

Acute *Plasmodium knowlesi* Infection in Olive Baboons (*Papio anubis*) Is Accompanied by High-Level of Gamma Interferon

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Abstract

Malaria is a major and growing threat to economic development and public health in developing countries. There are about 2.7 million malaria deaths annually. *Plasmodium knowlesi*, the fifth human malaria parasite, is an attractive model for malaria research and is also phylogenetically close to human malaria parasite *P. vivax*. It shares many vaccine candidate molecules with *P. vivax*. This study was carried out to determine immunological profiles mounted by Olive baboons during experimental infection with *P. knowlesi* H strain. Six Olive baboons (*Papio Anubis*) were infected with 1×10^6 *P. knowlesi* blood stage parasites. The infected baboons developed

either severe (acute) or mild (chronic) infection. High IgM titres were observed during primary infection in the baboons. Anti-IgG antibodies were raised after two weeks of chronic infection. Circulating IFN γ from serum rose fifty fold in acutely infected animals during the first two weeks post infection compared to a tenfold increase in chronically infected baboons. Recall proliferative responses were two fold higher in chronically infected animals by day 14 post infection. The highest stimulation index (10.06 ± 2.74) was observed in chronic animals at day 42 post infection. These results demonstrated that baboons infected with *P. knowlesi* mount an immune response that is characterized by antibody and cytokine responses involving IgG, IgM and IFN γ . These studies are important in validation of the *P. knowlesi*- baboon model for malaria drug and vaccine development.

Keywords: *Plasmodium knowlesi*, Olive Baboons, Immune responses

1. Introduction

Evaluation of new drugs and vaccines for use in humans requires testing in animal models that develop a disease comparable to that in humans. Similarities in biological mechanisms between human and non-human primates underlie the value of non-human primates as the final test systems preceding clinical trials in humans. The baboon is an attractive experimental model because it is well characterized and is also frequently used in biomedical research (King *et al.*, 1988). Baboons are fully susceptible to *Plasmodium knowlesi* infections (Coatney *et al.*, 1971; Ozwara *et al.*, 2003). In fact, the disease profile observed in baboons is similar to that in rhesus monkeys following infection with the same parasite strain (Ozwara *et al.*, 2003). *Papio anubis* infected with *P. knowlesi* shows various clinical characteristics that are also seen in human malaria, including cerebral involvement (Ozwara *et al.*, 2003; Aikawa *et al.*, 1990; Aikawa, 1988).

Plasmodium knowlesi infects animals on multiple occasions before immunity develops and interactions can be evaluated in immunological and metabolic environment similar to humans. The parasite was instrumental in the discovery and characterization of malaria blood stage vaccine candidate AMA-1 (Kocken *et al.*, 1999). In fact *P. knowlesi* has been used to identify, develop, and evaluate vaccine and drug candidates (Wengelnik *et al.*, 2002; Kocken *et al.*, 1999; Deans *et al.*, 1984). Protocols for genetic modification of *P. knowlesi* have been developed (Kocken *et al.*, 2002). These are powerful tools for drug and vaccine development because they enable the functions of target drug and vaccine candidates to be determined using *P. knowlesi* genes (Ozwara *et al.*, 2003).

As the largest monkey available for malaria research, the baboon provides a primate host for use in *in vivo* experiments for understanding malaria parasite biology and developing control measures against malaria. Host parasite interactions of both the wild type and the transfected *P. knowlesi* parasites can be readily studied (Ozwara, *et al.*, 2003; Munyao *et al.*, 2008).

At the moment the immune responses mounted by *P. anubis* against *P. knowlesi* have not been described. The analysis of the immune responses of the baboons to *P. knowlesi* infections is important in the development and validation of the baboon model for malaria research. The described study looks at some of the immune response profiles following experimental *P. knowlesi* H strain infections in baboons.

2. Methods

2.1 Parasites

P. knowlesi H strain (Ozwara *et al.*, 2003) blood stage parasites were used for inducing baboon infection. These parasites were retrieved from liquid nitrogen and quickly thawed at 37° C in a water bath, washed twice in 3.5% NaCl and twice in RPMI 1640 (sigma, UK) by spinning at 1200 revolutions per minute (RPM) for 10 minutes. The pellet was then transferred into a starting culture medium consisting 2.5% packed cell volume (PCV), 20% baboon serum and the rest RPMI 1640 having 20 μ g/ml of gentamycin (Sigma, UK). The parasites were cultured overnight before being inoculated into baboons.

2.2 Animals

Six adult baboons (*P. anubis*, weight range of 12-23kg) were used. Prior to the experiments, all animals were screened and determined to be free of infection with *Plasmodium* by giemsa-stained thick blood film. All the animals were fed on a standard diet for non-human primates and water was provided *ad libitum* (Olobo and Reid, 1990). The institutional animal care and use committee, and the institutions scientific and ethical review committee (ISERC) of the Institute of Primate Research approved the experiments. The baboons were selected and housed individually in single cages in a bio-containment facility to avoid possible transmission of *P. knowlesi* between experimental baboons.

2.3 Animal Infection, observation and sampling

All the six animals were sedated using ketamine hydrochloride (vetelar, parnke Davies co., ponypool Germany) at 10mg/kg body weight. They were then inoculated intravenously with 1×10^6 *P. knowlesi* blood stage parasites in 1ml of RPMI 1640 medium (Invitrogen, UK). Prior to inoculation, 13mls of blood was obtained from each animal, 10ml was obtained in alsevers solution and used for peripheral blood mononuclear cells preparation and the rest was processed for serum. This was used to get pre-infection values for IFN γ levels, antibody titres and recall proliferative responses. These formed the baseline values over the other subsequent values obtained from each animal in the other sampling points. Animals were then bled on day 7 and day 14 post infection. Acute animals (animals that had parasitaemia systematically rise to over 6%, reaching as high as 9.1% in some baboons at the time of treatment) were treated when parasitaemia reached above 6%; and chronic animals (animals that had peak parasitaemia levels ranging from 1.5%-5%, which thereafter decreased to less than 2% parasitaemia on average) were treated on day 11 post infection irrespective of parasitaemia. Each of the groups had three animals. Treatment was done using pyrimethamine (5-[4-chropheryl]-6-ethyl-2, 4-pyrimidinediamine; sigma aldrichemie, UK) at 1 mg/kg body weight once per day for four days. Finger pricks were taken from each animal on a daily basis from day three up to the day the animal was cured and used to prepare blood film which was used to determine the parasitaemia levels. Parasitaemia was determined as described in Ozwara *et al.*, 2003.

Chronic animals were then challenged two weeks after treatment and confirmation of cure. Challenge was done with a similar number of parasites as described above. Finger pricks and bioassay similar to those done for the initial infection were repeated. The general status of the animals was monitored by regularly determining weight, appetite and general behavior. Weight of the animals was measured on a sedated animal at the bleeding time points. Appetite was evaluated by closely monitoring the feeding habits of the animals.

2.4 Antibody detection

Polystyrene Micro ELISA plates (Dynatech laboratories, Sussex, UK) were coated overnight with 100 μ l of crude and solubilized whole parasite homogenised antigen at a concentration of 1×10^6 parasites/ml diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% bovine serum albumin (BSA Sigma, UK) in PBS for 1hr at 37°C. Unbound BSA was washed off six times with 0.05% Tween 20 (Sigma, UK) in PBS. Fifty microlitres of undiluted serum samples were then dispensed into the wells and incubated for 2 hour at 37°C. Control wells received 50 μ l of the blocking buffer. Unbound serum was washed off six times as above and 50 μ l of alkaline phosphate conjugated rabbit anti monkey IgG (Cappel, organon Teknika, Or,USA) diluted 1/2000 was added and followed by incubation for 1 hour at 37°C. Unbound conjugated rabbit anti monkey IgG was washed off as above before adding 50 μ l of p-nitrophenyl phosphate substrate (pNPP, Sigma, UK, final concentration 1mg/ml) in 10% diethanolamine buffer. The plates were incubated at 37°C for 30 minutes in the dark. Optical density (O.D) was read at 405nm in a microplate reader (Dynatech laboratories, UK). In assaying for IgM antibodies in serum, 100 μ l of Horse radish peroxidase conjugated rabbit anti monkey IgM (cappel Organon Teknika, OR, USA) diluted 1/2000 was added and incubated for one hour at 37°C. One hundred microlitres Orthophenyldiamine (OPD Sigma, UK, final concentration of 0.4 μ g/ml) in phosphate citrate buffer was used as the substrate. Optical density (O.D) was read at 450nm after 30 min of incubation.

2.5 Interferon gamma detection

Purified peripheral blood mononuclear cells (PBMC) were adjusted to 4×10^6 /ml in complete medium -RPMI 1640 medium supplemented with 10% heat inactivated FBS, 2-mercaptoethanol (5×10^{-5} M), L-glutamine (5mM/ml), and gentamycin (100 μ g/ml) and stimulated with either Con A or crude *P. knowlesi* whole parasite antigen. Culture supernatants were pooled from triplicate wells after the stimulation of PBMC. The concentration of IFN γ in the supernatants produced after the 72 hours of stimulation, and serum samples was determined in a sandwich ELISA as follows:

Polystyrene micro-Elisa plates (Dynatech laboratories, Sussex UK) were coated overnight with 50 μ l of monoclonal antibody which was rabbit anti-human recombinant IFN γ (2 μ g/ml; Genzyme Corporation MA, USA). The wells were washed 6 times with washing buffer (1%BSA and 0.05% Tween 20 in PBS). Non specific binding sites were blocked with 200 μ l of 3% BSA in PBS for 1 hr at 37°C. Excess blocking buffer was flicked off, and 50 μ l of test samples added. Wells where blocking buffer alone was added (blank wells) served as negative controls. These were incubated for 2hrs at 37°C and then washed as described above. 50 μ l biotinylated anti-human IFN γ (Mab-7B6-1-Biotin: MABTECH, Sweden) was then added at a dilution of 1:200 per well and incubated for 1hr at 37°C. Washing was done as before and 50 μ l of streptoavidin (ALP-PQ MABTECH, Sweden) was added at a dilution of 1:10,000 to each well, and incubated for 1hr at 37°C. Fifty microlitres of p-NPP in 10%

diethanolmine buffer was added to each well and plates incubated at 37°C for 30 minutes in the dark. The plates were read at an Optical density (O.D) of 405nm in a microplate reader. Optical density readings for the blank wells were subtracted from O.D readings of the sample wells to get the final O.D reading.

2.6 Recall proliferation assay

Peripheral blood was obtained from the femoral vein and diluted in an equal volume of sterile Alsevers solution. Peripheral blood mononuclear cells (PBMC) were isolated over Ficoll-hypaque by centrifugation at 3000 RPM for 30 minutes. A ratio of 4ml of diluted blood to 3ml of Ficoll-hypaque was used. Cells at the interface were aspirated and washed in Alsevers solution and complete RPMI 1640 medium. Cell viability was determined by trypan blue stain.

Purified PBMC were adjusted to 4×10^6 /ml in complete RPMI 1640 medium. Cell suspension (50 μ l) was distributed to each well of the 96 well flat bottomed micro titre plates (Nunc, Roskilde, Denmark). One hundred microlitres of crude *P. knowlesi* whole parasite antigen (1×10^6 parasites) or Concanavalin A (Con A; sigma UK) at a concentration of 10 μ g/ml were added to the wells. Control wells received 100 μ l of complete RPMI 1640. Cultures were set up in triplicates.

The cultures were then incubated at 37°C in humidified atmosphere containing 5% CO₂ for 72 hours. The cells were pulsed with 0.5 μ Ci of ³H-thymidine (5m Ci/mg: Amersham, UK) in 20 μ l RPMI 1640 medium and a further incubation was carried out for 18-24hrs. Cells were harvested onto fibre glass filters by using Titertek cell harvesters (Flow laboratories, UK). The filters were air dried and immersed in scintillation fluid (PPO, POPOP, Triton x-100, Germany). Incorporation of ³H-thymidine into DNA was determined by liquid scintillation spectrophotometry. Results were expressed as stimulation index (SI) that was obtained by dividing the proliferation of test cultures by proliferation of control cultures.

3. Results

3.1 Primary infection parasitaemia

All the baboons inoculated with *P. knowlesi* H strain blood stages developed patent parasitaemia with all the blood stages being manifested. Those that had acute parasitaemia, developed patent parasitaemia by day four post inoculation while chronic animals had patent parasitaemia by day five inoculation (Table 1). Acute animals had parasitaemia that systematically increased to over 6%, reaching as high as 9.1% in some baboons at the time of treatment. Chronic animals had peak parasitaemia levels ranging from 1.5%-5%, which thereafter decreased to less than 2% parasitaemia on average.

3.2 Secondary infection parasitaemia

Animals with chronic parasitaemia were cured and re-infected as described earlier. The animals developed patent parasitaemia by day six post infection. Peak parasitaemia levels were on average, lower than during the primary infection (Table 2). Thereafter parasitaemia levels decreased to sub patent levels by day 14 post re-infection. Animals that had developed acute parasitaemia during the primary infection were withdrawn from the experiment due to technical reasons unrelated to the experiment

3.3 Clinical symptoms

The clinical symptoms observed were as seen by Ozwara and others (Ozwara *et al.*, 2003).

3.4 Antibody responses

All baboons produced anti *P. knowlesi* IgG and IgM antibodies upon infection with *P. knowlesi* blood stage parasites (Figure 1 and Figure 2). During the first two weeks of infection, IgM responses were higher in acute animals (Mean O.D \pm SD: 0.238 \pm 0.135) than in the chronically infected animals (Mean O.D \pm SD: 0.173 \pm 0.057). Anti *P. knowlesi* IgG responses increased slowly upon infection. The increase in IgG responses however was higher in chronic animals (Mean O.D \pm SD: 0.113 \pm 0.04) than in acute animals (Mean O.D \pm SD: 0.097 \pm 0.014). Immunoglobulin G levels had increased by 18% in acute animals at day 14 post infection, whereas in chronic animals the increase at day 14 post infection was 48 %, and 72%. Immunoglobulin M levels in both the acutely and chronically infected baboons remained higher than IgG levels in all the sampling points, apart from day 42 where the IgG levels were higher in the chronic animals (Figure 2).

3.5 Cytokine responses

All the animals produced IFN γ by day 7 post infection (Figure 3). Acute animals showed a steady increase in serum IFN γ (Figure 3). Although the IFN γ produced by animals with chronic infection also rose in the course of the primary infection reaching a peak at day 14 (Figure 3), the levels in acutely infected animals was much

higher at the two sampling points (Figure 3). Upon treatment, the IFN γ levels in chronically infected animals subsided and then rose again one week later, only to decrease again by day 42 post infection (Figure 3). Interferon gamma detected in blast supernatants increased in both acute and chronically infected animals upon infection (Figure 4). The levels in acutely infected animals reached peak by day 14 post infection (Figure 4). In chronically infected animals, IFN γ in the supernatant increased gradually to reach a peak at day 42 post primary infection (Figure 4). Levels of IFN γ produced by cells from acutely infected animals remained higher than in those from animals with chronic infection (Figure 4).

3.6 Cellular immune responses

PBMC from the baboons showed mitogen and antigen specific proliferation, with the highest stimulation index observed from chronically infected animals by day 42 post primary infection 10.06 ± 2.74 (mean \pm S.D). Antigen proliferation observed in PBMC from acutely infected animals was lower compared to chronically infected animals at day 14 post inoculation (Figure 5). Upon re-infection, antigen specific proliferation responses increased in chronically infected animals by day 14 post re-infection (Figure 5).

4. Discussion

In this study immune responses (antibody, cytokine and recall proliferation responses) mounted by experimental *P. knowlesi* infection in olive baboons (*Papio anubis*) are presented for the first time. Our report confirms that *P. anubis* is fully susceptible to experimental *P. knowlesi* H strain infection since all infected animals developed patent parasitaemia. Infected baboons developed either acute or chronic infection. Acutely infected baboons mounted an immune response that failed to control parasitaemia. Previous work showed that *P. knowlesi* infected baboons develop either acute or chronic infection (Ozwarra *et al.*, 2003). Results in the current study are in support of the findings in the above mentioned previous study. The precise mechanism that predisposes a dual outcome in the infection profile warrants further investigations.

In baboons with severe *P. knowlesi* infection, patent parasitaemia developed earlier than in the chronically infected animals. The disease profiles reported here are similar to rhesus monkeys infected with *P. knowlesi* H strain (Ozwarra *et al.*, 2003; Ibiwoye *et al.*, 1993; Rosen *et al.*, 1998). However, our experiments are the first to show that *P. knowlesi* infected baboons can be infected, treated and re-infected. This suggests that baboons infected with *P. knowlesi* do not develop sterile infection following challenge exposure. Although it has been established that immune responses against malaria does not result in sterile infection (Chen *et al.*, 2000), we have shown that chronically infected *P. anubis* are able to control parasitaemia to sub patent levels upon re-infection. The profile of secondary infection of acutely infected baboons remains unknown. Secondary infection data from these animals indicates that protection is associated with pre-exposure as described in Voller and Rossan, 1969 and Richie and Saul, 2002.

During primary infection, antibody (IgG and IgM) levels increased in both infection groups. Immunoglobulin M titres increased steadily and remained higher than the IgG levels. Levels of IgM were raised more rapidly during the primary infection. In addition acutely infected animal showed higher IgM levels. *Plasmodium knowlesi* infections in monkeys show antigenic variations (Brown K and Brown I, 1995). This antigenic variation is possibly responsible for this persistently raised IgM levels seen in the *P. knowlesi* infected baboons. It is interesting to note that acutely infected animal produce higher IgM titres while chronically infected baboons show elevated IgG production. This pattern suggests that elevated production of IgM on its own is not sufficient to control parasitaemia and prevent severe infection. Previous studies have shown that IgM responses are mounted faster than IgG, especially in primary infections and IgM is seen to boost the initial response (Roit, 1988). This is similar to our findings. However, we can report here that control of parasitaemia is associated with elevated IgG response, as shown in chronically infected animals. The rise in IgG levels was modest during primary infection but elevated following challenge exposure of chronically infected animals, showing evidence of immunological memory.

Acutely infected animals developed higher parasitaemia levels as compared to the chronic animals during the primary infection. The fact that chronically infected animals were able to control the parasitaemia during the primary infection and reduce parasitaemia further to below detectable levels upon re-infection supports the role of acquired immune responses in protection against *P. knowlesi* infected baboons. It has been demonstrated in human malaria blood stage infection that antibodies are involved in protection (Miller *et al.*, 1998; Good *et al.*, 1998). Furthermore, T cell proliferation in response to blood stage antigens is also associated with protection (Miller *et al.*, 1998; Good *et al.*, 1998). In addition there is growing evidence for the protective role of IgG in *P. falciparum* (Christophe *et al.*, 2000). Passive transfer of IgG has provided protection against *P. falciparum* blood stage in South American monkeys (Fandeur *et al.*, 1984; Groux and Gysin, 1990), and in humans

(Bouharoun-Tayoun *et al.*, 1990). Furthermore, human antibodies efficiently inhibit *in vitro* *P. falciparum* merozoite proliferation (Bouharoun-Tayoun *et al.*, 1990), and mediate opsonisation of infected erythrocytes (Groux and Gysin, 1990). Our study indicates a role for IgG in protection against severe disease in baboons. However, further investigations are warranted to determine the role of increased IFN γ production in acute infection as shown in this study.

During the primary infection, proliferative cellular responses were higher in chronically infected baboons, especially after two weeks post-infection. This clearly demonstrates better immunological memory elicited in chronically infected baboons. Because antigen induced proliferative responses are pre-dominantly mediated by T cells, our results suggest that T cell memory develops in *P. knowlesi* infected baboons. This can be interpreted further to mean that T cell responses are required in order to control parasitaemia in these animals. Similar suggestions have been made for studies in humans and chimpanzees (Pombo *et al.*, 2002; Taylor *et al.*, 1985).

The role of cytokines in the regulation of immune responses against *Plasmodium* infections and pathogenesis of malaria has been extensively studied in rodent malaria models but there are few studies in primate malaria (Chunfu yang *et al.*, 1999). In one study in which human volunteers were infected experimentally with *Plasmodium falciparum*, there was increased production of IFN γ (Harpaz *et al.*, 1992). In a second study, *P. Coatneyi* infected Rhesus monkeys demonstrated an increased IFN γ production occurring in the early stage of infection, and is thought to have been associated with the pathologic changes that led to death of the infected animals (Chunfu yang *et al.*, 1999). This observation is supported by Riley (1990) who suggested that low amounts of IFN γ may be associated with resolution of infection. Therefore, excessive production of proinflammatory cytokines by human host has an adverse effect on disease progression (Chen *et al.*, 2000). High circulating levels of IFN γ and TNF α are more often found in patients with severe malaria than in uncomplicated cases (Kwiatkowski *et al.*, 1990). These studies, combined with ours, demonstrate a negative role for high IFN γ production during primary malaria infection. Resistance or susceptibility to malaria may depend on induction of appropriate levels of IFN γ and possibly other pro-inflammatory cytokines like TNF α as suggested by others (Riley, 1990). Factors that mediate high IFN γ production early in baboons, resulting in severe disease warrants further investigation. Similar mechanisms are likely operating in humans infected with *P. falciparum*. We can conclude that this phenomenon is linked to dual disease outcomes observed in baboons infected with *P. knowlesi*. Described results are important in validation of the new baboon model for drug and vaccine development against malaria.

5. Conclusion

In this study, baboons experimentally infected with *P. knowlesi* and showing acute infection profile were shown to produce higher IFN γ in the first two weeks of infection compared to chronically infected animals. This has indicated that high IFN γ production early in infection is associated with severe disease outcome. Although early infection in both acute and chronically infected baboons was associated with high IgM responses, antibody on its own was unable to prevent development of severe infection. Mechanisms resulting in moderated production of IFN γ at the start of malaria infection in baboons partly hold the key in understating control of severe infection. These studies are important in validation of the *P. knowlesi*- baboon model for malaria drug and vaccine research

Authors Contributions

T. Nyawira planned the study, undertook laboratory experiments and wrote the draft manuscript. M. Gicheru and Z. Ng'ang'a participated in supervising experiments and reviewing the manuscript. H. Ozwara developed the idea, obtained funding for the study and supervised planning, experiments and reviewed the manuscript.

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Table 1. Disease status of baboons infected with *P. knowlesi* H Strain during primary infection

Baboon number	Infection	profile	Day for patent parasitaemia
	Profile	Day post inoculation	
Pan 2730	Acute	9	3
Pan 2574	Acute	9	3
Pan 2677	Acute	10	4
Pan 2601	Chronic	10	5
Pan 2669	Chronic	10	5
Pan 2698	Chronic	11	5

Table 2. Comparison of parasitaemia in chronic animals during primary and secondary infections with *P. knowlesi*

Baboon number	Parameter	Primary infection	Secondary infection
Pan 2669	Day of patent parasitaemia	5	6
	Peak parasitaemia	5%	2.39%
	Treatment parasitaemia*	1.3%	<<
Pan 2601	Day of patent parasitaemia	5	6
	Peak parasitaemia	2.6%	1.5%
	Treatment parasitaemia*	1.94%	<<
Pan 2698	Day of patent parasitaemia	5	6
	Peak parasitaemia	1.5%	2.62%
	Treatment parasitaemia*	0.1%	<<

* Parasitaemia recorded at onset of treatment

<< Parasitaemia below detectable levels.

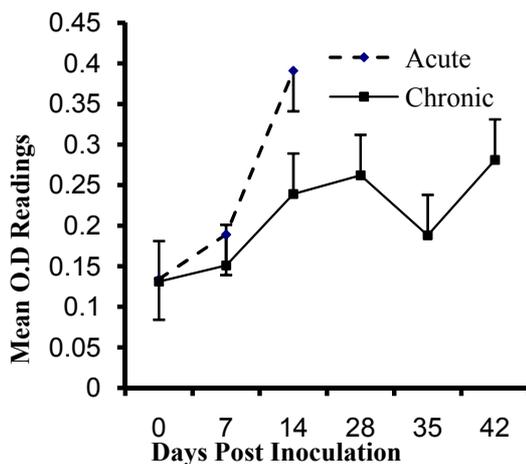


Figure 1. Mean IgM responses of baboons infected with *P. knowlesi*

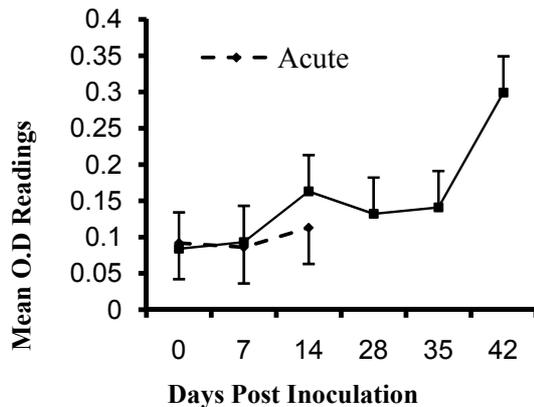


Figure 2. Mean IgG responses of baboons infected with *P. knowlesi*

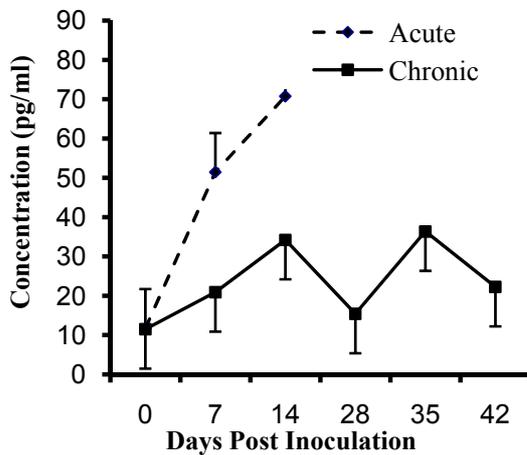


Figure 3. Concentration of IFN- γ in serum from baboons infected with *P. knowlesi*

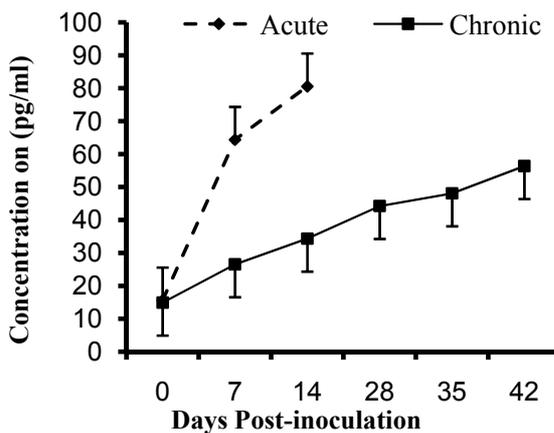


Figure 4. Interferon gamma levels in supernatants from PBMC stimulated with *P. knowlesi* crude whole parasite antigen

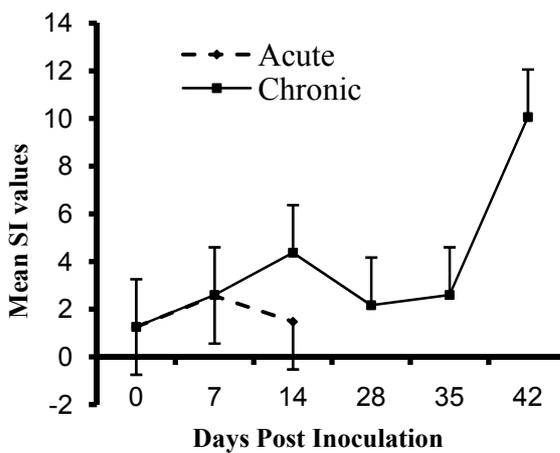


Figure 5. Antigen specific recall proliferative responses of PBMC from *P. knowlesi* infected baboons