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Plasmodium berghei is immunomodulated by transgenic mouse interferon gamma leading to enhanced malaria protection in mice

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Abstract: *The aim of this study was to explore immunomodulatory potential of immunopotentiated Plasmodium berghei parasites in a murine malaria model with a view of contributing to malaria vaccine development strategies. Transfection was used to generate immunopotentiated Plasmodium berghei parasites through expression of mouse interferon gamma (mIFN-γ) in wild-type Plasmodium berghei parasites. Mice were inoculated with mIFN-γ expressing Plasmodium berghei parasites and treated. Another group of mice was inoculated with the parasite expressed mIFN-γ culture supernatants. The mice were later intraperitonially challenged with wild-type parasites. Sampling for cytokine and antibody assays was done and ELISA performed on the collected samples. Parasitaemia was monitored daily and survival time (days) recorded for the two sets of experiments. Analysis of variance (ANOVA) was used to analyze the results using graphpad instat software. There was a significantly higher level of IFN-γ ($p < 0.001$). The level of IL-4 was significantly low ($p < 0.05$). There was no significant difference in the levels of IgG ($p = 0.0682$). There was a 3 to 4 day delay in patent parasitaemia accompanied by reduced mean parasitaemia and improved survival of the mice. This study showed that interferon gamma expressing Plasmodium berghei immunomodulates malaria infection in mice leading to enhanced protection during challenge infection.*
Key words: *immunomodulation, interferon gamma, parasitaemia, Plasmodium berghei, transfection.*

I. Introduction

Malaria, a parasitic infection caused by *Plasmodium* species causes up to 755,000 deaths per year (1), mainly in the tropical and subtropical regions of the world [2, 3, 4]. Although vaccination is regarded as the most effective strategy in control of infections, there is currently no registered vaccine available for the control of malaria [5, 6]. However, there is ample evidence that a malaria vaccine feasible [7, 8].

Most malaria vaccine development approaches have explored subunit [6, 9], DNA [10] or irradiated sporozoites [11]. These vaccines have faced challenges such as the choice of appropriate adjuvant and inconsistent efficacy results during trials [12]. Currently, the most advanced malaria vaccine candidate is the subunit vaccine RTS, S/AS01 [12]. The vaccine has undergone various field trials and has been shown to have low efficacy rates (30-50% prolonged protection from infection) [13, 14, 15, 16].

Use of whole-organism vaccines such as live attenuated or immunomodulated parasites could resolve this challenge [11, 12]. Although these types of vaccines raise interesting questions such as ability for mass production, possibility of generating a live infection as well as mode of delivery [12, 16,17,18, 19], they present a nearly total repertoire of antigens to the immune system [17]. In addition, antigen is delivered in native conformation and no precise antigen or epitope determination is required [18].

Increase in genomic and biological knowledge especially the genetic manipulation of *Plasmodium* parasites by transfection has facilitated the possibility of developing genetically attenuated malaria parasites through mechanisms such as immunopotentiation by expression of host cytokines [20]. One such cytokine is IFN-γ, which apart from its immunomodulating activity in viruses [21], is a key effector cytokine in protection against malaria [22]. Our previous work [22] has shown that *Plasmodium* transgenically expresses bioactive host IFN-γ. This study aimed at exploring immunommodulatory potential of mIFN-γ expressing *Plasmodium berghei* in a mouse malaria model.

II. Materials and Methods

2.1 Experimental mice and parasites

Inbred male BALB/c mice, 6 to 8 weeks old and weighing 20-24g were used in this study. These mice were bred at the rodent facility of the Institute of Primate Research (IPR), fed on standard pellet diet and water provided ad libitum. Care, use and disposal of animals were done according to the IPR guidelines on handling of experimental animals.

The study involved use of *P. berghei* ANKA parasites that were donated by Kenya Medical Research Institute (KEMRI).

2.2 Experimental groups

Two sets of experiments were performed in replicates. The first set of experiments constituted 3 groups of 5 mice each, randomly assigned to the experimental groups. The test group was inoculated with IFN-γ expressing *P. berghei*. Control groups constituted of a group of mice inoculated with wild type *P. berghei* and another group of uninfected mice. The groups were treated of the infection and challenged later with wild type *P. berghei*. The second set of experiments constituted 4 groups of 5 mice each. These mice were inoculated with *P. berghei* expressed mouse IFN-γ culture supernatants and challenged later with wild-type *P. berghei*.

Antibody (serum) and cytokine (splenocyte culture supernatant) responses were assessed and parasitaemia monitored daily as described elsewhere [23]. Survival time (days) was also recorded.

2.3 Preparation of the transfection constructs

The mouse IFN-γ expression transfection construct was prepared by isolating the open reading frame (ORF) of murine IFN-γ by restriction digestion of the murine IFN-γ cloning vector with the help of XbaI and SpeI restriction enzymes. Klenow polymerase was used to extend it and purification done using Qiagen gel extraction kit (Qiagen, Chartsworth, Calif.). The IFN-γ gene was cloned into the blunted BamHI site of the plasmid (pD.D_{TM}.D./D.-.D.) to generate (pD.D_{TM}.D./D.$_{\gamma MM}$.D). Multiplication of copies of the transfection constructs was achieved by transformation of E. coli cells, DH5-α, (Invitrogen ^{TM}), followed by isolation of the transformed cells. The competent DH5-α cells (Invitrogen subcloning efficiency) were transformed by heat shock method as described by Panja [24].

2.4 Extraction of the Plasmid DNA from DH 5-α Cells

The plasmid DNA extraction was done by the alkaline lysis method as described by Birnboim and Doly [25]. To confirm correct plasmid extraction, randomly picked samples were run on agarose gel to visualize DNA bands. Restriction digestion of the extracted DNA was done using two enzymes (EcoRI and EcoRV), separately, followed by Polymerase chain reaction, visualization and photography of the bands.

2.5 Propagation and purification of mature *P. berghei* parasites schizonts

This was done in accordance with the protocols described by Doolan [26]. Briefly, *P. berghei* ANKA parasites were quickly thawed in a water bath at 37°C and an equal volume of 1 x PBS added. This PBS/parasite mixture was then injected into 6 mice (100µl of parasite/PBS mixture per mouse) and parasitaemia monitored. At parasitaemia of between 4% and 5%, the mice were heart bled and the infected cells cultured for development into mature schizont stage. These were then subjected to Nycodenz density centrifugation [27] to isolate and concentrate the schizonts.

2.6 Electroporation of *P. berghei* and selection of the transformed parasites

A mixture of schizont and sterile cytomix containing the transfection plasmid DNA was aseptically transferred into a 0.4 cm electroporation cuvette and immediately transferred into the electroporation machine (Bio Rad gene Pulse^{TM}). This suspension was subjected to a single pulse (1000 V, 25 F) of the Bio-Rad gene pulser I electroporation machine and immediately transferred into ice for 5 minutes and then, about $5x10^9$ merozoites intravenously inoculated into the tail vein of BALB/c mice. Selection was done using pyrimethamine in drinking water as described by Doolan [26].

2.7 Confirming the success of *P. berghei* transfection

This was achieved by Plasmid DNA analysis. Plasmid rescue of circular transfection vectors from the transfected parasites was done and followed by restriction digestion of the plasmid DNA and PCR of the plasmid genes. Briefly, total parasite DNA was isolated and used to transform competent *E. coli* by electroporation. Then, LB broth was added and incubation done at 37°C for 1 hr with continued shaking after which single bacterial colonies were picked and cultured in LB broth containing 50µg/ml ampicilin for 18 hours. This was followed by plasmid isolation from the DH 5-α cells as described by Birnboim and Doly [24]. The isolated plasmid DNA was subjected to restriction digestion and polymerase chain reaction (PCR) to confirm

the presence of the mouse IFN-γ gene and the *T. gondii* DHFR gene. The primers used for the PCR of the mutagenised DHFR gene were (5' ATG CAT AAA CCG GTG TGT CTG 3') as forward primer and (5' CGT GAT CAA AGC TTC TGT ATT TCC GC 3') as the reverse primer. For the mouse IFN- γ gene, the forward primer was (5' CGG GAT CCA TGA ACG CTA CAC ACT GCA TC 3') while the reverse primer was (5' CGG GAT CCT CAG CAG CGA CTC CTT TTC CGC TTC 3').

2.8 Testing for IFN-γ expression in transfected *P. berghei* culture

This was confirmed by mouse IFN-γ ELISA. The cryopreserved, transfected *P. berghei* parasites were retrieved from liquid nitrogen, passaged in mice thorough intraperitonial injection and parasitaemia monitored daily as from day 3 post-infection. At parasitaemia of between 3% and 5%, the mice were euthanized and the parasitized cells collected, mixed with an equal volume of 1×PBS and injected into 6 mice for in vivo propagation under pyrimethamine pressure. Parasitaemia was monitored daily from day 3 post-infection. At parasitaemia of between 4% and 6%, the mice were bled via cardiac puncture and blood from all the mice pooled. The blood was washed thrice with an equal volume of plain RPMI 1640 (Gibco, Lot No.RNBC0616) at 200g for 10 minutes at 24°C. The supernatant and top layer of blood cells and other blood factors were discarded. The pellet (1ml at 5% parasitaemia) constituting of approximately 4.5×10^8 parasites was diluted at 2.5% hematocrit in RPMI 1640 medium (Gibco, Lot No.RNBC0616) supplemented with 25mM HEPES (Sigma, Lot No. BCBG2879B), 200 mM L-glutamine, and 25% fetal bovine serum and Neomycin (Sigma, Lot No. 097K2326). The culture was aseptically transferred into a T-75 culture flask. Then, it was gassed and incubated overnight at 37°C for 12 hours, with agitation at regular intervals, for development into schizont stage. After the 12 hours, the culture was washed once at 200g for 10 minutes at room temperature. The pellet (1ml) was resuspended at 20% hematocrit in the above supplemented RPMI culture medium, transferred into a T-25 culture flask, gassed and incubated for 6 hours for interferon gamma expression. The culture was then be spun at 200g for 10 minutes at 24°C and the supernatant collected (about 4ml). The supernatant was then filter-sterilized using 0.45um-pore membrane syringe filters and aliquots taken for interferon gamma expression confirmation and quantification by ELISA (Mabtech, Sweden) as per the manufacturer's instructions. The same protocol was adopted for culture of wild-type *P. berghei* (about 1ml pellet at 5.3%) and red blood cells (about 1ml pellet) from uninfected mice for control experiments. The culture supernatants were then used for immunization of mice.

2.9 In vivo growth of interferon gamma expressing *P. berghei*

The experimental group was infected with 2.0×10^6 IFN-γ expressing *P. berghei*. The control group was infected with the same number of wild-type *P. berghei* and parasitaemia checks done daily from day 3 onwards.

2.10 Survivorship of IFN-γ expressing *P. berghei* infected mice

Each mouse of the experimental group and the control group was infected with 2.0×10^6 IFN-γ expressing *P. berghei* and wild-type *P. berghei* respectively. Survival time (days) of the mouse was recorded for a period of two weeks.

2.11 Effects of IFN-γ gamma expressing *P. berghei* on parasitaemia in mice

The experimental group received 5×10^9 IFN-γ expressing *P. berghei* per mouse while the control group mice received the same number of the wild-type parasites. Parasitaemia was monitored daily for both groups and when it reached 4%, all the mice were cured using Artemisinin Lumefantrine combination therapy (COARTEM™). After two weeks, the animals were challenged with 5×10^9 wild-type parasites. At the point of challenge, a third group was introduced to act as a naive control.

2.12 Effects of *P. berghei* expressed IFN-γ culture supernatants on parasitaemia and mice survival

Mice were inoculated with 37.5pg/ml IFN-γ in 200µl *P. berghei* expressed mouse IFN-γ culture supernatants. Fourteen days later, the mice were challenged by intraperitonial injection of *P. berghei* suspension containing 1×10^6 parasites. Parasitaemia was monitored daily and survival time (days) recorded. The control groups comprised of a group of mice inoculated with culture supernatants from wild-type *P. berghei* and another one inoculated with culture supernatants from naïve red blood cells (RBCs). A final control group of mice was inoculated with RPMI.

2.13 Effects of *P. berghei* expressed IFN-γ culture supernatants on host antibody and cytokine response

Sampling for antibody and cytokine determination was done 3 weeks after inoculating mice with the *P. berghei* expressed IFN-γ culture supernatants (one week after challenge). Antibody (IgG) ELISA was done on serum prepared from blood obtained from the mice via cardiac puncture. Cytokine production was determined

by measuring the amount of the cytokines secreted into culture media during splenocyte culture. Splenocyte preparation and culture was done as described by Yole *et al*, [28]. The mouse IgG, IFN-γ and IL-4 were determined using ELISA according to the manufacturer's instructions (Mabtech AB, Sweden).

III. Results

3.1 Transfection of *P. berghei* and Analysis of mouse IFN-γ expression

In order to determine expression of transgenic mouse IFN-γ in *P. berghei*, wild-type *P. berghei* were episomally transfected with a pUC plasmid (Figure 1A) expressing mouse IFN-γ and pyrimethamine resistance (TgDHFR) genes. The TgDHFR gene facilitates selection of transformed parasites. A plasmid rescue experiment (to confirm the presence of the correct plasmid in the parasite) followed by plasmid DNA analysis (to confirm integrity of the plasmid) was performed. In addition, the transformed parasites were cultured and the supernatants subjected to ELISA to confirm IFN-γ expression by the parasites. A plasmid rescue recovered plasmids from the IFN-γ expressing *P. berghei* parasites (Figure 1B). A restriction digestion of these plasmids showed that both the mouse IFN-γ gene (Figure 1C) and the mutagenised DHFR gene (Figure 1D) were intact. ELISA of the culture supernatants showed expression of up to a 9 fold concentration of IFN-γ in the culture set up containing the transformed parasites as compared to the wild-type parasites (Table 1).

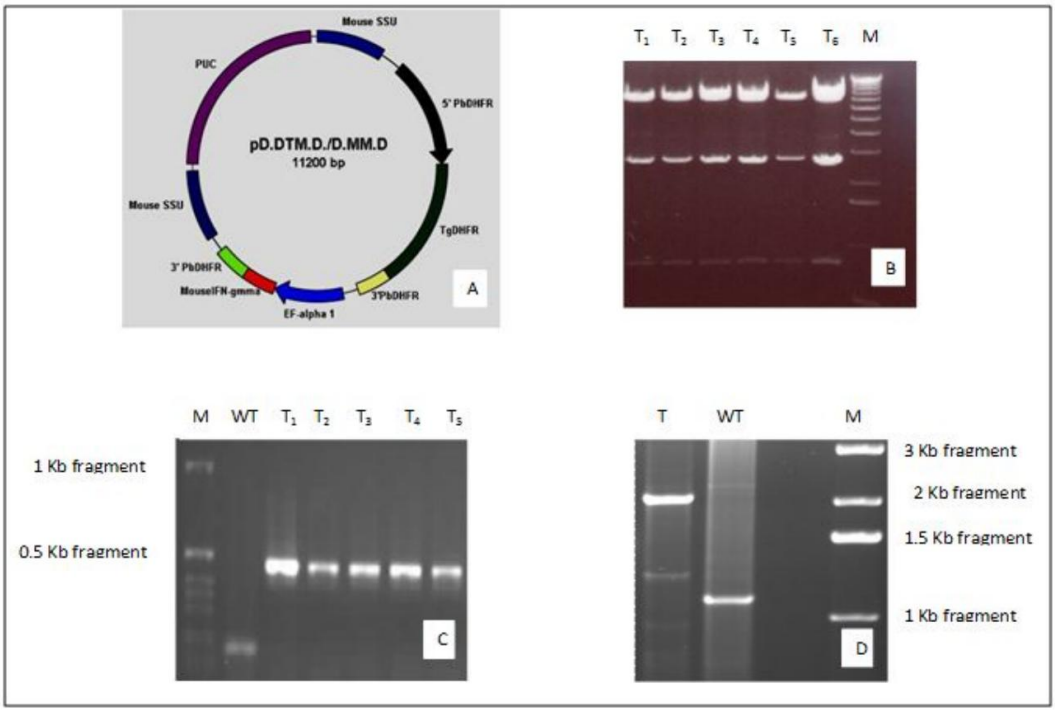

Figure 1: **Expression of mouse IFN-γ in *P. berghei***; A-map of plasmid used to transform *P. berghei*: the relative position of the genes and their relative sizes (Mouse SSU - 1 Kb, 5'PbDHFR- 1.5 Kb, TgDHFR-1.8 Kb, 3'PbDHFR - 0.5 Kb, EF-alpha 1 - 1Kb, Mouse IFN-γ -0.5Kb, 3'PbDHFR - 0.5Kb and pUC - 2.7Kb); **B** - Gel electrophoresis of the products of the plasmid rescue experiment (M-molecular marker; T_{1-6} - DNA samples isolated from IFN-γ expressing *P. berghei*); **C** - Gel electrophoresis of PCR product to show the integrity of mouse IFN-γ gene in the transformed *P. berghei*: (M-molecular marker, T_{1-5} - DNA samples isolated from the IFN-γ expressing *P. berghei*, WT- DNA sample isolated from wild-type *P. berghei*); **D** - Gel electrophoresis of PCR product to show the integrity of TgDHFR - TS gene in the IFN-γ expressing *P. berghei*: (M - molecular marker, T-DNA samples isolated from the IFN-γ expressing *P. berghei*, WT - DNA sample isolated from wild-type *P. berghei*).

Table 1. Concentration (pg/ml) of mouse interferon gamma in the different culture types

Culture type	IFN-γ concentration (pg/ml)
Transfected *P. berghei* culture supernatant	187.52
Wild type *P. berghei* culture supernatant	20
Red blood cells culture supernatant	< 20

3.2 IFN-γ expressing plasmid had no marked effect on in vivo growth of *P. berghei*

In order to determine the impact of plasmid expression on growth rate of IFN-γ expressing *P. berghei* parasites, mice were inoculated with IFN-γ expressing *P. berghei* and control group received an equal number of the wild-type parasites. Results showed a non significant reduction in growth rate of IFN-γ expressing parasites (Figure 2).

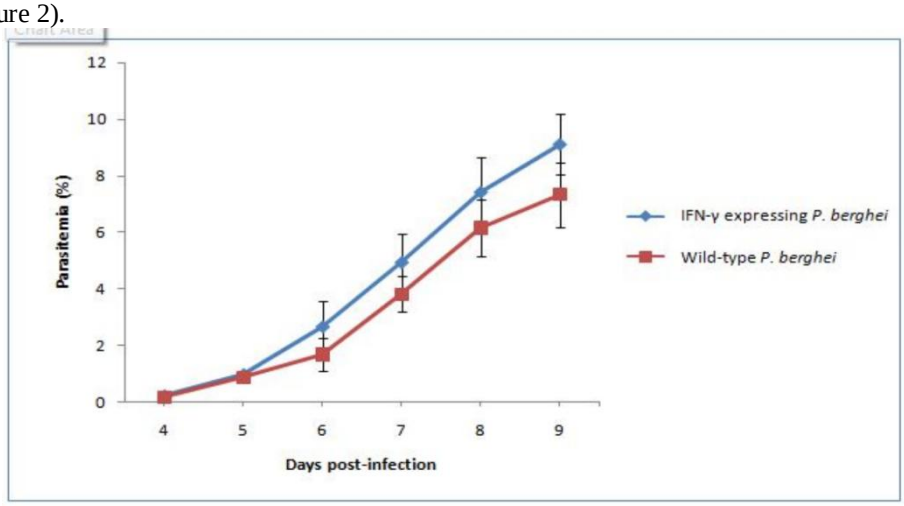

Figure 2: **Comparative growth rates of IFN-γ expressing and wild-type *P. berghei* in mice**. Mice were infected with wild-type and IFN-γ expressing *P. berghei* and parasitaemia monitored.

3.3 IFN-γ expressing *P. berghei* is less virulent in vivo

Impact of IFN-γ expressing *P. berghei* on survivorship of mice was compared to that of mice infected with wild-type parasites. These data would display protective potential of IFN-γ expressing *P. berghei* in mice. The mean survival time over a two week period was 4 days longer in the group infected with the IFN-γ expressing parasites compared to the controls (Figure 3). Eighty percent of mice in the IFN-γ expressing *P. berghei* group survived to day 14 compared to 100% death in controls.

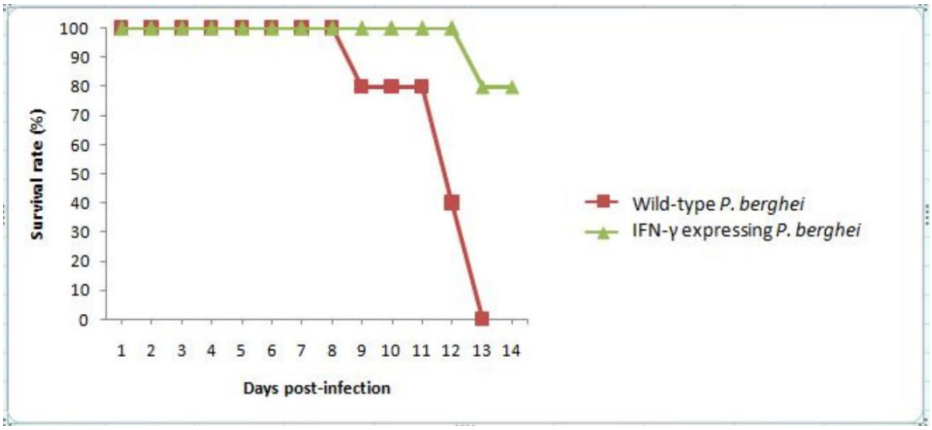

Figure 3: **Survivorship of mice infected with IFN-γ expressing and wild-type *P. berghei*.** Mice were infected with wild-type and IFN-γ expressing *P. berghei* and the survival time of the mice recorded.

3.5 IFN-γ expressing *P. berghei* delays parasitaemia patency and reduces mean parasitaemia during challenge infection

In order to determine how pre-exposure of mice to IFN-γ expressing *P. berghei* affects parasitaemia in a challenge infection, the experimental mice were inoculated with IFN-γ expressing *P. berghei*, cured of the infection, challenged with the wild-type parasites and parasitaemia monitored daily. Control groups constituted naïve mice and mice pre-exposed to wild-type parasites. All the groups of mice were infected. However, there was a 3 day delay in parasitaemia patency for the experimental group. In addition, mean parasitaemia was significantly reduced ($p = 0.023$) in the experimental group as opposed to the controls (Figure 4).

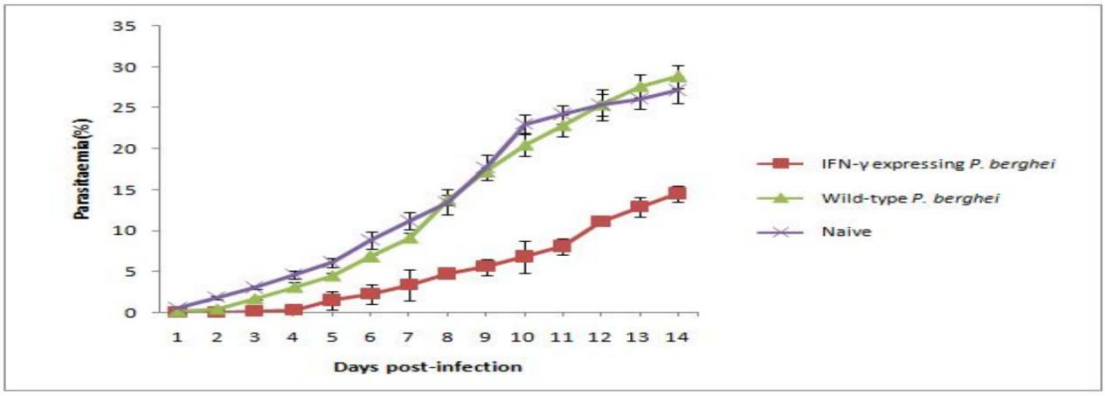

Figure 4: **Comparative mean parasitaemia curves for the different treatment groups upon challenge.** Mice were inoculated with mouse IFN-γ expressing *P. berghei*, cured of the infection and challenged by intraperitonial injection of wild-type *P. berghei*. Parasitaemia was then monitored.

3.6 *P. berghei* IFN-γ culture supernatants suppress parasitaemia in mice during challenge

We set to determine whether *P. berghei* expressed IFN-γ culture supernatants affects parasitaemia in a challenge infection as observed in the use of the IFN-γ expressing parasites. Culture supernatants from IFN-γ expressing *P. berghei* were inoculated into the experimental group of mice. Two weeks later, the mice were challenged with wild-type *P. berghei*. The group pre-exposed to *P. berghei* expressed IFN-γ culture supernatants exhibited a 5 day delay in onset of patent parasitaemia (Figure 5). There was reduced mean parasitaemia with parasitaemia suppression being 10 times higher in this group (94.15% on day 11), compared to the controls (8.81% on day 11). This showed that *P. berghei* IFN-γ culture supernatants delay parasitaemia patency and reduce mean parasitaemia during challenge infection.

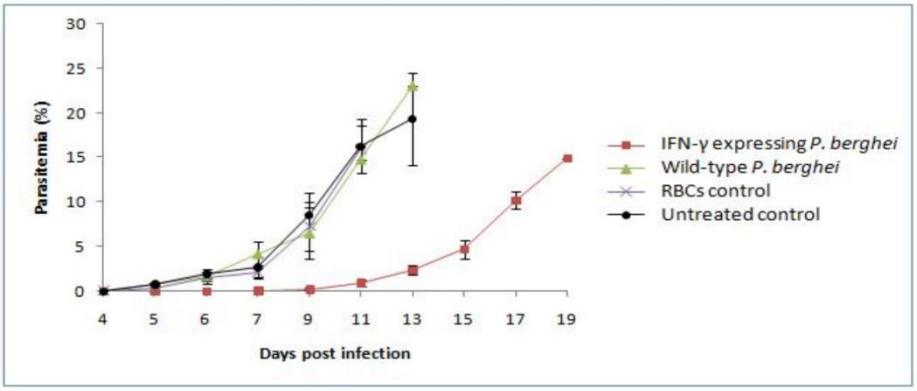

Figure 5: Comparative parasitaemia curves for the different treatment groups upon challenge. Mice were inoculated with 37.5pg/ml IFN-γ in 200µl *P. berghei* expressed mouse IFN-γ culture supernatants. Later, the mice were challenged by intraperitonial injection of wild-type *P. berghei* and parasitaemia was monitored.

3.7 *P. berghei* expressed mouse IFN-γ culture supernatants enhance protection of mice from malaria

Further experiments were done to determine if *P. berghei* expressed IFN-γ culture supernatants protect mice from death upon challenge with malaria parasites. Mice were challenged with wild-type *P. berghei* at two weeks post-inoculation of culture supernatants of IFN-γ expressing *P. berghei* and survival time (days) recorded. The group inoculated with *P. berghei* expressed IFN-γ culture supernatants survived five days longer than controls (Figure 6). This showed that *P. berghei* expressed IFN-γ culture supernatants have potential to protect mice from virulent infection.

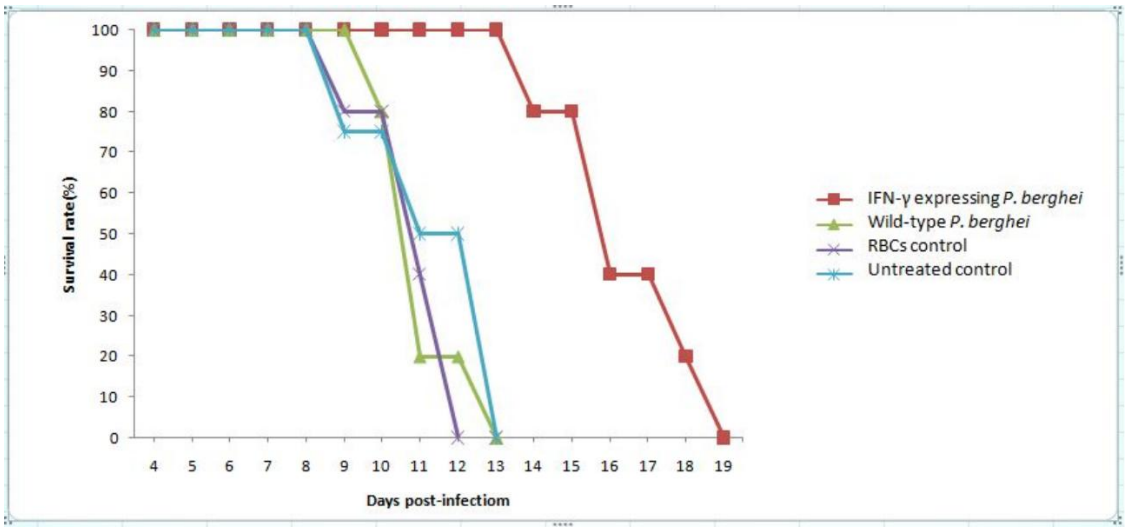

Figure 6: Survivorship curves for the different treatment groups upon challenge. Mice were inoculated with 37.5pg/ml IFN-γ in 200µl IFN-γ expressing *P. berghei* culture supernatants. Later, the mice were challenged with wild-type *P. berghei* and survival time (days) recorded.

3.8 *P. berghei* IFN-γ culture supernatants immunomodulate mouse response to malaria

In order to determine if the IFN-γ culture supernatants immunomodulate host immune response to virulent infection, mice were inoculated with the culture supernatants, challenged with wild-type *P. berghei* and sampling for total IgG, IFN-γ and IL-4 determination done. The ELISAs showed that after challenge (Figure 7), the group pretreated with IFN-γ *P. berghei* culture supernatants exhibited significantly higher levels of IFN-γ ($p < 0.001$). The level of IL-4 in this group was significantly low ($p < 0.05$). There was no significant difference in the levels of IgG ($p = 0.0682$) amongst all the treatment groups. This indicates that pre-exposure of mice to *P. berghei* IFN-γ culture supernatants immunomodulate the host cytokine responses to an active virulent infection.

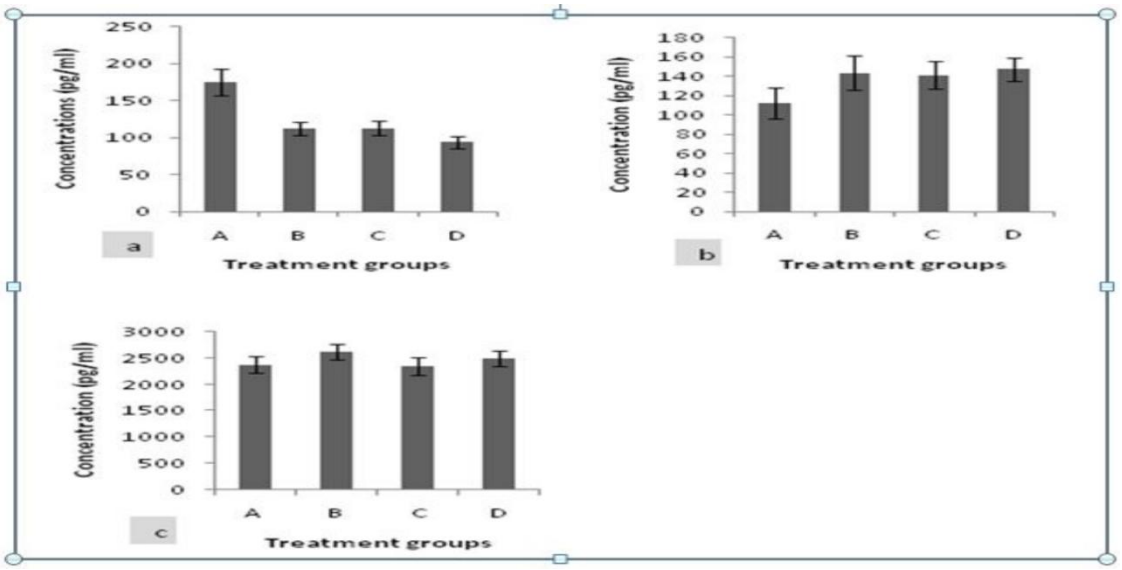

Figure 7: Concentrations (pg/ml) of IFN-γ (a), IL-4 (b) and IgG (c) after challenge (**A**-Pretreated with IFN-γ expressing *P. berghei* culture supernatants; **B**-Pretreated with wild type *P. berghei* culture supernatants; **C**-Pretreated with red blood cells culture supernatants; **D**-Untreated).

IV. Discussion

Previous studies have demonstrated the importance of IFN-γ in malaria infection [4, 22, 29, 30]. Additional studies have shown that it is feasible to generate cytokine immunopotentiated *Plasmodium* parasites through expression of bioactive host IFN-γ [22]. The present study aimed at expressing transgenic mouse IFN-γ in *P. berghei* and determining the immunomodulatory potential of the parasites expressing the cytokine in a mouse malaria model. This would provide more insight into the possibility of utilizing immunopotentiated

malaria parasites in malaria vaccine development strategies. Here, we report that transgenic mouse IFN-γ was successfully expressed in *P. berghei* and that the generated IFN-γ expressing parasites protected mice against malaria infection. The findings of this study have demonstrated further, the potential for culture supernatants of IFN-γ expressing *P. berghei* in protecting mice against virulent infection. These findings form an important initial step in understanding the use of cytokines to immunopotentiate malaria parasites, as a component of molecularly attenuated parasite vaccines.

P. berghei transfected with mouse IFN-γ expressing plasmid produces significant amounts of mouse IFN-γ. This shows that the parasite transcribes the mouse IFN-γ gene, assembles the protein in the right conformation and expressses mouse IFN-γ. This is in agreement with our previous study [22] where bioactive rhesus IFN-γ was expressed in *Plasmodium knowlesi*. In the study involving *P. Knowlesi* transfected with rhIFN-γ expressing gene [22], there was a similarly significantly higher production of IFN-γ in the culture containing the transfected parasites. However, it is not yet clear whether parasite expressed IFN-γ is secreted out by the erythrocyte, or released upon schizont rupture.

There was enhanced survival time of the mice infected with IFN-γ expressing *P. berghei* in comparison with wild-type parasite-infected mice. Because the in vivo growth rate of wild-type parasites was comparable to the cytokine expressing ones, differences in mice survival can be extrapolated to effects of IFN-γ expression by the parasites. Although precise mechanisms are unknown, this can be further interpreted as IFN-γ mediated immunomodulation of the parasites, leading to improved protection of the host [22]. Moreover, it could be that the cytokine immunopotentiates the parasites leading to potent host response as previously reported by Giavedoni [21] while using a viral model.

The delay in parasitaemia patency and increased parasitaemia suppression coupled with prolonged survival of the mice with high levels of IFN-γ upon challenge observed in this study point to a correlation of high levels of IFN-γ and reduced parasitaemia levels. Other studies have previously reported of an association between levels of IFN-γ and reduced parasitaemia in blood stage *Plasmodium* infection [4, 29]. It is probable that the cytokine enhances the ability of macrophages to destroy *Plasmodium* parasites thereby limiting parasite progression and thus prolonging the survival of the mice. Interferon gamma is a potent pro-inflammatory cytokine, offering protection against intracellular bacteria, some viruses as well as parasites [30]. The cytokine (IFN-γ) up-regulates the activity of phagocytes leading to elimination of bacteria and parasitic protozoans [4]. It has also been reported that IFN-γ up-regulates IL-12β2 expression and thus priming the IFN-γ-producing Th1 cells [31]. Therefore, it is possible that upon challenge, the primed Th1 cells produce cellular IFN-γ that acts on macrophages enhancing their ability to destroy the *Plasmodium* parasites [4].

Use of live attenuated parasites poses a challenge on production, optimization for vaccination and assurance against opportunistic revertants to virulent infection [31]. However, our studies provide proof of principle for molecular attenuation of parasite through cytokine production, leading to parasite immunopotentiation. This approach, possibly combined with others such attenuation that targets multiple genes of the parasite [32] could pose a real alternative to developing an effective malaria vaccine. There is real potential that with further refinement, such as developing a prime-boost strategy, improved if not complete protection of the host against virulent malaria parasite infection can be achieved.

V. Conclusion

This study demonstrated that IFN-γ expressing *P. berghei* on its own and culture supernatants from the parasites reduces parasite burden and prolongs survival of mice challenged with virulent *P. berghei* infection. The studies further provided proof of principle for molecular attenuation of malaria parasites through cytokine production, leading to parasite immunopotentiation.

Acknowledgements

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