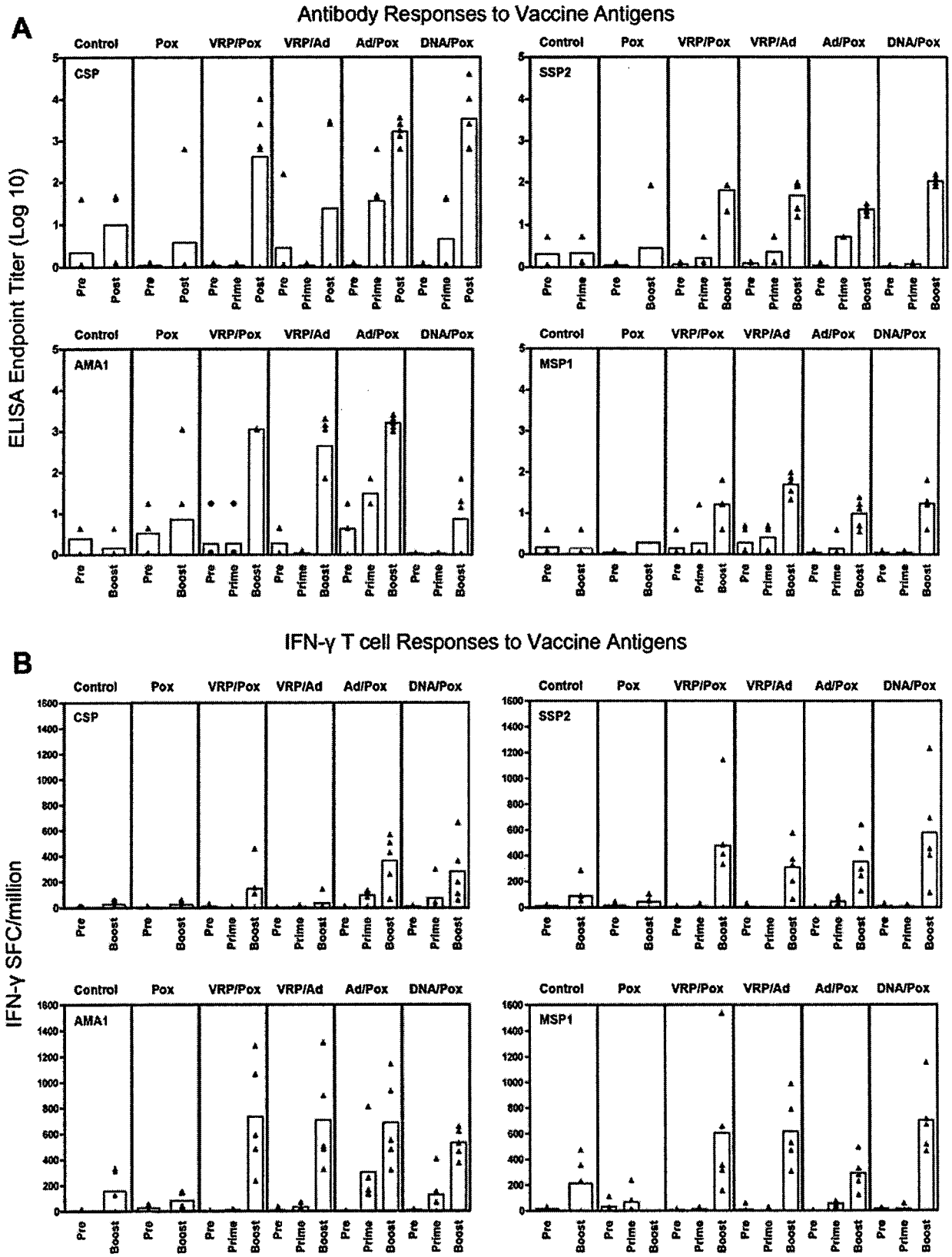


Figure 4. Immune responses induced by vaccinations against the four vaccine antigens. Plasma samples were assayed by ELISA (Panel A), and PBMCs were assayed -forming cells by Elispot (Panel B). Data is presented for 3 timeγfor IFN points: 'Pre' = pre-immunization; 'Prime' = 3 weeks after the last priming immunization; and 'Boost' = 7 days after viral boost immunization which was 5 days before challenge. Mean of 5 animals in each group was presented as a rectangle. Data for individual animals is presented as triangles. Immune responses in the Pox only group were lower than in all groups receiving prime/boost vaccines.
doi:10.1371/journal.pone.0006559.g004

Table 2. Immune responses of vaccine groups prior to challenge.

		Pox	VRP/Pox	VRP/Ad	Ad/Pox	DNA/Pox
ELISPOT	CSP				+	+
	SSP2			+	+	+
	AMA1		+	+	+	+
	MSP1		+	+	+	+
ELISA	CSP					+
	SSP2					
	AMA1		+	+	+	
	MSP1			+		

The five vaccine groups compared with the Control group for immune responses to each vaccine antigen. Analysis used Student's T test with Tukey's Adjustment for multiple comparisons. Crosses (+) indicate that the comparison with the Control group is statistically significant ($p<0.05$).

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three self-cure monkeys, the four sterilely protected monkeys and the eight unprotected monkeys in the DNA/Pox, Ad/Pox, and VRP/Pox groups (data not shown). Thus, neither analysis allows a clear dissection of the protective roles of the different vaccine antigens. This is possibly explained by the fact that all immune responses were highly correlated with each other, so statistical separation of effects was not possible.

Intracellular cytokine expression of CD4 and CD8 cells

To further understand the T cell responses to the four vaccine antigens, we used flow cytometry to study the CD4 and CD8 phenotype of T cells responding after in vitro restimulation using both IFN-γ and IL-2 production as measures of immune response. No increase in CD8+ T cell responses was detected for any of the four vaccine antigens (data not shown). In contrast, CD4+ T cells were detected producing IFN-γ, IL-2 or both cytokines together in a pattern similar to that seen in the IFN-γ ELISPOT assay (data not shown). We conclude that the ELISPOT responses from PBMC are primarily from CD4+ T cells, which has also been the case in our previous studies of the Pk4 vaccine in rhesus monkeys [28].

Table 3. Cox Proportional Hazards Analysis of immune responses and parasitemia.

		Day of 1st parasitemia	Day>2% parasitemia
ELISPOT	CSP		
	AMA1	+	+
	SSP2	+	+
	MSP1	+	+
ELISA	CSP		
	AMA1		
	SSP2	+	+
	MSP1	+	+

Crosses (+) show statistically significant effects on protective endpoints when immune responses are analysed separately. When responses to all antigens are analysed sim-ultaneously no single immune response is statistically associated with either protective endpoint.

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Protection against a second sporozoite challenge

Four months after the first sporozoite challenge, all animals received a second challenge with 100 P. knowlesi sporozoites given IV. Figure 5 shows the daily parasitemias for each monkey during the second challenge. All five Control monkeys became parasitemic at a mean 8.4 days after challenge, and all required drug treatment at mean day 11.4 (Figure 5 panel A). The four monkeys that were sterilely protected in the first challenge became parasitemic in the second challenge on day 9 and were treated on day 12 (Figure 5 panels E and F). Thus the vaccine responses that protected animals in the first challenge were not maintained long enough to protect them against the second challenge. Of the three monkeys which self-cured in the first challenge, two self-cured after the second challenge (Figure 5 panel D and E) and one required drug treatment (Figure 5 panel E). One monkey in the Pox group (223) was protected in the second challenge but not in the first (Figure 5 panel B). Protection of a monkey only in the second challenge but not in the first might seem paradoxical. We believe that this monkey did receive an adequate infectious challenge in the second round, as there were no technical problems with the injection. We believe that the most likely explanation is that the first challenge exposed monkeys to malaria parasites with multiple antigens that boosted vaccine induced immune responses. The complication of exposure to parasites after the first challenge makes further interpretation of protection data from the second challenge difficult.

Discussion

The goal of this study was to improve upon the Pk4 DNA/Pox vaccine by replacing the DNA components with VRP or Ad5 vaccines. Unfortunately, while both of these novel vaccines were able to prime immune responses for boosting neither gave as much protection as priming with DNA plasmids.

Vaccination with DNA plasmids is potent in mice but much less effective in primates and humans where very large amounts of DNA are required to induce small immune responses. In mouse malaria vaccine studies using PyCSP antigen, VRPs and DNA have been comparable in priming responses for boosting with recombinant adenovirus or poxvirus (Doolan, personal communication). Unfortunately, in the present experiment VRPs were minimally immunogenic in themselves, and did not prime for protection with a poxvirus boost as well as did DNA. Although VRPs were the least effective priming modality we tested, a comparison with the Pox alone group shows that VRP priming did enhance protection and immunogenicity.

Recombinant Pk4 adenovirus type 5 vaccines were given to two groups of monkeys in these experiments: in the Ad/Pox group they were the prime and in the VRP/Ad group they were the boost. Comparing immune responses after priming alone (Fig. 4a and b), we are impressed that after a single priming dose, the monkeys receiving the Ad5 vaccines were able to mount antibody and T cell responses to most of the Pk4 antigens. We suspect that the one month interval between prime and boost for the Ad/Pox group was not optimal, and that a longer interval might lead to even better immunogenicity and protection.

Comparing the VRP/Pox and VRP/Ad groups allows us to assess the value of the Ad5 virus as a booster vaccine. Protection was at least as good if not better in the VRP/Ad group than the VRP/Pox group, and immune responses were equivalent. Thus we believe that the Ad5 provided a boost as potent as the poxviruses

The most striking finding of this study is the high level of sterile protection in the monkeys receiving the Pk4 DNA prime/poxvirus

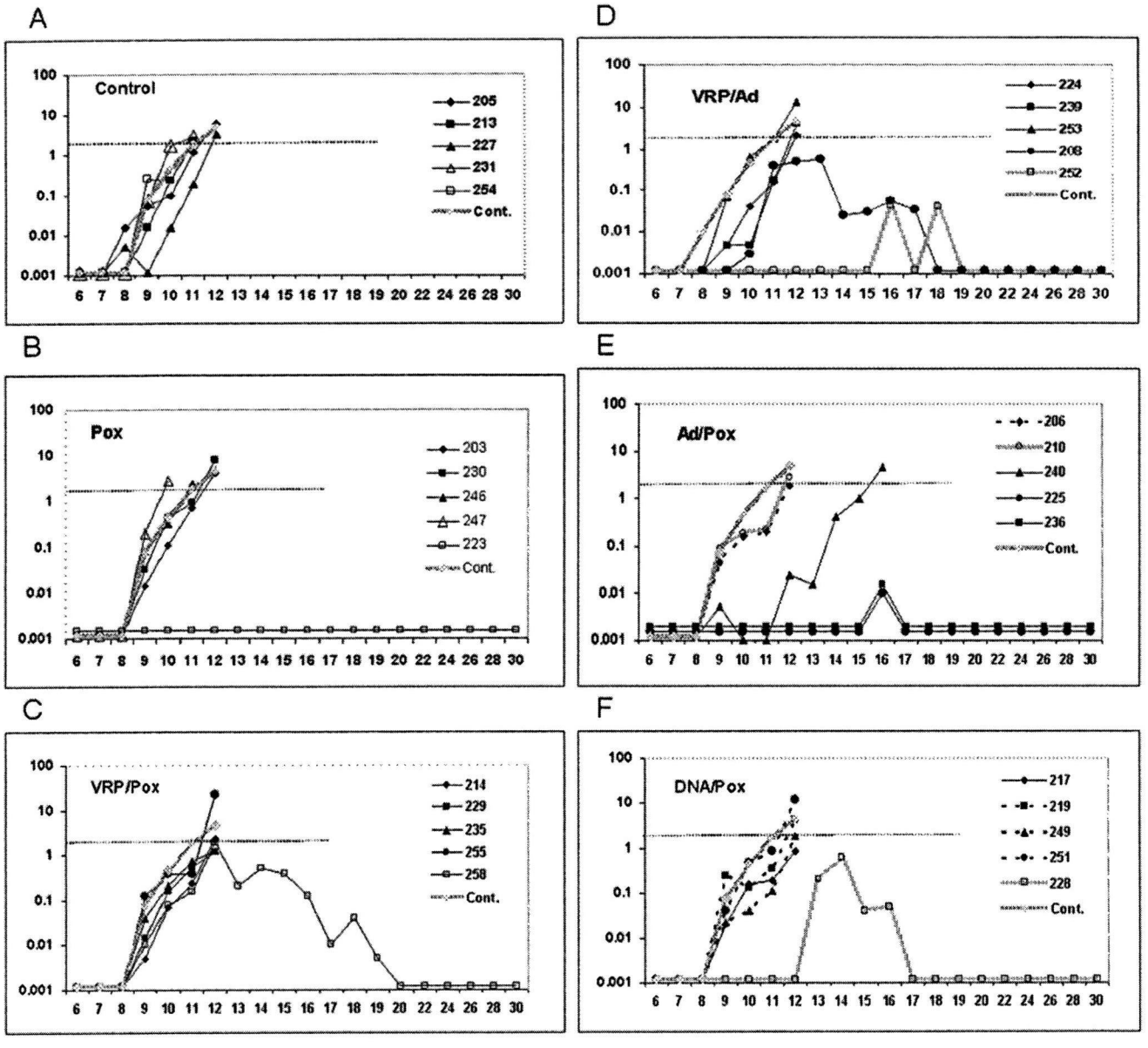

Figure 5. Daily parasitemias from individual monkeys after the second sporozoite challenge. Panel A, Control group: average parasitemia levels of 5 individual animals was presented as a thick grey line (Cont.) and is included in all 6 panels for comparison; Panel B, Pox group; Panel C, VRP/Pox group; Panel D, VRP/Ad group; Panel E, Ad/Pox group; Panel F, DNA/Pox group; The dotted line in each panel shows the 2% parasitemia level at which we treated animals with anti-malaria drugs. One monkey (223) in Panel C had no detectable parasitemia is shown as a horizontal line. The four monkeys which had no detectable parasitemias after the first challenge all developed parasites in the second challenge and are graphed with black interrupted lines. The three monkeys which self-cured their parasitemias after the first challenge are graphed with thick stippled lines.

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boost vaccine in the first challenge. Three of five monkeys (60%) never developed parasitemia after sporozoite challenge, and of the two monkeys that did become infected, one cured its parasitemia without the need for drug treatment. The fact that this protection was achieved using a DNA/poxvirus vaccine regimen suitable for humans is especially encouraging. In our previous four challenge experiments (Table 4), a total of 3/30 monkeys were sterilely protected by the Pk4 DNA/Pox vaccine, with protection ranging from 0 to 18%. Comparing the present study with the pooled results of our previous studies yields an Odds Ratio of 0.074 (95% CI 0.008, 0.636). Thus it is not likely that the improved protection is a random fluctuation due to the small number of experimental animals. Our hypothesis is that the increased protection may be due in part to the long intervals between vaccine doses used in this study, as has been seen in rodent malaria vaccine studies [32].

However there are several caveats to be considered when comparing the present study with our previously published results. Because we have been working over a period of years, different

Table 4. Summary of Sterile Protection in Five Pk4 DNA/Pox Vaccine Studies.

Trial							N =	Sterile #	Sterile %
a	DNA1	DNA2	DNA3			Pox	5	3	60
	day 0	28	96			420			
b	DNA1	DNA2	DNA3		DNA4	Pox	11	2	18
	day 0	30	60		280	310			
c	DNA1	DNA2	DNA3		Pox		5	0	0
	day 0	30	60		207				
d	DNA1	DNA2	DNA3	Pox			10	1	10
	day 0	30	60	156					
e	DNA1	DNA2	DNA3	Pox			4	0	0
	day 0	30	60	108					

Summary of five published vaccine studies in rhesus monkeys using the Pk4 DNA/Pox vaccine and challenge with 100 Pk sporozoites IV. Trial a is the present experiment. Trial b is from Rogers (18). Trials c and e are from experiment 3 in Weiss (19), Trial d is from experiments 1 and 2 in Weiss (19). N gives the number of animals receiving the Pk4 DNA/Pox vaccine, and Sterile gives the number of animals which did not develop parasites in the blood. Longer regimens give higher proportion of sterilely protected animals.

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production batches of plasmid and viral vaccines have been used. There are also some differences in the vaccine regimens, with the present study spreading the three priming DNA vaccinations over 4 months, and the study from Rogers et al. [18] including a fourth DNA dose before poxvirus boosting. Also, although challenge has always been with 100 P. knowlesi sporozoites the infectivity of those sporozoites no doubt varied between experiments. Finally, the rhesus monkeys in our studies have been obtained from several sources. In our previous studies using the Pk4 vaccine, we have used rhesus monkeys from breeding colonies founded with rhesus monkeys of Indian origin. In this study, we used rhesus monkeys of Chinese origin because Indian origin rhesus were not available at our institution. Although the immune responses to vaccines of genetic subgroups of rhesus monkeys may differ [33], in the absence of an immune correlate of protection we cannot know if genetic differences are responsible for the improved protection seen in this experiment. Because of all these concerns, the concept of longer vaccinations leading to better protection remains a conjecture which must be directly tested in a future experiment.

We were not able to identify an immune correlate of protection in this study. The two vaccines, DNA/Pox and Ad/Pox, which induced the most consistent immune responses to the P. knowlesi CSP were also the only two vaccines which sterilely protected monkeys (Table 2). From this, one might expect that the blood of protected monkeys would have higher antibody or T cell responses to CSP than non-protected monkeys. However, this was not the case (Table 3). One explanation for this seeming contradiction is that immune responses in the blood do not reflect protective immune responses in tissues. In mice, it has been shown that the immune responses that correlate with pre-erythrocytic malaria immunity occur within the liver tissue itself [34]. We think it likely that similar tissue specific liver immunity is occurring with pre-erythrocytic immunity in primates and humans as well, and that these immune events may not be easy to detect in the peripheral blood. We are undertaking studies of immune responses in the monkey liver to examine this concept.

Using flow cytometry, we were able to measure antigen specific responses from CD4+ T cells but we did not detect antigen specific CD8+ T cell responses. This is consistent with our previous studies of the Pk4 DNA/Pox vaccine [19,28]. We had hoped that the VRPs or Ad5 viruses would be able to induce CD8+ T cell responses but this was not the case. CD8+ T cells are important immune effectors against liver stages of malaria in mice [35–37] and monkeys (Weiss, unpublished data) protectively immunized with radiation-attenuated malaria sporozoites. We believe that a vaccine which induces CD8+ T cell effectors may have increased efficacy against malaria liver stages.

The biggest failing of the P. knowlesi vaccines has been the short duration of protection: no sterilely protected animals in the first sporozoite challenge were sterilely protected in the second challenge four months later. This has also been the case in all of our previous studies. Lacking an immune correlate of protection, our vaccine development strategy is to improve the magnitude and longevity of all immune responses to malaria vaccine antigens, and to induce CD8+ effector T cells. Our next plan is to replace DNA priming with recombinant malaria proteins in novel adjuvants [38,39]. We hope these next generation priming vaccines will allow stronger and longer lasting immune responses after boosting with recombinant viral vaccines, and a corresponding lengthening of vaccine efficacy.

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Author Contributions

Conceived and designed the experiments: GJ KIK JR JFS JB CRK TLR WRW. Performed the experiments: GJ SC NR GB HG AV PS JCA KL WRW. Analyzed the data: GJ MS DLD TLR WRW. Contributed reagents/materials/analysis tools: KIK JR JFS JB CRK TT ST YE. Wrote the paper: GJ TLR WRW.

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Evidence for the Transmission of *Plasmodium vivax* in the Republic of the Congo, West Central Africa

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***Plasmodium vivax* is not thought to be transmitted in western and central Africa, because of the very high prevalence of the red blood cell Duffy-negative phenotype in local populations, a condition which is thought to confer complete resistance against blood infection with *P. vivax*. There are, however, persistent reports of travelers returning from this region with *P. vivax* infections. To investigate whether transmission occurs in this region, the presence of antibodies specific to *P. vivax* preerythrocytic-stage antigens was assessed in individuals from the Republic of the Congo. A total of 55 (13%) of 409 samples tested by enzyme-linked immunosorbent assay had antibodies to *P. vivax*–specific antigens.**

Transmission of *Plasmodium vivax* is not generally thought to occur in western or central continental Africa, where 95%–99% of the human population is refractory to *P. vivax* blood infection because of the protective effect of the red blood cell (RBC) Duffy-negative condition [1, 2]. Despite this, reports of Duffy-positive nonimmune travelers returning from these areas with infections diagnosed as being due to *P. vivax* are common and have persisted over many years of surveillance [3]. Furthermore, a recent report has implied that *P. vivax* transmission may occur in a population consisting of very high percentages of Duffy-negative individuals, with the presence of *P. vivax*–specific proteins reported in 0.65% of mosquitoes from an area of western Kenya [4]. An additional study reported evidence of *P. vivax* infections in 2 Duffy-negative individuals in Brazil [5]. Some investigators have interpreted such findings as implying that the parasite may be in the process of evolving the ability to infect Duffy-negative individuals [6]. However, we have argued elsewhere [3] that *P. vivax* transmission can be expected in populations with high levels of RBC Duffy negativity and in which malaria transmission intensities are sufficiently high, as is the case in many areas of western and central Africa. Notwithstanding this expectation, a recent polymerase chain reaction (PCR)–based parasite species–typing survey of 2588 blood samples obtained from patients in 9 western and central African countries failed to find any *P. vivax* parasites, except on the island of Sao Tome, where *P. vivax* transmission is known to occur [3].

In the present study, we used serological testing to search for evidence of *P. vivax* transmission in Pointe-Noire, a city on the west coast of the Republic of the Congo, where >95% of the population is expected to be RBC Duffy negative and, thus, refractory to *P. vivax* blood infection. In September 2007, we collected blood samples from 415 Pointe-Noire residents and searched for the presence of antibodies to the *P. vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) and *P. vivax* merozoite surface protein 1 (PvMSP1). Both antigens are expressed in liver-stage parasites and induce antibodies even in the absence of *P. vivax* blood infection [7]. Detection of antibodies to these *P. vivax*–specific antigens in a largely Duffy-negative human population could be evidence of its transmission there.

Materials and methods. By means of passive case detection, 415 samples were collected from the Mbota health center in Pointe-Noire, located on the west coast of the Republic of

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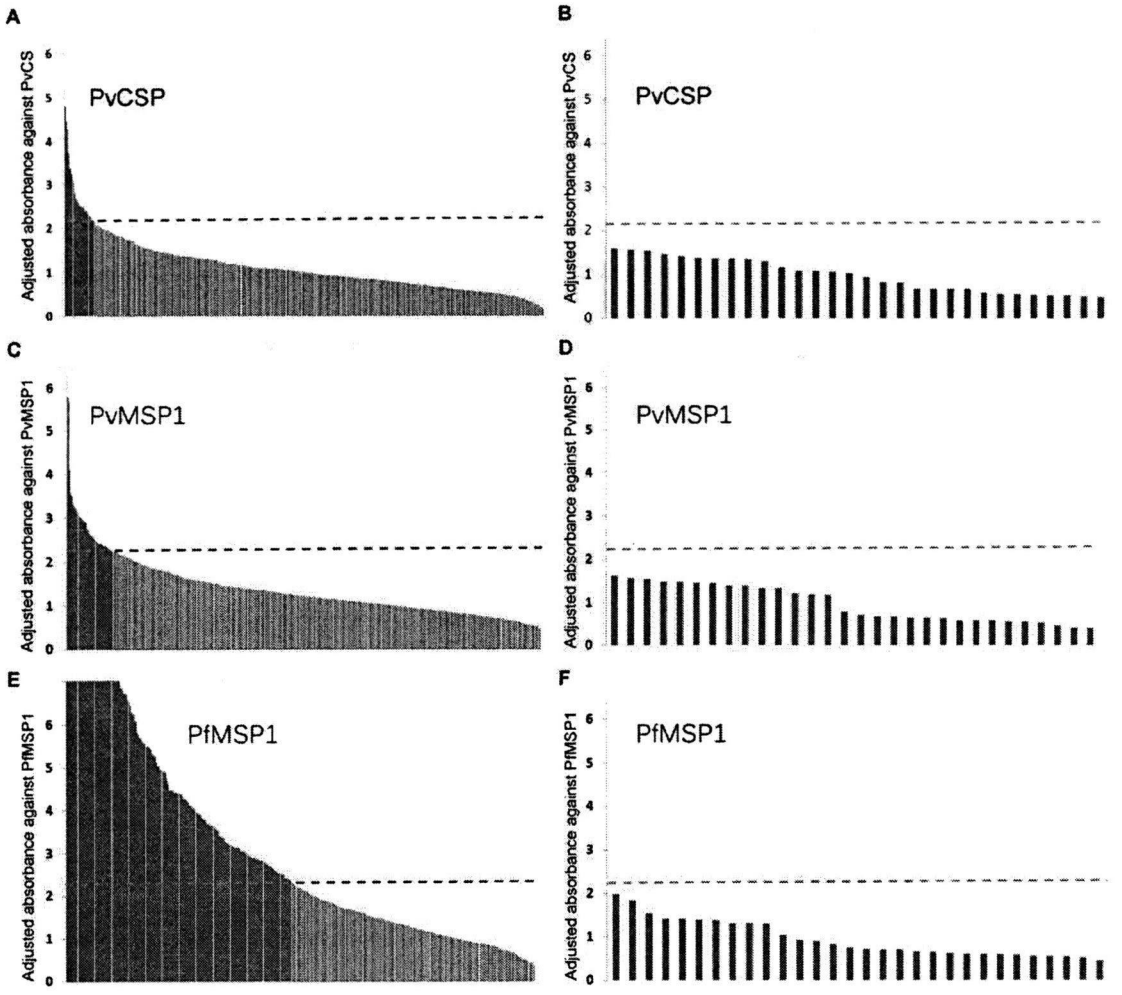

Figure 1. Adjusted absorbances against *Plasmodium vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) *(A), P. vivax* merozoite surface protein 1 (PvMSP1) *(C),* and the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) *(E)* for 409 individuals from Pointe-Noire, Republic of the Congo. *B, D,* and *F,* Corresponding adjusted absorbances for nonexposed individuals (from Japan and the United Kingdom). Cutoff values are denoted by horizontal dashed lines, and positive individuals are denoted by the areas shaded dark gray. The cutoff value was calculated as the mean value (+3 standard deviations) of the adjusted absorbances of 30 nonexposed individuals. All absorbances were measured at 405 nm.

the Congo, during September 2007. No age restrictions were applied to individuals from whom samples were obtained. The samples were collected on Whatman 31ETCHR filter paper. Travel histories were obtained from individuals before sample collection, and those who had traveled outside of the Republic of the Congo were excluded from the study ($n = 6$). Approval of the sample collection was obtained from the ethics committee at the Research Institute of Microbial Diseases, Osaka University (Osaka, Japan), and sampling was authorized by the administrative authority of the Ministry for Research and the Ministry for Health in the Republic of the Congo. Written informed consent was obtained from individual patients, and antimalarial treatment was provided when appropriate. An additional 10 blood samples were collected from *P. vivax*–infected patients from Siverek-Sanliurfa in the southeast of Turkey, for use as positive controls, and from 30 individuals from Japan and the United Kingdom with no previous exposure to *P. vivax* (ie, nonexposed individuals), for use as negative control samples (for collection details, see the description of supplementary methods in the Appendix, which appears only in the electronic version of the *Journal*).

All samples were screened by enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G antibodies to 3 *Plasmodium*-specific proteins. The first of these proteins was PvCSP recombinant protein. This *Escherichia coli*–expressed recombinant protein encompasses the N-terminal and C-terminal regions of PvCSP flanking a chimeric repeat region [8]. The second protein, PvMSP1 recombinant protein, was expressed using a wheat germ cell-free protein translation system [9] that encompasses N-terminal blocks 1 and 2 of

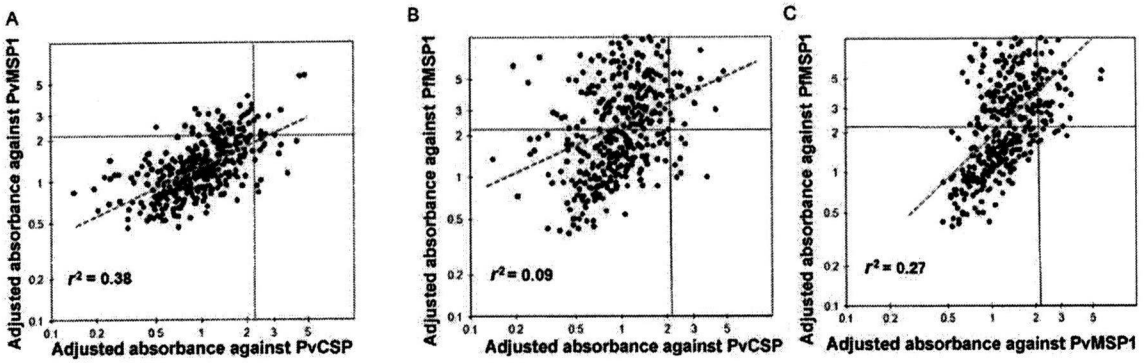

Figure 2. Linear regression analysis of the association between the adjusted absorbances against the *Plasmodium vivax* antigens *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. vivax* circumsporozoite protein (PvCSP) *(A),* the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) and PvCSP *(B),* and PfMSP1 and PvMSP1 *(C)* for 409 individuals from Pointe-Noire, Republic of the Congo. Coefficient of determination values (r^2) for each antigen pair are shown on the graph, and linear regression lines are denoted by dashed gray lines. The solid horizontal and vertical lines denote the positive cutoff values for each antigen.

PvMSP1 (Ser_{72} to His_{432} [based on the *SalI* sequence {GenBank accession number PVX_099980}]). The third protein, *Plasmodium falciparum* merozoite surface protein 1 (PfMSP1) recombinant protein, was an *E. coli*–expressed recombinant protein of N-terminal blocks 1–6 (M1/6) of the *P. falciparum* MAD20 sequence [10]. A detailed description of the ELISA protocol is provided in the Appendix.

To adjust for interplate variations, adjusted absorbances were obtained by dividing the mean optical density (OD) value of 2 repeats for each individual sample by the mean OD value for the same 4 nonexposed individuals assayed on the same 96-well plate (under identical conditions). Samples with adjusted OD values (+3 standard deviations) that were greater than the mean value for 30 nonexposed serum samples were considered to be positive for antibodies to the antigen tested. In the case of a positive result for PvCSP or PvMSP1, blood samples from the same patients were used for extraction of host and parasite DNA for parasite species typing and determination of host Duffy status.

Results. Figure 1 shows the results of ELISAs performed on the 409 samples collected from patients presenting to Mbota health center in Pointe-Noire, Republic of the Congo. For 25 (6%) of these samples, adjusted anti-PvCSP absorbance readings were greater than the mean value (+3 standard deviations) for 30 serum samples obtained from nonexposed individuals and were therefore considered to be positive for antibodies to this protein. For 39 (10%) of the samples, adjusted absorbance readings were greater than the cutoff value noted for PvMSP1. A total of 197 individuals (48%) were found to be positive for antibodies to PfMSP1, a *P. falciparum* antigen. All *P. vivax*–positive samples were independently tested twice more in duplicate, and the same positive results were obtained.

Of the 25 samples that were positive for PvCSP antibodies, 9 (36%) were also positive for antibodies to PvMSP1, and 16 (64%) were positive for antibodies to PfMSP1. Of the 39 samples that were positive for PvMSP1, 31 (79%) were also positive for PfMSP1. To investigate the possibility that there was cross-reactivity between antibodies to *P. falciparum* and *P. vivax* antigens, correlation and linear regression analyses were performed for the antigen pairs PvCSP/PvMSP1, PvMSP1/PfMSP1, and PvCSP/PfMSP1. Adjusted absorbance values were log transformed to meet the normality and homoscedasticity assumptions of the analysis, and coefficient of determination (r^2) values and linear regression lines were generated (Figure 2). There was a highly significant medium-strength positive correlation between antibody responses against PvCSP and PvMSP1 ($r^2 = 0.38$; 409 *df*; $P < .001$) but a much weaker, although still significant, low correlation between PvCSP and PfMSP1 ($r^2 = 0.09$; 409 *df*; $P < .001$). There was a stronger correlation between PvMSP1 and PfMSP1 ($r^2 = 0.27$; 409 *df*; $P < .001$), but this was also much weaker than the correlation between the 2 *P. vivax* antigens. Furthermore, serum antibody absorbance ELISA experiments performed with known positive serum samples incubated separately with *P. vivax* and *P. falciparum* MSP1 antigens showed no evidence of cross-reactivity between the respective antibodies (figure 3, which appears only in the electronic version of the *Journal*).

We assessed whether various factors (patient age or sex; pres-

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Figure 3. Enzyme-linked immunosorbent assay results for known *Plasmodium falciparum* antibody– and *Plasmodium vivax* antibody–positive serum samples incubated with *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. falciparum* merozoite protein surface protein 1 (PfMSP1) antigens and tested for antibody responses to PvMSP1 and PfMSP1 antigens.

Table 1. Descriptive Statistics for Adjusted Absorbencies against 3 Antigens for 409 Individuals from Pointe-Noire, Republic of the Congo

This table is available in its entirety in the online edition of *The Journal of Infectious Diseases.*

ence or absence of parasites; parasite species, if infected; and district of residence) were associated with an increased probability of seropositivity against *P. vivax* antigens. There was no association between the district of residence and the presence of antibodies to *P. vivax* or *P. falciparum* (Table 1, which appears only in the electronic version of the *Journal*). Similarly, there was no strong correlation between age and the presence of antibodies to PvCSP ($r^2 = 0.09$, 409 *df*; $P < .01$), PvMSP1 ($r^2 = 0.13$; 409 *df*; $P < .01$), and PfMSP1 ($r^2 = 0.16$; 409 *df*; $P < .01$), as determined by Spearman rank correlation tests. The presence or absence of parasites in blood, as detected by microscopy, as well as whether those parasites were *P. falciparum*, *Plasmodium malariae*, or *Plasmodium ovale*, was not correlated with the presence of antibodies to either *P. vivax* or *P. falciparum*; however, the numbers of *P. malariae*– and *P. ovale*–infected individuals were low ($n = 5$ and $n = 7$, respectively), precluding statistical analysis (Table 1). Interestingly, females were significantly more likely than males to be seropositive for *P. vivax* antibodies, with 45 (17%) of 269 females positive for antibodies to PvCSP or PvMSP1, or both, compared with 10 (7%) of 145 males (6.51, by χ^2 test; 1 *df*; $P = .01$). There was, however, no difference in seropositivity for *P. falciparum* antibodies between the sexes, with 137 (51%) of 269 females and 60 (41%) of 145 males having positive responses against PfMSP1 (1.81, by χ^2 test; 1 *df*; $P = .18$).

DNA was extracted from the 55 samples for which positive antibody responses against either of the 2 *P. vivax*–specific antigens were demonstrated by ELISA. *Plasmodium* species identification was performed by polymerase chain reaction (PCR), and *P. vivax* DNA was not detected in any samples. The Duffy genotype status of the 55 individuals was determined by PCR [11], and all these individuals were found to be homozygous carriers of the FY^*B^{null} allele and, thus, of the RBC Duffy-negative phenotype.

Discussion. We have shown that the serum samples from 55 (13%) of 409 individuals from Pointe Noire in the Republic of the Congo contained antibodies to the *P. vivax*–specific antigens PvCSP (25 samples [6%]), PvMSP1 (39 samples [9.5%]), or both (9 samples [2.2%]). These results suggest that *P. vivax* is transmitted in an area of west central Africa where the frequency of the Duffy-negative genotype is 95%–99% [1]. This finding goes against the current orthodoxy that *P. vivax* is not transmitted in western Africa and offers an explanation for the many cases of *P. vivax* contracted by Duffy-positive travelers in this region.

It has been established elsewhere [7] that Duffy-negative individuals who are refractory to the blood stages of *P. vivax* may develop antibodies to such antigens as CSP and MSP1, which are expressed in the preerythrocytic stages of this parasite in areas of endemicity. This finding is supported by evidence of the establishment of preerythrocytic immunity in individuals undergoing anti–blood-stage chemoprophylaxis for *P. falciparum* [12] and in mice with *Plasmodium yoelii* [13].

Although initial experiments indicated that there was no cross-reactivity between antibodies to the PvMSP1 and PfMSP1 antigens used in the present study, we did find a weak correlation between the antibody responses to the 2 species-specific versions of this antigen. There was also a very weak correlation between antibody responses to the PvCSP and PfMSP1 antigens. We do not consider, however, that these correlations are, in themselves, evidence for antigenic cross-reactivity between *P. vivax* and *P. falciparum* antigens. Indeed, if 2 species of malaria parasites are coendemic, this result is predicted from the fact that exposure to infection by one species of malaria parasite will be highly correlated with the risk of exposure to infection by other species.

Our data indicate that, in the region of study in western and central Africa, there is an endemic entity present that is inducing antibodies specific to the preerythrocytic stages of *P. vivax* in the RBC Duffy-negative human populations of the region. We suggest that this entity is most likely sporozoites of *P. vivax* itself, delivered by the local malaria vector mosquitoes. In conjunction with the frequent reports of travelers returning from western and central Africa with diagnosed *P. vivax* infections, these findings make a strong argument for the presence and continued transmission of *P. vivax* in this region. Given the very high malaria transmission intensity in this area, it is possible that the transmission of *P. vivax* is maintained within the local population by the ~1%–5% of Duffy-positive individuals who are presumed to be present in the local population.

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Identification of *Plasmodium malariae*, a Human Malaria Parasite, in Imported Chimpanzees

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Abstract

It is widely believed that human malaria parasites infect only man as a natural host. However, earlier morphological observations suggest that great apes are likely to be natural reservoirs as well. To identify malaria parasites in great apes, we screened 60 chimpanzees imported into Japan. Using the sequences of small subunit rRNA and the mitochondrial genome, we identified infection of *Plasmodium malariae*, a human malaria parasite, in two chimpanzees that were imported about thirty years ago. The chimpanzees have been asymptomatic to the present. In Japan, indigenous malaria disappeared more than fifty years ago; and thus, it is most likely inferred that the chimpanzees were infected in Africa, and *P. malariae* isolates were brought into Japan from Africa with their hosts, suggesting persistence of parasites at low level for thirty years. Such a long term latent infection is a unique feature of *P. malariae* infection in humans. To our knowledge, this is the first to report *P. malariae* infection in chimpanzees and a human malaria parasite from nonhuman primates imported to a nonendemic country.

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Introduction

Malaria is a major infectious disease prevalent in most tropical and subtropical areas in the world. Malaria parasites, genus *Plasmodium*, infect all classes of terrestrial vertebrates (i.e. mammals, birds, and reptiles)[1]. Of them, the four classical human malaria parasites, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, are widely believed to infect only man as a natural host. However, earlier studies described several human malaria parasite-like species from great apes: *Plasmodium reichenowi*, a *P. falciparum*-like parasite in chimpanzees, *Plasmodium schwetzi*, a *P. vivax*/*P. ovale*-like parasite in chimpanzees and gorillas, and *Plasmodium rodhaini*, a *P. malariae*-like parasite in chimpanzees [2,3]. Determination of host specificity or host range of human malaria parasites is of great importance not only for further understanding the parasite biology but also for better malaria control. Surveys of malaria parasites in great apes are thus required. Besides, the investigation of malaria infection in great apes should be helpful for the primates' health and biodiversity conservation efforts.

The *Plasmodium* species reportedly identified as great ape malaria parasites were described in the early 20th century [3]. These previous studies were ambiguous as to whether natural infections of great apes are due to human malaria parasite-like species or to human malaria parasites. Recently, evidence of human infections of *Plasmodium knowlesi*, an Asian simian malaria parasite, is accumulating with the aid of current molecular diagnostic tools [4–7]. Prior studies on great ape malaria parasites may consequently be supported or disputed by new surveys using molecular diagnosis. Of the great ape parasites, *P. reichenowi* is the only species that has been confirmed to be close to but independent from *P. falciparum* at the molecular level [8]. Other malaria parasites from great apes await species identification using molecular analysis and phylogenetic relationship to human malaria parasites.

Recently, a new species, *Plasmodium gaboni*, has been identified from chimpanzees, and defined as a close relative of *P. falciparum* [9]. *P. ovale* has also been identified in chimpanzees in Africa, suggesting that *P ovale* can infect chimpanzees as a natural host [10].

Here, we report malaria parasites in two chimpanzees imported into Japan thirty years ago. The parasites isolated from these chimpanzees were identified as *P. malariae* based on two gene markers. The infections have been asymptomatic to the present, and have persisted for about thirty years. This study also indicates that human malaria parasite has been maintained in nonhuman primates in a nonendemic country, which has significance to public health issues.

Results

Blood samples of 60 chimpanzees (*Pan troglodytes*) imported into Japan were examined. First, molecular diagnosis for the presence

of malaria parasites was carried out using polymerase chain reactions (PCRs) that specifically amplify the mitochondrial genome and nuclear-encoded small subunit (SSU) rRNA gene of all known malaria parasites. PCR diagnosis yielded malaria positives in two chimpanzees, Takaboh and Oumu.

Takaboh is a male chimpanzee (*Pan troglodytes verus*), assumed to have been born in 1978. He was imported into Japan from the Republic of Sierra Leone in April 1980. His blood samples were collected during routine health examinations in January 2003 and in September 2008. PCR diagnosis was positive on both occasions. Oumu is a female chimpanzee (*Pan troglodytes verus*), assumed to have been born in 1976. She was imported into Japan from Africa in March 1977. There is no record of her country of origin. It is likely that Oumu came from West Africa because she belongs to *P. t. verus*, a subspecies living in the western part of Africa. Her blood was obtained during routine health examination in June 2003. Importantly, Takaboh and Oumu have shown no symptoms of malaria to the present while in Japan.

Microscopic observations of Giemsa-stained thin blood smears were done for specimens available for Takaboh and Oumu. Blood smears of Takaboh were prepared in August 2000, January and June 2003, and September 2008, and those of Oumu were made in October 1986, October 1996, and June 2003. We were able to detect, at one instance, one erythrocyte infected with malaria parasite in a Takaboh specimen obtained in September 2008 (Figure 1), but detection was unsuccessful from the other samples. The parasite shows the morphology of an immature schizont having irregular masses of chromatin, resembling *P. malariae*. The typical band-form of *P. malariae* could not be seen (Figure 1).

To identify the malaria parasite species from Takaboh and Oumu, the complete nucleotide sequence of the mitochondrial genome and the near-complete SSU rRNA gene sequence were obtained from these chimpanzee parasites. We also obtained an additional sequence of the mitochondrial genome from human *P. malariae* (Thailand isolate) because only one sequence (Uganda I isolate; GenBank accession number AB354570) was available. The sequences obtained were used for the construction of phylogenetic trees together with those from diverse malaria parasite species including primate, rodent, bird, and reptile parasites (Figure 2). In the phylogenetic tree of the SSU rRNA gene, both parasites of Takaboh and Oumu are clearly located within the cluster of human *P. malariae* isolates. This indicates that the two chimpanzee parasites belong to *P. malariae* (Figure 2A). Likewise, the phylogenetic tree of the mitochondrial genome represents clustering of the chimpanzee parasites with human *P. malariae* isolates. A closer look shows that the parasites of Takaboh and

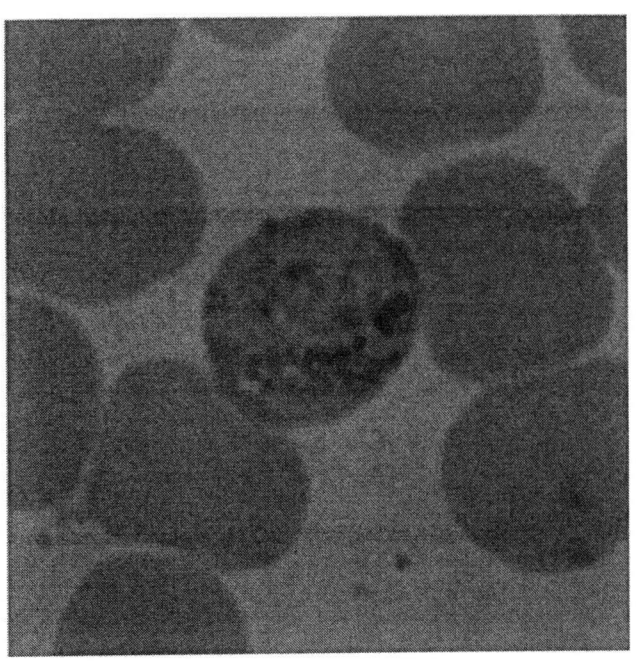

Figure 1. Giemsa-stained thin blood smear showing an immature schizont.
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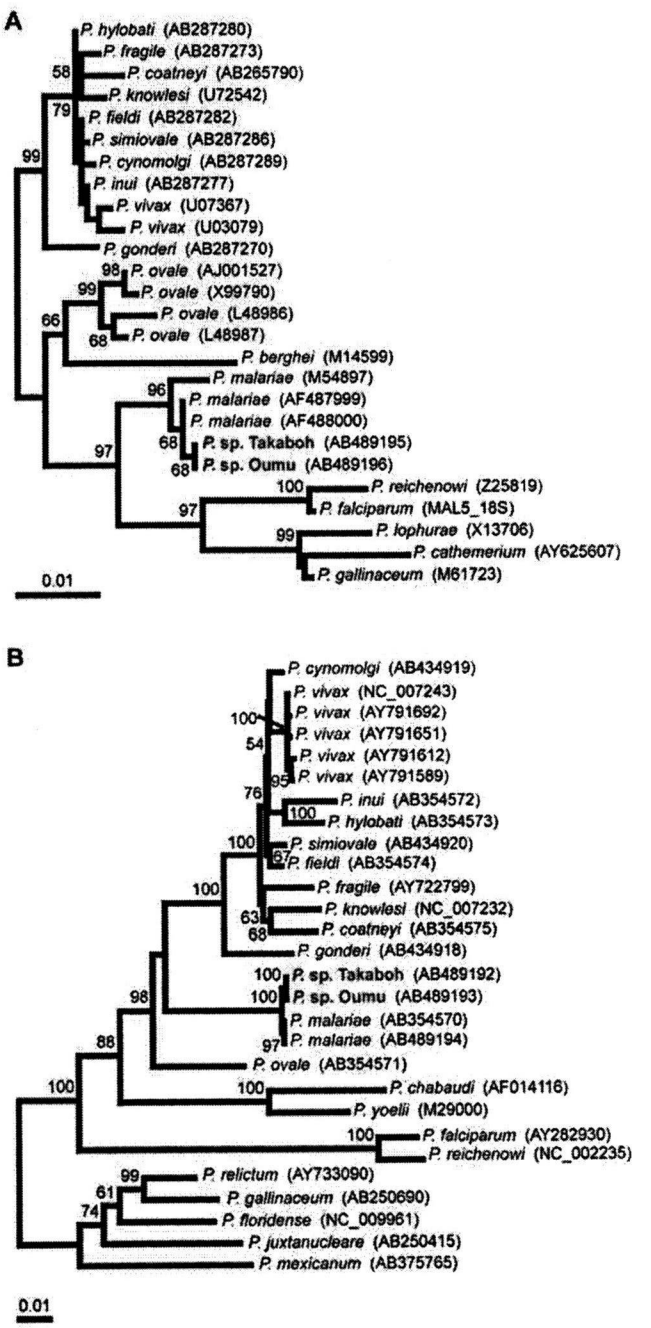

Figure 2. Phylogenetic trees of SSU rRNA gene (A) and mitochondrial genome (B). The numbers on the phylogenetic tree represent bootstrap values based on 1000 replications. GenBank accession numbers are in brackets.
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Oumu make a small sub-cluster and seem to be separated from the human *P. malariae* isolates (Figure 2B). This separation, however, does not indicate that the chimpanzee parasite is an independent species different from *P. malariae*. The genetic difference of the mitochondrial genome between the chimpanzee parasites and human *P. malariae* isolates should be regarded as an intraspecific variation (polymorphism): that is, the p-distances (the proportion of nucleotide sites at which two sequences being compared are different) between the parasites of Takaboh and Oumu and the human *P. malariae* isolates are at most 0.0023, smaller than the p-distance between the most distantly related isolates of *P. vivax* (at least 0.0026 is the observable polymorphism range in *P. vivax* populations; the calculation was based on the sequences in ref. 14; see also Figure 2B). We, therefore, conclude that the parasites from Takaboh and Oumu are isolates of *P. malariae*.

Discussion

The present finding of *P. malariae* isolates in two chimpanzees reinforces that *P. malariae* is able to infect chimpanzees as a natural host. Takaboh and Oumu were imported into Japan about thirty years ago at one or two years of age. In the mainland of Japan, there has been no indigenous malaria for more than 50 years [11]. These facts imply that the two chimpanzees were infected with *P. malariae* in Africa before their importation, and that *P. malariae* infection has persisted for about 30 years in the two chimpanzees. In addition, both Takaboh and Oumu have been asymptomatic to the present, and parasite densities were extremely low in their bloods. These circumstances are consistent with a long term latent infection, a unique feature of human *P. malariae* infection [12,13]. *P. malariae* therefore appears to have a similar course of infection in chimpanzees as in humans. A much larger survey in wild chimpanzees in Africa would present more details about *P. malariae* infection in chimpanzees.

It is known that, in humans, *P. malariae* infrequently recrudesces after tens of years of dormancy [13]. Takaboh and Oumu are currently under careful observation for such latency. One may argue that malaria parasites have been transmitted between chimpanzee hosts. But despite that Takaboh and Oumu currently live in the same facility, their parasites have distinct sequences of SSU rRNA and mitochondrial genome. Furthermore, since our PCR method can detect malaria parasites at very low parasite density (as few as 1 parasite/sample using PgeneralF3s and PgeneralR1s primers; see Materials and Methods; data not shown), more infection of other chimpanzees should have been detected. Thus, it is unlikely that the parasites have been transmitted among them. However, given the public health concern/issues, malaria monitoring may be proposed as a part of routine health examination for all captive chimpanzees.

P. rodhaini was previously described as a *P. malariae*-like quartan malaria parasite in chimpanzees [3,13]. Experimental transfer of *P. rodhaini* from chimpanzees to humans was successful by the inoculations of parasitized blood [3]. In addition, splenectomized chimpanzees are susceptible to *P. malariae* infection [3,13]. These experiments lead to note that *P. rodhaini* is synonymous with *P. malariae*. Our finding is consistent with this note. However, further findings from malaria survey in great apes are definitely needed to conclude that *P. rodhaini* is a variant of *P. malariae*.

Human malaria parasites have been widely supposed to be found only in humans; hence nonhuman primates are not included in the target for malaria control. The present finding necessitates a survey of human malaria parasites in nonhuman primates. In this context, it should be remembered that two New World monkey parasites, *Plasmodium brasilianum* and *Plasmodium simium*, are known as *P. malariae*-like and *P-vivax*-like parasites, respectively [3,13]. Evolutionary analysis represents that *P. brasilianum* and *P. simium* are very closely related to *P. malariae* and *P. vivax*, respectively [14,15]. Thus, both New World monkey parasites might be actually human parasites infected New World monkeys. In addition to chimpanzees, the New World monkey may therefore be a subject of malaria screening for public health.

Considering that great apes are endangered animals today [16], this study also has significance in their conservation biology. Further surveys may reveal the current situation of malaria infection in great apes, and help to assess risk of malaria in their life.

Materials and Methods

Chimpanzee samples

The chimpanzees are being kept in the Chimpanzee Sanctuary Uto, Kumamoto (n = 55) and Primate Research Institute, Kyoto University, Aichi (n = 5). During routine health examinations, chimpanzees were sedated with oral midazolam (1 mg/kg) or droperidol (0.2 mg/kg), and their bloods were collected under anesthesia by ketamine hydrochloride (7 mg/kg) or a combination of ketamine hydrochloride (3.5 mg/kg) and medetomidine hydrochloride (0.035 mg/kg). This study was approved by Research Institute for Microbial Diseases, Osaka University; Primate Research Institute, Kyoto University; and the Chimpanzee Sanctuary Uto. All animal work has been conducted according to the following guidelines: Guide for the Care and Use of Laboratory Primates, 2nd edn. (Primate Research Institute, Kyoto University), and Guideline for Care of Chimpanzees (the Chimpanzee Sanctuary Uto). Genomic DNA of blood samples was extracted using QIAamp DNA mini kit (Qiagen, Hilden, Germany).

SSU rRNA sequences

Genomic and nested PCRs using primers (PlaSSU5, PlaSSU3r, SSUF1, and SSUR1) were performed following methods reported in ref. 17. Briefly, genomic PCR conditions were as follows: denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, 68°C for 5 min, and extension at 68°C for 10 min, and the nested PCR conditions were as follows: denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 59.3°C for 30 s, 68°C for 5 min, and extension at 68°C for 10 min [17]. In addition, new primers [PmSSUF2 (5′-TCTC-AAAGATTAAGCCATGCAAGTG-3′), PmSSUR7 (5′-TTCAC-CGACGGAAACCTTGTTAC-3′), PmSSUF3 (5′-TTAAGC-CATGCAAGTGAAAGTATATG-3′), and PmSSUR2 (5′-TTA-AAAGATAGGATTTACGATTTTTC-3′)] were designed. PCR reactions using new primers (PmSSUF2 and PmSSUR7) were performed with 5 pmol of each primer and 1 μl of extracted genomic DNA solution in a total volume of 20 μl containing PrimeSTAR Max DNA polymerase (Takara, Otsu, Shiga, Japan). Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) was used to generate the following conditions: 35 cycles of 98°C for 10 s, 55°C for 5 s, and extension at 72°C for 3 min. The nested PCR were performed with 10 pmol of each new primer (PmSSUF3 and PmSSUR2) and 2 μl of PCR product in a total volume of 50 μl. The nested PCR conditions were as follows: 30 cycles of 98°C for 10 s, 55°C for 5 s, and extension at 72°C for 3 min. The PCR products obtained were cloned into pCR-Blunt II-TOPO vector according to the manufacture's instructions (Invitrogen, Carlsbad, CA, USA). The plasmids containing the SSU rRNA genes were prepared using QIAGEN Plasmid Mini kit (Qiagen), and then subjected to sequencing using an ABI 3130 genetic analyzer (Applied Biosystems).

Complete mitochondrial genome sequences

To amplify the mitochondrial genome sequences of chimpanzee parasites, the genomic PCRs using primers (PgeneralF2s, PgeneralR2, PgeneralF3s, and PgeneralR1s) were performed following methods reported in ref. 18. PCR conditions were as follows: denaturation at 93°C for 1 min followed by 40 cycles of 93°C for 20 s, 60°C for 1 min, 72°C for 3 or 5 min, and extension at 72°C for 10 min [18]. The nested PCRs were carried out using the nested primers [CP69F1 (5′-ATTTAGCGTGTATTGTTGCCTTG-TAC-3′), PgeneralR2 (ref. 18), PvmtF1001 (5′-CATGCAGGACG-GAGATTACCCGA-3′), and PgeneralR1s (ref. 18)] to obtain sufficient amount of PCR products for sequencing. The nested PCR reactions were performed with 10 pmol of each primer and 2 μl of PCR product in a total volume of 50 μl containing 400 μM dNTPs and 1 unit of LA-Taq DNA polymerase (Takara) in PCR buffer containing 2.5 mM $MgCl_2$. The nested PCR conditions were as follows: denaturation at 93°C for 1 min followed by 20 cycles of 93°C for 20 s, 60°C for 1 min, 72°C for 3 or 5 min, and extension at 72°C for 10 min.

In addition, we amplified mitochondrial genome sequences from one human *P. malariae* isolate (PVMS1229, Thailand). The genomic PCRs were performed with the primers [PgeneralF3s (ref. 18), PgeneralR1s (ref. 18), PmF1 (5′-CTAGCATGGGAC-TAAAAAATGTTATG-3′), PmR3 (5′-CTGTATCGTACCC-TAAAGGATTAG-3′), PmF3 (5′-AATTATGGAGTGGATGG-TGTTTTAG-3′), and PmR1 (5′-AGAAGTTAATATCTG-GAAGCGTCTG-3′)] and 2 μl of extracted genomic DNA solution under the following conditions: denaturation at 93°C for 1 min followed by 40 cycles of 93°C for 20 s, 59 or 60°C for 1 min, 72°C for 3 min, and extension at 72°C for 10 min. The nested PCRs were carried out using the nested primers [PvmtF1001 (5′-CATGCAGGACGGAGATTACCCGA-3′), PgeneralR1s (ref. 18), PmF2 (5′-TTAAGCCCTTTTTACCATA-CAAGAG-3′), PmR4 (5′-ATCTTTTTATAGTTGGATCACT-TACAG-3′), PmF4 (5′-TTACAGCTTTTATAGGTTATGTT-TTAC-3′), and PmR2 (5′-GTATCGTAAACGGTCCTAAGG-TAG-3′)] to obtain sufficient amount of PCR products for sequencing. The nested PCR conditions were as follows: denaturation at 93°C for 1 min followed by 20 cycles of 93°C for 20 s, 57 or 60°C for 1 min, 72°C for 3 min, and extension at 72°C for 10 min.

PCR products were purified using the QIA quick PCR purification Kit (Qiagen) and directly sequenced on an ABI 3130 genetic analyzer (Applied Biosystems).

Phylogenetic analysis

Maximum Likelihood trees of SSU rRNA gene and mitochondrial genome were inferred using PAUP 4.0 b [19] based on the GTR+Γ+I model from the selected 1419 sites and the GTR+Γ+I model from the selected 5837 sites, respectively. To construct trees, primate parasites (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium reichenowi*, *Plasmodium hylobati*, *Plasmodium knowlesi*, *Plasmodium cynomolgi*, *Plasmodium coatneyi*, *Plasmodium fieldi*, *Plasmodium fragile*, *Plasmodium gonderi*, *Plasmodium inui*, and *Plasmodium simiovale*), rodent parasites (*Plasmodium berghei*, *Plasmodium chabaudi*, and *Plasmodium yoelii*), bird parasites (*Plasmodium cathemerium*, *Plasmodium gallinaceum*, *Plasmodium juxtanucleare*, *Plasmodium lophurae*, and *Plasmodium relictum*), and reptile parasites (*Plasmodium floridense* and *Plasmodium mexicanum*) were used. The complete or nearly complete sequences of mitochondrial genome and SSU rRNA gene were obtained from the NCBI Web site (http://www.ncbi.nlm.nih.gov/; see also refs. 17 and 18).

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Author Contributions

Conceived and designed the experiments: TH KT. Performed the experiments: TH NA TT. Analyzed the data: TH NA. Contributed reagents/materials/analysis tools: TU HH JS TT. Wrote the paper: TH KT. Reviewed the paper: TH.

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Expert Opinion

Wheat germ cell-free technology for accelerating the malaria vaccine research

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Background: Malaria causes about 300 million illnesses and 1 million deaths annually. The likeliest scenario is the aggravation of this disease due to the re-emergence of drug-resistant parasites and insecticide-resistant mosquitoes. One of the promising solutions to this disease are vaccines. However, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts. Even the efficacy of RTS,S, the most advanced vaccine candidate in the pipeline of malaria vaccine development, is only around 50%. *Objective*: Against this backdrop, there is an urgency to rapidly enrich the pipeline of vaccine development with novel vaccine candidates that can be discovered by synthesizing and screening a multitude of malaria proteins. *Methods*: However, to achieve this objective, we require optimal technologies for high-throughput synthesis of quality malaria proteins. Among the various protein synthesis systems, the wheat germ cell-free protein synthesis system is advantageous and successful to this end. *Results/conclusion*: The wheat germ cell-free protein synthesis system is optimal for accelerating the decoding of malaria genome and hence characterization of malaria proteins and discovery of malaria vaccine candidates.

Keywords: malaria, *Plasmodium falciparum*, post-genome, vaccine candidate discovery, wheat germ cell-free protein synthesis

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1. Introduction

Malaria is a serious infectious disease caused by protozoan parasites of the genus *Plasmodium*. The most deadly form of the disease is caused by the inoculation of *Plasmodium falciparum* by infected mosquito bites. This disease causes deaths in millions of people annually and illness in about 300 million people and also puts the health of 3.3 billion people at risk [1]. The complex life cycle of the parasite starts when sporozoites are inoculated during the bite of female *Anopheles* sp. mosquito infected with *Plasmodium*. The sporozoites in the peripheral blood circulation then invade the liver and develop into schizonts containing thousands of merozoites. The schizonts in the hepatocytes then rupture and release into the blood stream the merozoites capable of invading red blood cells. This initiates an intraerythrocytic cycle which comprises the development of ring, trophozoite and schizont stage parasites, then rupture of schizonts, and release of free merozoites for reinvasion into the erythrocytes. This developmental cycle takes about 48 h. These asexual erythrocytic-stage parasites are responsible for the clinical manifestations and pathology of malaria, the most serious illnesses being anemia and cerebral malaria [2].

Despite the parasite's complex life cycle, high level of antigenic diversity and mechanism of immune evasion, naturally acquired immunity to malaria indeed develop after repeated exposure over a period of several years. Even though this natural protective immunity possibly never leads to sterile immunity as suggested by

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the fact that parasites can still be found in the circulation of adults living in high transmission settings, this immunity still confers protection against symptomatic disease, high-density parasitemia and death [3,4]. The naturally acquired immunity seems to predominantly target blood-stage parasites [5]. The importance of antibodies in passive immunization has been supported by the transfer of immunoglobulin conferring protection from immune individuals to non-immune individuals [6,7]. Furthermore, immunity against severe, life-threatening malaria is apparent after a few disease episodes, suggesting that the target antigens have limited diversity in parasite populations [8]. The immune effector mechanisms are poorly understood; however, it is widely understood that antibodies may have a role in preventing merozoite invasion, clearance of infected erythrocytes, prevention of adhesion and sequestration of parasitized infected erythrocytes in the vasculature, and prevention of schizont rupture. Functionally, antibodies could prevent erythrocyte invasion by opsonizing merozoites and facilitating phagocytosis by macrophages, activating complement-mediated damage of merozoites [3]. It is becoming increasingly clear, however, that cell-mediated immunity may also be a critical component of naturally acquired immunity to malaria [9]. In addition to the naturally acquired immunities mentioned above, there are encouraging experimental evidences that the sterile, long-lasting protective immunity was induced, after vaccination with radiation-attenuated sporozoites, in mice [10] and humans [11], or after monthly inoculation of sporozoites through mosquito bites to human volunteers under a prophylactic regimen of chloroquine which prevent only the growth of blood-stage parasites [12]. The above-mentioned evidences strongly support the strategy of malaria control through vaccination that elicits protective immunity.

2. Current situation on malaria vaccine development

Malaria is a leading cause of mortality and morbidity. The efficacy of malaria control through current interventions that use drugs and insecticides may not be sustained too long because they rely on too few compounds [4]. In fact, the disease is re-emerging mainly due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes [13]. Very recently, there is a disturbing report on the emergence of parasite's resistance even to artemisinin [14]. Therefore, we are now forced to endeavor to develop malaria vaccines as one of the essential components for the malaria eradication [15]. We should also keep in mind the fact that, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts [16].

Malaria vaccines are classically categorized into three groups based on the stage of the parasite in which the vaccine works. In other words, the parasite presents different proteins as antigens in different stages of life cycle. Pre-erythrocytic vaccines, either based on subunit vaccines or live attenuated parasite vaccines, have been designed to prevent infection and development of diseases. Protection is, to some extent, mediated by antibodies that prevent sporozoite invasion into hepatocytes [17]. Currently, the RTS,S, a pre-erythrocytic vaccine candidate that uses a fragment of *P. falciparum* circumsporozoite protein fused to the HBV surface antigen [18], is undoubtedly the most advanced and promising vaccine candidate based on a number of clinical trials [19-22]. However, the efficacy reported in these trials was around only 50%, suggesting that there is an urgent need to develop new adjuvant systems, test new boosting strategies and enrich the pipeline of vaccine development with additional promising vaccine candidates.

Asexual blood-stage vaccines are aimed at reducing parasite multiplication and growth in order to protect against clinical symptoms and particularly severe disease, and not infection. They are designed to induce antibody responses against the asexual blood stage of the parasites. Asexual blood stage parasite development is a repeating cycle of the release of new merozoites from schizonts and invasion of merozoites into naive erythrocytes. The ability of the merozoite to specifically attach to and invade erythrocytes is essential for blood stage development; for example, *Plasmodium vivax* must bind to the Duffy antigen to invade into reticulocytes [23]. This and other findings have inspired the search for merozoite antigens that elicit antibodies that block parasite invasion of erythrocytes. Immunization with blood-stage antigens, mainly with different merozoite antigens, has been shown to be protective in a number of animal models [24-27].

For example, Combination B, a vaccine combining merozoite surface protein-1 (MSP1) and MSP2 and a part of the ring-infected erythrocyte surface antigen when tested in humans conferred some level of strain specific protective effect. This vaccine showed promising effect in reducing the severity of malaria episodes but the effect is specific only to 3D7 type allele of MSP2, not to FC27 type allele. Because of this, this vaccine subsequently became ineffective in controlling morbidity, due to the selection pressure of this vaccine and subsequent emergence of the parasite with FC27 type allele of MSP2. This fact strongly argues for the development of vaccines comprising multiple components covering all major allelic types [4]. At present, the leading blood-stage vaccine candidates are all merozoite proteins, either located on the surface or within the apical organelles of merozoites. However, none of the merozoite antigens that have been tested in humans, including MSP1, MSP3 and apical merozoite antigen-1 (AMA1), has yet been shown convincingly to confer high levels of protection in clinical trials [28]. Unlike *P. vivax*, *P. falciparum* uses multiple redundant pathways to invade erythrocytes, complicating the effort to develop anti-invasion vaccines against *P. falciparum* [29,30].

Blood stage immunity might also target variant proteins encoded by the parasite multi-gene families (e.g., *var* gene families). PfEMP1, the most famous protein encoded by the *var* gene family, is exported by parasites onto the surface of the

infected erythrocytes (IRBC) and this protein is involved in IRBC adhesion to endothelium and immunoevasion [31]. The best example of this type of immunity has been demonstrated during pregnancy. In pregnant women, parasites emerge that express IRBC surface proteins, allowing these IRBC to bind chondroitin sulfate A (CSA) and sequester in the placenta [32]. First-time mothers lack antibodies specific for the IRBC surface proteins of these parasites and are highly susceptible to infection and disease. Women become resistant over successive pregnancies as they acquire antibodies that block IRBC binding to CSA [33]. Placental parasites express distinct genes and proteins, including an IRBC variant surface protein called VAR2CSA that is required for adhesion to CSA. A program to develop a vaccine based on VAR2CSA is underway [17,34-38]. This fact strongly argues for the development of vaccines comprising conserved epitopes covering all major variant genes [4].

Recently, there has been a renewed call for the 'eradication' of malaria [39]. An effective vaccine will probably be needed to achieve eradication, in addition to other existing drugs and insecticides-based measures. To achieve malaria eradication, the emphasis is on approaches that reduce not only clinical illness in humans but also parasite transmission from humans to mosquitos. Therefore, vaccines that confer sterile immunity and/or transmission-reducing activity would be preferred. The role of asexual blood-stage vaccine as part of this malaria eradication strategy has been questioned, as this vaccine is generally believed to reduce parasitemia and prevent clinical illness but not affect transmission. However, it is also possible that, in addition to preventing clinical illness, an effective blood-stage vaccine may also contribute to the reduction in the efficiency of the transmission by interrupting the blood-stage life cycle in the human body [28].

Transmission-blocking vaccines are aimed at blocking malaria transmission by interrupting the parasite life cycle in the mosquito. These vaccines elicit antibodies against antigens that are expressed by the sexual stage of the parasite and, thus, stop their subsequent development in the mosquito midgut [40]. The target antigens of this vaccine were shown to be sexual-stage specific surface molecules (e.g., P230 and P48/45) that are involved in the process of fertilization of male and female gametes. Subsequently, other antigens (e.g., P25 and P28) that are uniquely expressed by zygotes and ookinetes in the mosquito midgut were shown to be equally good for induction of transmission-blocking immunity. Antibodies in experimental animals against Pfs25 and Pvs25 have been successful at preventing parasite transmission [41,42] and, therefore, these vaccine antigens, Pfs25 [43] and Pvs25 [44], were tried out in Phase I clinical trials. Such vaccines will not provide any immediate direct benefit to the vaccinated individual, but their widespread deployment will help reduce transmission of the parasite and thus protect both the vaccinated individuals and their community as a whole [45]. These transmission-blocking vaccines, if used in combination with pre-erythrocytic or erythrocytic vaccines, might also prevent the transmission of mutant parasites and parasites expressing variant alleles that emerge by evading and escaping human protective immune responses. When used in combination with vector control measures, the transmission-blocking vaccine could play a key role in finally breaking the transmission of parasites, leading to eradication of the diseases [46].

An effective malaria vaccine is expected to target several antigens expressed at different stages of parasite [47] and induce protective immune responses equivalent to, or better than, those provided by naturally acquired immunity or immunization with attenuated whole parasite [48]. In order to accelerate the discovery of such vaccine candidates, we need two things. One is the optimal system for high-throughput synthesis of malaria proteins and the other is the efficient post-genomic high-throughput approaches for screening and selecting potential vaccine candidates from these synthesized proteins.

3. How the malaria genome data can be exploited profitably for malaria vaccine candidate discovery

Decades of research in the pre-genomic era have identified only a handful of vaccine candidates [4]. With the recent completion of the genome projects of human malaria parasites, *P. falciparum* [48], *P. vivax* [49], zoonotic primate malaria, *Plasmodium knowlesi* [50] and rodent malaria parasite, *Plasmodium yoelii* [51], we are now in the post-genome era. Thousands of novel genes have been annotated, providing new opportunities for vaccine research. In order to successfully identify putative novel vaccine candidates for downstream vaccine research, we now need to thoroughly analyze these genomic data using at least two post-genomic high-throughput approaches. One is the functional approach (otherwise called reverse vaccinology [52]) and the other is the immunoscreening approach.

In the functional approach, several direct and indirect functional criteria are used to objectively select and prioritize putative vaccine candidates for further functional studies [53]. For example, the putative candidates for vaccine research can be selected based on their role in erythrocyte invasion [54], essentiality for the parasite's survival revealed by gene knockout studies [30], putative secretory function predicted based on presence of signal peptides and transmembrane regions, localization on the surface or in the apical organelles of the merozoite, or signatures of immune selection pressure revealed by polymorphisms and diversity studies [55].

In the immunoscreening approach, hundreds or thousands of malaria proteins as antigens are screened, using immunoassays such as enzyme-linked immunosorbent assay and so on, with a large number of human serum samples obtained from non-immune, semi-immune and immune individuals. This profiling of immune responses that develop in humans, after natural or experimental infection, or after vaccination with irradiated or genetically attenuated organisms, will facilitate