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IMMUNIZATION WITH MUTANT *PLASMODIUM* PARASITES: HOW IS THAT ACHIEVED?

by

Bruno Gonçalo Douradinha Mateus

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Supervisor:
Prof. Doutora Maria Manuel Mota
Faculdade de Medicina da Universidade de Lisboa
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This thesis presents the results achieved during the research developed at I*nstituto de Medicina Molecular*, under the supervision of Prof. Doutora Maria Manuel Mota, from August 2004 to September 2006, aiming to be awarded a degree of Doctor of Philosophy in Biomedical Sciences by Faculdade de Medicina da Universidade de Lisboa.

This thesis is structured in 6 chapters, preceded by an abstract (both in English and Portuguese). The first chapter provides an insight on Malaria Liver Stage research, with an emphasized focus on Genomics and Vaccines, and the Aims of this work. The three following chapters (second to fourth) contain the original data attained regarding this project. The fifth chapter present an overall discussion of all data obtained. The last chapter concerns the methodologies, materials and experimental techniques used during the execution of this project. The scientific publications which outcome from this work are included in Appendixes 1 and 2.

The data presented in this dissertation is purely the result of my own work and it is clearly acknowledged in the text whenever data or reagents produced by others were utilized. This work has not been previously submitted for any degree at this or any other University.

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A Malária, causada pelo parasita *Apicomplexa* da espécie *Plasmodium*, ceifa a vida de mais de 1 milhão de crianças anualmente. Infelizmente, não existe nenhuma vacina contra esta doença está actualmente disponível, sendo crucial encontrar uma solução para diminuir as devastadoras consequências provocadas por esta doença, e é de consenso geral no mundo científico que novas intervenções são realmente necessárias para controlar a Malária. A infecção do fígado é o primeiro estadio que os parasitas *Plasmodium*, na forma de esporozoítos, têm de realizar (em hospedeiros mamíferos) para assegurar o seu desenvolvimento para o estadio seguinte, os merozoítos. Durante este estadio, com a duração aproximada de uma semana em humanos, os sintomas associados à doença não se manifestam, o que o torna ideal para uma terapia de intervenção, como uma vacina. Actualmente, os parasitas atenuados por radiação são, por excelência, uma referência nas vacinas contra o estadio hepático da Malária, uma vez que têm demonstrado uma elevada capacidade de immunizar humanos, outros primatas e ratinhos. Embora se consiga deste modo conferir uma protecção duradoura, esta metodologia foi considerada inapta para se conseguir immunizar pessoas em larga escala, devido a problemas de ordem logística, clínica e técnica. Este método apresenta ainda outra devantagem, não ser considerado seguro, dado que é necessário um controlo rígido da dose de radiação aplicada aos esporozoítos: se esta for demasiado pequena, os parasitas permanecerão infecciosos e, se for excessiva, o efeito protector induzido pelos esporozoítos irradiados é completamente anulado.

Neste projecto, estudamos a capacidade de conferir imunidade contra a Malária por parasitas que foram atenuados através de modificações genéticas, os *P. berghei* (um parasita que infecta ratinhos) *pb36p-*, *pbcrmp3-* e *pbcrmp4-*.

Os nossos resultados mostram claramente que os esporozoítos *pb36p-* são capazes de conferir protecção, podendo potencialmente ser aplicados como uma vacina experimental que previna contra a Malária. Estes parasitas são incapazes de produzir a proteina Pb36p, uma proteína de superfície encontrada nos micronemas, e actualmente, de função desconhecida. Caracterizámos a protecção conferida por estes parasitas mutantes e, sempre que possível, comparámo-la com a induzida por esporozoítos atenuados por radiação. Observámos que a protecção induzida pelos *pb36p-* é específica para o estadio hepático, uma vez que os ratinhos imunizados não estão protegidos contra a inoculação com eritrócitos

infectados com merozoítos *P. berghei*. Ratinhos das estirpes BALB/c e C57BL6, após imunização com *pb36p*-, ficaram protegidos contra sucessivas re-infecções até 18 e 12 meses, respectivamente. Adicionalmente, ratinhos BALB/c, após imunização, ficaram protegidos até 6 meses após a última inoculação de imunização, sem ser necessário recorrer re-infecções intermédias para continuarem protegidos. Imunizações com doses menores de esporozoítos *pb36p*- e utilizando métodos de administração clinicalmente aprovados (nomeadamente intramuscular, subcutanea e intradermal) também protegem ratinhos C57BL6 contra a doença, embora não sejam tão eficientes como as imunizações efectuadas pelo modo intravenoso, no que respeita aos níveis de protecção total. A capacidade de imunização do *pb36p*- e dos parasitas atenuados por radiação não se restringe apenas contra a infecção com a espécie homóloga *P. berghei*, mas também contra a espécie heterologa *P. yoelii*, um outro parasita de ratinho. O desenvolvimento intrahepático deste último é também fortemente inibida, levando a uma redução do nível de parasitémia máxima no sangue. Este estudo é o primeiro, do nosso conhecimento, a abordar este tópico.

Os mecanismos, despoletados pelos parasitas *pb36p*-, que induzem esta protecção foram também alvo de estudo. Observámos que os hepatócitos infectados com esporozoítos *pb36p- in vitro* apresentam uma elevada taxa de apoptose, bem como níveis bastante altos da proteina apoptótica Procaspase-3. Os níveis de apoptose *in vivo* também são bastante elevados nos fígados de ratinhos imunizados com parasitas *pb36p*- quando comparados com os imunizados com parasitas atenuados por radiação ou infectados com parasitas infecciosos, confirmando os resultados obtidos *in vitro*. A taxa de eliminação dos esporozoítos *pb36p*- é, consequentemente, bastante rápida. Imunização com parasitas *pb36p*- induz um aumento nas células T de memória $CD8^+$ no fígado, que eventualmente se dissipa nos meses seguintes.

Adicionalmente, foram também caracterizados os parasitas *pbcrmp3*- e *pbcrmp4*-. Estes parasitas não produzem proteinas que se sabe conterem domínios funcionais relacionados com adesão ou invasão. Estes parasitas não conseguem romper a membrana dos oocistos e, consequentemente, são incapazes de migrar para as glândulas salivares do mosquito. Dado este fenótipo aberrante, estes parasitas são incapazes de serem transmitidos para o hospedeiro através da picada do mosquito infectado. Nós observámos que os oocistos formados por ambos os parasitas

apresentam uma forma normal e sem defeitos aparentes, tal como os resultantes de parasitas da estirpe selvagem. Uma vez extraídos os esporozoítos *pbcrmp3*- e *pbcrmp4*- dos oocistos, observámos que eles, tal como os da estirpe selvagem, são capazes de executar o *gliding*, de migrar através de vários hepatócitos e de infectar uma célula final, iniciando assim o seu desenvolvimento para o próximo estadio. Estes parasitas mutantes infectam um número de células semelhante ao observado para a estirpe selvagem 24 horas após infecção. Contudo, os vacúolos parasitóforos dos parasitas *pbcrmp3*- e *pbcrmp4*- são menores que os da estirpe selvagem, e apresentam uma forma aberrante. Mesmo mantendo estes parasitas em cultura até 56 horas após infecção, não se verifica qualquer aumento no tamanho dos seus vacúolos parasitóforos.

Os parasitas *pbcrmp3*- e *pbcrmp4*-, tal como os parasitas atenuados por radiação e os *pb36p*-, são incapazes de se desenvolverem completamente no fígado. Visto apresentarem um fenótipo semelhante ao observado para os parasitas *pb36p*- e os atenuados por radiação, surgiu a hipótese de os esporozoítos *pbcrmp3*- e *pbcrmp4*- também poderem ser utilizados como uma vacina experimental contra Malária. Infelizmente, esta hipótese revelou não ser válida. Ratinhos BALB/c e C57BL6 imunizados com parasitas *pbcrmp3*- ou *pbcrmp4*- não ficaram protegidos contra uma infecção posterior com a estirpe selvagem. Pensamos que esta deficiência em conferir protecção advem do facto destes parasitas mutantes terem sido extraídos dos oocistos, estando ainda "imaturos", uma vez que não passaram pelo estadio de maturação nas glândulas salivares que alguns investigadores consideram ser fundamental para que os esporozoítos adquiram a sua capacidade infectiva. Ambos os parasitas *pb36p*- e atenuados por radiação necessitam de infectar e iniciar o desenvolvimento intrahepático, de modo a conferir imunidade contra a Malária. Outra alternativa derivaria do facto destes parasitas atenuados conseguirem inibir eficientemente a apoptose das células hospedeiras, tal como a estirpe selvagem. Dada a falta de corpos apoptóticos, os parasitas *pbcrmp3*- ou *pbcrmp4*- não conseguiriam montar uma resposta imune eficiente como os *pb36p*- ou os atenuados por radiação.Contudo, ainda estão a ser realizados os estudos que demonstrarão quais as verdadeiras razões responsáveis pela incapacidade dos parasitas *pbcrmp3*- e *pbcrmp4*- em conferir protecção contra a Malária. Embora estes parasitas não apresentem qualquer potencial como estratégia de vacinação contra a Malária, o seu fenótipo durante o estadio hepático é bastante interessante

e irá fornecer indubitavelmente novas perspectivas ácerca das interacções parasita-célula hospedeira a este nível.

Os nossos resultados demonstram claramente que os parasitas *pb36p*- possuem um forte potencial para serem aplicados como uma vacina experimental contra a Malária, ao contrário dos parasitas *pbcrmp3*- e *pbcrmp4*-. Os parasitas *pb36p*- conseguem conferir imunidade a longo termo e protecção contra outra espécie de parasita de ratinho, o *P. yoelii*. Imunizações utilizando rotas de administração utilizadas regularmente em vacinação humana, bem como doses com reduzido número de esporozoítos *pb36p*- são também eficazes. Torna-se agora fundamental compreender os mecanismos que permitem aos parasitas *pb36p*- conferir protecção. Os nossos esforços iniciais demonstram que a protecção induzida por esporozoítos *pb36p*- está dependente de interferão gama (IFN-γ) e de células T gama-delta. A apoptose também aparenta ter um papel primordial no efeito protectivo mediado por estes parasitas atenuados. Estes são apenas os passos iniciais nesta matéria. Novos esforços com o intuito de compreender e descobrir os mecanismos que levam à protecção induzida por estes e outros parasitas geneticamente modificados, o que, sem dúvida, fornecerá dados inovadores e de importância vital para o desenvolvimento de uma vacina dirigida ao estadio hepático da Malária. Esses esforços conduzirão a novas estratégias de vacinação contra esta doença.

Palavras-chave: Malaria, esporozoíto, parasitas geneticamente modificados, vacina, apoptose, mecanismos do sistema imune

Malaria claims the lives of more than 1 million children in Sub-Saharan Africa annually. Yet, no vaccine is currently available against this disease caused by the *Apicomplexa* parasites known as *Plasmodium*. There is no doubt that novel intervention strategies are required to control malaria. Liver infection is the first obligatory step, allowing parasites, in the form of sporozoites, to develop into the next infective stage, merozoites. This stage is clinically silent (no illness-related symptoms arise) and lasts around a week in human malaria, making it an ideal target for a vaccine. So far, the golden standard for a vaccine against the malaria liver stage is immunization with radiation attenuated sporozoites, a whole organism approach which has proved to be able to confer long lasting protection to humans, non human primates and mice, but considered unfeasible for mass immunization, due to logistical, clinical and technical obstacles. This approach was also considered unsafe, since strict control of radiation dose is vital to achieve effective protection: if parasites are underirradiated, they remain infectious, and if overirradiated, the protective effect elicited by them is completely lost.

In this work, we have focused on another whole organism approach immunization, by exploiting the potential of three *P. berghei* (a rodent parasite) genetically-attenuated sporozoites, *pb36p*-, *pbcrmp3*- and *pbcrmp4*-.

pb36p- based immunizations proved that these attenuated parasites may be used as an experimental vaccine against malaria. These parasites are unable to synthesize the protein Pb36p, a surface protein found in the sporozoite micronemes and without a known function. Thus, further studies were undertaken to characterize *pb36p*- mediated protection. We observed that the protection induced by *pb36p*- is stage specific, being unable to protect mice against challenge with *P. berghei* infected erythrocytes. BALB/c and C57BL6 mice are found to be protected against continuous challenges up to 18 months and 12 months post final immunization respectively (last time point tried). Moreover, BALB/c mice immunized with three doses of *pb36p*- sporozoites are protected up to 6 months after last immunization with no additional challenge. Protection in C57BL6 mice can be attained even with low doses of *pb36p*- sporozoites (1,000 sporozoites), a feature not observed in RAS based immunizations. *pb36p*- sporozoites still confer protection to C57BL6 mice when administered through routes commonly used as public health measures, namely intramuscular, subcutaneous and intradermal, although never attaining complete sterile

protection as seen with intravenous inoculation. *pb36p*- and RAS mediated immunity are not species specific, since mice immunized with these attenuated parasite have a high level of inhibition of intrahepatic parasite development and reduction in blood stage parasitemia when challenged with *P. yoelii* sporozoites, a heterologous species.

Preliminary studies regarding the mechanisms elicited by *pb36p*- to confer immunity to mice were also performed. We observed that *pb36p*- infected hepatocytes have a much higher rate of apoptosis and Procaspase-3 (the precursor of active caspase-3 which is necessary for apoptosis) was also detected *in vitro*. Apoptosis *in vivo* was also observed in *pb36p*- infected mice, and its clearance rate in livers of immunized mice is greatly increased to that observed under similar conditions in RAS immunized mice. Moreover, *pb36p*- immunization lead to an increase in $CD8^+$ T memory cells in the liver which eventually decreases with time.

In addition, we characterized *pbcrmp3*- and *pbcrmp4*- parasites, which are unable to express *Plasmodium* proteins containing motifs implicated in invasion or adhesion (*Plasmodium berghei* cysteine repeat modular protein). These parasites are unable to breach oocysts membrane and, therefore, to migrate to the salivary glands, abrogating transmission to host via mosquito bite. We observed that oocysts derived from *pbcrmp3*- and *pbcrmp4*- parasites have the normal shape seen for WT parasites. *pbcrmp3*- and *pbcrmp4*- sporozoites from oocysts are able to glide and migrate through several cells as WT sporozoites do. They also have the same infection level as WT 24 hours post infection. However, *pbcrmp3*- and *pbcrmp4*- EEFs are small and present aberrant shapes, compared to WT EEFs. Development in culture up to 56 hours does not promote any further increase in the size of these attenuated EEFs.

The characterization of *pbcrmp3*- and *pbcrmp4*- parasites' intrahepatic development led us to think these two attenuated parasites could be possible candidates for a genetically attenuated sporozoite vaccine. Like RAS and *pb36p*-, they arrest during liver development, never forming mature EEFs like WT sporozoites. Regrettably, this similarity is not reflected in their immunization potential. No protection is achieved with *pbcrmp3*- and *pbcrmp4*- parasites in either BALB/c or C57BL6 mice. The lack of protective effect demonstrated by *pbcrmp3*- and *pbcrmp4*- sporozoites could derive from their immature stage,

resulting in inefficient infection *in vivo*, an essential step attenuated sporozoites must perform to confer immunity, or from the ability of efficiently inhibit host cell apoptosis as WT, therefore not eliciting an immune response against the parasite as RAS and *pb36p*- do. However, this remains to be further investigated. Although these attenuated parasites lack potential as immunization agents, their phenotype in liver stage development is quite interesting and will hopefully provide undoubtedly new insights into parasite-host interactions.

Our results clearly confirm the potential of *pb36p*- as an experimental vaccine against malaria, whilst *pbcrmp3*- and *pbcrmp4*- failed to achieve the same goal. Furthermore, *pb36p*- is able to confer long-lasting immunity and cross-species protection against *P. yoelii*. This protection can be achieved even with low immunization doses and using the most common routes of immunization. Understanding the mechanisms by which *pb36p*- parasites induce immunity will help us to design a vaccine against the malaria liver stage. We have already made an attempt to initiate studies regarding this issue, and observed that *pb36p*-mediated immunity is dependent on IFN-γ and γδ T cells. Moreover, apoptosis seems to play a key role in the protection induced by these attenuated parasites. These are just the first steps on this subject, and further studies are already underway to unravel the mechanisms of this and other GAS mediated protection, which will no doubt provide ground-breaking relevant data for the development of a vaccine targeting the pre-erythrocytic stage of Malaria.

Keywords: Malaria, sporozoite, genetically attenuated sporozoites, vaccine, apoptosis, immune mechanisms

APC – Antigen Presenting Cells

CD – Cluster of Differentiation

CelTOS – Cell traversal Protein for Ookinete and sporozoite

CSP – Circumsporozoite Protein

DAPI – Diamidino-phenyl-indole

DC – Dendritic CellS

DMEM – Dulbecco's Modified Eagle Medium

EEF – Exoerythrocytic Form

FACS – Flow Activated Cell Sorting

FCS – Fetal Calf Serum

GAS – Genetically Attenuated Sporozoites

HGF – Hepatocyte Growth Factor

HLA – Human Leucocyte Antigens

HPRT – Hypoxanthine Guanine Phosphoribosyltransferase

HSPG – Heparan Sulfate Proteoglycan

i.d. – intradermal

IFN-γ – interferon γ

Ig – Immunoglobulin

IL – interleukin

i.m. – intramuscular

i.p. – intraperitoneal

ISIF – Infection Susceptibility-Inducing Factor

i.v. – intravenous

MHC – Major Histocompatibility Complex

NK – Natural Killer cells

PCR – Polymerase Chain Reaction

PS – Penicilin-Stretpomycin

PV – Parasitophorous Vacuole

RAS – Radiation Attenuated Sporozoites

RPMI – Roswell Park Memorial Institute (medium)

RT – Room Temperature

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

RTS,S – Repeat T cell epitopes Surface antigens plus S antigen from hepatitis B virus

s.c. – subcutaneous

SCS – solution of Sodium Cacodylate 0.1 M plus Sucrose 3.7% pH 7.4 (buffer)

SDS – Sodium Dodecyl Sulfate

SPECT – Sporozoite (micronemes) Protein Essential for Cell Traversal

spp. – species

TBS – Tris Buffered Saline (buffer)

TRAP – Thrombospondin-Related Adhesive Protein

WT – Wild Type

PREFACE **iii**

ACKNOWLEDGMENTS **v**

SUMÁRIO **vii**

ABSTRACT **xi**

ABBREVIATIONS **xv**

TABLE OF CONTENTS **xvii**

INDEX OF FIGURES xx

INDEX OF TABLES xxi

1 - INTRODUCTION **1**

1.1 – GENERAL OVERVIEW ON MALARIA 3

1.2 – *PLASMODIUM* LIFE CYCLE 4

1.3 – MALARIA LIVER STAGE 7

1.3.1 – Sporozoite Gliding Motility 7

1.3.2 – Next Stop: the Liver 8

1.3.3 – Migration 10

1.3.4 – Invasion and Development inside Hepatocytes 11

1.3.5 – Leaving the Hepatocyte 14

1.4 – VACCINE AGAINST MALARIA 15

1.4.1 – Aims and History 15

1.4.2 – Vaccine against *Plasmodium* Liver Stage 17

1.5 – COMPARATIVE GENOMICS IN *PLASMODIUM* 19

1.5.1 – General Features 19

1.5.2 – Applications 19

1.5.2.1 – Orthology 19

1.5.2.2 – Genetic Tools 20

1.5.2.3 – Genetic Modification elucidates the role of targeted genes in *Plasmodium* infection 21

1.6 – AIMS AND STRATEGIES 23

2 - *pb36p*- AS AN EXPERIMENTAL GAS BASED VACCINE AGAINST MALARIA **25**

2.1 – INTRODUCTION 27

2.2 – RESULTS 28

2.2.1 – Immunization with *pb36p*- sporozoites protects BALB/c and C57BL6 mice against subsequent infection 28

2.2.2 – *pb36p*- sporozoites induce long-lasting protection against continuous challenge with *P. berghei* sporozoites 30

2.2.3 – BALB/c mice immunized with *pb36p*- sporozoites do not require continuous challenge to maintain protection up to 6 months 32

2.2.4 – Lower doses of *pb36p*- sporozoites are still able to confer protection against subsequent challenge 33

2.2.5 – Different routes of administration of *pb36p*- and RAS sporozoites partially protect against infection 35

2.2.6 – *pb36p*- and RAS and confer partial protection against challenge with *P. yoelii* sporozoites 37

2.3 – CONCLUSIONS 44

3 - MECHANISMS OF PROTECTION INDUCED BY *pb36p*- 49

3.1 – INTRODUCTION 51

3.2 – RESULTS 53

3.2.1 – *pb36p*- sporozoites infected Hepatocytes enter Apoptosis more frequently than RAS or WT parasite-infected Hepatocytes 53

3.2.2 – *pb36p*- sporozoites have a higher Clearance Rate in Livers of infected BALB/c mice 55

3.2.3 – *pb36p*- induced protection is dependent on IFN-γ and γδ T cells 57

3.2.4 – Memory T and B Cells in *pb36p*- immunized BALB/c mice 58

3.2.4.1 – Increase in Memory T cells is observed in livers and spleens of *pb36p*- immunized BALB/c mice 58

3.2.4.2 – Memory B cells do not suffer an increase following *pb36p*- immunization in BALB/c mice 61

3.3 – CONCLUSIONS 62

4 - *pbcrmp*- AS POTENTIAL GAS BASED VACCINES FOR MALARIA 67

4.1 – INTRODUCTION 69

4.2 – RESULTS 71

4.2.1 – *pbcrmp3*- and *pbcrmp4*- sporozoites develop normally inside oocysts 71

4.2.2 – *pbcrmp3*- and *pbcrmp4*- sporozoites are able to glide and to migrate through several hepatocytes 73

4.2.3 – *pbcrmp3*- and *pbcrmp4*- sporozoites are not able to fully develop into mature EEFs 75

4.2.4 – *pbcrmp3*- and *pbcrmp4*- sporozoites are not able to confer protection to mice upon challenge with WT sporozoites 77

4.3 – CONCLUSIONS 79

5 - FINAL CONSIDERATIONS 83

6 - MATERIAL AND METHODS 97

6.1 – MATERIALS 99

6.1.1 – Chemical and General Reagents 99

6.1.2 – Cells 99

6.1.3 – Parasites 99

6.1.4 – Mice 100

6.2 – METHODS 101

6.2.1 – Immunizations 101

6.2.2 – Quantification of Liver Infection 101

6.2.3 – Sporozoite Infection *in vitro* 102

6.2.4 – Immunofluorescence Assay 103

6.2.5 – *In vitro* analysis of Apoptosis in RAS and *pb36p*- parasite-invaded Hepatocytes 103

6.2.6 – *In vivo* analysis of Apoptosis in RAS and *pb36p*- parasite-invaded Hepatocytes 104

6.2.7 – Western Blot for Caspase-3 Detection 104

6.2.8 – Memory Cells and FACS 105

6.2.9 – Electron Microscopy 106

6.2.10 - Gliding Assays for *pbcrmp3*- and *pbcrmp4*-sporozoites 106

6.2.11 - Migration Assays for *pbcrmp3*- and *pbcrmp4*-sporozoites 107

REFERENCES **109**

APPENDIXES **127**

Index of Figures

Figure 1.1 Endemic areas of Malaria worldwide 3

Figure 1.2 *Plasmodium* Life Cycle 6

Figure 1.3 Gliding 8

Figure 1.4 Sporozoites migrating in a liver blood vessel 9

Figure 1.5 *Plasmodium* sporozoites migration in HepG2 cells 10

Figure 1.6 Migration of sporozoites – Quantification 11

Figure 1.7 *P. berghei* developing inside a hepatocyte 13

Figure 1.8 Merosome leaving a hepatocyte 15

Figure 2.1 Level of inhibition of infection with *P. yoelii* 265 BY in mice immunized with *P. berghei* RAS and *pb36p*- sporozoites 39

Figure 2.2 Maximum blood-stage parasitemias levels achieved in *P. berghei p36p*- and RAS immunized BALB/c mice subsequently challenged with *P. yoelii* 265 BY sporozoites 41

Figure 2.3 Maximum blood-stage parasitemias levels achieved in *P. berghei pb36p*- and RAS immunized C57BL6 mice subsequently challenged with *P. yoelii* 265 BY sporozoites 42

Figure 2.4 Maximum blood-stage parasitemias levels achieved in *pb36p*- immunized BALB/c mice which were subsequently challenged with *P. yoelii* 17XL sporozoites 43

Figure 3.1 Apoptosis is increased in *pb36p*- parasitized liver cells 53

Figure 3.2 High levels of procaspase-3 are detected in HepG2 cells infected with RAS and *pb36p*- sporozoites 54

Figure 3.3 Hepatic Persistence of RAS and *pb36p*- sporozoites in BALB/c mice 56

Figure 3.4 Quantification of *P. berghei* liver load in *pb36p*- immunized $IFNg^{-/-}$ and $TCR\delta^{-/-}$ mice after challenge with infectious *P. berghei* sporozoites 58

Figure 3.5 Memory $CD8^+$ and $CD4^+$ in livers of naïve and *pb36p*- immunized mice 59

Figure 3.6 Memory $CD8^+$ and $CD4^+$ in spleens of naïve and *pb36p*- immunized mice 60

Figure 3.7 Memory $CD8^+$ and $CD4^+$ in lymph nodes of naïve and *pb36p*- immunized mice 60

Figure 3.8 Memory B cells in liver, spleen and lymph nodes of naïve and *pb36p*- immunized mice 62

Figure 4.1 *pbcrmp3*- and *pbcrmp4*- mature oocysts are still found in mosquitoes guts 18 days after infective blood meal 73

Figure 4.2 *pbcrmp3*- and *pbcrmp4*- mature oocysts are able to migrate through cells as WT sporozoites, although present different migration rates 75

Figure 4.3 *pbcrmp3*- and *pbcrmp4*- have the same infection rate in HepG2 cells than WT sporozoites 76

Figure 4.4 *pbcrmp3*- and *pbcrmp4*- infection lead to small and aberrant-like EEFs in HepG2 cells 76

Figure 4.5 *pbcrmp3*- and *pbcrmp4*- intrahepatic development is already Impaired at 24 hours post infection 77

Figure 4.6 *pbcrmp3*- and *pbcrmp4*- immunized C57BL6 mice develop blood-stage parasitemia similarly to naïve control 78

Index of Tables

Table 2.1 Immunization with *pb36p*- sporozoites protects against a subsequent infection with WT sporozoites 30

Table 2.2 Immunization with *pb36p*- sporozoites lead to protection against subsequent multiple challenges 31

Table 2.3 Protection elicited by *pb36p*- sporozoites does not require subsequent multiple challenges to remain fully protective up to 6 months after last immunization in BALB/c mice 33

Table 2.4 Protection elicited by *pb36p*- sporozoites can be achieved even with low immunization doses in C57BL6 mice 34

Table 2.5 *pb36p*- sporozoites administered intramuscularly, subcutaneously and intradermally are able to confer protection against intravenous challenge with infectious *P. berghei* sporozoites in C57BL6 mice 36

Table 2.6 *pb36p*- sporozoites administered intramuscularly, subcutaneously and intradermally are able to confer protection against WT *P. berghei* sporozoites infected mosquitoes bite challenge with in C57BL6 mice 37

Table 2.7 Immunization with *P. berghei* RAS and *pb36p*- sporozoites lead to a delay on parasitemia onset in BALB/c and C57BL6 mice challenged with the heterologous species *P. yoelii*, and some even achieve sterile protection 44

Table 4.1 Immunization with *pbcrmp3*- and *pbcrmp4*- sporozoites do not protect BALB/c and C57BL6 mice against a subsequent infection with WT sporozoites 79

Chapter I

Introduction

1. Introduction

1.1. General Overview on Malaria

Malaria exists for millennia. The name of this plague comes from the Italian word for "bad air". Nowadays, is still a major global health problem, which affects around 500 millions people worldwide. It is responsible for the high level of mortality and morbidity in emerging countries, killing more individuals than any other cause, including civil wars (Sherman, 1998). In addition, it is a huge obstacle to the economical development of those regions (Fig. 1.1).

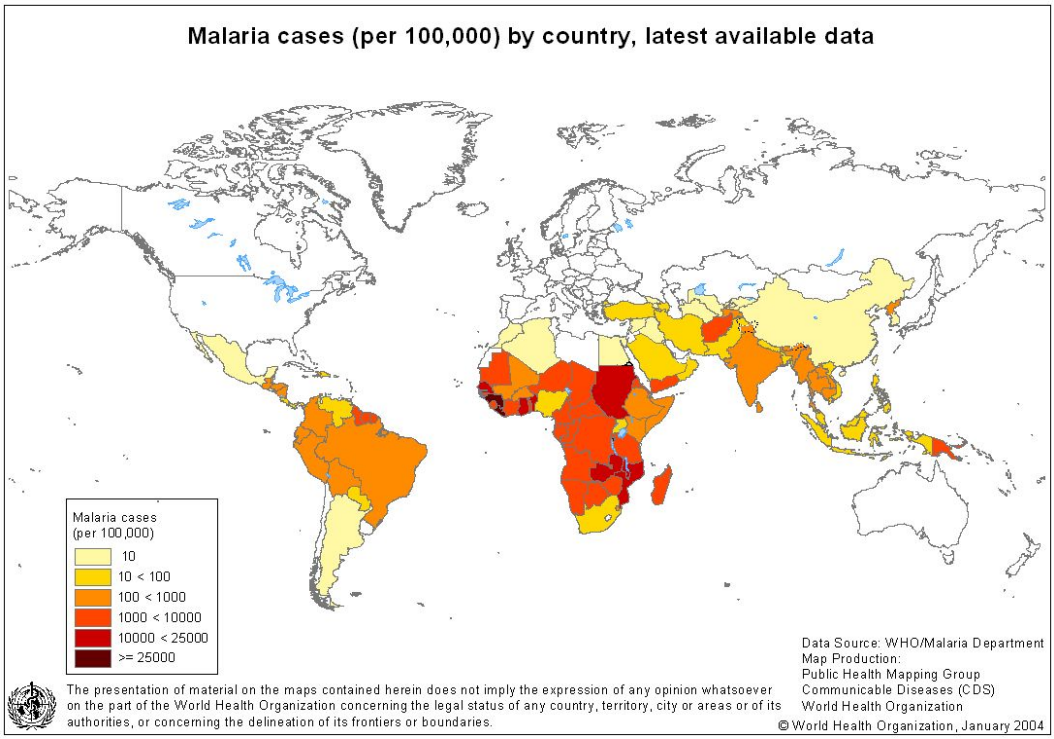

Figure 1.1. Endemic areas of Malaria worldwide according World Health Organization, in January of 2004 (adapted from http://www.who.int/)

Malaria alone is considered the "public health enemy number one" in Africa, where 90% of all global cases of this disease occur, leading to the loss of 2 billion of euros annually. Every 30 seconds, an African child dies from malaria (http://www.who.int/). Several attempts of eradicating the disease were made, such as the use of drugs and insecticides. However, these strategies were not successful due to advent of the *Plasmodium* parasites resistance to drugs widely

used to prevent and fight malarial infection, as chloroquine, and of the *Anopheles* mosquitoes to insecticides such as dichlorodiphenyltrichloroethane (DDT) and others. In fact, in the most recent years, malaria has re-emerged in many parts of the world where it was assumed to be eradicated (http://www.who.int/). The global failure to effectively reduce malaria supports the need of finding a cost-effective vaccine against this disease.

The genus *Plasmodium*, the causative agent of malaria, is a member of the *Apicomplexa* phylum of protozoa that contains more than 5000 named species (spp.), mostly obligatory intracellular parasites, and all with a complex of organelles at the apical end of the invasive stages. The phylum includes other human pathogens like *Toxoplasma* and *Cryptosporidium*, which cause opportunistic infections, or *Babesia* and *Theileria*, that cause huge animal stock losses (Sherman, 1998).

Different *Plasmodium* spp. can infect different vertebrates, including human, other primates, rodents, birds, and even reptiles. Four distinct *Plasmodium* spp. are known to be pathogenic to man, *Plasmodium falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. A significant amount of research has been made in *Plasmodium* spp. that infect rodents, due to the many limitations of working with humans samples and/or subjects, such as lack of clinical history of infections in patients, legal authorization procedures, amongst others. These are *P. berghei*, *P. yoelii* and *P. chabaudi* (Sherman, 1998).

1.2. *Plasmodium* Life Cycle

The *Plasmodium* life cycle is quite complex and comprises two hosts: (i) the definitive host, which is the female *Anopheles* mosquito, and (ii), a vertebrate host.

In the mosquito, the sporogonic stage is initiated in the *Anopheles* mosquito after a blood meal from a vertebrate infected host that contains gametocytes in the blood. The gametocytes will develop into male and female gametes inside the gut of the mosquito, a process that is triggered by the shift of temperatures between vertebrate host (37ºC, in the case of mammal hosts), and invertebrate hosts (around 20ºC, for the mosquitoes). Fusion of the gametes occurs, forming an ookinete. This new form traverses the gut wall of the mosquito (part of digestive

tract that resembles to stomach), developing into an oocyst which can reach 30 to 40 μm diameter (Sherman, 1998; Vanderberg, 1967). Inside this new form, sporozoites (around 10 μm length per 1 μm width) will be formed, being released after 3-4 days of maturation (which corresponds to 10-14 days after the blood meal). The sporozoites aim towards mosquitoes salivary glands, from where they will be injected into a new vertebrate host (along with anticoagulants and vasodilators), when the anopheline mosquito collects its next blood meal (Sherman, 1998).

Sporozoite migration from gut to salivary glands occurs through the haemolymph (circulatory fluid of molluscs and insects that have an 'open' circulatory system) and is mediated by a major surface protein of the parasite, the circumsporozoite protein (CSP). CSP can be found all over *Plasmodium* sporozoite surface, being highly conserved between the different species and presenting homology with other apicomplexan parasite proteins (Myung, 2004). Antibodies against CSP can block sporozoite infection of mosquito salivary glands (Sidjanski, 1997). Besides, this, CSP seems also to be essential for sporozoite formation inside oocysts. Parasites with a targeted disruption for this gene produce a normal number of oocysts like wild-type (WT), but sporozoite formation is blocked (Ménard, 1997).

In the mammalian host, the schizogonic stage takes place after the injection of sporozoites. Here, the parasites proliferate in two different sites, first on liver cells (exoerythrocytic site) and later, in a series of cycles, on red blood cells (erythrocytic site) (Sherman, 1998). Once inside the host, the sporozoites make their journey to the liver through blood vessels. Each mosquito bite injects around 15 to 200 sporozoites under the skin of the host, which will remain in that area approximately 2 minutes (on mice) or 15 up to 30 minutes (on humans). Once in the liver, *Plasmodium* schizont development only occurs inside hepatocytes. The schizont will develop progressively until it gives rise to tens of thousands merozoites (Sherman, 1998). During the schizont development in the liver, no symptoms of the disease are observed in infected individuals. After complete maturation in the hepatocytes (around 2 days on mice and 5.5-7 days in humans), merozoites leave these cells and enter the bloodstream. The Exoerythrocytic Stage is now complete, and the parasite development will proceed from now on in the Erythrocytic Stage. Here, unlike exoerythrocytic stage, the malaria symptoms arise, which comprise fever, anaemia, nausea and headaches (reviewed in

Stevenson, 2004). Once in the blood stream, merozoites will invade erythrocytes. This is a cyclic process, progressively amplified by repeated cycles of invasion, intracellular growth, multiplication and re-invasion, which varies from 24 to 72 hours, depending on the *Plasmodium* spp, being 24 hours in *P. falciparum*. Each mature parasite develops into 8 up to 32 new merozoites (depending on the *Plasmodium* spp., being around 20 in *P. falciparum* and *P. vivax*) (Bannister, 2003; Sherman, 1998). Some merozoites will not develop into more merozoites, instead they form gametocytes (male, or microgametocyte, and female, or macrogametocyte). When a mosquito collects blood for feeding, and ingests gametocytes, the cycle continues, as mentioned before. The entire life cycle can be observed in figure 1.2.

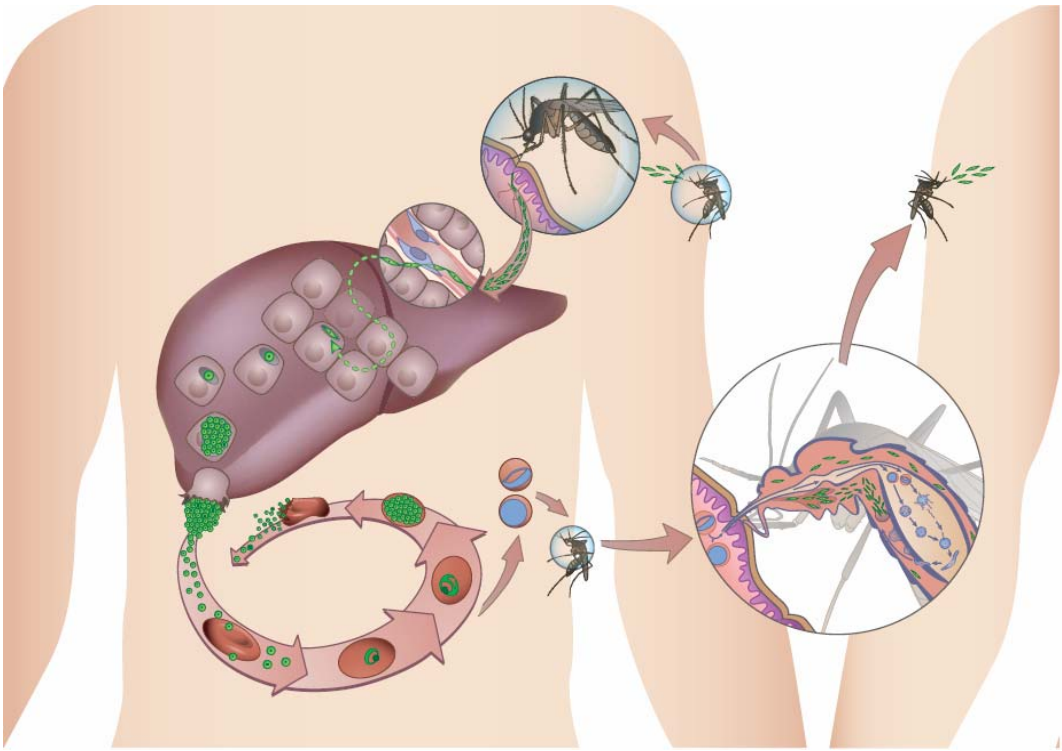

Figure 1.2. *Plasmodium* Life Cycle. An infected *Anopheles* mosquito feeds on a host, injecting *Plasmodium* sporozoites into the blood stream. Sporozoites will reach the liver within minutes, infecting a hepatocyte for further development. There, they multiply into the new infective form, merozoites, which will leave the liver and aim to the blood. Once there, they will infect cyclically erythrocytes, developing into more merozoites. However, some of them will develop not in more merozoites, but in gametocytes (male and female). When an *Anopheles* mosquito take a blood feed on this host, it will collect these gametocytes, which will fecundate and develop into an ookinete, and later in an oocyst. Oocysts will attach to midgut for maturation, giving rise to sporozoites, which will burst the oocyst and migrate to the mosquito salivary glands, ready to infect a new host upon the next mosquito blood feed.

1.3. Malaria Liver Stage

The liver infection is the first obligatory step for an efficient malaria infection. Shortly after injection into mammalian host, sporozoites are rapidly found in the liver, a crucial step for their further development into merozoites. This step is clinically silent, since no symptoms of illness are observed. Thus, it is an attractive target for a vaccine against malaria, since it represents the first step in infection, presents no symptoms and the development of sporozoites into merozoites has a time span which would allow an immune response to stop the infection at an early stage. However, the hepatic stages of infection are poorly known and understood, and almost none *Plasmodium* molecules are known to have an impact on the parasite development inside hepatocytes (Sherman, 1998).

1.3.1. Sporozoite Gliding Motility

Sporozoites, as the majority of the invasive stages of all apicomplexan parasites, present a form of locomotion, which is not based on cilia or flagella (which they do not possess). Instead, they move in a substrate-dependent manner, named gliding motility. This feature seems to be related with secretion of certain proteins (namely CSP and Thrombospondin-Related Adhesive Protein – TRAP) by the apical complex of *Plasmodium* parasites, translocated to its surface by an actin-myosin dependent process, spreading this way to its posterior end, reaching by this mean the substrate. As the substrate, in parasite membrane, is immovable, the posterior translocation of the receptor-ligand complexes results in the forward movement of the parasite. This process is easily observed *in vitro*, using antibodies against CSP (Fig. 1.3). CSP and TRAP are expressed by all *Plasmodium* spp. members, and it was observed not only actin-depolymerizing agents, such as cytochalasin-D or anti-CSP monoclonal antibodies as well as *Plasmodium* lines containing null mutations for TRAP, are unable to move and, consequently, of invading liver cells or mosquitoes salivary glands (Mota, 2002; Mota and Hafalla, 2002; Sultan, 1999). Thus, it has been shown unequivocally that gliding motility is required for invasion of both the mosquito and the hepatocytes.

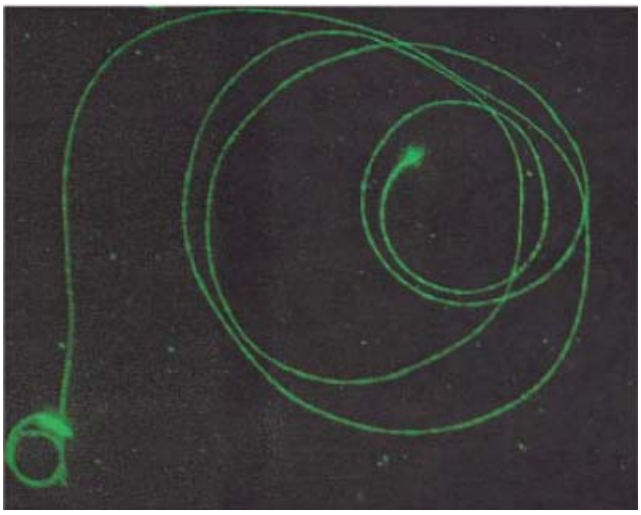

Figure 1.3. Gliding sporozoite, leaving a trail of CSP behind (Adapted from Mota, 2002)

1.3.2. Next stop: the Liver

Once inside the host, sporozoites enter in circulatory system. Only those which enter the blood capillaries will reach the liver. The remaining will be drained by lymphatic vessels and degraded by leukocytes (Amino, 2006). Specific recognition of hepatocytes by sporozoites is very efficient and TRAP and CSP also play a pivot role in this process. Both are essential for parasite migration to mosquito salivary glands, and also for invasion of liver cells (Akhouri, 2004; Kappe, 2003; Rathore, 2003). These proteins are crucial during this period of the parasite life cycle, a vital step for further parasite development and the establishment of infection therefore they represent ideal targets for vaccines against malaria (Bodescot, 2004; Bruña-Romero, 2001).

In order to leave the blood vessels and reach the liver, the sporozoites have to traverse either endothelial cells (cells which form those vessels wall) or Kupffer cells, and the space of Disse, which separates the endothelial layer from liver cells (Fig. 1.4).

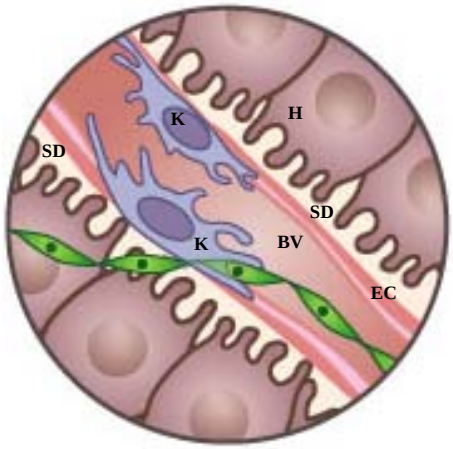

Figure 1.4. Schematic representation of sporozoites migrating in a liver blood vessel. Sporozoites (in green), in order to leave the blood vessel (BV) and reach the hepatocytes (H), need to traverse either endothelial cells (EC) or Kupffer cells (K) and the space of Disse (SD).

It has been demonstrated that CSP binds to the glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs), present in the basolateral pole of hepatocytes. Liver endothelial cells possess fenestrations, but not wide enough to allow sporozoite passage. It has been proposed that HSPGs would be extended through these fenestrations, allowing the sequestration of sporozoites. Although this process is necessary for the attachment to the hepatocytes, it is not required for cell invasion (Kappe, 2003; Mota, 2002). CSP and TRAP recognize distinct cell type-specific surface proteoglycans, not only on hepatocytes, but also on Kupffer cells, the resident macrophages in the liver, and stellate cells. Moreover, because stellate cells synthesize 8× more sulfated proteoglycans than hepatocytes and incorporate twice the amount of sulfate into HSPGs, stellate cell proteoglycans protruding through the endothelial fenestrations have been suggested to mediate the initial arrest of *Plasmodium* sporozoites in the liver sinusoid (Pradel, 2004; Pradel, 2002). It has been proposed that sporozoites migrate to the space of Disse through Kupffer cells. Sporozoites have been found preferentially inside Kupffer cells shortly after sporozoites infection, but mostly surrounded by a vacuole. However, it is likely that sporozoites may use more than one cellular pathway to cross the sinusoidal cell layer, as a proportion of cell traversal deficient parasites can still infect the liver and propagate in mice (Ishino, 2004; Mota, 2002).

1.3.3. Migration

Plasmodium sporozoites migrate through several cells before infecting a final host cell. During this migration, sporozoites enter and leave the cells by disrupting cell membrane. Usually, cells can repair this damage and survive, but, in some cases, death occurs (Fig. 1.5). This process does not lead to the formation of a Parasitophorous Vacuole (PV) inside the cell, which is only observed when the parasite reaches a final hepatocyte for infection.

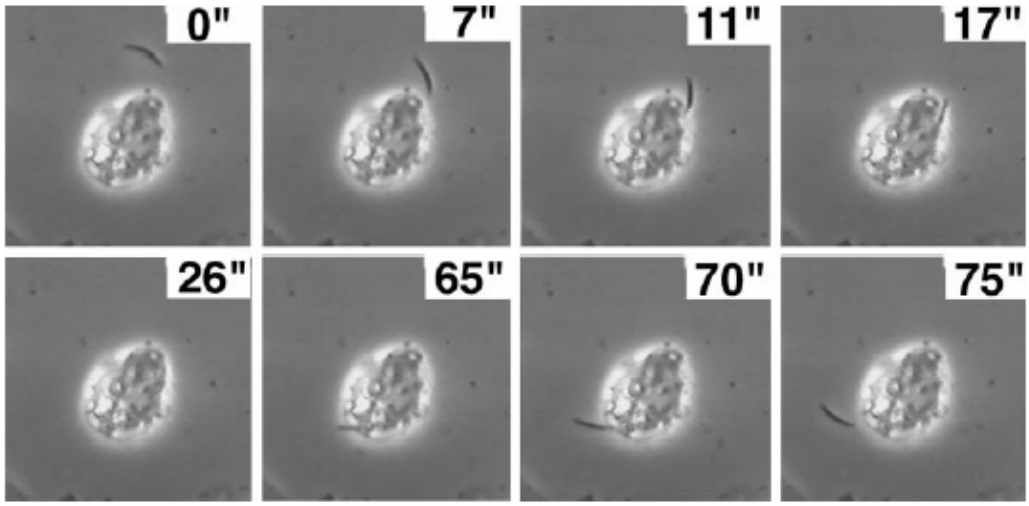

Figure 1.5. *Plasmodium* sporozoites migration in HepG2 cells. *Plasmodium* sporozoites enter and exit a host cell within a minute, as seen above in these time-lapse video images of a *P. berghei* sporozoite entering and exiting a HepG2 hepatocyte (adapted from Mota, 2001)

Migration was confirmed in experiments *in vitro* (using human and mouse hepatoma cell lines) and *in vivo* (using a mouse model for malarial infection) (Fig. 1.6), and only viable and motile sporozoites were able of migrate through cells. In fact, heat-inactivated, as well as sporozoites treated with anti-CSP monoclonal antibody or cytochalasin-D were unable to migrate through cells (Mota, 2002; Mota and Hafalla, 2002; Mota, 2001; Mota and Rodriguez, 2001).

It has been proposed that sporozoites migration through cells might provide a biological advantage in establishing a successful infection. It might supply an unimpeded view of the local host cytoplasmic environment, allowing the sporozoites to confirm whether or not they reached the liver, and triggers pathways required for invasion and infection. Migration is commonly observed in

other apicomplexan parasites at similar stages of the life cycle. *Toxoplasma* and *Eimeria bovis* (a cattle pathogen) sporozoites are also able to migrate through host endothelial cells, disrupting the cell membrane and without the formation of a parasitophorous vacuole (Mota, 2002; Mota and Hafalla, 2002; Mota, 2001; Mota and Rodriguez, 2001).

Breaching of the cell membranes by the *Plasmodium* sporozoite is likely to involve specific lipases, proteases or pore-forming proteins. Four distinct *P. berghei* proteins have been shown to play important roles during cell traversal. They are sporozoite microneme protein essential for cell traversal (SPECT), SPECT2, cell traversal protein for ookinete and sporozoite (CelTOS) and a phospholipase (PbPL) (Kariu, 2006; Bhanot, 2005; Ishino, 2005A; Ishino, 2004). At least two of these, SPECT2 and PbPL, seem to be involved in pore formation activity while CelTOS has been proposed to be required for movement through host cell cytosol.

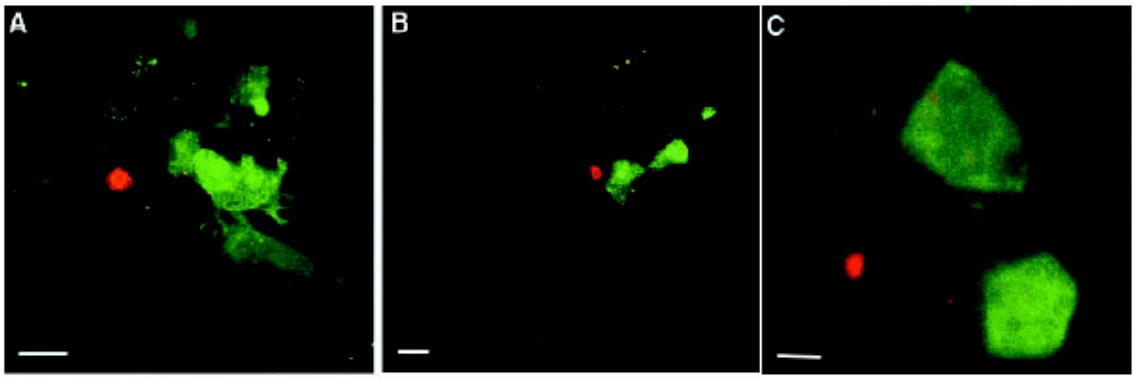

Figure 1.6. Migration of *Plasmodium* sporozoites in hepatoma cell lines can be quantified using a cell-impermeant fluorescent tracer. Cells incubated with *P. yoelii* sporozoites (A and B, Hepa 1-6 cell line, C, Balb/c 6 weeks old mice). Dextran-FITC, a marker for wounded cells, was used, so, cells traversed by sporozoites appear with a bright green colour (adapted from Mota, 2001)

1.3.4. Invasion and Development inside Hepatocytes

After migration through several cells, the sporozoite enters the host cell, with the formation of a PV. Once inside, the sporozoite starts a new stage of infection, commonly named Exoerythrocytic Form (EEF). Little is known about the process of invasion and formation of EEFs in *Plasmodium*. Usually, host cell invasion by

Toxoplasma tachyzoites is used as a model for *Plasmodium*. Gliding is crucial at this point. Molecules excreted at the apical end of the parasite bind to a fixed substrate on cell surface, being transported for the posterior, leading to forward locomotion. During this process, when the parasite finds and binds to host cell receptors, invasion takes place. The anterior end forms a tight junction with the host cell surface. Penetration occurs, and a vacuole is formed around the parasite, being sealed once invasion process is complete (Silvie, 2004; Mota, 2002).

Another host molecule that seems to interact directly or indirectly with sporozoites is tetraspanin CD81, a membrane protein that is expressed on the surface of hepatocytes and a putative receptor for hepatitis C virus (Silvie, 2003). CD81 seems to play an essential role in the invasion of mouse hepatocytes by *P. yoelii* and human hepatocytes by *P. falciparum* but is not required for cell traverse by sporozoites. CD81 mechanism is exclusive in hepatic stage of malarial infection but, most interesting, were not reproducible in another rodent parasite, *P. berghei*, a model widely used in malaria research. *P. berghei* sporozoites seem to invade hepatocytes by using a CD81-independent pathway. Interestingly, in the presence of CD81, *P. berghei* sporozoites can use this molecule to invade hepatocytes. However, they are still able to invade cells if CD81 is absent suggesting, again, that *P. berghei* sporozoites can use alternative invasion pathways. The same authors have also shown that host cell membrane cholesterol contributes to CD81-dependent infection by *P. yoelii* and *P. falciparum* sporozoites but not to CD81-independent infection by *P. berghei* sporozoites (Silvie, 2006).

Another strategy used by *Plasmodium* parasites explains the need for a migration process prior to the final invasion. In order to be ready to infection, hepatocytes must contact with an Infection Susceptibility-Inducing Factor (ISIF). This was first observed when cells in culture presented a higher level of infection if treated with medium where previously other cells have been subjected to infection instead of fresh medium. Later, this ISIF was identified as been hepatocyte growth factor (HGF). HGF is released from hepatocytes after wounding, and traversed cells present a higher level of production of this factor. Cells incubated with antibodies against HGF show a decrease in sporozoite infection of around 90% than the matched untreated control. MET is the tyrosine kinase receptor for HGF, and is activated when HGF is released from neighbour cells. Activation of MET

by HGF enhances sporozoite infectivity in hepatocytes. Downmodulation of MET (e.g., using RNA interference) leads to a reduction in infection. It was seen that HGF/MET signalling might be required for early parasite establishment and development inside the host cell. The HGF/MET requirement in infection was also shown *in vivo* (Carrolo, 2003). Recently, it has been shown that *Plasmodium* parasites exploit HGF to activate anti-apoptotic signalling pathways in the host cell through HGF/MET signalling (Leirião, 2005). HGF/MET signalling is able to protect cells from apoptosis by using mainly PI3-kinase/Akt pathway, contributing to a successful infection by *Plasmodium* sporozoites. Interestingly, in the context of infection, apoptosis is being redefined based on a number of studies demonstrating that apoptotic death of host cells after pathogen infection can trigger powerful innate and adaptive immune responses. In fact, apoptosis-induced inflammation is actively being investigated as a way of enhancing vaccine function improving accessibility of the effector cells of the immune system to the site of infection (reviewed in Restifo, 2000). The mechanisms and (parasite) molecules involved in preventing host cell apoptosis by intracellular parasites including apicomplexans are being investigated (Blaho, 2004; Luder, 2001).

Once inside the final hepatocyte, the sporozoite starts its EEF formation procedure. Its nucleus increases progressively, giving rise to a spherical form. Inside an EEF, a single sporozoite develops into a schizont, which contains tens of thousands uninucleate merozoites (Hoffman, 2000) (Fig. 1.7).

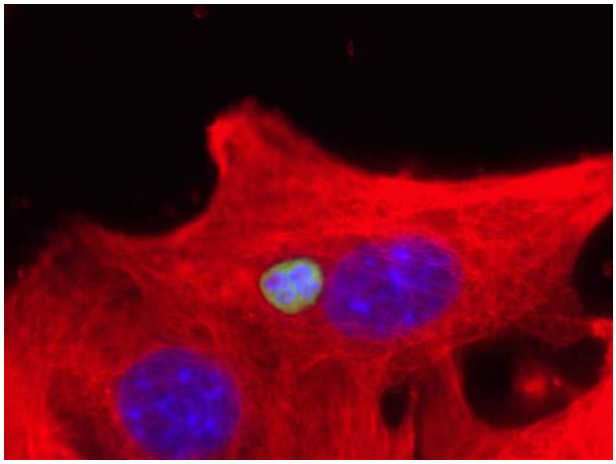

Figure 1.7. *P. berghei* developing inside a hepatocyte. *P. berghei* EEF (green) developing inside a HepG2 cell. Inside the EEF, merozoites in formation can be observed (blue). The nucleus of host/neighbour cell (great blue circles) and cells cytoskeleton (red staining for actin) also can be observed (adapted from Mota, 2002)

The process of formation of the *Plasmodium* parasites intracellular vacuole is widely studied in the erythrocytic stage, but very little is known how it occurs in hepatocytes. The two types of host cells are completely different, as are their surrounding environment (Sherman, 1998). So, very few assumptions that what occurs in one cell type can be made for the other. Only recently have host molecules that influence parasite development inside the hepatocytes started to be identified. Signalling pathways are abundant within cells and represent potential targets upon which a pathogen can interact with its host as well as likely key elements of the host's response to infection. Intracellular signalling mechanisms rely heavily on phosphorylation, a process that is enzymatically catalyzed by kinases. A genome wide RNA interference screen of all the human kinases and kinase associated proteins is currently being performed in our lab to identify signalling pathways that are involved in the interactions that occur between the parasite and its host. Our preliminary results indicate that infection is significantly modulated by kinases involved in cytoskeleton regulation, suggesting a crucial role for cytoskeletal dynamics in infection. An unbiased microarray approach to identify genes that are differentially expressed at various time points following *Plasmodium* infection of a mouse hepatoma cell line is also being undertaken by us. Interestingly, it was observed that the number of differentially expressed host genes decreases as infection proceeds. A host molecule that seems to play an important role is alipoprotein A1 (ApoA1), which localizes 24 hours post infection at the parasitophorous vacuole, and seems to interact with the parasite molecule UIS4 (A. K. Mueller, 2005 Molecular Parasitology Meeting). The implications of such behaviour remain to be elucidated.

1.3.5. Leaving the Hepatocyte

A final important step in the intracellular life of pathogens is their ability to exit the host cell after replication, to continue their life cycle. Nevertheless, the mechanisms which mediated such processes are still poorly understood. The release of *Plasmodium* merozoites from hepatocytes is usually referred to as occurring after hepatocyte rupture. Still, this has never been directly observed and the signal(s) that trigger their exit remain unknown. Very recently, it has been

reported that *Plasmodium* merozoites are released not by rupture of the hepatocyte but by the formation of merozoite-filled vesicles (merosomes), which bud off the infected hepatocyte into the lumen of liver sinusoids (Fig. 1.8). Initially, merozoites are released from the parasitophorous vacuole membrane and mix freely with the host cell cytoplasm (Meis, 1985). The plasma membranes of merosomes are of host cell origin and are thus not recognised by Kupffer cells, which guide the parasites safely, back to the blood stream. The molecular basis of the process is not fully understood but it has been shown that proteases mediate liberation of merozoites from the PV and the formation of merosomes (Sturm, 2006).

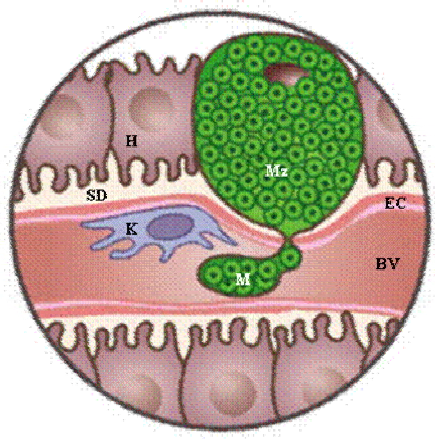

Figure 1.8. Schematic representation of a merosome leaving a hepatocyte. Merozoites (Mz), in order to abandon hepatocytes (H) and reach blood vessel (BV), traverse the layer of endothelial cells (EC) and the space of Disse (SD) in a merozoites-filled vesicle, or merosome (M), thus avoiding elimination by Kupffer cells (K).

1.4. Vaccine against Malaria

1.4.1. Aims and History

Vaccination, in principle, is an attempt to mimic certain aspects of an infection for the purpose of causing an immune response that will protect the individual from that infection. Vaccine research has three goals: induction of strain-transcending and durable immune responses, identification of protective antigens for stage

specific immunity and successful combination of candidate immunogens (Artavanis-Tsakonas, 2003; Kilama, 2003). Promising candidate biomolecules, with proven potential by research institutions, can be selected by industry for manufacture, and posterior validation phases, till they reach clinical trials (Kilama, 2003).

In 1967, mice immunized with X-radiation-attenuated rodent malarial sporozoites have shown protection against challenge with live and infectious sporozoites (Nussenzweig, 1967). In 1973, this approach had the same result in humans (Clyde, 1973). Volunteers infected with irradiated *P. falciparum* sporozoites were protected against challenge within nine weeks of immunization. This protection lasted 23-42 weeks post immunization, in five of the six volunteers. However, this vaccination consists in the bite of several irradiated mosquitoes that is an unpractical mean when planning a mass vaccination. Many potential candidate vaccines against hepatic and also blood stage have been tested in clinical trials in the last decade, and other warrant preclinical assessment. But, so far, there is not a single effective vaccine available against malaria (reviewed in Hill, 2005). In the 90s, vaccination with a synthetic peptide, Spf66, against malarial blood stage parasites, generated much speculation and excitement, but it revealed lack of efficacy in protection against this disease (Moorthy, 2004; Molano, 1992).

The aim of most vaccines is to induce antibody and T-cell responses to one or a few antigens, but for effective vaccination, these will need to be of greater magnitude, duration (and not strain-specific) than in naturally acquired immunity. At the moment, it is unknown and not understood which responses are needed, except that is necessary to produce T-cell help for an antibody response. An alternative approach is to use a cocktail of several antigens, attempting to mimic natural immunity, but it is a complex and costly process (Moorthy, 2004). Other problems, which arose from combination with multiple antigens, are interference and competition. In the last decade a huge effort been allocated to design a subunit vaccine, which means, to use partial or complete antigens (previously identified by a proteomics approach from host-pathogen interactions), which could induce immunity to the whole pathogen in vaccination, e.g., the hepatitis B vaccine. Nevertheless, these type of vaccine are usually ineffective in induce long-term immune responses, as effector T cells responses, like CD8+ cytotoxic T lymphocytes (Moorthy, 2004). Another subunit vaccine type is DNA based. DNA

sequences of *Plasmodium* parasites (namely *P. falciparum*) have been inserted into plasmid DNA molecules (DNA vaccine) or various recombinant attenuated DNA viruses (recombinant vaccines), to generate vaccine candidates. The delivered DNA molecules are uptaken by host cells, parasites then proteins are expressed, and T cell epitopes bound to Human Leukocyte Antigens (HLA, the human Major Histocompatibility Complexes) molecules prime naïve T cells to form memory T cell population. Recombinant subunit vaccines act similarly, but are more active in infecting cells and expressing parasite proteins, before infection process is aborted. DNA based vaccines can induce high levels of effector T cell immune responses, and permits the combination of multiple antigens, but poor antibody responses are observed (reviewed in Hill, 2005). The development of an effective vaccine also requires research into antigenic polymorphism, duration of efficacy, and means of antigen combination. For a vaccine be licensed for use, the entire process (from first observations to demonstrations of high level of efficacy) can take more then a decade (reviewed in Hill, 2005).

1.4.2. Vaccine against *Plasmodium* Liver Stage

The hepatic stage of the life cycle is an ideal target for vaccine-induced protective immune responses, because this stage lasts around 5.5-7 days and is not associated with symptoms. A vaccine that would efficiently target the infected hepatocytes would prevent both the clinical symptoms of the disease, since they arise only in the erythrocytic stage, and the transmission of malaria, since formation of gametocytes occurs during gametogenesis, also in the blood stage (Hoffman, 2000).

An ideal vaccine against parasites at this stage would induce high titres of functional antibody against sporozoites, to prevent their entry inside hepatocytes, and stimulate potent cytotoxic T lymphocyte immunogenicity against infected liver cells, while not harming remaining host tissues. Actually, the leading candidate vaccine is a recombinant protein vaccine, named RTS,S/A02, developed by GlaxoSmithKline, in which Hepatitis B surface antigen was fused to DNA encoding CSP, and mixed in an adjuvant AS02. Although has proven efficacy, it confers only 30% of protection against parasites of the same strain after infectious sporozoite challenge. In field trials held in Mozambique, none of the vaccinees

inoculated with RTS,S was protected 6 months post immunization, being a case of a successful vaccine which misses its target unfortunately (reviewed in Hill, 2005). Although more than 10 vaccines designed to induce protective antibody- or cell-mediated immune responses against the infected hepatocyte have been evaluated in humans, only a small number of humans have been protected (reviewed in Hill, 2005). Other vaccines using viral vectors such as adenoviruses and modified vaccinia virus Ankara are being developed (Ophorst, 2006; Hill, 2005), by other biotech companies, such as Crucell Holland BV (http://www.crucell.com/) and Oxxon Therapeutics (http://www.oxti.com/), respectively.

So far, the golden standard for an exoerythrocytic malaria vaccine is protection conferred by immunization with radiation attenuated sporozoites (RAS). It has conferred protection to more than 90% of animal subjects and human volunteer hosts who were subjected to that immunization. Irradiated sporozoites enter hepatocytes, but only partially develop within these cells (Surhbier, 1990; Sigler, 1984). As so, the host will never suffer from the symptoms caused by parasites in blood stage. There is no immunogenic response against blood stage malarial antigens, because they are not expressed in liver stage. Sexual differentiation, which also occurs in blood, will not take place as well and consequently, nor will transmission. Immunization of human volunteers with radiation-attenuated sporozoites demonstrated that this type of vaccine is feasible in developing protection against malaria, and provides foundation for further studies aiming to achieve a subunit pre-erythrocytic stage malaria vaccine. The actual data indicates that this type of vaccine is not strain-specific, meaning an immunization with irradiated *P. falciparum* sporozoites will induce responses not only against that strain, but that will also confer protection against other strains within the same spp. (Hoffman, 2002; Doolan, 2000; Hoffman, 2000). RAS mediated protection is dependent on $CD8^+$ and $CD4^+$ T cells, nitric oxide, cytokines (such as interleukin 12 (IL-12) and γ interferon (IFN-γ)) and natural killer cells (NK cells), although this varies according with the background of the host (Doolan, 2000). Immunization strategies using *P. falciparum* RAS have been considered impossible due to technical, clinical and logistical hurdles: route of administration, large quantities of sporozoites needed and regulatory, potency and safety requirements (Luke, 2003). However, some are pursuing this approach and

already have overcome some of those obstacles (S. L. Hoffman, personal communication; http://www.sanaria.com/).

1.5. Comparative Genomics in *Plasmodium*

1.5.1. General Features

In October 2002, fruit of seven years of labour resulted in the publication of an almost complete annotated genome sequence of *P. falciparum* (PlasmoDB, http://www.plasmodb.org/; Gardner, 2002). Other simultaneous initiatives have allowed comparative genomics of this human parasite with *P. vivax* and the rodent parasites *P. yoelii*, *P. berghei* and *P. chabaudi* (Thompson, 2001). Comparative analysis of these genomes can provide substantially more information on parasites physiology and biology, and expand the ability to assign putative functions to predicted coding sequences. More than 60% of expressed genes have no known function. Comparative genomics has given the opportunity to deduce the function of many of the predicted proteins through the identification of orthologous genes (genes with high rate of homology, in different species or strains) and motifs in other organisms, and to analyse genome organization and the phylogenetic profiles of protein families. It will be also possible to discover orthologues and paralogues (genes with high rate of homology, within the same specie or strain) of less well conserved genes, and enhance studies that seek to discover and evaluate the vaccine and drug target potential of *Plasmodium* proteins (Hall, 2005; Waters, 2003; Thompson, 2001).

1.5.2. Applications

1.5.2.1. Orthology

Model malaria parasite orthologues of *P. falciparum* have an important use as vaccine and drug targets research and development. Some of those orthologues were previously identified by biochemical, molecular biology and immunological assays. Accessing to PlasmoDB resources, the identification of orthologues between *P. falciparum* and other *Plasmodium*, as well as paralogues, may be

performed *in silico*, using BLAST analysis. Once those genes have been identified, the biology and the function of the encoded proteins can be investigated in the most appropriate *Plasmodium* species, using mice as animal models, to test their potential as vaccines or drug targets. Another rich source of information will be parasite mutants that fail to undergo specific developmental phases, such as intrahepatic growth, enabling specific transcription programs to be elucidated (Waters, 2003; Thompson, 2001).

1.5.2.2. Genetic Tools

In the 90s, several genetic tools for the study of malaria parasite biology were developed (van Dijk, 1995). Transfection systems for this haploid unicellular organism are now available, for human and animal *Plasmodium* spp. Transient transfection has been used to provide insight into the regulation of gene expression, while stable transfection has given the opportunity of expressing transgenes, as well as elucidating the function of proteins by disrupting, modifying or replacing the genes encoding them. These genetic tools, added to the information now available of the *P. falciparum* genome, represent an important breakthrough for malaria research, contributing significantly to the understanding of the hugely complex biology of this parasite. Their potential is vast, since more rational approaches to vaccine and drug design can be achieved, by altering the parasite genome (Koning-Ward, 2000).

Stable transfections in *P. falciparum*, *P. berghei*, *P. yoelii* and the nonhuman primate parasites *P. knowlesi* and *P. cynomolgi* have proven feasibility, by drug selection (Mota and Thathy, 2001; Koning-Ward, 2000). Unlike transient transfections, this system requires that transfected DNA contain a selectable marker gene encoding a protein that confers a selectable phenotype, such as drug resistance (pyrimethamine is the commonly used), on the recipient parasite. Mature schizonts containing fully developed merozoites (in *P. berghei*, *P. yoelii* and *P. knowlesi*) or intraerythrocytic blood stage parasites (in *P. falciparum*) are chosen as target cells for introduction of DNA, because these stages are not dependent on the erythrocyte for survival, minimizing the problem of host cell damage during the process. This technology has provided the opportunity to produce specific gene knockout parasites and to express foreign genes within the

parasite. Transient transfections, per turn, provided the opportunity to identify elements that control gene expression, and to temporarily express or overexpress genes thought to be lethal to *Plasmodium*. Although its efficiency is fifty times higher than in stable transfections, this method is often of little value to researchers performing transfections experiments with *Plasmodium* spp., namely *P. falciparum* (Skinner-Adams, 2003; Koning-Ward, 2000).

1.5.2.3. Genetic Modification elucidates the role of targeted genes in *Plasmodium* infection

Thanks to genetic modifications, a powerful tool was finally accomplished: GFP-expressing constitutively throughout all life cycle *P. berghei* parasites were achieved (Franke-Fayard, 2004). These GFP-expressing lines will, for example, provide a means to purify by flow sorting cultured, infected hepatocytes or erythrocytes containing parasites allowing a detailed analysis of the transcriptome and proteome of both parasite and host cells and as a consequence shed more light on the parasite strategies used to influence host cell pathways. Luciferase expressing *P. berghei* parasites are also available and can be used when GFP technology fails to provide the insights about host-*Plasmodium* interactions (Franke-Fayard, 2005; Koning-Ward, 1999).

Depletion of genes using these tools also led to important findings about *Plasmodium* biology. An example is P48/45 gene, member of a gene superfamily with 10 elements containing six-cysteine (6-cys) structural domain, unique to *Plasmodium*. This family appears to be well distributed among *Plasmodium* spp. Most of the members are conserved in the genome of *P. vivax*, *P. yoelii*, *P. chabaudi* and *P. berghei*. To date, all the members of 6-cys domain superfamily are conserved. Disruption of *p48/45* gene in *P. berghei* parasites led to the formation of infertile male gametocytes, making it an excellent candidate for transmission blocking vaccines (van Dijk, 2001). Other members of this family, namely P47, the paralogue of P48/45 and P230 are also being studied as transmission blocking vaccines (Thompson, 2001).

Our previous work with another member of this family, P36p led to a completely different and unexpected phenotype. Unlike seen for *p48/45* gene, disruption of *p36p* in *P. berghei* does not impair the parasite development inside the mosquito.

However, when injected in mice, these attenuated parasites do not give rise to a patent blood stage infection. We observed that *p36p*- sporozoites perform all features necessary for a successful hepatocyte infection (gliding motility, activation of apical complex and invasion), at the same level WT sporozoites do. Nevertheless, their intrahepatic development is strongly arrested, never leading to formation of merozoites and, consequently, to a blood stage infection (van Dijk, 2005). This phenotype is similar to the one observed for RAS (Suhrbier, 1990; Sigler, 1984) and to other *P. berghei* genetically-attenuated sporozoites (GAS) lacking either *uis3* (upregulated in sporozoites gene 3) or *uis4* genes (Mueller, 2005; Mueller and Camargo, 2005). As mentioned before, RAS can be used as an immunization strategy against malaria (Nussenzweig, 1967). Interestingly, *uis3*- and *uis4*-, with a similar aborted intrahepatic development also confer protection to mice against later challenge with WT *P. berghei* sporozoites (Mueller, 2005; Mueller and Camargo, 2005). The reasons which lead to this phenotype are still unclear. So far, we only know that ApoA1 co-localizes with UIS4 protein at the PV membrane inside the hepatocyte, most probably due to the cholesterol need for parasite development (A. K. Mueller, 2005 Molecular Parasitology Meeting). *pb36p*- sporozoites are able to reduce parasite liver burden in mice upon challenge with infectious WT sporozoites (van Dijk, 2005). P36p protein localizes in sporozoites micronemes, and is excreted during gliding (Ishino, 2005B). The function of P36p is still unknown and is currently being investigated. Interestingly, another study regarding *pb36p*- sporozoites demonstrate that they have a much smaller infectivity in HepG2 cells, leading to a continuous traversal of cells. Consequently, these attenuated parasites possess a 6× higher rate of migration through hepatocytes than WT sporozoites (Ishino, 2005B). The discrepancy between this study and our results is presently unknown.

The examples just described show that the use of these genetic tools in *Plasmodium* can lead to groundbreaking discoveries about aspects still unidentified of this parasite biology and interaction with its host molecules, and provide insight about novel vaccine and drug targets candidates to fight this plague.

1.6. Aims and Strategies

Our previous results have shown that *P. berghei* GAS lacking a gene denominated *pb36p* were not fully capable of developing inside hepatocytes and demonstrate potential to be used as a novel vaccine strategy. In this project, we decided to focus on *pb36p*- parasites immunization potential, characterizing several features of this GAS based vaccine, using the mouse strains BALB/c and C57BL6. More precisely, we proposed (i) to determine the number of doses required to confer sterile protection, (ii) to determine the minimum dose of attenuated parasites required to elicit a full protective immune response, (iii) to elucidate the administration routes by which these parasites (as well as RAS) are still able to confer protection, (iv) to access the potential of *pb36p*- and RAS on conferring protection to a distinct rodent *Plasmodium* species, *P. yoelii*, (v) to elucidate the mechanisms by which *pb36p*- confers immunity and (vi) to determine whether two other GAS, *pbcrmp3*- and *pbcrmp4*- were also able to protect against subsequent challenge.

Chapter II

pb36p- as an experimental GAS based vaccine against Malaria

2.1. Introduction

The current rationale for the characterization of *Plasmodium* molecules involved in liver infection lies in the development of a (subunit) vaccine that protects against liver and subsequent blood stage infection (Tsuji, 2001). However, vaccination studies with subunit vaccines that contain only (parts of) single proteins of sporozoites have failed so far to provide any significant, long-lasting protective immunity (reviewed in Hill, 2005). Contrastingly, immunization studies using complete radiation-attenuated sporozoites (RAS) showed full protection against subsequent challenge with infectious sporozoites in both animal models of malaria (Gwadz, 1979; Nussenzweig, 1967) and in human volunteers (Clyde, 1975). So far, only *P. falciparum* RAS immunizations have conferred long-lasting protection to human volunteers against challenge with infectious sporozoites (lasting up to 10.5 months) but failed to give protection against cross-species challenges in humans, not protecting against a challenge with another human malaria parasite, *P. vivax* (Hoffman, 2002). The lack of significant progress with subunit vaccines stimulated recent attempts to produce a vaccine based on non-replicating, metabolically active RAS (Luke, 2003). Nonetheless, some major hurdles may impair the development a RAS-based *Plasmodium* sporozoites vaccine, namely production of adequate number of sporozoites for such a vaccine and viability of applying delivery routes commonly used as public health measures (Luke, 2003). Furthermore, RAS-based approach suffers from significant drawbacks, not least the question of safety and reproducibility because the amount of radiation that generates the attenuated state is strictly defined. Parasites that are underirradiated remain infectious, and those that are overirradiated do not induce protective immunity (Suhrbier, 1990). Recently, it has been shown that genetically attenuated sporozoites (GAS) that lack sporozoite specific conserved genes (*uis3*- and *uis4*-) that are apparently important for sporozoite development in the hepatocyte can induce significant or complete protective immunity in the *P. berghei* rodent model of malaria when different immunization protocols are used (Mueller, 2005; Mueller and Camargo, 2005). Generation and analysis of biological and immunogenic characteristics of multiple

GAS models are essential for further development of GAS as a potential malaria vaccine.

In this study, we characterized the immunization potential of a recent GAS in *P. berghei*, which lacks the protein P36p, a sporozoite-specific microneme protein that is excreted to surface during gliding (Ishino, 2005B; van Dijk, 2005). P36p is a member of the 6-cys protein family, which comprises 10 members, some of them constituting potential vaccine candidates (van Dijk, 2001; Thompson, 2001). Development of *pb36p*- parasites aborts within the hepatocyte and immunization with *pb36p*- sporozoites reduces significantly or even abolish completely parasites liver burden after subsequent challenges with wild-type sporozoites, (van Dijk, 2005).

Here, we show that *P. berghei p36p*- sporozoites are able to induce full protection in rodent models against Malaria, as seen before for *P. berghei* RAS, *uis3*- and *uis4*- parasites, and can last up to 18 months. Furthermore, *pb36p*- sporozoites require low immunization doses to confer complete immunity, and confer protection when administered through feasible routes for human vaccination, such as intramuscular, subcutaneous and intradermal. But, more surprisingly, this protection is not restricted to challenges with *P. berghei* homologous parasites. Both RAS and *pb36p*- sporozoites also protect mice against challenge with a heterologous rodent species, *P. yoelii*, strongly inhibiting this species intrahepatic development, and some mice even achieve sterile protection. To our knowledge, our results show for the first time that whole parasite attenuated malaria sporozoite vaccine is capable of inducing cross-species protection.

2.2. Results

2.2.1. Immunization with *pb36p*- sporozoites protects BALB/c and C57BL6 mice against subsequent infection

We have shown that *pb36p*- development within hepatocytes is arrested, similarly to which occurs with RAS sporozoites (van Dijk, 2005). Because RAS has been successfully used in immunization studies for generation of protection against subsequent challenge with WT sporozoites, we investigated the immunization

potential of *pb36p*- sporozoites. Mice were i.v. immunized with *pb36p*- sporozoites by using different immunization protocols and subsequently challenged with WT parasites. Our previous results, using real-time PCR assays to quantify A-type ribosomal RNA transcripts produced by developing trophozoites in the liver (van Dijk, 2005), show that mice immunized with RAS and *pb36p*- sporozoites had a very strong and comparable reduction (>99%) in liver stage development of WT parasites compared to unimmunized mice. In several independent experiments groups of BALB/c and C57BL6 mice were immunized with a single dose of 50,000 *pb36p*- sporozoites or multiple times (one to three immunizations), with a prime of 50,000 and subsequent boosts of 20,000 *pb36p*- sporozoites, respectively, and challenged i.v. with 10,000 WT sporozoites on different days after immunization (Table 2.1). Protection was determined by monitoring mice daily for blood stage parasitemia in blood smears or using FACS analysis from day 3 to 3 weeks after challenge. A single immunization dose of *pb36p*- sporozoites fully protected BALB/c mice, whereas three subsequent immunizations with *pb36p*- sporozoites were required to completely protect C57BL6 mice, as shown by the absence of subsequent blood stage infection after challenge with WT sporozoites. C57BL6 mice were partially protected when immunized only twice (Table 2.1), with either RAS (33%) or *pb36p*- sporozoites (25%). The protective immune response induced by *pb36p*- sporozoites as well as RAS sporozoites seemed to be parasite stage-specific, because BALB/c mice were not protected from a challenge with 1×10^6 parasite-infected erythrocytes and developed a normal blood stage infection comparable to the control group (Table 2.1).

Occasionally, some mice from both host strains (10% C57BL6, i.e., 5 of 48 mice; 4% BALB/c, i.e., 1 of 26 mice) did develop a 7-day delayed blood stage infection upon i.v. injection of *pb36p*- sporozoites. Interestingly, blood stage infections were never observed when *pb36p*- sporozoites were transmitted naturally through mosquito bites (van Dijk, 2005). The resulting blood stage parasites still contained the knockout genotype, as analyzed by PCR in Leiden University Medical Center, The Netherlands, by Milly van Dijk (van Dijk, 2005).

TABLE 2.1. Immunization with *pb36p*- sporozoites protects against a subsequent infection with WT sporozoites

Mouse Strain	Immunization[a], RAS/*pb36p* - × 1,000	Challenge[b], × 1,000	No. protected (no. challenged)		
			Control	RAS	*pb36p*-
BALB/c	50	10	0 (5)	ND	5 (5)
C57BL6	50	10	0 (3)	0 (3)	0 (1)
C57BL6	50/20	10	0 (3)	1 (3)	1 (4)
C57BL6	50/20/20	10	0 (5)	5 (5)	4 (4)
BALB/c	50	1,000 iRBC	0 (3)	0 (5)	0 (4)

[a] Groups of mice were immunized i.v. with one dose of RAS or *pb36p* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. ND, not determined.

[b] Mice were challenged intravenously (i.v.) with WT *P. berghei* sporozoites, isolated from different mosquitoes batches, or intraperitoneally (i.p.) with infected red blood cells (iRBC), 10 days after last immunization. The pre-patent period was monitored by FACS analysis. All unimmunized mice become positive on day 3 or 4 after challenge.

2.2.2. *pb36p*- sporozoites induce long-lasting protection against continuous challenges with *P. berghei* sporozoites

BALB/c animals shown protected when challenged 10 days after last immunization (Table 2.1) were rechallenged, 1, 2, 4 and 6 months after immunization, respectively, with 10,000 WT sporozoites and did not develop blood stage infections up to 3 weeks after challenge (Table 2.2, group 1; adapted from van Dijk, 2005).

In contrast, blood stage parasites could be observed in control mice on day 3.0 after challenge (Table 2.2, group 1). We next sought to test if this protection could last up to 1 year. The same group of protected BALB/c mice was again challenged i.v. with 20,000 WT sporozoites nearly 12 months (351 days) after immunization. A higher dose was used, since although age has no effect on lethality in *P. berghei* in mice, older rats are able to completely control the infection (Pierrot, 2003; Greenberg, 1953; Singer, 1955). Interestingly, mice were fully protected and failed to develop blood stage parasites up to 3 weeks after the challenge, while blood stage parasites could be detected on day 3.5 in control animals (Table 2.2; group 1).

TABLE 2.2. Immunization with *pb36p*- sporozoites lead to protection against subsequent multiple challenges

Group	Mouse Strain	Immunization[a], RAS/*pb36p* - × 1,000	Challenge[b], × 1,000	Challenge at ... days post last immunization	No. protected (no. challenged)		
					Control	RAS	*pb36p*-
1	BALB/c	50	10/10/10	30/60/120	0 (5)	ND	5 (5)
1	BALB/c[c]	50	10/20	180/351	0 (5)	ND	4 (4)
1	BALB/c[d]	50	20	531	0 (5)	ND	3 (3)
2	C57BL6	50/20/20	10/10/10/20	30/60/90/180	0 (5)	5 (5)	4 (4)
2	C57BL6	50/20/20	20	365	0(5)	5 (5)	2 (4)
3	BALB/c	50/20/20	10/20	60/180	0 (5)	3 (3)	4 (4)
3	BALB/c[e]	50/20/20	20	365	0 (5)	3 (3)	1 (2)
4	BALB/c	50/20/20	10/20	90/180	0 (5)	3 (3)	3 (3)
4	BALB/c[f]	50/20/20	10/20	365	0 (5)	1 (1)	2 (2)
5	BALB/c	50/20/20	20	365	0 (5)	ND	1 (2)

[a] Groups of mice were immunized i.v. with one dose of RAS or *pb36p* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. ND, not determined.
[b] Mice were challenged with WT *P. berghei* sporozoites, isolated from different mosquitoes batches. The pre-patent period was monitored by FACS analysis. All unimmunized mice become positive on day 3 or 4 after challenge.
[c,d,e,f] Mice were found dead during the time which experiments were performed, but cause of death is not related with malaria

And, most remarkable, these mice were still fully protected when re-challenged i.v. with 20,000 WT *P. berghei* sporozoites 18 months after last boost, unlike matched naïve control mice (all positive on day 3.0 after challenge). The immune response elicited by *pb36p*- sporozoites was still able to fully protect BALB/c mice after 18 months (Table 2.2, group 1).

C57BL6 mice immunized i.v. with three doses of RAS or *pb36p*- were protected against subsequent challenge with infectious sporozoites (Table 2.1). Like in BALB/c mice, we propose to determine the protective immune response persistence. After the final immunization, these mice received i.v. challenges of 10,000 WT *P. berghei* parasites 1, 2 and 3 months post final immunization and, of 20,000 WT sporozoites 6 months post final immunization. A complete absence of blood stage parasites up to 3 weeks post challenge was observed in mice immunized with either RAS or *pb36p*- sporozoites regarding all three subsequent challenges (Table 2.2, group 2; adapted from van Dijk, 2005). Blood stage parasites were detected in control mice after 3.0 days, for each challenge. *pb36p*- sporozoites were able to confer, like RAS-based immunizations, sterile protective

immunity that lasts up to 6 months in C57BL6 mice. These mice were re-challenged, 12 months after last immunization, and 50% of *pb36p*- immunized mice were still fully protected (Table 2.2, group 2). Nevertheless, *pb36p*-immunized mice which developed parasitemia have a longer pre-patency period (4.5 days), when compared with naïve matched control (3.0 days).

2.2.3. BALB/c mice immunized with *pb36p*- sporozoites do not require continuous challenges to maintain protection up to 6 months

All the previous immunization experiments were performed with subsequent multiple challenges within the same groups of mice. Therefore, we investigated whether the long-lasting protection observed was due to the constant strengthening of the immune response, elicited by the continuous presentation of antigens during infectious sporozoite challenge. To test whether RAS and *pb36p*-based protective immunity could still be evoked when i.v. challenges were performed after long periods post immunization. Groups of BALB/c mice were immunized i.v. with three doses (50,000/20,000/20,000 weekly apart) of either RAS or *pb36p*- parasites, and only challenged once with 10,000 WT *P. berghei* sporozoites 2 or 3 months (60 or 90 days, respectively) or with 20,000 WT *P. berghei* sporozoites 6 months (180 days) after final immunization (Table 2.3). None of these immunized mice became ever positive for blood stage parasites whereas all control mice for each challenge experiment developed blood-stage parasitemias as expected. These results show that full and long-lasting protection does not require continuous challenges to be maintained up to 6 months in BALB/c mice, when using a prime plus 2 boosts immunization regimen.

Furthermore, immunized mice which have shown to be protected 2 and 3 months after last immunization were re-challenged with 20,000 infectious parasites 6 months (180 days) after last immunization (Table 2.2, groups 3-4). Not surprisingly, these mice were still protected and have never developed blood stage parasites, unlike their matched control. In addition they, as well as those which shown to be protected 6 months after final immunization were challenged again 12 months subsequent to last immunization (Table 2.2, groups 3-5). After one year, protection starts waning, since only 50% of mice which suffer challenges at

2/6/12 months (Table 1, group 3) and at 6/12 months (Table 1, group 5) were still fully protected. Even so, pre-patency periods for immunized mice which became infected (Table 2.3, groups 3 and 5) is longer when compared with naïve control mice (6.0 and 5.0 days, respectively, *versus* 3.0 days for naïve matched control), as observed above for *pb36p*- immunized C57BL6 mice challenged 12 months after last immunization (Table 2.2, group 2).

TABLE 2.3. Protection elicited by *pb36p*- sporozoites does not require subsequent multiple challenges to remain fully protective up to 6 months after last immunization in BALB/c mice

Immunization[a], RAS/*pb36p* - × 1,000	Challenge[b], × 1,000	Challenge at ... days post last immunization	No. protected (no. challenged)		
			Control	RAS	*pb36p*-
50/20/20	10	60	0 (5)	3 (3)	4 (4)
50/20/20	10	90	0 (5)	3 (3)	3 (3)
50/20/20	20	180	0 (5)	4 (4)	4 (4)

[a] Groups of mice were immunized i.v. with one dose of RAS or *pb36p*- sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals

[b] Mice were challenged with WT *P. berghei* sporozoites, isolated from different mosquitoes batches. The pre-patent period was monitored by FACS analysis. All unimmunized mice become positive on day 3 or 4 after challenge.

2.2.4. Lower doses of *pb36p*- sporozoites are still able to confer protection against subsequent challenge

It is known that low doses of *P. berghei* RAS are sufficient to confer protection against malaria in mice. A single dose of 1,000 RAS is enough to protect BALB/c mice against a subsequent challenge with 10,000 WT sporozoites, while multiple doses of 10,000 RAS (but not of 1,000 RAS) are able to protect C57BL6 mice against a similar concurrent challenge with 10,000 sporozoites (Jaffe, 1990).

Thus, we next sought to determine the minimum dose required of these attenuated parasites to protect against challenge with infectious WT sporozoites. C57BL6 mice were divided in groups, and each group was immunized i.v. with three equal doses (with one week interval between them). Doses used were of 10,000, 5,000 or 1,000 sporozoites, either RAS or *pb36p*-. Control mice were immunized i.v.

with a prime of 50,000 and two boosts of 20,000 (weekly apart) of either RAS or *pb36p*- sporozoites. Ten days after last immunization, mice were challenged i.v. with 10,000 WT sporozoites. A group of naïve mice was added at this point. As expected, mice immunized with three doses of 10,000 RAS sporozoites were fully protected against challenge with 10,000 WT sporozoites (Table 2.4). Similarly, mice immunized with three doses of 5,000 RAS sporozoites and challenged also with 10,000 *P. berghei* WT sporozoites, are also entirely protected against disease. Not surprisingly, immunizations using the same doses of *pb36p*- sporozoites (10,000 and 5,000) and challenged likewise were also able to prevent completely the emergence of blood stage parasites (Table 2.4). Matched controls became blood-stage positive 3.2 days post challenge.

TABLE 2.4. Protection elicited by *pb36p*- sporozoites can be achieved even with low immunization doses in C57BL6 mice

Immunization[a], RAS/*pb36p*- × 1,000	Pre-patency (days post infection)[b]		No. protected/no. challenged (%)	
	RAS	*pb36p*-	RAS	*pb36p*-
50/20/20	-	-	5/5 (100%)	4/4 (100%)
10/10/10	-	-	5/5 (100%)	5/5 (100%)
5/5/5	-	-	5/5 (100%)	5/5 (100%)
1/1/1	6.2	-	0/5 (0%)	5/5 (100%)
None (Control)	3.2		0/5 (0%)	

[a] Groups of mice were immunized i.v. with RAS or *pb36p* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. Data from three independent experiments are shown.
[b] Mice were challenged with 10,000 WT *P. berghei* sporozoites, isolated from different mosquitoes batches. Pre-patency is defined as the time until the first appearance of blood-stage parasites, and it was monitored by FACS analysis.

As one could expect, three doses of 1,000 RAS sporozoites were not sufficient to protect mice against a challenge with 10,000 sporozoites. Nevertheless, a delay in pre-patency was observed (6.2 days), when compared to naïve control infected mice (3.2 days), indicating some extent of protection, which is able to reduce intrahepatic parasite burden (although not enough to provide complete immunity). But, most amazing, and unlike occur in RAS immunized mice, three doses of 1,000 *pb36p*- sporozoites confer full protection to mice posterior infected with 10,000 *P. berghei* sporozoites. Low immunization doses of *pb36p*- (1,000 *pb36p*-

sporozoites per dose) are, therefore, required to fully immunize mice against subsequent challenge with infectious *P. berghei* sporozoites.

2.2.5. Different routes of administration of *pb36p*- and RAS sporozoites partially protect against infection

All vaccines used as public health measures are administered using intradermal, subcutaneous or intramuscular routes. Studies are being done to prove that a RAS-based vaccine can be safely administered using one of the conventional routes (Luke, 2003). Previous studies in A/J mice (Kramer, 1975) demonstrated that protection can be achieved administrating RAS sporozoites intramuscularly (but not subcutaneously), being some mice fully protected. To verify if a *pb36p*-mediated vaccine fulfils this requirement, we immunize C57BL6 mice intramuscularly (i.m.), subcutaneously (s.c.) and intradermally (i.d), using a prime plus 2 boots regimen (50,000/20,000/20,000 *P. berghei* RAS or *pb36p*-sporozoites, one week interval between them). Control mice were immunized i.v. using the same regimen. Ten days after last boost, mice were challenged i.v with 10,000 WT *P. berghei* sporozoites. At this time, a group of naïve mice was added. All mice immunized i.v with either RAS or *pb36p*- sporozoites were fully protected against challenge with infectious *P. berghei* sporozoites, as expected (Table 2.5). Some sterile protection was achieved in i.m. immunizations (20% and 13% for RAS and *pb36p*- immunized mice, respectively) and for RAS i.d. immunized mice (20%), as can be observed on Table 2.5. The remaining immunized mice (both RAS and *pb36p*-) which became positive had a strong delay in pre-patency period, ranging from 4.5 to 5.3 days, while naïve mice developed blood-stage parasitemia 3.2 days after challenge (Table 2.5). The delay in pre-patency of immunized mice which became blood-stage positives strongly suggests a partial inhibition of intrahepatic development of parasites (as seen above with mice which were immunized with 1,000 RAS sporozoites), indicating therefore these mice are partially protected against challenge with *P. berghei* infectious parasites.

TABLE 2.5. *pb36p-* sporozoites administered intramuscularly, subcutaneously and intradermally are able to confer protection against intravenous challenge with infectious *P. berghei* sporozoites in C57BL6 mice

Type of immunization[a]	Pre-patency (days post infection)[b]		No. protected/no. challenged (%)	
	RAS	*pb36p-*	RAS	*pb36p-*
intravenous	-	-	5/5 (100%)	4/4 (100%)
intramuscular	4.5	5.3	1/5 (20%)	1/8 (13%)
subcutaneous	4.8	5.0	0/5 (0%)	0/5 (0%)
intradermal	4.5	4.8	1/5 (20%)	0/5 (0%)
None (Control)	3.2		0/5 (0%)	

[a] Groups of mice were immunized as indicated with RAS or *pb36-* sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p-* sporozoites were performed with weekly intervals. Data from two independent experiments are shown.
[b] Mice were challenged with 10,000 WT *P. berghei* sporozoites, isolated from different mosquitoes batches. Pre-patency is defined as the time until the first appearance of blood-stage parasites, and it was monitored by FACS analysis.

None of these routes conferred the same level of sterile protection which can be achieved with intravenous route. However, the challenges with WT parasites were performed intravenously, which does not correspond to the natural route of infection, and therefore not providing practical data about the efficiency of these different routes of immunization with attenuated parasites if applied in the field. Since malaria is naturally transmitted through infected *Anopheles* mosquitoes bite, we decided to repeat *pb36p-* immunizations applying these different routes (intramuscular, subcutaneous and intradermal) as before, and challenging the mice by allowing *P. berghei* infected mosquitoes to feed on them. Consequently, the efficacy of these routes of administration using these genetically-attenuated sporozoites against the natural course of infection *versus* challenge performed intravenously could be assessed. C57BL6 mice were immunized i.v., i.m., s.c. and i.d with *pb36p-* sporozoites (50,000/20,000/20,000, weekly apart) as above. Ten days later, *P. berghei* infected mosquitoes were allowed to feed on these mice, as well as in a control unimmunized group. Mice were checked daily for blood stage parasites up to two weeks post blood feed. As expected, mice immunized i.v. were fully protected, and naïve control mice became all positive. Mice immunized i.m., s.c. and i.d. reached much higher levels of sterile protection (83%, 80% and 75%,

respectively) when compared to mice immunized likewise which suffer i.v. challenge, as one would expected (Table 2.6).

TABLE 2.6. *pb36p*- sporozoites administered intramuscularly, subcutaneously and intradermally are able to confer protection against WT *P. berghei* sporozoites infected mosquitoes bite challenge with in C57BL6 mice

Type of immunization[a]	No. protected/no. challenged[b] (%)
intravenous	4/4 (100%)
intramuscular	5/6 (83%)
subcutaneous	4/5 (80%)
intradermal	3/4 (75%)
None (Control)	0/4 (0%)

[a] Groups of mice were immunized as indicated with *pb36p*- sporozoites isolated from different mosquitoes batches. Multiple immunizations were performed with weekly intervals. Data from two independent experiments are shown.
[b] Six highly infected mosquitoes (around 50,000-80,000 sporozoites per salivary gland, as confirmed posterior) were allowed to feed on mice. Sterile protection is defined as the complete absence of blood-stage parasites, and it was monitored by FACS analysis.

2.2.6. *pb36p*- and RAS confer partial protection against challenge with *P. yoelii* sporozoites

Until now, our knowledge regarding cross-species protection with attenuated sporozoites relies on a preliminary study, where one volunteer was immunized with *P. falciparum* RAS infected mosquitoes and subsequently infected by mosquitoes harbouring infectious *P. vivax* sporozoites (Hoffman, 2002). The volunteer eventually developed blood-stage parasites, meaning, in this particular episode, *P. falciparum* RAS administered through mosquitoes bite were unable to confer sterile protection against another *Plasmodium* species, *P. vivax*.

We tested whether cross-species protection could be conferred by *P. berghei pb36p*- sporozoites, by immunizing mice i.v. with these attenuated parasites and challenging them with another rodent *Plasmodium* species, *P. yoelii*. BALB/c mice were divided in different groups, according to immunization protocol used: single immunization dose of 50,000 *pb36p*- sporozoites, prime-boost regimen (50,000/20,000 *pb36p*- sporozoites, weekly apart), and full immunization regimen (50,000/20,000/20,000 *pb36p*- sporozoites, one week interval between them).

Cross-species protection was also assessed for RAS immunizations (prime-boost and prime-2 boosts regimens). As seen above, a single dose of 50,000 RAS or *pb36p*- sporozoites is able to confer sterile protection against a homologous challenge with 10,000 *P. berghei* sporozoites in BALB/c mice, so we chose this dose as a start point for cross-species protection experiments. Additional boosts (up to two) were also performed, in order to confirm if protection against *P. yoelii* requires multiple immunizations. Three weeks after last boost, mice were challenged with 100, 1,000 or 10,000 *P. yoelii* 265 BY sporozoites. *P. yoelii* is known to be 2,000× more infective in BALB/c mice than *P. berghei* (Khan, 1991), so we also used lower number of *P. yoelii* sporozoites for challenge. Naïve controls were challenged with the same number of *P. yoelii* sporozoites. Forty hours after infection, half of the animals of groups which received a single immunization and prime-boost regimen (n=3) was sacrificed and liver collected for assess the parasite burden by RT-PCR (Fig. 2.1). Prime-boost immunization with RAS reduces significantly parasite burden in liver compared with naïve controls, achieving levels of inhibition of 99.9% ($P<0.001$) and 94.8% ($P=0.042$), against challenges of 100 and 1,000 *P. yoelii*, in that order (Fig. 2.1A and 2.1B, respectively). Mice which suffered only a single immunization with *pb36p*- sporozoites and challenged with 100 and 1,000 *P. yoelii* sporozoites, parasite burden suffered a reduction of 58.5% and 51.1%, correspondingly, (Fig. 2.1A and 2.1B, respectively), although not statistically significant ($P>0.05$). However, mice which suffered an extra boost of *pb36p*- sporozoites, like in RAS immunized mice, had a significant higher level of inhibition of *P. yoelii* intrahepatic development, reaching 94.6% ($P=0.001$) and 88.6% ($P=0.049$) *versus* challenge with 100 and 1,000 *P. yoelii*, correspondingly (Fig. 2.1A and 2.1B, respectively).

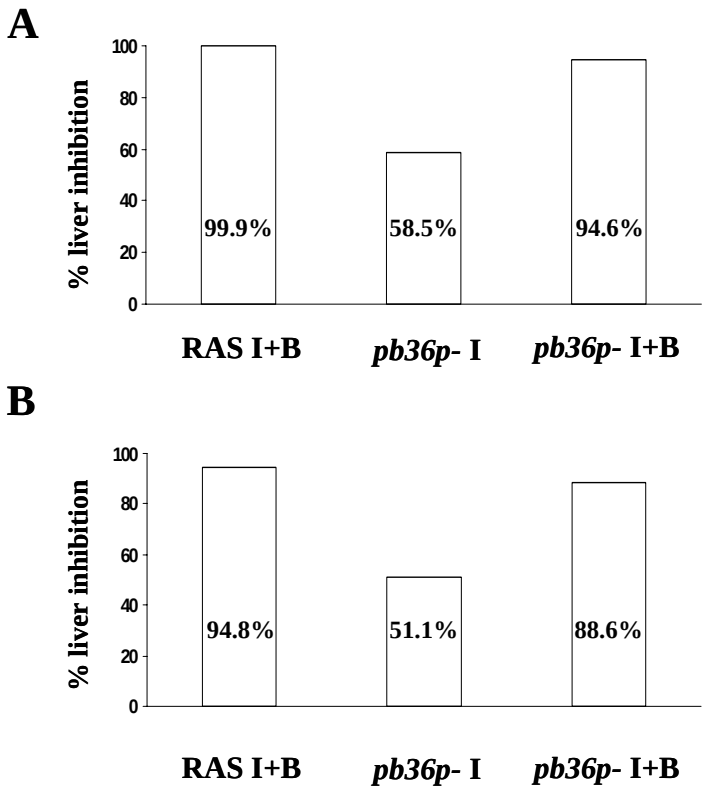

Figure 2.1. Level of inhibition of infection with *P. yoelii* 265 BY sporozoites in mice immunized with *P. berghei* RAS and *pb36p*- sporozoites. BALB/c mice were immunized with RAS sporozoites (prime plus boost) or with *pb36p*- sporozoites (single immunization and prime plus boost) and challenged 3 weeks after last immunization with 100 (A) or 1,000 (B) *P. yoelii* sporozoites. Livers were collected 40 hours post challenge and parasite burden was assessed by Real Time PCR. Control mice have no inhibition in infection.

Blood-stage parasitemia were evaluated daily up to 2 weeks post infection, for all immunization regimens tried in this mouse strain. In mice challenged with 100 *P. yoelii* sporozoites, control naïve infected mice had a maximum parasitemia average of 7.57% ± 0.75 and a pre-patency period of 5.2 days (Fig. 2.2 and Table 2.7, groups 1-3). Mice which suffered a single immunization with *pb36p*- sporozoites were all blood-stage positive on day 5.0, reaching a maximum parasitemia of 5.47% ± 3.67, not presenting a significant decrease when compared with non-immunized controls (Fig. 2.2A and Table 2.7, group 1). On other hand, mice which received an extra boost of *pb36p*- sporozoites only reached a maximum parasitemia of 1.30% ± 2.08 (Fig. 2.2A), showing an significant decrease in blood-stage parasitemia (P=0.019). Furthermore, one out of three of these mice failed to develop blood-stage parasitemia, being fully protected against a challenge with *P. yoelii* sporozoites. The remaining mice immunized this way

had a delay in pre-patency period (7.0 days), when compared to naïve control (Table 2.7, group 2). But most remarkable, all mice immunized likewise with RAS presented sterile protection against *P. yoelii* challenge (Fig. 2.2B and Table 2.7, group 2). These mice never developed parasitemia. BALB/c mice *pb36p*- immunized using a prime-2 boosts regimen did not present a delay on blood stage parasitemia (Table 2.7, group 3) when compared to naïve counterpart (5.0 days), although a significant marked inhibition in maximum parasitemia (Fig. 2.2A) is also observed (2.72% ± 0.87; P=0.047). In RAS immunized mice with a similar regimen, 75% of sterile protection was attained, and the only mouse which became positive had a pre-patency period of 6.0 days (Table 2.7, group 3). In addition, maximum parasitemia reached for this group was 0.88% ± 1.75 (Fig. 2.2B), which is significantly lower (P=0.008), when compared to naïve control mice.

Concerning challenge with 1,000 *P. yoelii* sporozoites, blood-stage parasites were already observed on day 4.0 post infection for both naïve (Table 2.7, groups 4-6) and *pb36p*- single-immunized mice (Table 2.7, group 4). In naïve mice, parasitemia reached a maximum of 12.17% ± 1.87 (Fig. 2). *pb36p*- single-immunized mice present an decrease in maximum parasitemia, only reaching 6.00% ± 2.01 (Fig. 2.2A), although not significant (P>0.05). In *pb36p*- prime-boost immunized mice, this value reaches 5.60% ± 1.45 (Fig. 2.2A), presenting a significant decrease (P=0.035), as well as a higher pre-patency period (4.7 days), when compared with naïve control mice (Table 2.7, group 5). RAS correspondingly immunized mice had a similar delay (5.5 days) on blood-stage parasites arise (Table 2.7, group 5). Maximum parasitemia achieved was 2.07% ± 1.79 (Fig. 2.2B), significantly lower than matched unimmunized controls (P=0.004). Furthermore, one out of the three RAS immunized mice failed to develop blood-stage parasites, clearly indicating some sterile protection are still achieved with this immunization method when a 10× higher challenge dose of *P. yoelii* is used. Moreover, *pb36p*-and RAS prime-2 boosts immunizations also led to higher pre-patency periods, 5.2 and 7.0 days, respectively, when compared to naïve control (Table 2.7, group 6). Maximum parasitemia value achieved was also significantly lower for both types of attenuated parasites. *pb36p*- immunized mice reached 2.22% ± 1.24 (P=0.02), as can be observed in Fig. 2.2A. Like in RAS prime-2 boosts immunized which suffered a challenge with 100 *P. yoelii*, RAS

likewise immunized mice reached 75% of sterile protection against a challenge of 1,000 *P. yoelii*, and the maximum parasitemia average was 0.28% ± 0.55 (*P*=0.003) (Fig. 2.2B).

In mice challenged with 10,000 *P. yoelii* sporozoites, control mice have a pre-patency period of 4.0 days (Table 2.7, group 8) and a maximum parasitemia of 14.38% ± 4.60 (Fig. 2.2). Not surprisingly, *pb36p*- and RAS prime-2 boosts immunized mice have longer pre-patency periods of 4.6 and 5 days, respectively (Table 2.7, group 8), as well as significant lower values for maximum parasitemia, only achieving 2.32% ± 0.52 (*P*=0.01) and 1.30% ± 0.34 (*P*=0.008), respectively.

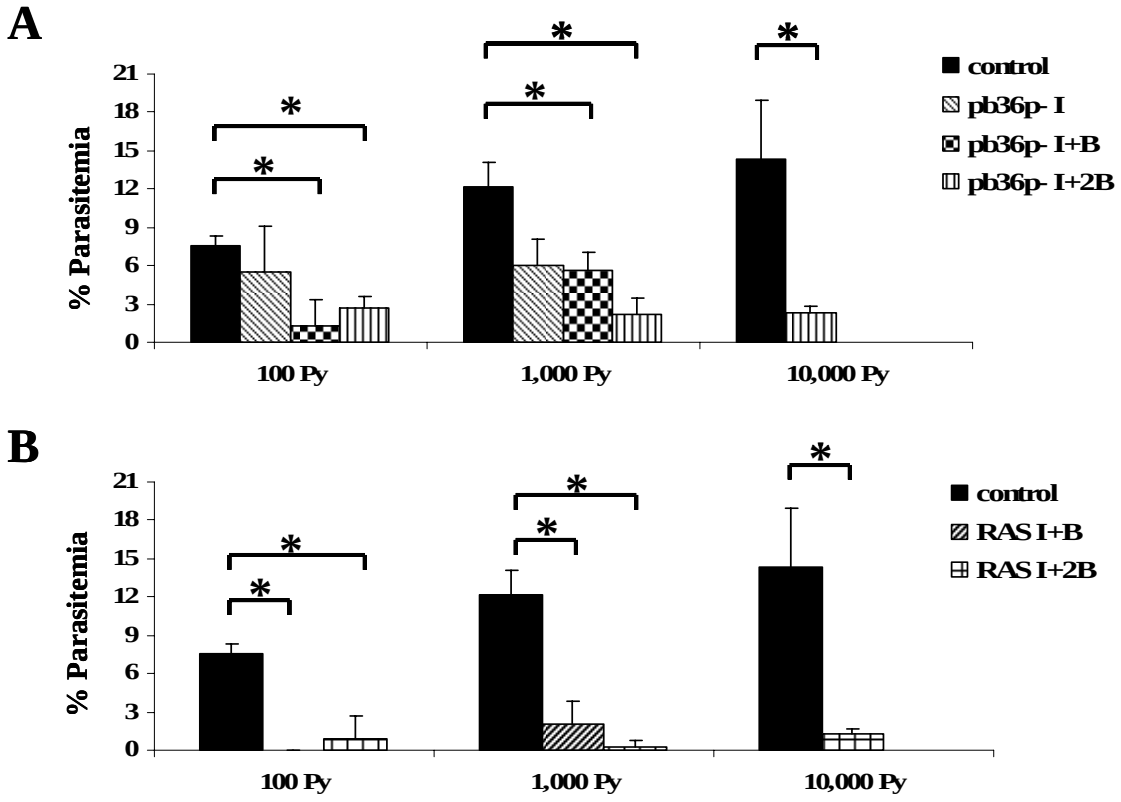

Figure 2.2. Maximum blood-stage parasitemias levels achieved in *P. berghei p36p*- and RAS immunized BALB/c mice which were subsequently challenged with *P. yoelii* 265 BY sporozoites. BALB/c mice were immunized with *pb36p*- sporozoites (single immunization, prime plus boost and prime plus 2 boosts) and with RAS sporozoites (prime plus boost and prime plus 2 boosts) and challenged 3 weeks after last immunization with 100, 1,000 or 10,000 *P. yoelii* sporozoites (only prime plus 2 boosts for the later). *pb36p*- (A) is able to decrease maximum blood-stage parasitemia after challenge with different doses of *P. yoelii* sporozoites in BALB/c mice, although at least two immunization doses are required to achieve significant reduction. RAS (B) equally induce diminution on maximum parasitemias observed in the same conditions, even achieving sterile protection in some cases. Parasitemias are expressed as the mean ± the standard deviation of individual animals. Data from two independent experiments are shown. * $P<0.05$.

Partial protection against challenge with *P. yoelii* is also observed in C57BL6 mice immunized with either *P. berghei* RAS or *pb36p-* sporozoites. C57BL6 mice were immunized according to a regimen of prime plus two boosts with either *P. berghei* RAS or *pb36p-* sporozoites (50,000/20,000/20,000), weekly apart. Unlikely BALB/c, C57BL6 mice require this regimen, as mentioned previously, to become fully protected against a challenge with WT *P. berghei* sporozoites. As so, we immunize mice according to this routine, to assess its degree of protection against challenge with the heterologous species *P. yoelii*. No additional boots were performed, since that treatment would be too fierce to mice. 3 weeks later, mice were challenged i.v. with 1,000 *P. yoelii* sporozoites. Naïve unimmunized mice were challenged likewise. As in BALB/c mice, blood-stage parasites were verified daily from day 3 to day 14 post infection. Naïve control mice were all blood-stage positive already at day 4.0 post infection (Table 2.7, group 7) and reached a maximum parasitemia of 14.60% ± 3.36 (Fig. 2.3). RAS and *pb36p-* immunized mice, as expected, had longer pre-patency periods (6.3 and 5.3 days, respectively) and reaching significant lower values for maximum parasitemia observed (5.1% ± 1.16, P=0.038 and 4.5 ± 1.14, P=0.033, correspondingly), when compared to naïve control mice (Fig. 2.3).

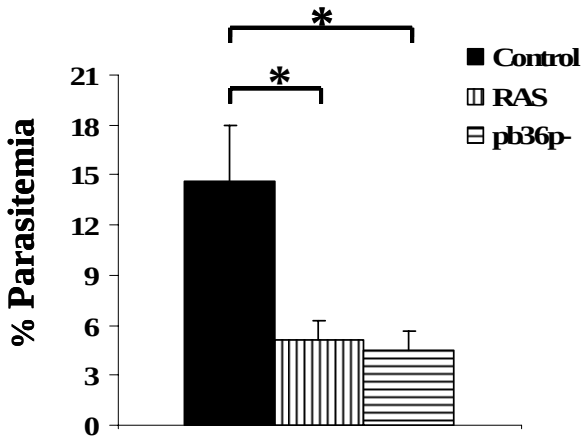

Figure 2.3. Maximum blood-stage parasitemias levels achieved in *P. berghei* RAS and *pb36p-* immunized C57BL6 mice which were subsequently challenged with *P. yoelii* 265 BY sporozoites. C57BL6 mice were immunized with either RAS or *pb36p-* sporozoites (prime plus 2 boosts) and challenged 3 weeks after last immunization with 1,000 *P. yoelii* sporozoites. Both RAS and *pb36p-* are able to decrease maximum blood-stage parasitemia when challenged with *P. yoelii* sporozoites, attaining significant reduction. Parasitemias are expressed as the mean ± the standard deviation of individual animals. Data from two independent experiments are shown. * P<0.05

Our preliminary observations show that *pb36p*- mediated cross-species protection is also observed against another (lethal) strain of this parasite, *P. yoelii* 17XL. BALB/c mice were immunized with a single dose of 50,000 *pb36p*- sporozoites. Three weeks later, these mice were challenged with 10,000 *P. yoelii* 17XL sporozoites. Naïve control mice were infected likewise. Blood-stage parasitemia were assessed from days 3 to 11 days post infection. Control mice have a pre-patency period of 3.0 days (Table 2.7, group 9) and a maximum parasitemia of 57.11% ± 10.23 (Fig. 2.4). Not surprisingly, *pb36p*- immunized mice have lower pre-patency period of 4.6 days (Table 2.7, group 9), and present a decrease in maximum parasitemia achieved, only reaching 36.50% ± 10.14 (Fig. 2.4). However, this reduction in parasitemia is not statistically significant.

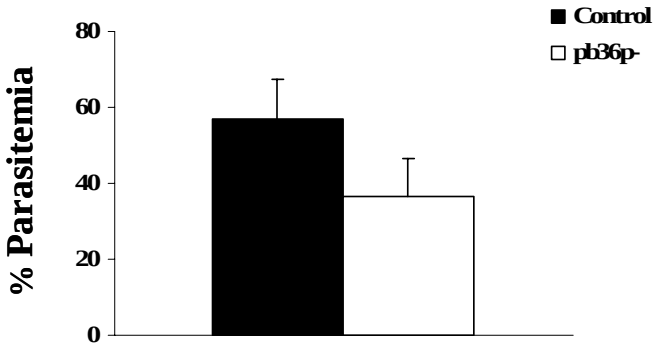

Figure 2.4. Maximum blood-stage parasitemias levels achieved in *pb36p*- immunized BALB/c mice which were subsequently challenged with *P. yoelii* 17XL sporozoites. BALB/c mice were immunized with *pb36p*- sporozoites (single immunization) and challenged 3 weeks after last immunization with 10,000 *P. yoelii* 17XL sporozoites. *pb36p*- are able to decrease maximum blood-stage parasitemia when challenged with a lethal strain of *P. yoelii* sporozoites, although not significantly. Parasitemias are expressed as the mean ± the standard deviation of individual animals.

Consequently, immunization with these both types of *P. berghei* live-attenuated parasites can confer protection against challenge with another rodent *Plasmodium* species, *P. yoelii*. Both delays in pre-patency periods and maximum parasitemia observed indicate a partial inhibition in *P. yoelii* liver burden, as clearly confirmed by RT-PCR (Fig. 2.1), strong enough in some cases to even induce sterile protection against this heterologous-species challenge.

Additionally, we intended to assess the level of *pb36p*- cross-species protection against another rodent heterologous species, *P. chabaudi chabaudi* AS and *P.*

vinckei petteri, but we did not obtained an adequate amount of sporozoites to perform this kind of experiment.

TABLE 2.7. Immunization with *P. berghei* RAS and *pb36p*- sporozoites lead to a delay on parasitemia onset in BALB/c and C57BL6 mice challenged with the heterologous species *P. yoelii*, and some even achieve sterile protection.

Groups	Mouse Strain	Immunization × 1,000[a]	Challenge Dose (*P. yoelii* sporozoites)[b]	Pre-patency (days)			No. protected (no. challenged)		
				pb36p-	RAS	Control	*pb36p*-	RAS	Control
1	BALB/c	50	100	5.0	ND	5.2	0 (3)	ND	0 (3)
2	BALB/c	50/20	100	7.0	-	5.2	1 (3)	3 (3)	0 (3)
3	BALB/c	50/20/20	100	5.0	6.0	5.2	0 (5)	3 (4)	0 (5)
4	BALB/c	50	1,000	4.0	ND	4.0	0 (3)	ND	0 (3)
5	BALB/c	50/20	1,000	4.7	5.5	4.0	0 (3)	1 (3)	0 (3)
6	BALB/c	50/20/20	1,000	5.2	7.0	4.0	0 (5)	3 (4)	0 (5)
7	C57BL6	50/20/20	1,000	4.8	5.5	4.0	0 (5)	0 (5)	0 (5)
8	BALB/c	50/20/20	10,000	4.6	5.0	4.0	0 (5)	0 (4)	0 (5)
9	BALB/c	50	10,000	4.6	ND	3.0	0 (6)	ND	0 (5)

[a] Groups of mice were immunized as described before with *P. berghei* RAS or *pb36p*- sporozoites. ND, not determined.
[b] Mice were challenged with *P. yoelii* 265 BY sporozoites, a non lethal strain (groups 1-8). Data from two independent experiments are shown for these groups. Group 9 was challenged with *P. yoelii* lethal strain 17XL sporozoites. This experiment was only performed once.

For all experiments described above, control mice groups (both BALB/c and C57BL6, 3-5 animals per group) immunized likewise with either RAS or *pb36p*- sporozoites from the same mosquitoes' batch and challenged with 10,000 homologous *P. berghei* sporozoites each fail to develop blood stage parasites, confirming that these sporozoites were fully protective in homologous challenge in these conditions. All control naïve mice (3-5 animals per group) challenged equally with WT *P. berghei* sporozoites developed blood stage parasitemia 3-4 days later, as expected.

2.3. Conclusions

The lack of significant progress with (recombinant) subunit vaccines that contain only (parts of) single proteins of sporozoites has led to renewed interest in vaccines based on live, attenuated whole sporozoites (Luke, 2003). Recently, it has been shown that GAS, like RAS, produced by engineered inactivation of sporozoite-specific genes can induce protective immunity in the *P. berghei* rodent

model of malaria (Mueller, 2005; Mueller and Camargo, 2005; van Dijk, 2005). Specifically, our studies demonstrate that a GAS produced through the inactivation of the sporozoite-specific protein P36p induces protective immunity. P36p is a member of the P48/45 family of surface proteins (Thompson, 2001) that include a number of promising vaccine candidate antigens, such as P48/45 and P230 that are expressed on the surface of gametes (Thompson, 2001; van Dijk, 2001). P36p has no function in sporozoite motility and invasion of both salivary glands and hepatocytes (Ishino, 2005B; van Dijk, 2005), and instead plays an essential role during development of the liver trophozoite (van Dijk, 2005). *pb36p*- parasites apparently fail to maintain a parasitophorous vacuole (PV), arresting early during intrahepatocytic development (van Dijk, 2005). Importantly, immunization with *pb36p*- sporozoites induces a fully protective immune response against subsequent challenge with WT sporozoites. Interestingly, as has been shown for RAS (Chatterjee, 2001), only a single immunizing dose of *pb36p*- sporozoites is required to elicit a protective immune response in BALB/c mice. As in the immunization studies performed with *uis3*- and *uis4*- GAS (Mueller, 2005; Mueller and Camargo, 2005) and RAS (Chatterjee, 2001), three immunizing doses of *pb36p*- sporozoites are required to completely protect C57BL6 mice. This protective immune response, as shown before for RAS, *uis3*- and *uis4*- (Mueller, 2005; Mueller and Camargo, 2005; Clyde, 1975), is parasite stage specific, not preventing infection when mice are challenged with infected erythrocytes.

Intriguingly, *pb36p*- sporozoites, like *uis4*- GAS (Mueller and Camargo, 2005), albeit at a lower frequency, initiated a delayed blood stage infection in some mice. The reasons for this are unclear and are currently under investigation (Milly van Dijk, personal communication).

The protection elicited by *pb36p*- sporozoites may last up to 12 and 18 months in C57BL6 and BALB/c, respectively. The length of the protection induced by these parasites is quite outstanding. Mice live in average 18 months and can reach 3 years-old (Fox, 1984). Thus, experiments were stopped due to the low number of mice that reached the last time points. On the later time points, protection conferred by RAS was always stronger than by *pb36p*- sporozoites. The prominent differences observed above between BALB/c and C57BL6 regarding immunization with RAS and *pb36p*- clearly demonstrate that the choice of the

host strain in studying the mechanisms of protective immunity induced by attenuated sporozoites is critical, as has been shown by earlier studies (Chatterjee, 2001).

Similarly to RAS-induced protection, lower doses than those currently tested of *pb36p*- sporozoites are sufficient to confer sterile protection. Immunization of C57BL6 mice with 3 doses of 10,000 or 5,000 *pb36p*- sporozoites or RAS fully protects mice against challenge with 10,000 sporozoites. However, while 3 doses of 1,000 *pb36p*- sporozoites are able to confer fully protection to challenge with 10,000 sporozoites, similar RAS immunizations only confer partial protection, confirming previous reports for RAS (Jaffe, 1990). This suggests that lower doses of *pb36p*- sporozoites are required than those required for RAS sporozoites, to induce the same level of protection. Moreover, RAS immunized mice present a higher pre-patency period when compared with naïve controls (6.2 days vs. 3.2 days, correspondingly), which lead to think that even low doses as 1,000 RAS sporozoites are sufficient to induce a partial inhibition on parasites intrahepatic development (most probably, induced by apoptosis, although that remains to be elucidated), but unable to enough to confer sterile protection, as clearly demonstrated.

All vaccines used as public health measures are administered intramuscularly, subcutaneously or intradermally (Luke, 2003). A vaccine based on live attenuated sporozoites should also accomplish this requirement. Our results, as well as other studies for RAS (Kramer, 1975), clearly demonstrated that protection can be achieved administrating RAS and *pb36p*- sporozoites intramuscularly, being some mice fully protected. Protection is also achieved using subcutaneous route in both types of attenuated parasites, as clearly demonstrated by delay in patency period. This is not been observed in previous studies (Kramer, 1975), but different mouse strains and immunization regimens were used, which could explain these differences. Another GAS, *P. berghei uis3*-, contrastingly, when administered subcutaneously, confer full sterile protection against intravenous challenge with WT *P. berghei* sporozoites (Mueller, 2005), which leads to think its protection mechanisms differ greatly from those induced by RAS or *pb36p*- itself. To our knowledge, no studies regarding intradermal immunization with live attenuated *Plasmodium* sporozoites were described previously. Our results show that both RAS and *pb36p*- sporozoite confer some level of protection when administered by

this route, with only a small proportion of RAS immunized mice being fully protected.

Mice immunized with *pb36p*- sporozoites through all these routes followed by mosquito bite challenge (the natural course of infection) have a higher level of sterile protection, around 80%, which supports the potential as an experimental vaccine against malaria of this particular GAS. Unfortunately, none of these routes confer the same level of sterile protection as immunizations performed intravenously, which is undoubtedly desirable. Although at the moment these routes do not lead to complete protection, the results are quite encouraging and promising, strengthening the potential of a human live attenuated parasites-based vaccine. Optimization of the conditions used (e.g., higher immunization doses) may increase (or even lead to full) protection, which ought to be further investigated.

Our results show for the first time the potential of attenuated malaria sporozoites to induce cross-species protection. We clearly demonstrated that immunization with *P. berghei* RAS and *pb36p*- sporozoites is able to partially inhibit the development of *P. yoelii* in both BALB/c and C57BL6 mice. Immunization with either RAS or *pb36p*- sporozoites lead to a significant decrease both in *P. yoelii* liver burden and of values of maximum blood-stage parasitemia, achieving, in some cases, sterile protection. However, significant levels of partial cross-species protection are only attained with, at least, one extra boost. These results confirm the potential of live-attenuated parasites as a multi-species vaccine against Malaria.

Here, we show the immunization potential of *P. berghei p36p*- as an experimental vaccine against Malaria, able to induce long-lasting immunity and cross-species protection against *P. yoelii*. Furthermore, protection with this GAS can be achieved even with low immunization doses and using practicable routes of immunization commonly used as public health measures in humans.

Chapter III

Mechanisms of protection induced by *pb36p*-

3.1. Introduction

In the previous chapter, we have described the potential of *pb36p-* sporozoites as an experimental vaccine against the preerythrocytic stage of Malaria. To make it more accurate, whenever possible, we compared it with the golden standard for Malaria vaccine, Radiation-Attenuated Sporozoites (RAS). For decades, RAS induced immunity has been widely studied and is now known to be mediated by complex mechanisms involving antibody responses that inhibit sporozoite motility and host cell invasion and T cell responses directed against intrahepatocytic stages. Cytotoxic $CD8^+$ T and $CD4^+$ T helper cells lymphocytes recognizing Major Histocompatibility Complex I and II (MHC I and II), respectively, presented parasite-derived peptides, as well as cytokines (IL-2, IFN-γ, and IL-12; TNF- α, IL-1, and IL-6) and nitric oxide, have been shown to be critical effectors in protection against preerythrocytic malaria (reviewed in Morrot, 2004 and Tsuji, 2003; Doolan, 1999). γδ T cells, although a component of early immunity against preerythrocytic malaria parasites, are not required for RAS mediated protection (McKenna, 2000). Moreover, RAS induced long-lasting protection is characterized by the establishment of a specific subset of memory $CD8^+$ T cells in the liver (but not of $CD4^+$ T cells), $CD44^{high}CD45RB^{low}CD62L^{low}CD122^{low/high}$ (Berenzon, 2003; Guebre-Xabier, 1999). No increase in splenic memory T cells is observed in RAS long lasting immunizations in C57BL6 mice (Guebre-Xabier, 1999). B cells are known to be important in the establishment of immune protective mechanisms against liver stage malaria, since antibodies are paramount in conferring sterile protection against challenge with sporozoites (Schofield, 1987). However, to the extent of our knowledge, nothing no studies have been done regarding the role of memory B cells in immunity against malaria liver stage. Although some authors suggest that memory B cells might be involved in liver protection using other immunization strategies, such as adenovirus and modified vaccinia virus (Bruña-Romero, 2001), that remains to be elucidated in RAS mediated immunity. Another mechanism which is most likely to be involved in RAS mediated protection is apoptosis of infected host cells. Recently, it has been shown that both *P. berghei* sporozoites are able to prevent apoptosis of the hepatocytes they infect, ensuring so their survival during intrahepatic development (Leirião, 2005; van de Sand, 2005). Furthermore, *P. yoelii* RAS,

which are unable to inhibit apoptosis pathways at the same level than non irradiated sporozoites, have be identified as a source of antigens for antigen presenting cells (APC), such as dendritic cells and macrophages (Leirião and Mota, 2005), initiating a protective immune response.

As stated before, RAS mediated protection presents some concerning disadvantages. The use of GAS for vaccination might remove the uncertainty associated with RAS once a more thorough understanding of the mechanisms of immunity invoked by GAS and their developmental defect(s) are available. In this chapter, we describe an initial study of potential mechanisms which could lead to *pb36p-* induced immunity. Two main features were studied: apoptosis and immune factors required for mediate/maintain protection. We found evidence that *pb36p-* sporozoites have a much higher rate of apoptosis in HepG2 cells, when compared to RAS and WT sporozoites. *pb36p-* parasitized cell numbers are also progressively reduced *in vivo* and apparently fail to prevent host cell apoptosis, consequently leading to parasite clearance.

Using deficient mice for either IFN-γ and TCRδ (T cell receptor δ), we assessed the requirements of these two factors involved in immune response against pathogens in *pb36p-* mediated protection. Like in RAS induced immunity, *pb36p-* requires IFN-γ to elicit an immune protective response. However, most surprisingly and, unlike observed in RAS protection (McKenna, 2000), *pb36p-* protective immunity is also dependent of γδ T cells. Moreover, *pb36p-* immunized BALB/c mice have an increase in intrahepatic memory $CD8^+$ T cells ($CD44^{high}CD62L^{low}$), but not of memory $CD4^+$ T cells, 10 days after immunization, which eventually fades at 1 month and being scarcely detected 6 months later. Splenic memory $CD8^+$ T cells suffer similar increase, although at a lower rate. Contrastingly to what happens in RAS long lasting protection, *pb36p-* immunized BALB/c mice have a huge increase in splenic memory $CD4^+$ T cells ($CD44^{high}CD62L^{low}$) 1 month after immunization. TO CONTINUED W/ 6 MTHS DATA…Memory B cells are not increased in liver, spleen or lymph nodes following *pb36p-* immunization up to 6 months later.

Our results, although preliminary, clearly demonstrate that *pb36p-* and RAS mediated protection have some prominent differences which need to be dissected in detail, so a vaccine based on this GAS may be accomplished.

3.2. Results

3.2.1. *pb36p-* sporozoite infected Hepatocytes enter Apoptosis more frequently than RAS or WT parasite-infected Hepatocytes

Host hepatocyte apoptosis is normally inhibited by WT parasites upon invasion and establishment of the PV (Leirião, 2005; van de Sand, 2005). Therefore, it was possible that the observed rapid disappearance of *pb36p-* sporozoite-infected HepG2 cells in culture was due to the inability of the parasite to prevent the hepatocyte from undergoing apoptosis. Thus, the level of apoptosis in *pb36p-* sporozoite invaded cells was examined (Fig. 3.1A).

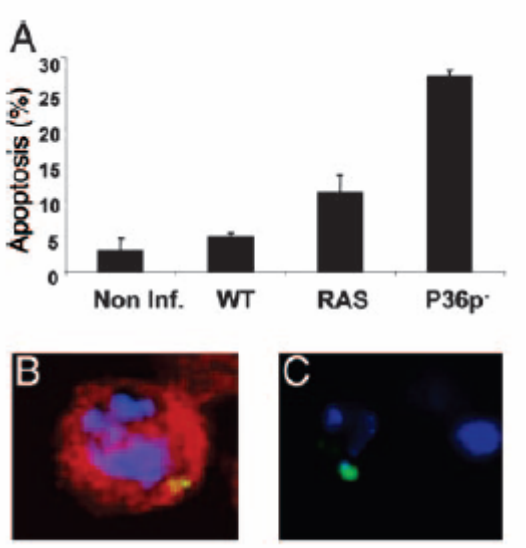

Figure 3.1. Apoptosis is increased in *pb36p-* parasitized liver cells. (A) Apoptosis rates represent the percentage of parasite-invaded cells that undergo apoptosis 6 hours after infection of HepG2 cells with WT, RAS, or *pb36p-* sporozoites. Non. Inf. indicates non infected HepG2 cell cultures. Rates are defined as the mean ± the standard deviation of each well. Data from three independent experiments is shown. (B and C) Visualization of *pb36p-* sporozoite (green, anti-HSP70) infected hepatocytes displaying typical apoptotic signs as detected by DAPI staining (blue) *in vitro* (B) as well as *in vivo* (C). Active caspase-3 (red) detection was also performed in B. (Original magnification, × 1,260) (adapted from van Dijk, 2005).

HepG2 cells were incubated with WT, RAS, or *pb36p-* sporozoites (20,000 sporozoites per well), and apoptosis was determined 6 hours after infection by detection of active caspase-3 (the factor which will ultimately lead to apoptosis) in the infected host cell cytoplasm and by analysis of the nuclear morphology of the

infected cells after DAPI staining by observing chromatin condensation and/or nuclear fragmentation (Fig. 3.1B). Interestingly, we also detected apoptosis of *pb36p-* parasitized hepatocytes *in vivo* 6 hours after infection (Fig. 3.1C). The level of apoptosis in *pb36p-* parasitized cells was significantly higher ($P<0.05$) compared to that in RAS-infected cells, which was higher than the level observed in WT-infected cells (Fig. 3.1A). The different levels of apoptosis are consistent with the observed longer survival time of RAS in culture (Scheller, 1995).

Our results also show that in HepG2 cells during infection with RAS or *pb36p-* parasites, high levels of procaspase-3, the precursor of active caspase-3 are detected (Fig. 3.2).

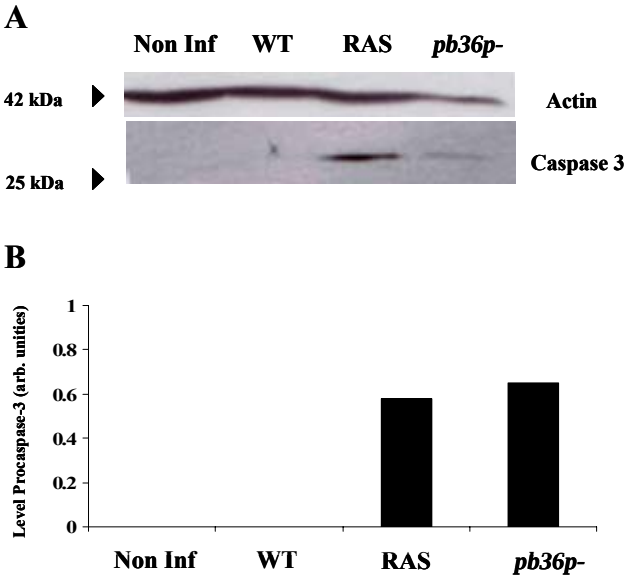

Figure 3.2. High levels of procaspase-3 are detected in HepG2 cells infected with RAS and *pb36p-* sporozoites. A) High levels of procaspase-3 (MW 32 kDa), the precursor of active caspase-3, which will lead to apoptosis, are detected in HepG2 cells infected with RAS or *pb36p-* sporozoites, but not in non infected neither in WT-infected HepG2, after 24 hours in culture, using Western Blot. B) Quantification of Procaspase-3 shows that this apoptotic protein is highly expressed in RAS and *pb36p-* infected HepG2, but not in WT *P. berghei* infected cells. Non Inf indicates non infected HepG2 cell cultures. To assure samples were equally loaded with the same quantities of protein, detection of actin (MW 42 kDa) was performed. The density of each band was determined using ImageJ (National Institute of Health, Bethesda, Maryland, USA). The values were normalized as the ratio of Procaspase-3 density to the Actin density in the corresponding sample. Results are expressed in arbitrary units.

HepG2 cells were infected with either 150,000 WT, RAS or *pb36p-* sporozoites. After 24 hours, cells were collected and quantified. 50,000 cells of each type (including non infected HepG2) were used for detection of caspase-3 by Western Blot. Procaspase-3 (MW 32 kDa) is detected in cells infected by RAS and *pb36p-*

sporozoites, but not in uninfected and WT infected hepatocytes (Fig. 3.2), strengthening the hypothesis that live attenuated parasites are unable to inhibit apoptosis in host cells, as others have shown (Leirião, 2005; van de Sand, 2005).

3.2.2. *pb36p-* sporozoites have a higher Clearance Rate in Livers of infected BALB/c mice

We determined *in vivo* the persistence of WT, RAS and *pb36p-* infected hepatocytes in whole liver extracts of BALB/c mice by using real-time PCR for liver schizont detection. WT parasites have a continuous growing liver burden up to 40 hours post infection, as expected (Fig. 3.3A). RAS parasites show an increasing development up to 18 hours after infection, although at a much slower rate compared to WT, which begun to decrease from that period onwards (Fig. 3.3B). *pb36p-* parasites are hardly detected at 6 hours post infection and even barely at further time points (Fig. 3.3 C). Our results confirm the significantly more rapid clearance of *pb36p-* parasitized hepatocytes within 6 hours after infection compared to RAS-infected hepatocytes as has been observed *in vitro* (P=0.002). However, RAS and *pb36p-* infected hepatocytes are barely detected 1 week after infection (Fig. 3.3B and C).

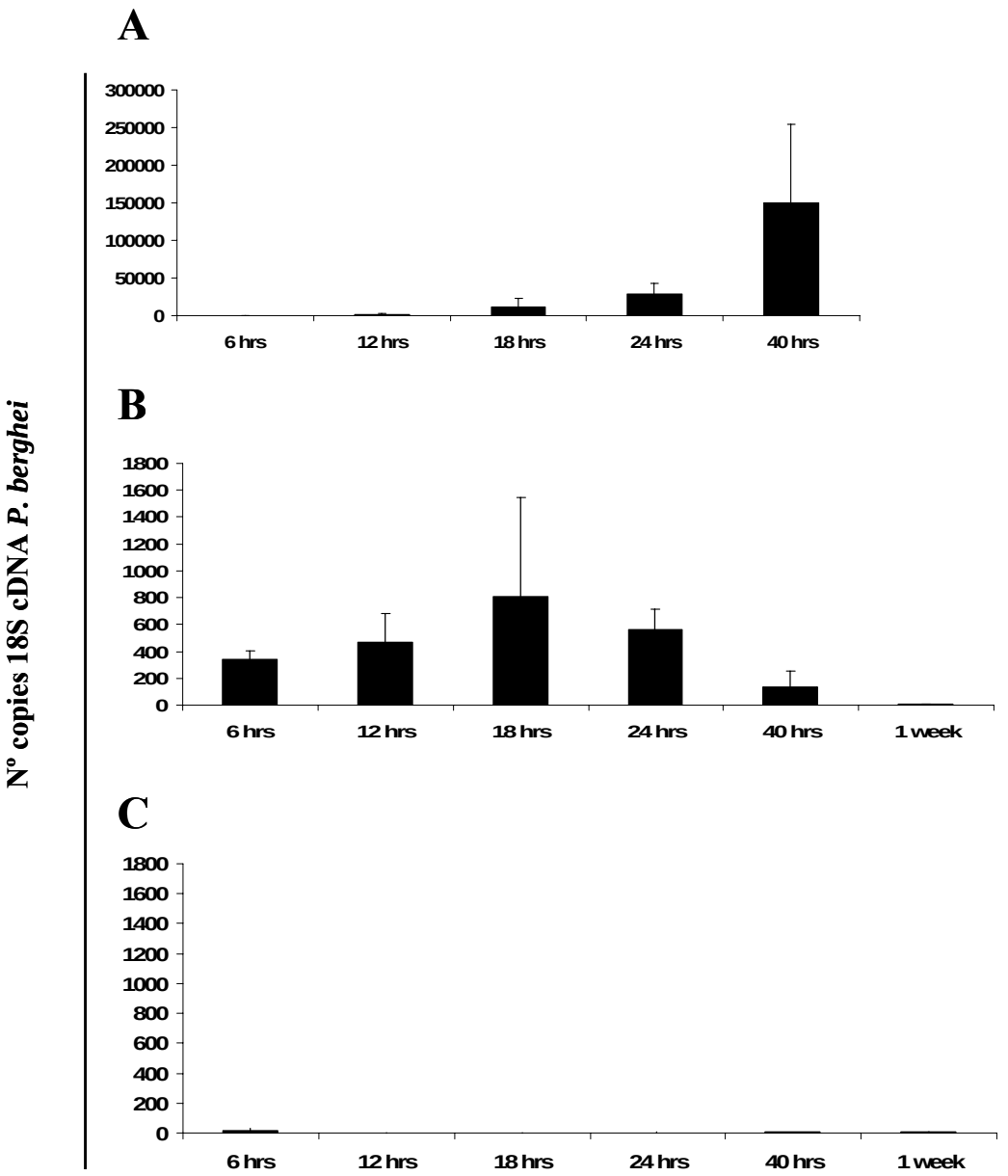

Figure 3.3. Hepatic Persistence of RAS and *pb36p-* sporozoites in BALB/c mice. Liver burden of *P. berghei* was quantified in BALB/c mice infected with (A) WT, (B) RAS and (C) *pb36p-* sporozoites, at several time points. WT parasites continuously grow up to 40 hours. RAS parasites also initiate intrahepatic development up to 18 hours post infection, although decreasing onwards. *pb36p-* parasites are only hardly detected at 6 hours post infection. After 1 week, very low numbers of copies of both attenuated parasites are detected. Number of copies of *P. berghei* 18S cDNA are defined as the mean ± the standard deviation of each animal (n=3 per time point). Data from two independent experiments are shown.

3.2.3. *pb36p-* induced protection is dependent on IFN-γ and γδ T cells

Our previous work has shown that a single immunization with 50,000 *pb36p-* sporozoites led to a reduction of parasite liver burden up to 90%, after challenge with infectious WT *P. berghei* sporozoites in C57BL6 mice (van Dijk, 2005). Using deficient mice for either IFN-γ ($IFNg^{-/-}$) or γδ T cells ($TCR\delta^{-/-}$) in the same background, we confirmed if these two features were required to establish *pb36p-* induced immunity. $IFNg^{-/-}$ and $TCR\delta^{-/-}$ C57BL6 mice (as well as a WT mice group) were immunized once with 50,000 *pb36p-* sporozoites and challenged with 10,000 WT *P. berghei* sporozoites 10 days later. Mice were sacrificed 40 hours after challenge and livers collected and homogenised for quantification of parasite load by real time PCR. As expected, *pb36p-* single immunization reduced significantly liver parasite burden, in 90% ($P<0.001$), when compared to WT parasite load (Fig. 3.4). Not unanticipated, protection induced by this GAS in $IFNg^{-/-}$ is abolished (Fig. 3.4), as in RAS based immunizations (Doolan, 2000). These mice achieve no reduction in parasite liver burden, implicating a complete failure in attaining protection when immunized with *pb36p-* sporozoites. However, and quite unexpected, *pb36p-* induced protection requires also the presence of γδ T cells to became fully effective (Fig. 3.4). Like in $IFNg^{-/-}$ mice, no reduction in parasite load is observed in $TCR\delta^{-/-}$ mice after immunization with *pb36p-*, indicating a similar lack of protection. RAS based immunization does not require this particular subset of T cells to confer full protection in C57BL6 mice (McKenna, 2000), which suggests that the immune mechanisms elicited by these two types of attenuated parasites present major differences. However, previous studies have been done using a different *Plasmodium* species (*P. yoelii*), which may also contribute to the observed differences.

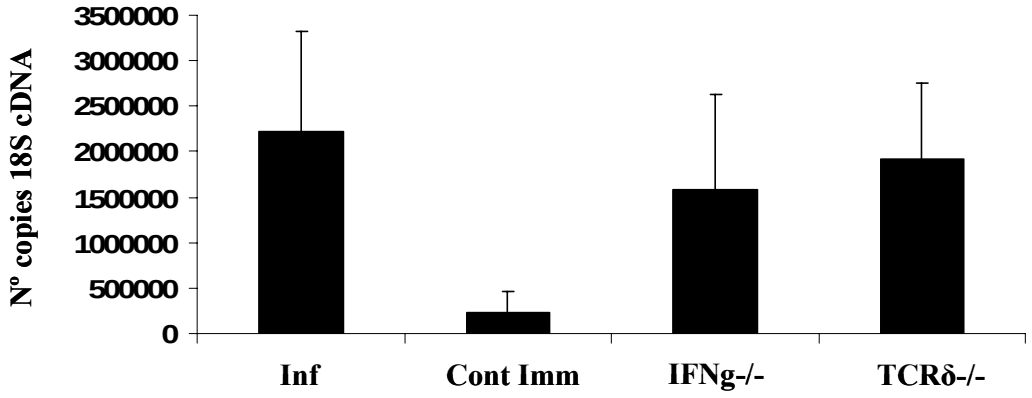

Figure 3.4. Quantification of *P. berghei* liver load in *pb36p*- immunized IFNg$^{-/-}$ and TCRδ$^{-/-}$ mice after challenge with infectious *P. berghei* sporozoites. Liver burden of 50,000 *pb36p*- sporozoites immunized IFNg$^{-/-}$ (n=8) and TCRδ$^{-/-}$ (n=6) C57BL6 mice was quantified 40 hours after a challenge with 10,000 WT *P. berghei* sporozoites. Inf indicates naive infected mice (n=3) and Cont Imm WT immunized mice (n=5). Lack of either IFN-γ or γδ T cells abrogate *pb36p*- induced protective effect on C57BL6 mice. Parasite Loads are defined as the mean ± the standard deviation of each animal.

3.2.4. Memory T and B Cells in *pb36p*- immunized BALB/c mice

3.2.4.1. Increase in Memory T cells is observed in livers and spleens of *pb36p*- immunized BALB/c mice

Memory in RAS immunized C57BL6 mice is associated with the establishment of a specific subset of memory CD8$^+$ T cells (but not of CD4$^+$ T cells) can be found in the liver, CD44^{high}CD45RB^{low}CD62L^{low}CD122$^{low/high}$ (Berenzon, 2003; Guebre-Xabier, 1999). However, no difference was observed between unimmunized and immunized mice regarding splenic memory T cells (Guebre-Xabier, 1999).

Our results show that, concerning *pb36p*- immunizations, BALB/c mice are protected longer than C57BL6 mice (previous chapter), suggesting that immune memory cells might be easily established in that mouse strain. So, we immunize BALB/c mice with 50,000 *pb36p*- sporozoites (single dose) and memory CD8$^+$ and CD4$^+$ T and B cells were quantified at later time points in liver, spleen and lymph nodes of non immunized and immunized mice. Memory T cells were quantified as CD44^{high}CD62L^{low} (Berenzon, 2003; Guebre-Xabier, 1999), using Flow Activated Cell Sorting (FACS).

$CD8^+$ memory cells ($CD44^{high}CD62L^{low}$) have an increase of nearly three-fold in the liver of immunized mice, compared to naïve mice, 10 days after immunization (Fig. 3.5). However, 1 month after immunization, $CD8^+CD44^{high}CD62L^{low}$ cell population declines, being only 50% higher than in naïve mice (Fig. 3.5). DATA FOR 6 MONTHS. Contrastingly, $CD4^+$ liver memory cells present no different phenotype in both unimmunized and immunized mice up to 6 months (Fig. 3.5).

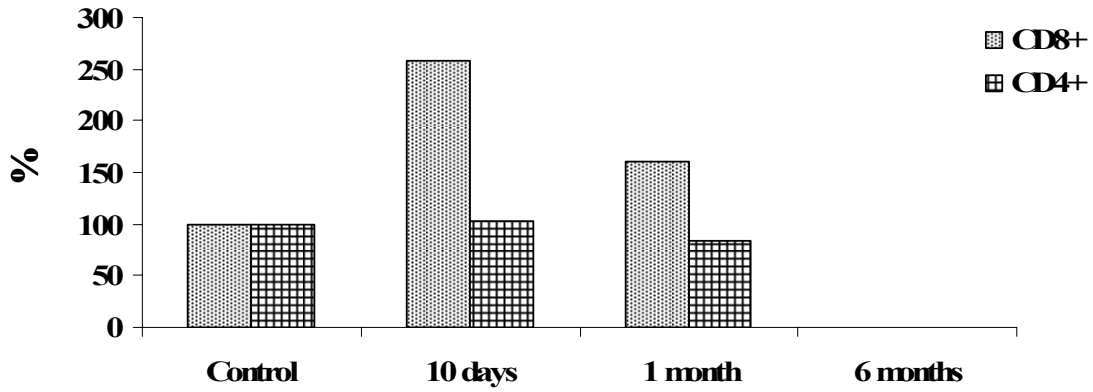

Figure 3.5. Memory $CD8^+$ and $CD4^+$ in livers of naïve and *pb36p-* immunized mice. Memory T cells in livers of 50,000 *pb36p-* sporozoites immunized BALB/c mice (n=3 per group) were assessed 10 days, 1 month and 6 months after immunization. $CD8^+$ memory cells have a huge increase 10 days in immunized mice, eventually declining. $CD4^+$ memory cells do not present major differences between non immunized and immunized mice. Cells were quantified by Fluorescence Activated Cell Sorting (FACS). A pool of cells from the three animals within the same group was analysed. Cell population is defined as percentage, being control (cell populations of naïve mice) stipulated as 100%.

In spleens of *pb36p-* immunized mice, 10 days after immunization, both $CD8^+$ and $CD4^+$ memory cells suffer minor increases (Fig. 3.6), compared to naïve mice. $CD8^+CD44^{high}CD62L^{low}$ cell population remains stable at further time points (1 and 6 months) in immunized mice (Fig. 3.6). However, $CD4^+CD44^{high}CD62L^{low}$ cell population present a huge increase, being 150% superior to what is observed in naïve mice. DATA FOR 6 MONTHS.

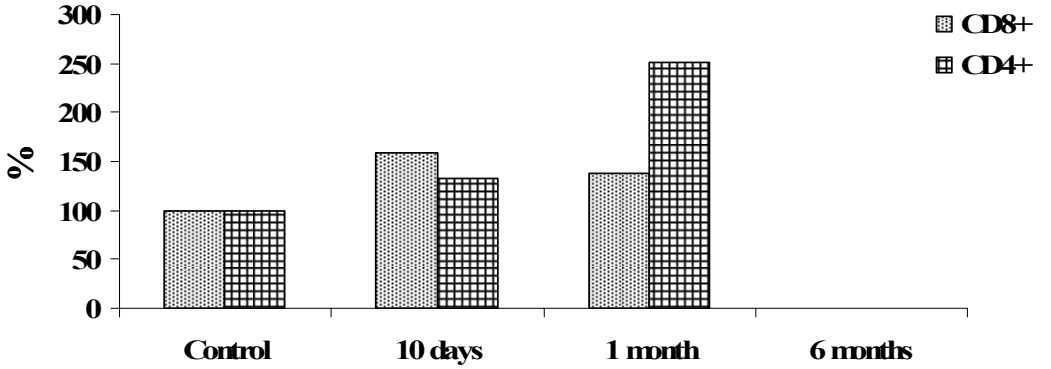

Figure 3.6. Memory CD8$^+$ and CD4$^+$ in spleen of naïve and *pb36p-* immunized mice. Memory T cells in spleens of 50,000 *pb36p-* sporozoites immunized BALB/c mice (n=3 per group) were assessed 10 days, 1 month and 6 months after immunization. CD8$^+$ memory cells present a small increase 10 days in immunized mice, remaining stationary at later time points. CD4$^+$ memory cells do not present major differences between non immunized and immunized mice 10 days later, but a huge increase is observed 1 month after immunization. Cells were quantified by FACS. A pool of cells from the three animals within the same group was analysed. Cell population is defined as percentage, being control (cell populations of naïve mice) stipulated as 100%.

The initial site of induction of memory T cells resident in the liver is unclear. While some defend it is in the liver itself, others claim they are induced on a draining lymph node, migrating to the liver upon sporozoite challenge (reviewed in Krzych, 2005). So, we also assess memory T cells in lymph nodes of non immunized and *pb36p-* immunized mice, 10 days after immunization. No striking differences are observed in memory CD8$^+$ and CD4$^+$ T cells for each condition studied (Fig. 3.7). As so, we decided not to analyse memory populations at later time points in these organs.

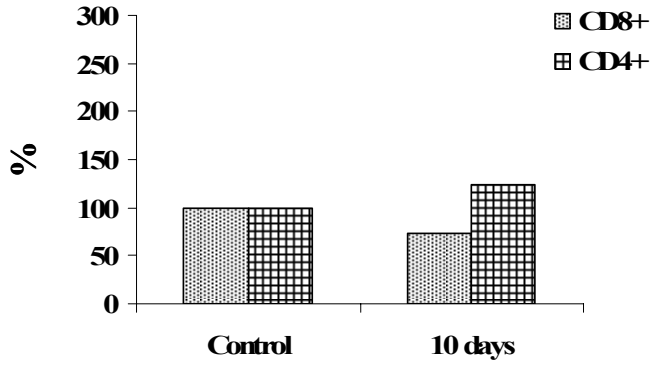

Figure 3.7. Memory CD8$^+$ and CD4$^+$ in lymph nodes of naïve and *pb36p-* immunized mice. Memory T cells in lymph mnodes of 50,000 *pb36p-* sporozoites immunized BALB/c mice (n=3 per group) were assessed 10 days after immunization. No striking differences were observed in both CD8$^+$ and CD4$^+$ memory cells between non immunized and immunized mice. Cells were quantified by FACS. A pool of cells from the three animals within the same group was analysed. Cell population is defined as percentage, being control (cell population of counter parties' naïve mice) stipulated as 100%.

3.2.4.2. Memory B cells do not suffer an increase following *pb36p*-immunization in BALB/c mice

Memory B cells have scarcely been studied in immunization regarding liver stage of malaria. In RAS immunizations, nothing is known concerning this cell population behaviour. However, it has been proposed that they play a prominent role in immunization with adenoviruses and recombinant vaccinia virus expressing malaria antigen *P. yoelii* CSP (Bruña-Romero, 2001). To observe if memory B cells are increased after *pb36p*- immunization, we quantified them by FACS, in the same organs (liver, spleen and lymph nodes) and time points (10 days, 1 month and 6 months) as done for memory T cells. Memory B cells were defined as $CD19^{+}CD27^{high}$ (Fecteau, 2003). After 10 days, no difference in memory B cell populations is observed in non immunized and *pb36p*- immunized mice (Fig. 3.8), in all organs analysed. Memory B cells were also quantified 1 and 6 months after immunization in liver and spleen of both unimmunized and *pb36p*-immunized mice. Again, no differences were observed between naïve and immunized mice (histograms are similar to those on Fig. 3.8, so we decided not to include them, since they would not provide further essential data). CONFIRM FOR 6 MONTHS

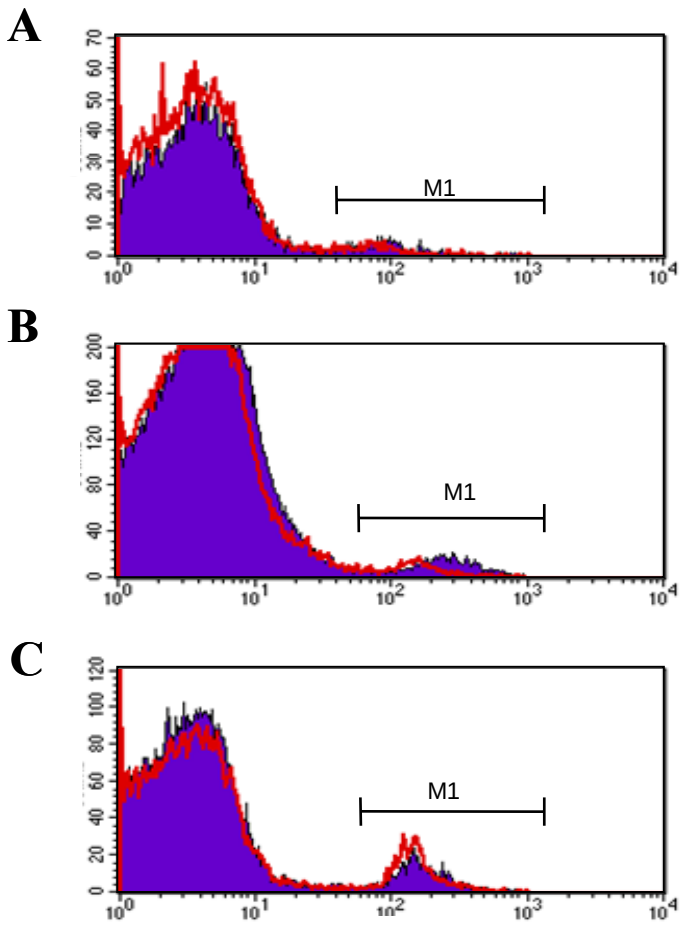

Figure 3.8. Memory B cells in liver, spleen and lymph nodes of naïve and *pb36p-* immunized mice. Memory B cells in (A) liver, (B) spleen and (C) lymph mnodes of 50,000 *pb36p-* sporozoites from naïve (filled histograms) and immunized (open histograms) BALB/c mice (n=3 per group) were assessed 10 days after immunization. Memory B cells were defined as $C19^{+}CD27^{high}$ (subpopulation defined as M1). No striking differences were observed in B memory cells between non immunized and immunized mice, in all organs analysed. Cells were quantified by FACS. A pool of cells from the three animals within the same group was analysed.

3.3. Conclusions

It is now possible to consider a comparative study of the intrahepatocytic development and the mechanism(s) of immunity invoked by different GAS and RAS. Like GAS, RAS sporozoites invade liver cells, although trophozoite development is arrested at a later stage and produces a visible PV compared to *pb36p-* sporozoites. Moreover, RAS-infected hepatocytes persist in culture longer than their GAS-infected counterparts, which, in the case of *pb36p-* parasites, results from their failure to prevent the host cell from entering apoptosis. *Plasmodium* sporozoites have the ability to prevent host cells from entering

apoptosis during intrahepatic development, to avoid their elimination (Leirião, 2005; van de Sand, 2005). *pb36p-* sporozoites, in turn, are severely impaired in protecting hepatocytes from apoptosis, these cells being eliminated from the liver very early during parasite development (van Dijk, 2005). Host cell apoptosis has also been shown at early stages during RAS development (Leirião and Mota, 2005). Although RAS populations provoke apoptosis to a greater degree than WT sporozoites, they do so to a significantly lower degree than *pb36p-* sporozoites. Accordingly, high levels of procaspase-3, the precursor of active caspase-3 which plays a prominent role in promoting apoptosis, are detected in HepG2 cells infected by both types of attenuated parasites, but not in WT infected cells. We also determined *in vivo* the persistence of RAS and *pb36p-* infected hepatocytes in whole liver extracts of BALB/c mice by using real-time PCR for liver parasite load detection/quantification. Our results confirm the significantly more rapid clearance of *pb36p-* parasitized hepatocytes within 6 hours after infection compared to RAS-infected hepatocytes, similarly to what occurs *in vitro*. One week after immunization, similar low parasite loads of both attenuated parasites infected hepatocytes are detected.

The differing biological characteristics of RAS and GAS described here suggest that the immune responses elicited by RAS and GAS could be different. Presentation of parasite antigens in the context of host cell apoptosis has recently been shown as an alternative mechanism involved in the induction of protective immunity by RAS (Leirião and Mota, 2005). As apoptosis of *pb36p-* infected hepatocytes was also detected *in vivo*, it might well be that host-cell apoptosis is associated with the ability of *pb36p-* sporozoites and other GAS to induce protective immune responses and needs further investigation. Since apoptotic infected-hepatocytes can provide a huge array of antigens to professional antigen presenting cells, like dendritic cells, (Leirião and Mota, 2005), one can consider that the protective effect mediated by *pb36p-* immunization might be at least partially initiated by these apoptotic infected hepatocytes.

Immune responses against RAS are complex and involve both cell-mediated and humoral immunity (reviewed in Morrot, 2004 and Tsuji, 2003). One can only speculate about possible immune mechanisms that may lead to the protection induced by *pb36p-* against both homologous and heterologous species. Unlike RAS, which immune response in mice is well studied and documented (Doolan,

2000), the features that lead to *pb36p*- induced protection are still unknown (van Dijk, 2005), as well as for other GAS parasites (Mueller, 2005; Mueller and Camargo, 2005). Whether *pb36p*- induced immunity is dependent or not on $CD8^+$ T cells, IFN-γ, $CD4^+$ T cells, IL-12, NK cells and nitric oxide as seen for RAS (Doolan, 2000; Sedegah, 2002) in different genetic backgrounds remains to be elucidated. Our preliminary data points that *pb36p*- protection is dependent, like in RAS, of IFN-γ, but also of γδ T cells, which are known to not exert any effect on *P. yoelii* RAS-mediated protection (McKenna, 2000), suggesting therefore that some striking differences regarding the immune responses evoked by these two different live-attenuated parasites may occur. However, more detailed experiments must be undertaken to fully access all immunological features needed for *pb36p*- mediated protection and eventual differences when compared to RAS induced immunity.

As seen for RAS, *pb36p*- immunizations induce a huge increase of $CD8^+CD44^{high}CD62L^{low}$ memory T cells in the liver (Guebre-Xabier, 1999) 10 days after immunization, fading at 1 month and barely detectable 6 months later. This subset of $CD8^+$ T cells most probably is responsible for the long-lasting protective effect of *pb36p*- sporozoites described in the previous chapter. On contrast, memory T cells in the spleen of RAS immunized mice present no phenotypic differences when compared to counterparts from naïve mice up to 6 months (Guebre-Xabier, 1999). However, our preliminary data shows that $CD4^+CD44^{high}CD62L^{low}$ memory T cells in the spleen of *pb36p*- immunized mice increase at later time points after immunization, reaching values nearly three-fold higher. One could say that this is in agreement with the previous data, supporting that *pb36p*- and RAS immunizations are mediated through different mechanisms. Nonetheless, this experiment was done in a different mouse strain (BALB/c mice) than the one used in previous studies (Guebre-Xabier, 1999) and, moreover, it was only performed once. Memory T cells populations in lymph nodes in *pb36p*- immunized mice 10 days later do not suffer any considerable change. Furthermore, memory B cells in all organs analysed in *pb36p*- immunized mice show no differences from naïve mice up to 6 months after immunization. Nevertheless, only preliminary studies were performed and further analysis regarding *pb36p*- immune memory cells using both mouse strains (BALB/c and

C57BL6) must be performed so feasible data and final conclusions may be accomplished.

Chapter IV

pbcrmp- as potential GAS based vaccines for Malaria

4.1. Introduction

Intracellular parasites are able to manipulate the host cell pathways into their own benefit, making them successful pathogens (reviewed in Nyalwhide, 2003). Nevertheless, these mechanisms are poorly known and understood for *Plasmodium* parasites, especially in the liver stage. In fact, our group was the first to elucidate one strategy maneuver performed by *Plasmodium* parasites during intrahepatocytic development in order to survive, the manipulation of apoptosis of infected cells (Leirião, 2005). In the last decade, several genetic tools for the study of malaria parasite biology were developed. Transfection systems for this haploid unicellular organism are now available, for human and animal *Plasmodium spp*, providing insight into the regulation of gene expression and elucidating the function of proteins by disrupting, modifying or replacing the genes encoding them. These genetic tools, added to the information now available of the *P. falciparum* genome, represent an important breakthrough for malaria research, contributing significantly to the understanding of the hugely complex biology of this parasite. Their potential is vast, since more rational approaches to vaccine and drug design can be achieved, by altering the parasite genome (Koning-Ward, 2000). Intracellular development is essential for *Plasmodium* in the vertebrate host and, consequently two features are crucial for this parasite: invasion and adhesion. Using the recently genetic approaches to search for *Plasmodium* proteins containing motifs implicated in invasion or adhesion, a new family of genes was identified, *pbcrmp* (*Plasmodium berghei* cysteine repeat modular protein), consisting in 4 members, *pbcrmp1-4*, with homologues in *P. falciparum* (Thompson, submitted). *pbcrmp1* and *pbcrmp2* are transcribed in mature blood-stage parasites, while *pbcrmp3* and *pbcrmp4* are only transcribed in gametocytes. Furthermore, in the mosquito stage, *pbcrmp1* and *pbcrmp2* are transcribed in sporulating oocysts. *pbcrmp2* is also transcribed in sporozoites. *pbcrmp* parasites showed normal growth rates and formed ookinetes both *in vitro* and *in vivo*. Interestingly, sporozoites lacking *pbcrmp1* and *pbcrmp2* genes (*pbcrmp1*- and *pbcrmp2*-) are not able to efficiently invade mosquito salivary glands, most remaining in the haemolymph. On the other hand, parasites whose *pbcrmp3* or *pbcrmp4* genes were disrupted (*pbcrmp3*- and *pbcrmp4*-) are severely impaired in

disrupting the oocyst's membrane being scarcely found in haemolymph and never in salivary glands (Thompson, submitted; K. D. Augustijn, personal communication). Thus, *pbcrmp* parasites are not able to transmit from the vector to the host, since they never reach salivary glands. It is anticipated that *pbcrmp* family members can mimic the structure of Tumor Necrosis Factor Receptors (TNFR), which would mean that *Plasmodium* parasites would be able to bind to host immune molecules and thereby modify the course of the immune response (Thompson, submitted). Nevertheless, this remains to be confirmed experimentally.

We decided to extract *pbcrmp3*- and *pbcrmp4*- directly from oocysts and see if this attenuation presented a particular phenotype in the hepatic stage. We observed that *pbcrmp3*- and *pbcrmp4*- sporozoites develop normally inside oocysts in mosquitos' midgut like WT do. Nevertheless, *pbcrmp3*- and *pbcrmp4*- mature oocysts are still observed in midguts on day 18 after infective blood meal, unlike WT.

To efficiently reach the liver, sporozoites need to be motile. Since they possess no cilia or flagella, they do so in a substrate-dependent manner, named gliding motility. This allows them, once in the liver, to migrate through several cells (hepatocytes and Kupffer cells), a crucial step which will permit an efficient infection (Mota, 2002; Mota, 2001). Our results show that *pbcrmp3*- and *pbcrmp4*- sporozoites extracted from oocysts are able to glide, migrate through several hepatocytes and infect a final one for further development. However, *pbcrmp3*- and *pbcrmp4*- intrahepatic development lead to small and aberrant-shape EEFs, although these are found in similar number compared to WT control, namely EEFs derived from WT sporozoites from salivary glands.

Recently, it has been shown *P. berghei* sporozoites which suffered genetic modifications are as efficient as radiation-attenuated sporozoites in immunization against malaria (Mueller, 2005; Mueller and Camargo, 2005; van Dijk, 2005). Importantly, these parasites are severely impaired in intrahepatocytic development, making them unable to progress to erythrocytic stages and, consequently, they do not cause disease, making them ideal candidates for a GAS based vaccine. Since *pbcrmp3*- and *pbcrmp4*- sporozoites also present an impaired development inside hepatocytes, we decided to evaluate their immunization potential. Both BALB/c and C57BL6 mice were immunized with

either *pbcrmp3*- and *pbcrmp4*- oocysts-derived sporozoites, according to regimens proved efficacious using *pb36p*- immunizations in these mouse strains. Both *pbcrmp3*- and *pbcrmp4*- oocysts-derived sporozoites immunizations failed completely in conferring protection to all mice tested, confirming therefore that these attenuated parasites are not suitable candidates for a GAS-based malaria vaccine.

This chapter provides our results concerning the role of *pbcrmp* in parasite-host interactions and development, and is the first study regarding these deficient parasites in liver stage. Determining the role of this family may not only shed light on its mechanisms of infection but also provide potential new targets for therapy.

4.2. Results

4.2.1. *pbcrmp3*- and *pbcrmp4*- sporozoites develop normally inside oocysts

When an anopheline feeds on an infected host, gametes fertilization occurs in the midgut. The resulting zygote, known as ookinete, will penetrate the midgut epithelium of the mosquito and continues its sporogonic development as an encapsulated oocyst attached to the epithelium. As described, a full grown oocyst is a spherical cell with 30-40 μm in diameter, limited by a plasma membrane and a thick capsule. It contains numerous dividing nuclei and cytoplasmatic membranes. Sporozoites will begin to fill oocysts days 10-12 after infective blood meal (Sherman, 1998). Upon extraction of oocysts, *pbcrmp3*- and *pbcrmp4*- sporozoites could be observed in light microscope, looking similar to WT salivary glands or haemolymph sporozoites (K. D. Augustijn, personal communication). So, we hypothesized that this attenuation in *P. berghei* could lead to deficient oocysts, presenting morphological differences which could be responsible for the differences observed between WT, *pbcrmp3*- and *pbcrmp4*- parasites. In order to confirm so, we extracted guts from WT, *pbcrmp3*- and *pbcrmp4*- infected mosquitoes (~5 guts per parasite type) at days 10 and 18 after infective blood meal and, using Electron Microscopy, checked for any differences between WT

and attenuated parasites oocysts. At day 10 after blood meal, for either WT or deficient parasites, we observed several oocysts in the external surface of mosquitoes gut. Like in WT, *pbcrmp3*- and *pbcrmp4*- oocysts were still in the formation process, containing already various nuclei, which would give rise to sporozoites (Fig. 4.1A-C, respectively). No morphological differences between WT and attenuated parasites oocysts were detected. *P. berghei* sporozoites usually leave oocysts around day 14, and the vast majority of them are found in salivary glands day 15-16 onwards. At day 18 post infection, we observe almost none oocyst in WT infected mosquitoes, and those observed had very few nuclei and, mostly probably, had suffered melanization (Fig. 4.1D). Melanization is a standard defense mechanism for insects against microbial and parasitic invaders, leading to their destruction (Sherman, 1998). On the other hand, at the same time post infection, many *pbcrmp3*- and *pbcrmp4*- oocysts were still found, and full of mature sporozoites (Fig. 4.1E-F, respectively), looking structural-like WT *P. berghei* sporozoites found in guts days 12-13 after infective blood meal have been previously described (Sherman, 1998; Vanderberg, 1967). This leads to conclude that the attenuation done in *pbcrmp3*- and *pbcrmp4*- parasites does not induce any morphologically changes which render them unable to breach oocysts but, almost certainly, acts in some molecular pathway, strongly impairing their ability to burst oocysts and migrate to the salivary glands.

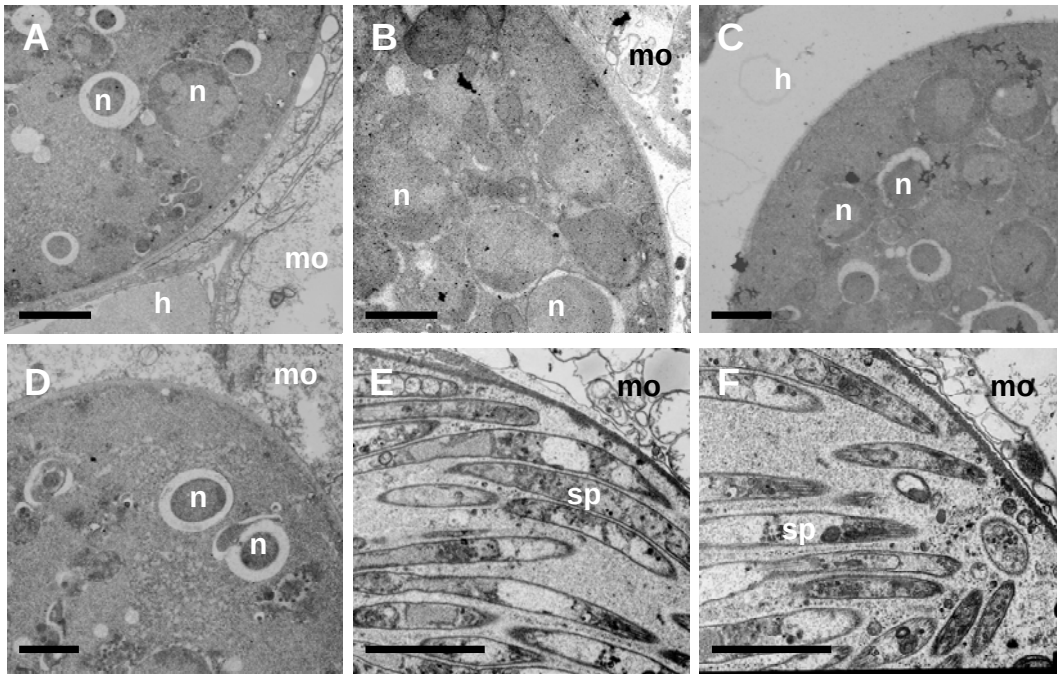

Figure 4.1. *pbcrmp3*- and *pbcrmp4*- mature oocysts are still found in mosquitoes gut 18 days after infective blood meal. WT (A), *pbcrmp3*-(B) and *pbcrmp4*-(C) oocysts are found in mosquitos' guts 10 days after infective blood meal. Oocysts rely on the external part of the gut, between mosquito epithelium (mo) and haemocoel (h). Several nuclei (n), from which sporozoites will derive, can be observed. No morphological changes are observed between *pbcrmp3*- and *pbcrmp4*- oocysts when compared to their WT counterparts At day 18 post infection, very few WT oocysts were observed (D), and those which still remain in the gut, present very few nuclei and most probably are melanized. Per turn, several *pbcrmp3*- and *pbcrmp4*- mature oocysts are still found in gut at this time (E and F, respectively), full of sporozoites (sp). These attenuated parasites, unlike WT, are not able to breach oocysts membrane and, therefore, to migrate to salivary glands. (bar 5µm).

4.2.2. *pbcrmp3*- and *pbcrmp4*- sporozoites are able to glide and to migrate through several hepatocytes

WT sporozoites have the ability of gliding, which they use as a motility mechanism, characterized by spiral movements across the substrate that propel the parasite forward (Mota, 2002; Mota, 2001). While executing this process, they leave a trail of CSP behind. This last feature has been routinely used to detect and quantify *Plasmodium* sporozoites motility. After incubating *pbcrmp3*- and *pbcrmp4*- sporozoites (extracted from oocysts 21 days after infective blood meal) in gelatin-coated coverslips (6,000 sporozoites per coverslip), they were stained for CSP (which became attached to gelatin). *pbcrmp3*- and *pbcrmp4*- sporozoites performed several spiral trails (around 2-6 per sporozoite), as in WT salivary

glands matched control. These results show that *pbcrmp3*- and *pbcrmp4*- sporozoites are motile as WT sporozoites.

As mentioned before, *Plasmodium* sporozoites migrate through several cells before the invasion of a last one. This feature is dependent on sporozoite motility (Mota, 2002; Mota, 2001). Thus, we decided to check whether *pbcrmp3*- and *pbcrmp4*- sporozoites are able to migrate through cells as WT sporozoites. WT salivary glands and *pbcrmp3*- and *pbcrmp4*- oocysts sporozoites (extracted from mosquitoes 21 days after infective blood meal) were incubated 2 hours with HepG2cells (seeded 24 hours before in coverslips in 24 wells plate), in the presence of a cell-impermeant fluorescent tracer, rhodamine-dextran (6,000 sporozoites per coverslip). Sporozoites enter and leave hepatocytes by disrupting their membrane, which is rapidly repaired, while in infection, sporozoites entry occurs with formation of a parasitophorous vacuole surrounding the parasite. Thus, it is possible to determine which cells were traversed, using rhodamine-dextran, a tracer which enters the cell immediately after membrane disruption, and becomes trapped inside after its repair (Mota, 2002; Mota, 2001). Rhodamine-dextran uninfected positive cells, which were those traversed by sporozoites, were quantified (Fig. 4.2). Surprisingly, these two attenuated parasites lead to distinct results. Both are able to efficiently perform migration. However, *pbcrmp3*- sporozoites present a significant decrease in traversed cells compared to WT sporozoites (P=0.003), while *pbcrmp4*- sporozoites significantly migrate through more cells than their WT counterparts (P=0.03).

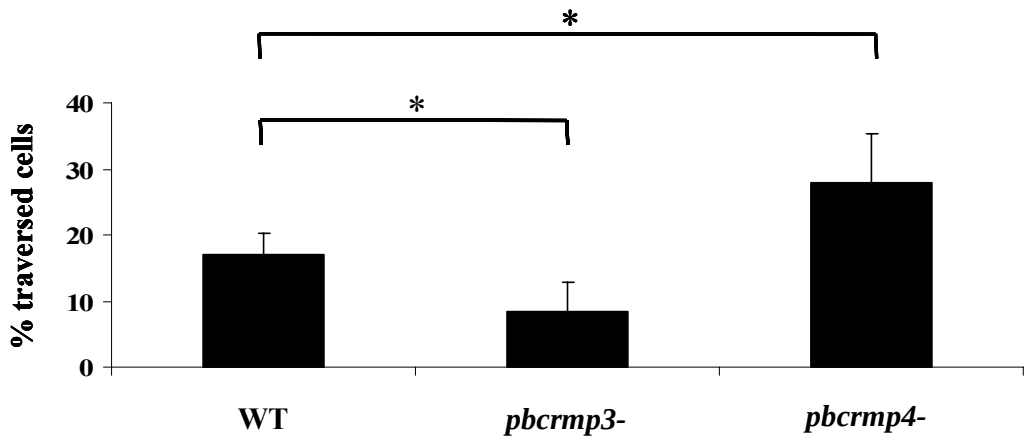

Figure 4.2. *pbcrmp3*- and *pbcrmp4*- sporozoites are able to migrate through cells as WT sporozoites, although present different migration rates. HepG2 cells were incubated with either WT salivary glands sporozoites, *pbcrmp3*- or *pbcrmp4*- oocysts sporozoites. Using a cell-impermeant fluorescent tracer, rhodamine-dextran, the migration rate of these attenuated parasites was quantified. *pbcrmp3*- sporozoites have a significant decrease in their migration ability compared to WT sporozoites. Contrastingly, *pbcrmp4*- sporozoites migrate through more cells significantly compared to WT sporozoites. Traversed cells are expressed as the mean ± the standard deviation for each coverslip. * $P<0.05$.

4.2.3. *pbcrmp3*- and *pbcrmp4*- sporozoites are not able to fully develop into mature EEFs

Our results show that *pbcrmp3*- and *pbcrmp4*- sporozoites are able to move and migrate through host cells. In order to reach the next step, WT sporozoites need develop into EEFs once inside the hepatocyte, forming, trophozoites, schizonts and, at a later stage, merozoites, which will reach the bloodstream. Thus, we decided to determine whether *pbcrmp3*- and *pbcrmp4*- sporozoites were able to develop regular-form EEFs. *pbcrmp3*- and *pbcrmp4*- oocysts sporozoites, as well as a control (WT salivary glands sporozoites) were incubated for 24 hours with HepG2 cells (as done for migration essays; 6,000 sporozoites per coverslip), and the number of EEFs was quantified (Fig. 4.3).

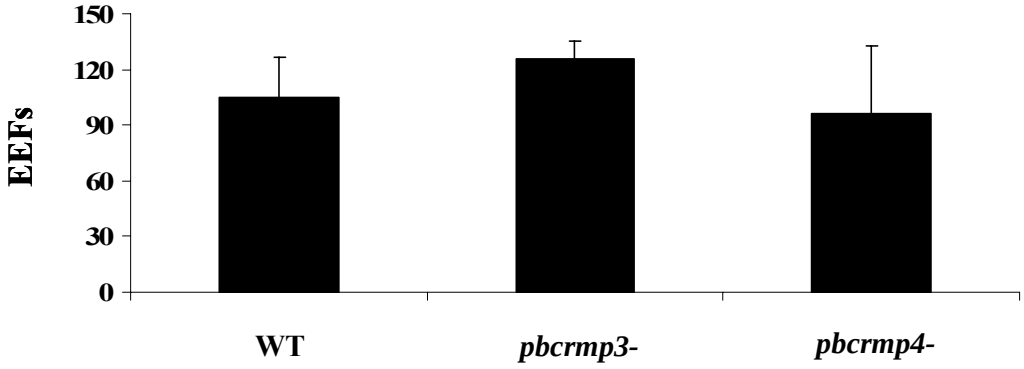

Figure 4.3. *pbcrmp3*- and *pbcrmp4*- sporozoites have the same infection rate in HepG2 cells than WT sporozoites. HepG2 cells were incubated with either WT salivary glands sporozoites, *pbcrmp3*- or *pbcrmp4*- oocysts sporozoites. 24 hours later, infection was quantified. *pbcrmp3*- and *pbcrmp4*- parasites present no difference in infection rate when compared to WT matched control. Infected cells are expressed as the mean ± the standard deviation for each coverslip.

No major differences were observed in the level of infection regarding WT salivary glands and these attenuated oocysts sporozoites, being quite similar. However, attenuated parasites EEFs are quite small and present aberrant forms, when compared to WT EEFs (Fig. 4.4), similar to what occurs with *pb36p*- sporozoites (van Dijk, 2005).

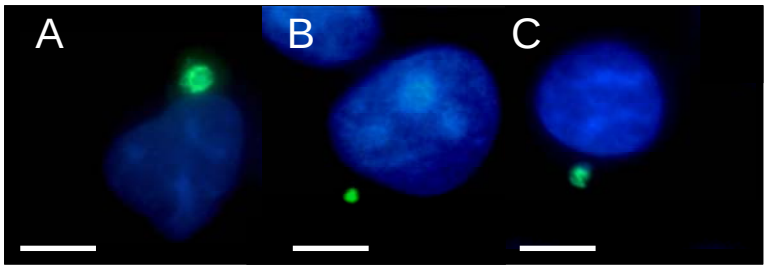

Figure 4.4. *pbcrmp3*- and *pbcrmp4*- infection lead to small and aberrant-like EEFs in HepG2 cells. Although the level of infection of *pbcrmp3*- and *pbcrmp4*- in HepG2 cells is quite similar to WT, EEFs derived from both attenuated parasites have a smaller compared to WT and possess abnormal forms. WT EEFs (A) are quite large and present a normal spherical form, contrasting with *pbcrmp3*- (B) and *pbcrmp4*- (C) EEFs. Fluorescence pictures of EEF (green), hepatocyte nucleus (blue), DNA labeled with DAPI. (bar 5µm).

However, and unlike what happens with *pb36p*- infections, the level of infection of *pbcrmp3*- and *pbcrmp4*- in HepG2 cells is quite similar to WT. Thus, the small size observed of these attenuated parasites could simply mean that they had a delay in intrahepatic development. As so, we decide to check the intrahepatocytic stage of these deficient parasites at later time points, to confirm whether or not

they could still fully develop. As done previously, HepG2 cells were infected with WT salivary glands and *pbcrmp3*- and *pbcrmp4*- oocysts sporozoites, and incubated at 24, 48 and 56 hours post infection. WT parasites, as expected, develop normally, their size increasing through the time (Fig. 4.5A-C). At 56 hours, WT merozoites are easily observed within the Parasitophorous Vacuole (Fig. 4.5C). Differently, no change in size is observed from 24 to 56 hours in *pbcrmp3*- and *pbcrmp4*- parasites (Fig. 4.5D-F and G-I, respectively). EEFs originated by these attenuated parasites possess a small size and anomalous form, which is maintained up to 56 hours post infection.

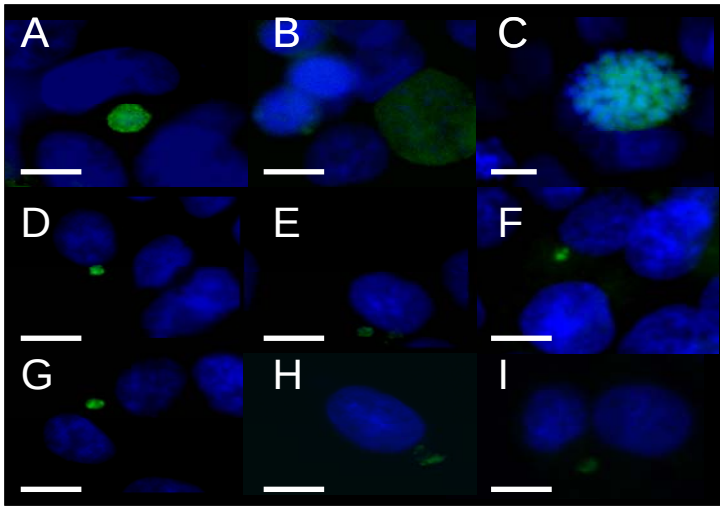

Figure 4.5. *pbcrmp3*- and *pbcrmp4*- intrahepatic development is already impaired at 24 hours post infection. WT parasites show normal development in EEFs at 24 hours post infection (A), increasing successively size at 48 hours (B) and 56 hours (C) post infection. *pbcrmp3*- (D-F) and *pbcrmp4*- (G-I), at the same time points, correspondingly, do not show any difference in size throughout all the infection, remaining small and with irregular shapes. Fluorescence pictures of EEF (green), hepatocyte nucleus (blue), DNA labeled with DAPI. (bar 5µm).

4.2.4. *pbcrmp3*- and *pbcrmp4*- sporozoites are not able to confer protection to mice upon challenge with WT sporozoites

Immunization with radiation-attenuated sporozoites (RAS), which are still able to invade cells and form a parasitophorous vacuole, but do not develop into mature EEFs, leads to full protection against a consequent infection (Luke, 2003; Hoffman, 2000). More recently, genetically-attenuated sporozoites (GAS) in *P.*

berghei, which are also impaired in liver development but are capable of conferring immunity to mice against challenge with infectious sporozoites have been described (Mueller, 2005; Mueller and Camargo, 2005; van Dijk, 2005). Since *pbcrmp3*- and *pbcrmp4*- parasites present an *in vitro* infection phenotype similar to the one observed with RAS and other GAS, we decided to confirm if these two attenuated parasites could be used as a vaccine strategy against the pre-erythrocytic stage of malaria,

Balb/c and C57BL6 mice were immunized intravenously (i.v.) with *pbcrmp3*- and *pbcrmp4*- sporozoites accordingly to regimens proven effective to other GAS (single dose of 50,000 attenuated sporozoites for BALB/c and 50,000/20,000/20,000 attenuated sporozoites, weekly apart, for C57BL6). Mice immunized with *pbcrmp3*- and *pbcrmp4*- sporozoites were checked before challenge for blood stage parasites, and all were negative, which confirms the inability of these attenuated parasites to develop patent blood stage infection. Mice were challenged i.v. with 10,000 WT *P. berghei* sporozoites per mouse 10 days after last immunization. Parasitemia was determined by thin smearing tail blood from the infected mice daily day 4 after infection onwards (Fig. 4.6).

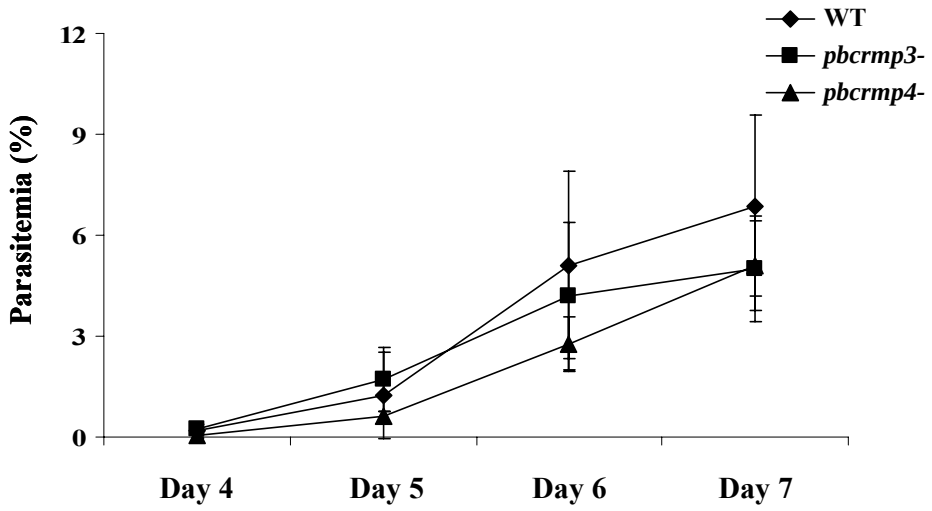

Figure 4.6. *pbcrmp3*- and *pbcrmp4*- immunized C57BL6 mice develop blood-stage parasitemia similarly to naïve control. C57BL6 mice immunized with *pbcrmp3*- (-■-) and *pbcrmp4*- (-▲-) are not protected against posterior challenge with WT infectious sporozoites, and develop a patent blood stage infection similar to the one observed in naïve challenged mice (-♦-).

All mice immunized with *pbcrmp3*- and *pbcrmp4*- sporozoites developed parasitemias similar to the ones observed in the control groups used (both Balb/c

and C57Bl6). Data regarding development of blood stage establishment parasitemia in BALB/c immunized mice is similar to the one observed in C57BL6 mice and we consider redundant to show it.

Thus, *pbcrmp3*- and *pbcrmp4*- sporozoites are not able to confer protection against challenge with infectious sporozoites (Table 4.1), and, therefore, do not present any immunization potential to be used as an experimental GAS-based malaria vaccine.

TABLE 4.1. Immunization with *pbcrmp3*- and *pbcrmp4*- sporozoites do not protect BALB/c and C57BL6 mice against a subsequent infection with WT sporozoites

Mouse Strain	Immunization[a], *pbcrmp3*-/ *pbcrmp4*- × 1,000	Challenge[b], × 1,000	No. protected (no. challenged)		
			Control	*pbcrmp3*-	*pbcrmp4*-
BALB/c	50	10	0 (6)	0 (6)	0 (6)
C57BL6	50/20/20	10	0 (6)	0 (6)	0 (6)

[a] Groups of mice were immunized i.v. with one dose of *pbcrmp3*- and *pbcrmp4*- sporozoites isolated from different mosquitoes batches. Multiple immunizations with *pbcrmp3*- and *pbcrmp4*- sporozoites were performed with weekly intervals.

[b] Mice were challenged with WT *P. berghei* sporozoites, isolated from different mosquitoes batches 10 days after last immunization. Parasitemias were monitored by giemsa staining/blood smear. All mice become positive on day 4 after challenge.

4.3. Conclusions

In this work, we characterized two genetically modified *P. berghei* parasites, *pbcrmp3*- and *pbcrmp4*-, unable to express proteins containing motifs implicated in invasion or adhesion (*P. berghei* cysteine repeat modular protein). These attenuated parasites are unable to breach oocysts membrane, never being found in mosquitoes salivary glands and, therefore, unable to be transmitted to the mammalian host (Thompson, submitted). Using Electron Microscopy, we observed that *pbcrmp3*- and *pbcrmp4*- oocysts have a normal structure similarly to what as been described to WT oocysts (Vanderberg, 1967), which lead us to think their inability to burst oocysts membrane rely in a molecular pathway(s) which is(are) strongly impaired due to the attenuation performed in these parasites.

The hepatic stage of malaria infection *in vitro* was also studied, to observe if these deficient parasites present an unusual phenotype compared to WT sporozoites.

Using the hepatoma cell line HepG2, we demonstrate that these mutant sporozoites were able, as WT sporozoites to perform the known features which lead to hepatocytes invasion and infection: they are motile and migrate through several cells before reaching one for further development. *pbcrmp3*- parasites present a lower rate of hepatocyte traversion, which is not reflected in their infectivity (these disruptants parasites rate of EEF formation is similar to matched control). *pbcrmp4*- parasites behave differently, presenting a higher rate of migration through cells. That may be due to the fact of PbCRM 4 protein be a large protein (687 kDa), and its disruption originate lighter (and, consequently, swifter) parasites and, as so, able of migrate throughout more cells (J. Thompson, personal communication). However, the number of EEFs 24 hours post infection is quite similar to the WT salivary glands.

Nevertheless, both *pbcrmp3*- and *pbcrmp4*- are not able to form regular EEFs. It was noticed impairment in the growth of these attenuated parasites EEFs. Longer time points post infection were also observed (48 and 56 hours), to determine whether there was a real impairment in these deficient parasites, or simply a delay in intrahepatic development. We confirmed that these deficient parasites can infect HepG2 cells, but impairment in EEF growth was noticed. Even at longer time-points, these EEFs do not show a significant increase in size. We assume these parasites lack the ability to mature once inside the hepatocytes, and arrest development at a very early stage post infection.

It is known that immunization with RAS and GAS can confer protection against posterior challenge with infectious sporozoites (Mueller, 2005; Mueller and Camargo, 2005; van Dijk, 2005; Hoffman, 2000). Since *pbcrmp3*- and *pbcrmp4*- parasites are also arrested during intrahepatic development like RAS and GAS, we considered the hypothesis of they could confer protection as well. Regrettably, *pbcrmp3*- and *pbcrmp4*- parasites failed to confer protection against challenge with WT *P. berghei*, These deficient parasites may simply not have the same potential as other GAS studied (*uis3*-, *uis4*- and *pb36p*-), or maybe sporozoites from oocysts are just too immature to confer protection. We intended to use γ irradiated WT oocyst sporozoites (an irradiator was not available in the facilities where these experiments were performed) in immunization assays, to investigate its ability or not of inducing protection against malaria in mice. If so, it will be pointless to carry on these immunizations with *pbcrmp3*- and *pbcrmp4*- parasites.

Otherwise, if protection will be achieved using WT RAS from oocysts, lack of maturation of sporozoites is not what is abrogating the protection using *pbcrmp3*- and *pbcrmp4*- parasites and higher doses and/or other regimens of immunization should be tried.

The crucial question now is if *pbcrmp3*- and *pbcrmp4*- sporozoites are or not able to develop due to simple lack of maturation, since they do not reach the salivary glands. Some authors considered that this feature may be essential for maturation and consequently, to confer infectivity to *Plasmodium* sporozoites (Vanderberg, 1975). The answer to this question will, with no doubt, contribute for a better understanding of the biology of *Plasmodium*-host interactions that until now are a "black hole" in our knowledge. This work is an example how genetic manipulations in *Plasmodium* can help to understand the several and complex mechanisms that underlie this parasite's genome.

Chapter V

Final Considerations

There are no doubts that novel intervention strategies are required to control malaria. For decades, since its discovery (Nussenzweig, 1967), immunization with RAS has been considered the golden standard for a malaria preerythrocytic vaccine. So far, they conferred sterile protection to 93% of vaccinees (human, non-human primates and mice), making them the most promising vaccine of all which are being (or have been) developed (Luke, 2003). Due to its immunization potential, the immune response elicited by these attenuated parasites has been widely studied and described (Berenzon, 2003; Doolan, 2000; Guebre-Xabier, 1999; Schofield; 1987). However, large scale immunization with RAS was considered unfeasible and clinically, technically and logistically impracticable since sporozoites had to be delivered alive through mosquito bite or intravenously in order to immunize large numbers of individuals (Luke, 2003). Shortly after the discovery of the immunization potential of RAS, scientists have discovered the potential of genetic manipulation: the DNA recombinant era was born. Performing genetic engineering seemed to be the answer for all major hurdles in medicine, including the synthesis of new vaccines, containing parts of genes which code for major virulent proteins of certain pathogens. Malaria vaccine was not excluded. Subunit vaccines against this disease were developed, but all failed in conferring sterile protection at the same level RAS did years before (reviewed in Hill, 2005). Even the leading candidate, RTS,S/AS02, containing the B cell epitope of CSP fused with the hepatitis B virus surface antigen only achieved 30% of protection, which faded rapidly in the following months (Alonso, 2004). The constant failures and disappointments in subunit vaccines against the preerythrocytic stage of malaria led some researchers to focus again on a *P. falciparum* RAS based vaccine (Luke, 2003). They are currently developing a vaccine using cryopreserved RAS, and studying the efficacy of this vaccine, in addition to overcome the major obstacles presented, meaning routes of administration, production of adequate sporozoites numbers and physical requirements (namely safety and potency), to achieve such a vaccine (Luke, 2003). Nevertheless, this approach suffers from significant disadvantages, not least the question of safety and reproducibility because the amount of radiation that generates the attenuated state is strictly defined. Parasites that are underirradiated remain infectious, and those that are overirradiated do not induce protective immunity (Suhrbier, 1990). Recently, an alternative for RAS has been

found. Genetically attenuated sporozoites (GAS) that lack sporozoite specific conserved genes (*uis3*- and *uis4*-) that are apparently important for sporozoite development in the hepatocyte can induce significant or complete protective immunity in the *P. berghei* rodent model of malaria when different immunization protocols are used (Mueller, 2005; Mueller and Camargo, 2005). Like RAS, these attenuated sporozoites are nonreplicating and metabolically active during intrahepatocytic development. Moreover, since attenuation is done using genetic engineering, they do not require strict control of radiation dose as RAS do, making them more feasible for a vaccine based on attenuated sporozoites.

In this study, we characterized three other *P. berghei* GAS, *pb36p-, pbcrmp3*- and *pbcrmp4*-, to assess their potential as an experimental vaccine against malaria. Our previous work has shown that *pb36p*- sporozoites are not able to fully develop after hepatocytes infection and, moreover, previous inoculations led to a huge reduction in parasites liver burden upon posterior challenge with infectious WT sporozoites (van Dijk, 2005). Here, we also observed that *pbcrmp3*- and *pbcrmp4*- parasites are arrested during intrahepatic development. Thus, since *pb36p*-, *pbcrmp3*- and *pbcrmp4*- parasites are strongly impaired in intrahepatic development like RAS and other GAS with proven immunization potential, we ought to confirm if they could also confer protection to mice against challenge with WT parasites. BALB/c and C57BL6 mice were immunized with *pb36p*-, *pbcrmp3*- or *pbcrmp4*- sporozoites. We observed that *pb36p*- sporozoites where able to protect both BALB/c and C57BL6 mice from later challenge with infectious WT parasites. BALB/c mice require only one single dose to be fully protected, while C57BL6 need three immunizations to achieve sterile protection. On the contrary, all mice immunized with *pbcrmp3*- or *pbcrmp4*- sporozoites develop a patent blood stage infection upon challenge with infectious WT sporozoites at the same level than naïve challenged mice.

Since *pbcrmp3*- and *pbcrmp4*- sporozoites have no immunization potential, no further studies were done regarding these attenuated parasites. We focused next in characterizing *pb36p*- mediated protection and the mechanisms that could lead to such protection. Whenever possible, we establish comparisons between RAS and *pb36p*- induced protections and observed that, although many features are shared by both attenuated parasites, some differences were also observed. Like RAS, *pb36p*- confers full protection to two distinct mouse strains, BALB/c and

C57BL6. Moreover, like in RAS based immunizations, different immunizations regimens are required to confer protection to those different mouse strains (Chatterjee, 2001). Previous studies have shown that RAS immunity is stage specific (Clyde, 1975). Mice immunized with RAS sporozoites were not protected against posterior challenge with *Plasmodium* infected red blood cells. We observed the same phenotype using BALB/c mice immunized with either RAS or *pb36p*- sporozoites and challenged later with infected erythrocytes. Immunized mice developed a patent blood stage parasitemia like naïve challenged mice, showing no degree of protection, leading to conclude *pb36p*- induced protection is also stage-specific.

Protection induced by these attenuated parasites is long lasting. BALB/c mice challenged with infectious sporozoites several times up to 18 months after last immunization were always protected. Moreover, BALB/c mice immunized with *pb36p*- using a prime plus 2 boosts regimen were found protected up to 6 months without any previous challenge. However, when those mice are re-challenged 12 months after last immunization, some of them are no longer protected. RAS immunized mice in the same conditions, contrastingly, were always protected. Thus, BALB/c mice require no additional challenges to become fully protected up to 6 months. To achieve protection at longer time points, antigen presentation (through challenges) seems to be required, so the immune response against *Plasmodium* sporozoites can be maintained. Most likely, a source of antigens is necessary to keep the protective effect after 6 months. Berenzon and colleagues have shown that presence of antigen is needed to maintain the persistence of intrahepatic memory T cells (Berenzon, 2003), and, therefore, immunity against the parasite. An antigen depot in the liver would obviously re-stimulate the protective immune response and it may supplied either by undeveloped RAS/*pb36p*- parasites arrested in hepatocytes or through continuous challenges with WT sporozoites. *P. berghei* RAS can be found in livers of immunized mice 6 months after immunization (Scheller, 1995), acting as a persistent source of antigens, at least, during that period. Moreover, when mice are treated with primaquine to eradicate these RAS persisting liver forms, protection is completely abrogated (Scheller, 1995), supporting that an antigen depot is in fact crucial for RAS based immunizations to be completely effective. Most probably, *pb36p*- parasites remain long enough in the liver, inducing the protection achieved 6

months after final immunization without the need of performing any additional challenge. The presence of *pb36p*- would elicit an immune response, strong enough to fully protect mice after 6 months without any other type of antigen presentation, and eventually, starts fading from this period onwards. Moreover, a higher number of challenges appear to be required to maintain full protection longer than 6 months in BALB/c mice, almost certainly acting synergistically with persistent *pb36p*- in the liver, thus eliciting a strong long lasting immune response. We have also shown that *pb36p*- has a higher apoptosis rate than RAS, being cleared in the liver sooner than RAS parasites. Most likely, these parasites would not last in the liver as long as RAS do in the same conditions, therefore exhausting sooner the supply of antigens and, consequently, the length of induced protection. Our results also show a decline in $CD8^{+}CD44^{high}CD62L^{low}$ T cells (memory cells) in the liver from 10 days up to 6 months in *pb36p*- immunized BALB/c mice. As mentioned above, it has been shown that in RAS immunized C57BL6 mice, an antigen depot is necessary for maintenance of these $CD8^{+}$ T memory cells (Berenzon, 2003). Although done in a different mouse strain, the results are quite similar, leading us to think that *pb36p*- induced immunity is maintained while these attenuated parasites remain in the liver (up to six months) and additional antigen presentation derived from challenges is required to achieve longer periods of sterile protection. Furthermore, intrahepatic memory $CD8^{+}$ T cells in *pb36p*- immunized mice decrease with time, most probably due to exhaustion of antigen depot of these parasites in the liver. Therefore, as for RAS induced protection, *pb36p*- mediated immunity also relies in continuous antigen presentation, derived either from persisting attenuated parasites in the liver or, at a later stage, from WT parasites inoculated. Long lasting protection elicited by *pb36p*- is also observed in another mouse strain. *pb36p*- immunized C57BL6 mice are also protected against several challenges up to 6 months after last immunization, and 50% of these mice are still fully protected against a re-challenge performed 12 months later. RAS immunized mice, in contrast, were always fully protected in these conditions. As in BALB/c mice, continuous antigen presentation must elicit a protracted immune response, which eventually declines, and lasting longer in RAS immunized mice most probably also due to *pb36p*- higher clearance in the liver. Even so, the length of this protection is quite

amazing for both mice strains studied, and for both types of attenuated parasites, strengthening their potential as a vaccination strategy against malaria.

Nowadays, the development of a *P. falciparum* RAS based vaccine faces some hurdles, namely production of adequate quantities of sporozoites and administration through a route considered clinically practical (Luke, 2003). Thus, it is fundamental to determine the minimum quantity of attenuated sporozoites which can confer protection, since that will have serious implications in cost evaluation. Previous studies have shown that low immunizations doses of RAS (multiple doses of 10,000) can protect C57BL6 mice against a challenge with 10,000 WT sporozoites (Jaffe, 1990). Our results confirmed these previous results and, furthermore, showed that protection can be achieved with even lower doses of either RAS or *pb36p*-. C57BL6 mice immunized with prime plus two boosts regimen with equal doses of 10,000 or 5,000 of either attenuated parasite were fully protected against a challenge of 10,000 WT *P. berghei* sporozoites. Using the same immunization protocol, we immunized C57BL6 with an even lower dose, 1,000 RAS or *pb36p*- sporozoites, and challenged them likewise with 10,000 WT sporozoites. As some have already described (Jaffe, 1990), this dose is unable to protect C57BL6 mice against infection. Our results with RAS confirmed such, since all mice immunized this way developed blood stage infection. A strong delay (3 days) in the onset of blood stage parasitemia has been observed compared to naïve challenged mice, indicating some extent of protection, although obviously not enough to confer sterile protection. However, and most surprisingly, mice immunized similarly with *pb36p*- sporozoites achieved fully protection. Such difference is quite remarkable and clearly shows that *pb36p*- sporozoites require lower doses than RAS to attain similar level of protection.

So far, mostly of immunizations using attenuated sporozoites have been performed either intravenously or through irradiated mosquitoes bite (Luke, 2003). None of these is clinically accepted as a route of administration for a human vaccine. So far, public health measures state that a vaccine can only be administrated intramuscularly, subcutaneously or intradermally. Our studies show that all these routes conferred some extent of protection for either RAS or *pb36p*- sporozoites upon intravenous challenge with infectious parasites, as suggested by the delay on blood stage parasitemia onset in immunized mice. Furthermore, a proportion of sterile protection is achieved for both attenuated parasites using

intramuscular route and by RAS only in intradermally challenged mice. Previous studies concerning RAS immunized A/J mice intramuscularly showed similar results (Kramer, 1975). However, in the same study, mice immunized subcutaneously presented no protection, developing a patent blood stage infection as unimmunized challenged mice (Kramer, 1975). We assume that the discrepancy observed between such and our results derives from the fact of two distinct mouse strains have been used in these immunizations, leading to the differences observed. However, that remains to be elucidated. Contrastingly, another *P. berghei* GAS, *uis3*-, is able to confer full sterile protection to C57BL6 when administered subcutaneously, using the same regimen (Mueller, 2005), suggesting that immune mechanisms elicited by *uis3*- differ greatly from those attained by either RAS or *pb36p*-. So far, to our knowledge, no studies regarding intradermal immunizations with RAS or other GAS have been done, being this the first study using this immunization route. However, these results are obtained by challenging with infectious sporozoites i.v. and not through the natural course of a malaria infection, the bite of an infected mosquito. Thus, to assess more realistically the efficacy of these routes, immunizations with *pb36p*- sporozoites were repeated, using all these clinically acceptable routes and mice were then challenged via mosquito bite. Immunized mice were shown to have a higher level of sterile protection, around 80% for all routes, which supports *pb36p*- parasites potential as an experimental vaccine against malaria. Unfortunately, none of these routes confer the same level of sterile protection as immunizations performed intravenously, which is undoubtedly desirable. Although at the moment these routes do not lead to complete protection, the results are quite encouraging and promising, strengthening the potential of a human live attenuated parasites-based vaccine.

Our results show for the first time that protection elicited by attenuated sporozoites is not species specific. *P. berghei* RAS and *pb36p*- can confer protection to BALB/c and C57BL6 mice challenged with a different rodent *Plasmodium* species, *P. yoelii*, even achieving in some cases sterile protection (the later only observed in BALB/c so far). Cross-protection induced against one *Plasmodium* species by other different species has already been observed for the blood-stages of infection. Primary infection with *P. chabaudi chabaudi AS* infected erythrocytes led to protection against mortality and lower parasitemia

levels after a challenge with a lethal strain of *P. yoelii* (Legorreta-Herrera, 2004). Mice infected with blood stage *P. berghei* parasites and later re-infected with *P. yoelii*, *P. vinckei* and *P. chabaudi* parasitized erythrocytes show partial resistance against mortality (McColm, 1983; Jarra, 1985). In humans, there are similar evidences for this type of protection. Prior infections with *P. malariae* and *P. vivax* prevent the symptoms associated to subsequent *P. falciparum* infection, although not other forms of morbidity (Smith, 2001). Regarding *Plasmodium* liver stages of infection, it has been observed that, during treatment of neurosyphilis, previous infection with *P. malariae* sporozoites resulted in reduction of parasitemia levels and fever frequencies following secondary infection with *P. falciparum* sporozoites (Collins, 1999). However, there are no reports showing that immunization with attenuated parasites, including RAS, is able to elicit any type of cross-protection. So far, to the extent of our knowledge, immunization with *P. falciparum* RAS and subsequent challenge with *P. vivax* sporozoites has only been performed once, to a single subject, whom did not became protected (Hoffman, 2002). Our results show that the immune response elicited by both RAS and GAS *P. berghei p36p-* sporozoites also acts partially against another murine parasite, *P. yoelii*. Considering that BALB/c mice are 2,000 times more sensitive to *P. yoelii* than *P. berghei* sporozoites (Khan, 1991), the partial protection attained with *pb36p-* against *P. yoelii* sporozoites challenge is quite notable. One can only speculate about the mechanisms which are responsible for inducing cross-species protection. Most probably, $CD8^+$ T cells are a key feature in this type of immunity. It has been reported that a cytotoxic T cell clone obtained from a BALB/c mouse immunized with *P. yoelii* irradiated sporozoites recognizes an epitope on both *P. yoelii* and *P. berghei* CSP (Weiss, 1992). When this clone is transferred to naïve mice, it confers protection against posterior challenge with either *P. berghei* or *P. yoelii* sporozoites, leading to believe that cross-species immunity elicited by RAS (and most likely also by *pb36p-* sporozoites) relies on $CD8^+$ T cells.

Apoptosis must also play a prominent role on cross-species protection. It is known that, in order to survive, *Plasmodium* sporozoites have the ability to prevent host cells from entering apoptosis during intrahepatic development (Leiriao, 2005; van de Sand, 2005). We have shown that *pb36p-* sporozoites, in turn, are severely impaired in protecting hepatocytes from apoptosis, these cells being eliminated

from the liver very early during parasite development. Host cell apoptosis has also been shown at early stages during RAS development (Leiriao and Mota, 2005) and we observed a similar phenotype, with a higher extent, during *pb36p*- intrahepatic growth. Since apoptotic infected-hepatocytes can provide a huge array of antigens to APC (e.g. dendritic cells) (Leiriao and Mota, 2005), one can consider that the protective effect mediated by *pb36p*- immunization against *P. yoelii* might be at least partially initiated by these apoptotic infected hepatocytes. *P. berghei* and *P. yoelii* have an average protein identity of 88.2% (Hall, 2005), which suggests that through apoptosis several conserved antigens common to *P. berghei* and *P. yoelii* would be presented to immune system, therefore eliciting an protective response strong enough to act against both parasite species. Since *P. berghei* and *P. chabaudi* have also a high average protein identity, 83.2% (Hall, 2005), one might speculate this *P. berghei* attenuated sporozoite induced immune response might also exert a protective effect against infection with *P. chabaudi* sporozoites. Most likely, optimized immunization protocols, i.e., using other immunization protocols and/or higher doses of RAS or *pb36p*- sporozoites per immunization, might lead to complete sterile protection against *P. yoelii* and other murine *Plasmodium* species, and we are currently investigating those approaches.

Immunization strategies using *P. falciparum* RAS have been considered impossible due to technical, clinical and logistical hurdles: route of administration, large quantities of sporozoites needed and regulatory, potency and safety requirements (Luke, 2003). However, some are pursuing this approach (Luke, 2003), which would be quite useful in a possible implementation of *P. falciparum* GAS immunization strategy. Orthologues of *pb36p*- in *P. falciparum* and *P. vivax* have already been described (Thompson, 2001), therefore being possible to perform this type of genetic modification in human parasites. Unfortunately, as observed in *uis4*- parasites (Mueller and Camargo, 2005), *pb36p*- sporozoites initiate a delayed blood in some mice, although at a lower frequency. GAS vaccine efficacy and safety could be improved by developing more complex GAS strains that lack multiple genes in a single parasite, as some defend (Matuschewski, 2006; Waters, 2005). There is a close paralogue of *pb36p*, *pb36* that is also expressed in sporozoites (Thompson, 2001) and located directly adjacent to *pb36p* in the parasite genome. Improved levels of protection may be achieved by creating double mutants, like *pb36*-/*pb36p*- or *uis3*-/*pb36p*- parasites.

Each form of GAS must be evaluated for its immunization potential and the best single or combination one, taken forward to human trials.

The characterization of *pbcrmp3*- and *pbcrmp4*- parasites intrahepatic development in the liver led us to think these two attenuated parasites could also be candidates for a GAS experimental vaccine. Like RAS and *pb36p*-, they arrest during liver development, never forming mature EEFs like WT sporozoites. Regrettably, this similarity is not reflected in their immunization potential. No protection is achieved with *pbcrmp3*- and *pbcrmp4*- parasites in either BALB/c or C57BL6 mice. *pbcrmp3*- and *pbcrmp4*- sporozoites were extracted from oocysts and, consequently never passed through the maturation stage in mosquito salivary glands, which some authors consider crucial to confer to sporozoites their infectivity (Vanderberg, 1975). So, the lack of protective effect demonstrated by *pbcrmp3*- and *pbcrmp4*- sporozoites could derive of their immature stage, not allowing an efficient infection *in vivo*, an essential step attenuated sporozoites must perform to confer immunity. However, that remains to be further investigated. The level of infection observed in *pbcrmp3*- and *pbcrmp4*- infections is quite similar to the one observed in the WT, which mean these attenuated parasites are able to efficiently inhibit apoptosis pathways as WT parasites do. The lack of apoptotic infected hepatocytes may also explain the deficiency on conferring protection of *pbcrmp3*- and *pbcrmp4*- sporozoites, but this ought to be confirmed. Nevertheless, although these attenuated parasites do not present any immunization potential, their phenotype in liver stage development is quite interesting and will provide undoubtedly new insights into parasite-host interactions.

Our results clearly confirm the potential of *pb36p*- as an experimental vaccine against malaria, while *pbcrmp3*- and *pbcrmp4*- failed in achieve the same goal. Furthermore, *pb36p*- is able to confer long-lasting immunity and cross-species protection against *P. yoelii*. This protection can be achieved even with low immunization doses and using the most commonly used routes of immunization. Understanding the mechanisms by which *pb36p*- parasites induce immunity will help us to design a vaccine against malaria liver stage. We have already made an attempt to initiate studies regarding this issue, and observed that *pb36p*- mediated immunity is dependent on IFN-γ and γδ T cells. Moreover, apoptosis seems to be paramount in these attenuated parasites induced protection. These are just the first

steps on this subject, and further studies are already in course to unravel the mechanisms of this and other GAS mediated protection, which will no doubt provide ground-breaking relevant data for the development of a vaccine aimed for the pre-erythrocytic stage of Malaria.

Chapter VI

Material and Methods

6.1. Materials

6.1.1. Chemical and General Reagents

Analytical or molecular grade chemicals were purchased from Sigma, Merck, BioRad, Calbiochem, Roche, Riedel-de Haen, Fluka AG, BDH AnalaR, Carlo Erba Reagenti, Mallincrkodt and TAAB. Molecular biological reagents and enzymes were purchased from Roche, Applied Biosystems, Promega and New England Biolabs. Culture media was purchased from Gibco or Sigma. Primers were acquired from MWG. Antibodies for immunofluorescence were obtained from Molecular Probes or Jackson Biolabs. Antibodies for Blotting were purchased from Amersham or Zymed. Antibodies for flow activated cell sorting (FACS) were purchased from Pharmingen or Serotec.

6.1.2. Cells

For all *in vitro* experiments, the hepatoma cell line HepG2 (ATCC, HB8065)., which is efficiently infected by *P. berghei* parasites and sustains their complete development (Hollingdale , 1983) was used. Cells were maintained in DMEM supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicilin-Streptomycin (P/S) (complete DMEM), in an atmosphere containing 5% CO_2. Cells were periodically tested for mycoplasma infections, as previously described (Uphoff, 2002).

6.1.3. Parasites

Anopheles stephensi mosquitoes infected with *P. berghei* parasites (clone 15cy1; ANKA strain) expressing GFP (Franke-Fayard, 2004) and *pb36p- P. berghei* parasites (clone 1; ANKA strain) expressing GFP (van Dijk 2005) were either reared in our own insectary or supplied by Dr. Robert Sauerwein (Department of Medical Microbiology, University Medical Center St. Radboud, Nijmegen, The Netherlands.). *Anopheles stephensi* mosquitoes infected with *P. yoelii* (265 BY strain) were supplied by Dr. Dominique Mazier (Inserm U511, Immunobiologie

Cellulaire et Moléculaire des Infections Parasitaires, Centre Hospitalo-Universitaire Pitié-Salpêtrière, Université Pierre and Marie Curie, Paris, France). *Anopheles stephensi* mosquitoes infected with *P. yoelii* (17XL strain), *P. chabaudi chabaudi* (AS strain) and *P. vinckei petteri* were supplied by Dr. Richard Culleton (Institute of Immunology and Infection Research, Ashworth Laboratories, School of Biological Science, University of Edinburgh, Edinburgh, United Kingdom). Parasites were maintained by alternating passage of the parasites in *A. stephensi* mosquitoes and BALB/c or C57BL6 mice (Gwadz, 1980).

Disruption of *pb36p-* or *pbcrmp3-*/*pbcrmp4-* in *P. berghei* were performed by Dr. Milly van Dijk or Dr. Kevin Augustijn (Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands), respectively.

Sporozoites were obtained by hand dissection of infected mosquitoes 17-25 days (*P. berghei, P. chabaudi* and *P. vinckei*) or 14-18 days (*P. yoelii*) after mosquitoes had taken an infectious blood meal. Salivary glands were collected in an eppendorf containing RPMI medium, disrupted and centrifuged thrice, 5 minutes at 800 rpm RT each. The supernatant containing the sporozoites was collected and placed in other eppendorf. Sporozoites were counted using a haemacytometer (Ozaki, 1984).

6.1.4. Mice

BALB/c (H-2K^{d}) and C57BL6 (H-2K^{b}) were supplied by Instituto Gulbenkian de Ciência (Oeiras, Portugal) or purchased to Harlan Ibèrica (Barcelona, Spain). C57BL6 IFN-γ and TCRδ deficient mice (*Ifng*^{tm1Ts} and *Tcrd*^{tmMom} targeted mutation, respectively) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and have been described earlier (Itohara, 1993; Dalton, 1993). For all experiments, 6-8 weeks old mice were used (except for long-lasting protection, due to obvious reasons). Animal were kept in pathogen-free environment facilities. All animal care and procedures were in accordance with European regulations.

6.2. Methods

6.2.1. Immunizations

Balb/c and C57Bl/6 mice were immunized with either RAS or GAS (*pb36p*-, *pbcrmp3*- and *pbcrmp4*-). RAS were previously submitted to a radiation dose of 16,000 rad in a Co source, using a γ irradiator (Oris Industrie IBL 437C, Gif-sur-Yvette, France). Immunizations were performed according to a standard protocol (Orjih, 1982). Briefly, intravenous administration was performed (ocular vein), with a single dose of 50,000 of attenuated sporozoites for BALB/c and three doses of 50,000/20,000/20,000 of attenuated sporozoites for C57BL6 (weekly apart). Other administration routes (intramuscular, subcutaneous and intradermal) were also performed for RAS and *pb36p*- immunizations. Immunized mice were challenged 10 days later with 10,000 WT *P. berghei* sporozoites (standard procedure). A naïve control mice group was added at this time for all experiments performed. As described in chapter 2, different doses and parasites were also used for challenge of RAS and *pb36p*- immunized mice.
Parasitemias in blood were detected/measured by FACS analysis (when GFP expressing parasites were available) or by blood smears and Giemsa staining (van Dijk, 2005).

6.2.2. Quantification of Liver Infection

Livers were dissected 40 hours post infection, and immediately suspended in 4 ml volume of Denaturing Solution (4M Guanidium Thiocyanate, 25mM Sodium Citrate pH 7, and 0.5% N-Laurosyl-sarcosine) plus 0.1M β-mercaptoethanol, and stored at 4ºC until homogenization. Livers were homogenized with a Tissue Tearer bladder. Liver homogenates were aliquoted (3×300µl per sacrificed animal) and stored at -80ºC (Witney, 2001). Total RNA of the samples obtained was purified using the RNeasy Mini Kit (Qiagen), according to standard protocols supplied by the manufacturer. RNA was quantified using a Nanodrop Spectrometer. 1 µg of total RNA (per sample) was reverse transcribed using a First Strand cDNA Synthesis Kit for RT-PCR, in a 20µl reaction volume,

according to standard protocols supplied by the manufacturer. cDNA synthesis was performed at 25ºC for 10 minutes, then 42ºC for 60 minutes, following heat inactivation at 99ºC for 10 minutes (Witney, 2001), in a BioRad MyCycler Thermal Cycler. 2 µl of sample cDNA was submitted to real time fluorescent detection of PCR products, using a Power SyBrGreen PCR Master Mix (12.5 µl), *P. yoelii* 18S rRNA specific forward (5' – GGG GAT TGG TTT TGA CGT TTT TGC G – 3', Tm 64.6ºC) and reverse (5' – AAG CAT TAA ATA AAG CGA ATA CAT CCT TAT – 3', Tm 59.2ºC) or *P. berghei* 18S rRNA specific forward (5' – GGA GAT TGG TTT TGA CGT TTA TGT G – 3', Tm 59.7ºC) and reverse (5' – AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC – 3', Tm 59.9ºC) primers 10 mg/ml (2 µl of each) and nuclease free water (6.5 µl), in a total 25µl reaction volume. RT-PCR was performed using a ABI Prism 7000 thermocycler (Applied Biosystems), using the following thermocycling conditions: 1 cycle of 95ºC for 10 minutes and 45 cycles of 95ºC for 15 seconds (denaturation) and 60ºC for 1 minute (annealing/extension) (Bruña-Romero, 2001; Witney, 2001). Control plasmids with standard concentrations of 18S rRNA gene, from 10^1 to 10^8 copies were used in the assay and were synthesized as described elsewhere. A similar RT-PCR was performed for a house-keeping gene, HPRT, for standardization of values obtained (Witney, 2001).

6.2.3. Sporozoite Infection *in vitro*

P. berghei sporozoites were added to cell monolayers (seeded previously and used when confluence was ~80-90%) in complete DMEM, in 24 well plates, and centrifuged 10 minutes at 3000 rpm. Plates were then incubated at 37ºC, in an atmosphere containing 5% CO_2. At different time points after infection, such as 2 hours (migration essays), 6 and 24 hours (Apoptosis detection and EEF development) and 48 and 56 hours (EEF development), cells were fixed with paraformaldehyde 2% (PFA) and absolute methanol at room temperature (RT), and stained afterwards.

6.2.4. Immunofluorescence Assay

After fixation, coverslips containing infected cells were incubated with a protein blocking solution (3% Bovine Serum Albumin, 100mM Glycine, 10% FCS) to avoid unspecific reaction, containing 0.1% Saponin (Sigma) for permeabilization, for 30 minutes, rinsed twice shortly in PBS, and incubated 45 minutes RT with a monoclonal antibody (mAb) against anti HSP70 (also known as 2E6, produced by a hybridoma cell line; Tsuji, 1994) and, when apoptosis detection was required, simultaneously with a mAb against active caspase-3. After smooth rinsing of the coverslips to remove exceeding mAb, they were incubated with mAb Cy2 Alexa Fluor® 488 for mice IgG (dilution 1:2000) and, when apoptosis detection was performed, simultaneously with mAb anti Rabbit IgG Texas Red, for 45 minutes RT, light protected. Following removal of exceeding mAb, the coverslips were incubated with 20 ng/µl of diamidino-phenyl-indole (DAPI), a DNA stain, up to 2 minutes RT. Then, the coverslips are mounted in glass slides, with a mounting medium, Mowiole and then observed in a Fluorescence Microscope. Infection rate was quantified by counting the number of parasite infected cells per coverslip. Fluorescence pictures were taken using Metamorph software (Molecular Devices, Sunnyvale, California, USA).

6.2.5. *In vitro* Analysis of Apoptosis in RAS and *pb36p*- Parasite-Invaded Hepatocytes

Two distinct fluorescent methods for apoptosis detection were used: (i) active caspase-3 detection, and (ii) nuclear morphology by DAPI staining as described (Leirião, 2005). *P. berghei* sporozoites (WT, *pb36p*- or RAS) were added to HepG2 cells for 6 h before staining with anti-HSP70 mAb (as described in 6.2.3 and 6.2.4) for parasite detection. Apoptotic cells were visualized 6 h after infection by active caspase-3 detection and DAPI staining. Infection and apoptosis rates were quantified by counting the number of parasite infected cells per coverslip and apoptotic parasite-infected cells per coverslip, respectively.

6.2.6. *In vivo* Analysis of Apoptosis in *pb36p-* Parasite-Invaded Hepatocytes

C57BL6 mice were infected with 500,000 *pb36p-* or WT *P. berghei* sporozoites by i.v. injection. Livers were removed 6 h after infection and snap-frozen in Tissue Tek (Sakura) in dry ice. Twenty histological sections from each mouse were obtained with a cryostat (Leica CM 3050 S), and placed in Super Frost Plus slides (Menzel-Glaser). Afterwards, these slides were fixed and stained for both parasite detection and nuclear morphology by DAPI staining (as described in 6.2.4 and 6.2.5). All sections were examined for the presence of infected cells with signs of apoptosis.

6.2.7. Western Blot for Caspase-3 detection

Protein separation was accomplished using a polyacrylamide gel electrophoresis system composed by a stacking and a resolving gel in tris-glycine running buffer with 20%. SDS. Protein sample from whole cell lysates (HepG2 uninfected or incubated 24 hours with 150,000 WT, RAS or *pb36p-* sporozoites, as described in 6.2.3) in 2x Laemmli buffer were boiled (5 minutes, 100ºC) and loaded into 12 % SDS-PAGE gels (Burnette, 1981). Pre-stained SDS-PAGE protein standards were also loaded to determine molecular mass of analyzed proteins. Proteins were resolved by running the gel using Mini Protean II (BioRad) at 110 V. After separation by electrophoresis proteins were transferred to a nitrocellulose membrane through a wet electroblotting (200 mA 90 minutes) using mini protean II (BioRad) (Burnette, 1981). Membranes were washed in tris-buffered saline (TBS) and incubated for 1 hour in blocking solution (5% fat-free powder milk in TBS). Membranes were washed in TBS and probed with primary antibodies in blocking solution (90 minutes, RT). The primary antibodies used were anti procaspase-3 and actin (loading control) (both 1:5000), identified by a molecular weight around 32 and 42 kDa, respectively. After incubation with the primary antibodies, membranes were washed in TBS (3 times 10 minutes) and all primary antibodies were detected using horseradish peroxidase conjugated donkey anti–rabbit (1:4000) or goat anti-mouse (1:4000) secondary antibodies (60 minutes,

RT). After membranes were washed in TBS (3 times 10-15 minutes), HRP enzymatic activity was visualized using the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer instructions. Results were obtained in the form of photo-radiographs (Amersham Hyperfilm) in a developer (Curix 60, Agfa).

6.2.8. Memory cells and FACS

BALB/c mice immunized as described (6.2.1) were sacrificed (at 10 days, 1 and 6 months after immunization) to collect livers, spleens and lymph nodes for memory cells quantification.

Livers were perfused via the hepatic portal vein using a Liver Perfusion Medium (Gibco) and digested using Liver Digestion Medium (Gibco). Livers were then resected and cells were teased from the tissue using a stainless steel wire mesh. Cells were centrifuged at 10 minutes and 1500 rpm (4ºC) in complete RPMI (supplemented with 10% FCS, 1% PS, 1% HEPES, 1% Sodium Pyruvate and 0.1% β-mercaptoethanol). The pellet was ressupended in complete RPMI and, to eliminate hepatocytes, centrifuged twice over Percoll (60% and 30%, respectively) 2000 rpm and 10 minutes (20ºC), without brake. Pelleted lymphocytes were washed once in complete RPMI and treated with ACK (ammonium, chloride and potassium; 0.15M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2 EDTA, pH 7.2), to eliminate erythrocytes (Guebre-Xabier, 1999).

Spleens and lymph nodes suffered similar treatment as just described for liver: after removal, they were teased in a stainless steel wire mesh. Cells were centrifuged 1500 rpm for 15 minutes RT, no brake, over Ficoll, for elimination of red blood cells. Interfase was collected and washed (Guebre-Xabier, 1999).

For FACS analysis, lymphocytes were resuspended PBS 0.2% FCS and incubated with FACS antibodies for 15 minutes RT. Per group, a pool of cells was analyzed. Stainings were done with the following conjugated antibodies: $CD8^+$ PerCP Cy5/$CD4^+$ PE/ CD44 APC/ CD62L FITC (memory T cells) or $CD19^+$ APC/CD27 PE (memory B cells). Cells were washed and resuspended in 200 µl for FACS acquisition on flow cytometer FACSCalibur (BD Biosciences) and data was analyzed afterwards using CellQuest software (Becton Dickinson, Immunocytometry Systems, San Jose, California, USA).

6.2.9. Electron Microscopy

Guts from mosquitoes infected with WT, *pbcrmp3*- or *pbcrmp4*- parasites were hand dissected, as previously described (Sinden, 1976). Guts were then washed twice with PBS, and fixed at 4ºC, 30 minutes, with a solution of Sodium Cacodylate 0.1 M, Sucrose 3.7% and Glutaraldehyde 2%. Next, guts were washed 3 times with a solution of Sodium Cacodylate 0.1 M plus Sucrose 3.7% (buffer SCS), pH 7.4, and stored overnight in fresh SCS. Then, guts were fixed with Osmic Acid 1% in buffer SCS, during 10 minutes RT. This process is followed by 3 washes, 10 minutes each, with buffer SCS, and incubation with a solution of 0.2% Tannic Acid in buffer SCS, 30 minutes RT. Guts were washed 3 times briefly with Sodium Cacodylate 0.1 M pH 7.4, and, next, treated with ethanol 70º twice, 5 minutes each. In a radioactive handling chemicals facility, guts were treated with a solution of 0.5% Uranyl Acetate plus 1% Phosphotungstic Acid in ethanol 70º, during 30 minutes, light protected. Afterwards, guts were dehydrated, with 2 washes with ethanol 70º, 2 washes with ethanol 95ºC and 3 washes with absolute ethanol, 5 minutes each, and treated with an pure epoxy resin, EPON, first once during 10 minutes, and then 4 times 30 minutes each. Then, guts were incubated at 60ºC overnight, with the remaining EPON. 24 hours later, the capsules were filled with fresh EPON, and incubated more 24 hours at 60ºC. After that, the guts were treated with liquid nitrogen, and remove the capsules containing the cells. The capsules were, afterwards, cut (Leica Reichter Ultracuts), mounted in Electron Microscopy 200 Mesh copper grids and observed in the Electron Microscope (TEM Jeol JEM – 100 CX II) (Vanderberg, 1967).

6.2.10. Gliding Assays for *pbcrmp3*- and *pbcrmp4*- sporozoites

WT, *pbcrmp3*- and *pbcrmp4*- sporozoites were spun for 10 minutes at 3000 rpm on glass coverslips, previously coated with gelatin 0.02% in water (for 30 minutes, excess removed and left to dry overnight) in sterile conditions and incubated 2 hours, at 37ºC. After 2 hours incubation in complete DMEM, coverslips with sporozoites were stained for mAb anti CSP (produced by a

hybridoma cell line; Charoenvit, 1991), as described in 6.2.3 and 6.2.4, except for the DNA stain with DAPI, which was omitted. Sporozoites motility ability is considered, when CSP trails originated by parasite gliding trail on the gelatin are observed (Mota and Hafalla, 2002).

6.2.11. Migration Assays for *pbcrmp3-* and *pbcrmp4-* sporozoites

Sporozoite migration through cells was quantified by detection of parasite-wounded hepatocytes using a cell-impermeant fluorescent tracer macromolecule, rhodamin-dextran. Sporozoites were incubated for 2 hours at 37ºC (as described in 6.2.3 and 6.2.4), in complete DMEM containing 1 mg/ml of rhodamine-dextran (red). After 2 hours, cells were washed twice in PBS, fixed with 2% PFA for 30 minutes (followed by three washes in PBS, 10 minutes each) and in absolute methanol overnight. Cells were stained afterwards as described in 6.2.10 for CSP protein. Migration through host cells is quantified as percentage of dextran positive non infected cells, and the number of sporozoites that reach a final hepatocyte for infection and further development, as the percentage of parasites inside dextran negative cells (Mota and Hafalla, 2002).

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Appendix I

Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells

Melissa R. van Dijk*†, Bruno Douradinha†‡, Blandine Franke-Fayard*, Volker Heussler§, Maaike W. van Dooren*, Ben van Schaijk¶, Geert-Jan van Gemert¶, Robert W. Sauerwein¶, Maria M. Mota‡, Andrew P. Waters*‖, and Chris J. Janse*

*Department of Parasitology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands; ‡Instituto Gulbenkian de Ciencia, Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal; §Section of Immunology, Bernhard Nocht Institute for Tropical Medicine, D-20359 Hamburg, Germany; and ¶Department of Medical Microbiology, University Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Immunization with *Plasmodium* sporozoites that have been attenuated by γ-irradiation or specific genetic modification can induce protective immunity against subsequent malaria infection. The mechanism of protection is only known for radiation-attenuated sporozoites, involving cell-mediated and humoral immune responses invoked by infected hepatocytes cells that contain long-lived, partially developed parasites. Here we analyzed sporozoites of *Plasmodium berghei* that are deficient in P36p (*p36p*$^-$), a member of the P48/45 family of surface proteins. P36p plays no role in the ability of sporozoites to infect and traverse hepatocytes, but *p36p*$^-$ sporozoites abort during development within the hepatocyte. Immunization with *p36p*$^-$ sporozoites results in a protective immunity against subsequent challenge with infectious wild-type sporozoites, another example of a specifically genetically attenuated sporozoite (GAS) conferring protective immunity. Comparison of biological characteristics of *p36p*$^-$ sporozoites with radiation-attenuated sporozoites demonstrates that liver cells infected with *p36p*$^-$ sporozoites disappear rapidly as a result of apoptosis of host cells that may potentiate the immune response. Such knowledge of the biological characteristics of GAS and their evoked immune responses are essential for further investigation of the utility of an optimized GAS-based malaria vaccine.

apoptosis | attenuated vaccines | host–pathogen interaction | *Plasmodium*

Upon introduction into the bloodstream by the female Anopheline mosquito during blood feeding, the infectious sporozoite of *Plasmodium* invades and multiplies within the hepatocyte. Recognition and invasion of a hepatocyte is a complex process involving traversal through macrophage-like Kupffer cells (1) and several hepatocytes (2) before forming a parasitophorous vacuole (PV) in the final hepatocyte. Currently, only a few proteins of sporozoites have been described that play an essential role in establishing infection of the liver but are thought to be conserved in all species of *Plasmodium*. These include circumsporozoite protein (CS), thrombospondin-related anonymous protein (TRAP), microneme proteins essential for cell traversal (SPECT1, SPECT2 or PPLP1, and CelTOS) and PbIMC1a, which variously are involved in motility of sporozoites, recognition of surface receptors on host cells, and traversal and invasion of host cells (3–10). Within the PV, the sporozoite transforms and grows (trophozoite stage) and multiplies (schizont stage) for a period of a few days, resulting in the generation and release of thousands of merozoites that invade red blood cells.

The current rationale for the characterization of *Plasmodium* molecules involved in liver infection lies in the development of a (subunit) vaccine that protects against liver and subsequent blood stage infection (11). Immunization studies using complete radiation-attenuated sporozoites (RAS) showed full protection against subsequent challenge with infectious sporozoites in both animal models of malaria and in human volunteers (12). The protective immunity that is observed after immunization with RAS requires that the sporozoites infect hepatocytes and transform into the trophozoite stage (13). Such immunity is mediated by complex mechanisms involving antibody responses that inhibit sporozoite motility and host cell invasion and T cell responses directed against intrahepatocytic stages. $CD4^+$ T helper cells and cytotoxic $CD8^+$ T lymphocytes recognizing MHC presented parasite-derived peptides, as well as cytokines (IL-2, IFN-γ, and IL-12; TNF-α, IL-1, and IL-6) and nitric oxide, have been shown to be critical effectors in protection against preerythrocytic malaria (13–15).

However, vaccination studies with subunit vaccines that contain only (parts of) single proteins of sporozoites have failed so far to provide any significant, long-lasting protective immunity (11, 12). The lack of significant progress with subunit vaccines stimulated recent attempts to produce a vaccine based on the nonreplicating, metabolically active RAS (12). However, such vaccines suffer from significant drawbacks, not least the question of safety and reproducibility because the amount of radiation that generates the attenuated state is strictly defined. Parasites that are underirradiated remain infectious, and those that are overirradiated do not induce protective immunity. Recently, it has been shown that genetically attenuated sporozoites (GAS) that lack sporozoite-specific conserved genes (*uis3* and *uis4*) that are apparently important for sporozoite development in the hepatocyte can induce significant or complete protective immunity in the *P. berghei* rodent model of malaria when different immunization protocols are used (16, 17). The use of GAS for vaccination might remove the uncertainty associated with RAS once a more thorough understanding of the mechanisms of immunity invoked by GAS and their developmental defect(s) are available.

In this study, we generated a GAS in *P. berghei* that lacks the sporozoite-specific protein P36p (refs. 18 and 19, and E. Lasonder, personal communication), which is a member of a small family of 10 *Plasmodium* surface proteins, the P48/45 family, that include other promising vaccine candidate antigens (20–22). Development of *p36p*$^-$ parasites aborts within the hepatocyte and immunization with *p36p*$^-$ sporozoites induces protective immune responses against subsequent challenges with wild-type sporozoites. P36p-deficient parasitized cell numbers are progressively reduced after 24 h in culture and *in vivo* and apparently fail to prevent host cell apoptosis, consequently leading to parasite clearance. Generation and analysis of biological and immunogenic characteristics of

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Abbreviations: PV, parasitophorous vacuole; RAS, radiation-attenuated sporozoite; GAS, genetically attenuated sporozoite; SC, selection cassette.

†M.R.v.D. and B.D. contributed equally to this work.

‖To whom correspondence should be addressed. E-mail: waters@lumc.nl.

multiple GAS models are essential for further development of GAS as a potential malaria vaccine.

Materials and Methods

Generation of the *p36p*$^-$ Parasite Lines. To disrupt the *p36p* locus a *p36p* replacement vector was constructed in vector b3D.D$_T$./\H./\D$_b$ containing the pyrimethamine-resistant *Toxoplasma gondii* (*tg*) *dhfr/ts* gene. To introduce *gfp* into the genome of *p36p*$^-$ parasites, a vector was constructed with the human (*h*) *dhfr* selectable marker and *gfp* under control of the constitutive *pbef-1aa* promoter (23–25) and a fragment of 2 kb of the D-type small subunit (*dssu*) rRNA gene of *P. berghei* (22). The linearized vector can integrate in C-type small subunit (*cssu*) and/or *dssu.* Integration into *cssu* does not affect the phenotype of the parasites (26). For further details, see *Supporting Text*, which is published as supporting information on the PNAS web site.

P. berghei wild-type (WT) parasites (clone 15cy1; ANKA strain) were used to generate *p36p*$^-$ parasites. Transfection, selection, and cloning of *p36p*$^-$ parasites was performed as described (27). Two independent transfection experiments were performed, and two clones (KO1 and KO2) were selected for further analysis. *p36p*$^-$ parasites (KO1) were transfected with the *gfp* vector to create *p36p*$^-$ mutants expressing *gfp* constitutively throughout the life cycle. Selection of transformed parasites was performed by treating infected animals with WR99210 (16 mg/kg bodyweight) as has been described (25). One parasite clone (KOGFP) in which the *gfp* was integrated into the *cssu* was selected for further analysis. Correct integration of constructs into the genome of transformed parasites was analyzed by RT-PCR and Southern analysis of restricted DNA or separated chromosomes by field inversion gel electrophoresis (27). PCR on DNA of WT and *p36p*$^-$ parasites was performed by using primers specific for the WT (L1362 5′-CCGCTCGAGACCTTAGGACACTTTGAAATTTG-3′ and L1363 5′-CCGCTCGAGCTACTCATAATAAGAAGAAGAGGTAC-3′; amplifying a fragment of 1.2 kb) and disrupted (L1389 5′-ATTTTGCAACAATTTTATTCTTGG-3′ and L313 5′-ACGCATTATATGAGTTCATTTTAC-3′; amplifying a fragment of 1.0 kb) locus. PCR on DNA of WT and *p36p*$^-$*:gfp* parasites was performed by using primers specific for WT (L270 5′-GTGTAGTAACATCAGTTATTGTGTG-3′ and L271 5′-CTTAGTGTTTTGTATTAATGACGATTTG-3′, amplifying a fragment of 3 kb) and disrupted *cssu* (L270 and L635 5′-TTTCCCAGTCACGACGTTG-3′, amplifying a fragment of 3 kb). Primers 1389 and 313 amplified the expected fragment of 1.0 kb of the disrupted *p36p* locus in KOGFP parasites. RT-PCR was performed on RNA isolated from WT and *p36p*$^-$ sporozoites as described by Invitrogen. To amplify cDNA derived from the *p36p*$^-$ or *circumsporozoite* (CS) gene, primers L1425 (5′-GAAATGAATATGTCGGTACTATG-3′) and L1363 (5′-CCGCTCGAGCTACTCATAATAAGAAGAAGAGGTAC-3′), amplifying a fragment of 0.5 kb, and L1502 (5′-AGTCAACAGATTATTGCCGATG-3′) and L1503 (5′-TACAAATCCTAATGAATTGCTTAC-3′), amplifying a 0.8-kb fragment, were used, respectively.

Phenotype Analysis of the *p36p*$^-$ Parasite During Blood Stage and Mosquito Stage Development. The phenotype of blood stage development was analyzed in asynchronous infections in Swiss mice and during standardized synchronized development *in vivo* and *in vitro* as described (28). Gamete formation, fertilization and ookinete production were studied *in vitro* as described (20). Oocyst formation and sporozoite development were investigated by using *Anopheles stephensi* and standard methodologies (29). The number of sporozoites per salivary gland was determined by mixing the salivary glands of 10 infected mosquitoes in 300 μl of PBS and counting the numbers of sporozoites in duplicate in a cell counter.

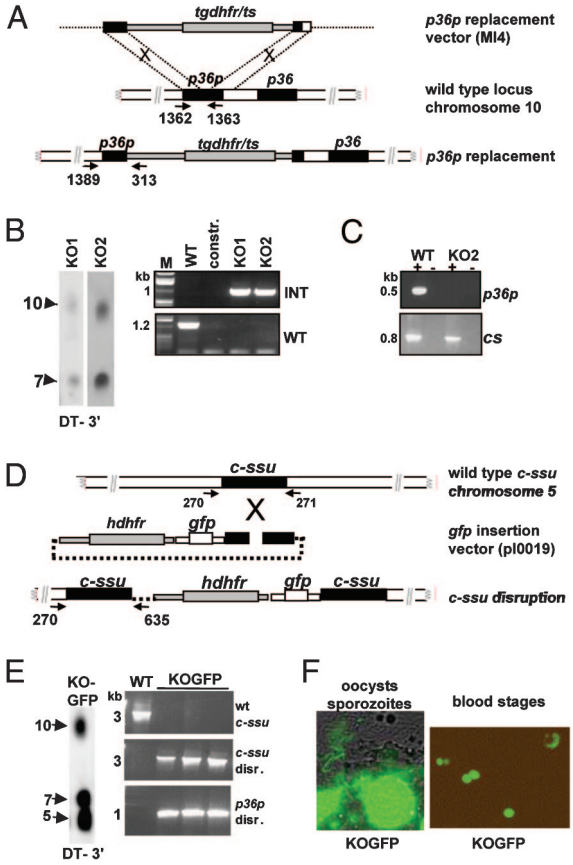

Fig. 1. Generation of the *p36p*$^-$ parasite lines. (*A*) Schematic representation of the *p36p*-locus on chromosome 10 (containing *p36* and the paralogue *p36p*) (20) and the replacement vector MI4. Correct integration of the construct results in the disrupted *p36p* gene as shown. Open box, untranslated regions; black box, *pb36p* and *pb36* coding regions; gray box, *tgdhfr/ts* SC. (*B*) Disruption of *p36p* was shown by PCR (*Right*) and by Southern analysis of separated chromosomes (*Left*). PCR on DNA of WT and *p36p*$^-$ clones (KO1 and KO2) results in the amplification of a 1.2-kb WT fragment and a 1.0-kb disrupted fragment. Chromosomes hybridized to a *P. berghei* (*pb*) *dhfr/ts* 3′ UTR region (DT-3′) specific probe detect the endogenous *dhfr/ts* copy on chromosome 7 and the integrated construct on chromosome 10. (*C*) The absence of *p36p* transcripts in *p36p*$^-$ parasites as shown by RT-PCR on RNA from WT and *p36p*$^-$ sporozoites with (+) or without (−) reverse transcriptase. (*D*) Schematic representation of the *gfp* insertion construct pl0019 and the *cssu* of the rRNA gene unit on chromosome 5 that serves as target locus for integration of the *gfp* vector by a single crossover. Black box, *cssu*; gray box, *hdhfr* SC; open box, *gfp* expression cassette. (*E*) Correct integration of the *gfp* construct in the genome of *p36p*$^-$ parasites (KO1) shown by PCR (*Right*) and by Southern analysis of separated chromosomes (*Left*). PCR on DNA of WT and *p36p*$^-$ *gfp* parasites (KOGFP) results in the amplification of a 3-kb WT fragment, a 3-kb fragment of the disrupted *cssu*, and a 1-kb fragment of the disrupted *p36p* locus. Separated chromosomes were hybridized to the DT-3′-probe, detecting *pbdhfr/ts* on chromosome 7, the *gfp* construct on chromosome 5, and the MI4 construct on chromosome 10. (*F*) GFP-fluorescent oocysts and sporozoites (*Left*) and blood stages (*Right*) of the *p36p*$^-$*gfp* parasites.

Analysis of Characteristics of the Infectivity of *p36p*$^-$ Sporozoites. The capacity of *p36p*$^-$ sporozoites to establish blood stage infections was established by infection of mice (female BALB/c and C57BL6, 15–20 g) through bites of infected mosquitoes or i.v. injection of 5×10^4 purified sporozoites, dissected from infected mosquito salivary glands (30), per mouse. Twenty to 40 infected mosquitoes were allowed to feed on each mouse 20 days after the infectious blood meal. Blood stage infections were monitored in Giemsa-stained bloodsmears or by FACS analysis of tail blood when *p36p*$^-$*:gfp* parasites were used (23) on days 4–14 after infection. Infection with WT sporozoites results in a 1–10% para-

Table 1. Mosquito development, gliding motility, hepatocyte traversal, and invasion of *p36p*$^-$ sporozoites

Parasite	Ookinete production *in vitro*,* %	Oocyst, no. mean (range)†	Sporozoite no. per salivary gland†	Mosquito infection, no of mice infected‡	Sporozoite injection: no of mice infected§	Gliding motility¶	Traversal through cells‖**	Hepatocyte invasion**	Liver trophozoites at 24 h**††
WT	78% (60–85)	165 (8–200)	114,000	4 (4)	5 (5)	4.1 ± 1.2	38.3 ± 7.6	32.4 ± 14.9	248 ± 8.1
		174 (21–230)	72,000	4 (4)	15 (15)	6.4 ± 2.1	26.2 ± 5.6	38.5 ± 12.9	330 ± 20.1
KO1	69% (60–75)	210 (120–350)	128,000	0 (5)	0 (3)	4.3 ± 1.3	37.3 ± 15.4	30.1 ± 9.2	1.3 ± 1.5
		183 (112–240)	99,000	0 (3)	0 (15)				
KO2	75% (65–80)	120 (35–160)	108,000	0 (4)	0 (3)				
KOGFP	66% (55–70)	160 (20–260)	98,000	0 (2)	0 (20)	6.1 ± 2.3	27.6 ± 1.2	39.7 ± 16.3	0

*Percentage of female gametocytes that transform into ookinetes *in vitro* under standard culture conditions as described (19).

†Mean number (and range) of oocysts in mosquitoes at day 10 after feeding on infected mice and mean number of sporozoites per salivary gland in glands dissected from mosquitoes at day 20 after the infectious blood meal.

‡Number of mice that became positive after feeding of 20–40 infected mosquitoes at day 20 after the infectious blood meal. The total number of mice that were exposed to mosquito infection are shown in parentheses. In experiments using WT parasites, all mice became positive with a parasitemia of 1–10% on day 4 or 6 after infection.

§Number of mice that became positive after i.v. injection of 5×10^4 sporozoites. The total number of mice that were injected with sporozoites are shown in parentheses. In experiments using WT sporozoites, all mice became positive with a parasitemia of 0.5–5% on day 4 or 5 after infection.

¶The gliding motility of sporozoites is defined as the average number of circles performed by a single sporozoite.

‖The capacity of sporozoites to traverse hepatocytes is defined as the percentage of dextran positive hepatocytes *in vitro* 2 h after adding sporozoites to hepatocytes.

**Hepatocyte invasion was determined by counting the number dextran-negative hepatocytes containing sporozoites, 2 h after adding sporozoites to hepatocytes *in vitro.*

††Parasite development in hepatocytes was determined by counting trophozoites present at 24 h after invasion of sporozoites into hepatocytes *in vitro.*

sitemia at day 4–6 after mosquito feeding and a 0.5–5% parasitemia on day 4 or 5 after i.v. injection.

Gliding motility of sporozoites was analyzed by counting the average number of circles performed by single sporozoites (31). Sporozoites (4×10^4) were spun for 10 min at $1,800 \times g$ onto glass coverslips previously coated with 0.02% gelatin in water, followed by incubation for 2 h at 37°C and staining with anti-CS 3D11 antibody (Ab) for sporozoite and trail visualization. Quantification was performed by counting the average number of circles performed by 100 sporozoites in three independent coverslips. Hepatocyte invasion and traversal were studied *in vitro* by adding purified sporozoites to confluent monolayers of HepG2 cells in supplemented MEM medium as described (32). Cell traversal was quantified by counting parasite-wounded hepatocytes using a cell-impermeant fluorescent tracer macromolecule, rhodamine-dextran (1 mg/ml) (2). Hepatocyte invasion was determined by counting the percentage of sporozoites inside dextran-negative cells as described (33). Sporozoite development within HepG2 cells *in vitro* was determined by staining cells using different Abs: anti-PbEXP-1, detecting a PVM-resident protein (V.H., unpublished results); and anti-HSP90 (V.H., unpublished results) or anti-HSP70 (2), detecting the parasite cytoplasmic heat shock protein 90 or 70, respectively. Cells were stained with DAPI to visualize the nuclei. Trophozoite development was quantified by counting the numbers of trophozoites 24 h after invasion of sporozoites in a whole coverslip.

***In Vitro* Analysis of Apoptosis in RAS and *p36p*$^-$ Parasite-Invaded Hepatocytes.** Two distinct fluorescent methods for apoptosis detection were used: (*i*) active caspase-3 detection, and (*ii*) nuclear morphology by DAPI staining as described (34). A total of 3×10^4 sporozoites (WT, *p36p*$^-$, or RAS; gamma source, 16 Krad, ref. 35) were added to monolayers of 2×10^5 HepG2 cells (DMEM/10% FCS/1% penicillin/streptomycin/1 mM glutamine) for 6 h before staining with anti-HSP70 Ab (2) for parasite detection. Apoptotic cells were visualized 6 h after infection by using an active caspase-3 detection kit (Promega) and DAPI staining. Infection and apoptosis rates were quantified by counting the number of parasite-infected cells per coverslip and apoptotic parasite-infected cells per coverslip respectively, in triplicate.

***In Vivo* Analysis of Apoptosis in *p36p*$^-$ Parasite-Invaded Hepatocytes.** Two C57BL/6 mice per group were infected with 5×10^5 *p36p*$^-$ or WT sporozoites by i.v. injection. Livers were removed 6 h after infection and snap-frozen, and 20 histological sections from each mouse were stained for both parasite detection (anti-HSP70; ref. 2) and nuclear morphology by DAPI staining. All sections were examined for the presence of infected cells with signs of apoptosis.

Analysis of the Immunization Potential of *p36p*$^-$ Sporozoites. Groups of BALB/c and C57BL6 mice were immunized by i.v. injection of *p36p*$^-$ sporozoites or RAS (35) or PBS and monitored for blood stage parasitaemia in Giemsa-stained bloodsmears. Mice were challenged with different doses of WT sporozoites on different time points. Animals were either monitored for blood stage parasitemia in bloodsmears every other day starting from day 3 to 3 weeks after challenge or killed 40 h after challenge for liver extraction and quantification of infection by real-time PCR quantification of A-type 18S ribosomal RNA copies (36). For each set of experiments, groups of naïve mice were included to verify infectivity of the sporozoite challenge dose.

Results

Generation and Characterization of *p36p*$^-$ and *p36p*$^-$*:gfp P. berghei* Parasites. To investigate the function of P36p, we created two independent, cloned lines of *P. berghei* parasites that are deficient in P36p (*p36p*$^-$) by using targeted disruption of the P36p gene through double crossover homologous recombination (27) (Fig. 1). In addition, to better visualize and count the *p36p*$^-$ parasites, we introduced *gfp* under control of a constitutive promoter in the genome of one of the *p36p*$^-$ lines (*p36p*$^-$*:gfp*; Fig. 1). The inability of the *p36p*$^-$ parasites to produce transcripts of *p36p* and thus express P36p in sporozoites was shown by RT-PCR (Fig. 1). All three *p36p*$^-$ lines show no phenotype that is different from WT parasites during blood stage development (results not shown) and development in the mosquito. The characteristics of fertilization and zygote development as well as oocyst and sporozoite production in the mosquito were not affected in the independent *p36p*$^-$ lines (Table 1).

P36p Is Essential for Sporozoite Development within the Hepatocyte. The infectivity of *p36p*$^-$ sporozoites to the host (BALB/c and C57BL6 mice) was strongly affected, as was shown by the complete absence of a blood stage infection after infection through the bite of mosquitoes or after i.v. inoculation of purified salivary gland sporozoites (Table 1). However, occasionally, some mice from both host strains (10% C57BL6, i.e., 5 of 48 mice; 4% BALB/c, i.e., 1 of

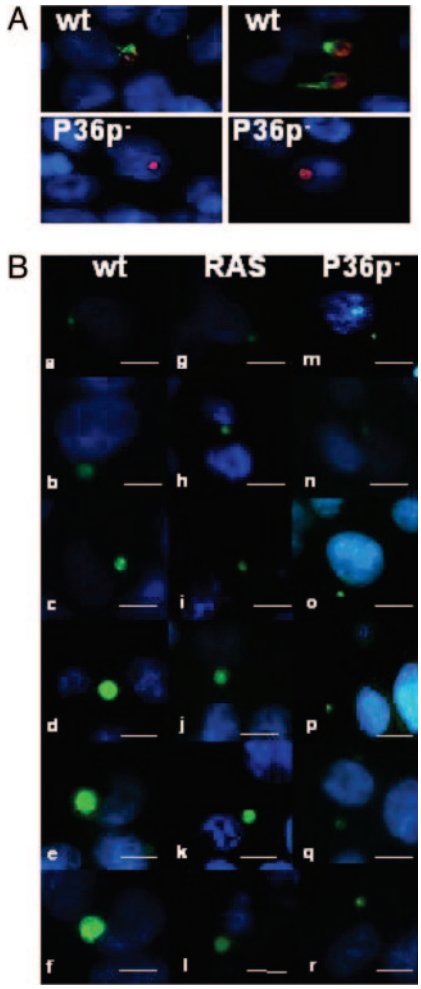

Fig. 2. Development of WT, RAS, and *p36p*$^-$ sporozoites in hepatocytes *in vitro*. Cells were stained by using anti-PbEXP-1 to detect the PVM, anti-HSP90, or HSP70 to visualize the parasite cytoplasm and DAPI to stain the nuclei (blue fluorescence). (*A*) Visualization of the PVM (green, anti-PbEXP-1) in trophozoites of WT and *p36p*$^-$ parasites (red, anti-HSP90) at 15 and 24 h after infection, respectively. (*B*) Trophozoite development of WT (*a–f*), RAS (*g–l*), and *p36p*$^-$ (*m–r*) parasites at 6, 12, 15, 18, 21, and 24 h after invasion using anti-HSP70 (green; bar, 10 μm).

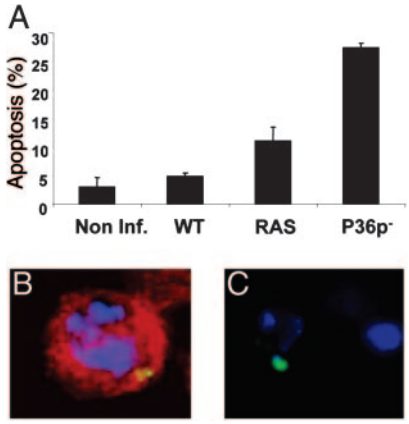

Fig. 3. Apoptosis is increased in *p36p*$^-$ parasitized liver cells. (*A*) Apoptosis rates represent the percentage of parasite-invaded cells that undergo apoptosis 6 h after infection of HepG2 cells with WT, RAS, or *p36p*$^-$ sporozoites. Non. Inf. indicates noninfected HepG2 cell cultures. Error bars represent SD. (*B* and *C*) Visualization of *p36p*$^-$ sporozoite (green, anti-HSP70) infected hepatocytes displaying typical apoptotic signs as detected by DAPI staining (blue) *in vitro* (*B*) as well as *in vivo* (*C*). Active caspase-3 (red) detection was also performed in *B*. (Original magnification, ×1,260.)

26 mice) did develop a 7-day delayed blood stage infection upon i.v. injection of *p36p*$^-$ sporozoites in a parasite dose-independent fashion (data not shown). Interestingly, blood stage infections were never observed when *p36p*$^-$ sporozoites were transmitted naturally through mosquito bites. The resulting blood stage parasites still contained the knockout genotype, as analyzed by PCR (data not shown).

Gliding motility is a feature of *Plasmodium* sporozoites and associated with invasion of both salivary glands and hepatocytes (4). *p36p*$^-$ sporozoites are unaffected in their ability to glide (Table 1) and, therefore, the loss of infectivity of these sporozoites is not due to disrupted motility.

We analyzed the ability of *p36p*$^-$ sporozoites to traverse and infect human hepatocyte cells (HepG2) in culture. HepG2 cells support full exoerythrocytic development of *P. berghei* sporozoites into mature schizonts containing erythrocyte-infectious merozoites (37). *p36p*$^-$ sporozoites were able to traverse and infect hepatocytes as well as WT sporozoites (Table 1).

Sporozoite invasion of hepatocytes requires sporozoite apical secretion, permitting the subsequent formation of a PV within which the developing parasite resides (38). Induction of apical secretion by incubation of sporozoites with HepG2 cell extracts showed that both WT and *p36p*$^-$ sporozoites present a similar level of activation of the apical complex (data not shown). Immunofluorescent antibody test analysis revealed that WT sporozoites generate a clearly visible PV membrane (PVM) formation containing PbExp1, a PVM-resident protein, in infected HepG2 cells that is completely absent in HepG2 cells at 15 and 24 h after infection by *p36p*$^-$sporozoites (Fig. 2). In sharp contrast to WT sporozoites, which gave rise to mature schizonts after 60–72 h of *in vitro* culture in HepG2 cells, *p36p*$^-$ sporozoites develop poorly (Fig. 2), and most parasitised hepatocytes could no longer be detected after 24 h (Table 1). Equal numbers of *p36p*$^-$, RAS, and WT sporozoites were observed in dextran-impermeant hepatocytes; therefore, *p36p*$^-$ sporozoites might initiate but not maintain a PVM in HepG2 cells.

The aborted development of *p36p*$^-$ sporozoites shows several similar characteristics to the aborted development of RAS, yet trophozoite development of *p36p*$^-$ parasites is aborted at an earlier stage compared to RAS (Fig. 2).

***p36p*$^-$ Sporozoite-Infected Hepatocytes Enter Apoptosis More Frequently than RAS or WT Parasite-Infected Hepatocytes.** Host hepatocyte apoptosis is normally inhibited by WT parasites upon invasion and establishment of the PV (34). Therefore, it was possible that the observed rapid disappearance of *p36p*$^-$ sporozoite-infected HepG2 cells in culture was due to the inability of the parasite to prevent the hepatocyte from undergoing apoptosis. Consequently, the level of apoptosis in *p36p*$^-$ sporozoite-invaded cells was examined (Fig. 3*A*). In three independent experiments, HepG2 cells were incubated with WT, RAS, or *p36p*$^-$ sporozoites, and apoptosis was determined 6 h after infection by detection of active caspase-3 in the infected host cell cytoplasm and by analysis of the nuclear morphology of the infected cells after DAPI staining by observing chromatin condensation and/or nuclear fragmentation (Fig. 3*B*). Interestingly, we also detected apoptosis of *p36p*$^-$ parasitized hepatocytes *in vivo* 6 h after infection (Fig. 3*C*). The level of apoptosis in *p36p*$^-$ parasitized cells was significantly higher ($P < 0.05$) compared to that in RAS-infected cells, which was higher than the level observed in WT-infected cells (Fig. 3*A*). The different levels of apoptosis are consistent with the observed longer survival time of RAS in culture (39).

Immunization with *p36p*$^-$ Sporozoites Protects BALB/c and C57BL6 Mice Against Subsequent Infection. Because RAS has been successfully used in immunization studies for generation of protection

Table 2. Immunization with *p36p*$^-$ sporozoites protects against a subsequent infection with WT sporozoites

Experiment no.	Mouse strain	Immunization,* RAS/*p36p*$^-$ × 10^3	Challenge dose†, WT × 10^3	Time of challenge, days after final immunization	No. protected (no. challenged)		
					Control	RAS	*p36p*$^-$
1	BALB/c	100	50	10	0 (10)	ND	18 (20)
2	BALB/c	50	25	10	0 (15)	15 (15)	15 (15)
3	BALB/c	50	10	10	0 (5)	ND	5 (5)
3	BALB/c	50	10	30	0 (5)	ND	5 (5)
3	BALB/c	50	10	60	0 (5)	ND	5 (5)
3	BALB/c	50	10	120	0 (5)	ND	5 (5)
4	BALB/c	50	1,000 iRBC	10	0 (3)	0 (5)	0 (4)
1	C57BL6	50	10	10	0 (3)	0 (3)	0 (1)
1	C57BL6	50/20	10	10	0 (3)	1 (3)	1 (4)
1	C57BL6	50/20/20	10	10	0 (5)	5 (5)	4 (4)
1	C57BL6	50/20/20	10	30	0 (5)	5 (5)	5 (5)

*Groups of mice were immunized intravenously with one of PBS (control), RAS, or *p36p*$^-$ sporozoites isolated from different mosquito batches. Multiple immunizations with RAS or *p36p*$^-$ sporozoites were administered at 7-day intervals.

†Mice were challenged with WT sporozoites or parasite infected red blood cell (iRBC) stages and the prepatent period monitored by either counting Giemsa-stained bloodsmears or FACS analysis. All control, unimmunized mice became positive on day 4 or 5 after challenge. Sporozoites were isolated from different mosquito batches.

against subsequent challenge with WT sporozoites, we investigated the immunization potential of *p36p*$^-$ sporozoites. Mice were i.v. immunized with *p36p*$^-$ sporozoites by using different immunization protocols and subsequently challenged with WT parasites. Protection was determined by using two different detection assays. (*i*) In three independent experiments, groups of four BALB/c mice were infected with either 10^5 *p36p*$^-$ sporozoites or 10^5 RAS. After 10 days, mice were challenged with 5×10^4 WT sporozoites and assessed for liver stage development by using real-time PCR assays to quantify A-type ribosomal RNA transcripts produced by developing trophozoites (36). Mice immunized with RAS and *p36p*$^-$ sporozoites showed a very strong and comparable reduction (>99%) in liver stage development of WT parasites compared to nonimmunized mice (Fig. 4, which is published as supporting information on the PNAS web site). (*ii*) In several independent experiments groups of BALB/c and C57BL6 mice were immunized with a single dose of 10 to 5×10^4 *p36p*$^-$ sporozoites or multiple times (one to three immunizations) with 5 to 2×10^4 *p36p*$^-$ sporozoites, respectively, and challenged with different doses of WT sporozoites on different days after immunization (Table 2). Protection was determined by monitoring mice intermittently for blood stage parasitemia in bloodsmears or using FACS analysis from day 3 to 3 weeks after challenge.

A single immunization dose of *p36p*$^-$ sporozoites fully protected BALB/c mice (53 of 55) and induced a protective immune response that lasted for at least 4 months (120 days), whereas three subsequent immunizations with *p36p*$^-$ sporozoites were required to completely protect C57BL6 mice (9 of 9), resulting in a protective immune response that lasted for at least one month (30 days), as shown by the absence of subsequent blood stage infection after challenge with WT sporozoites. Furthermore, in two nonprotected BALB/c mice (Table 2; experiment 1) liver development was strongly inhibited ($\approx 10^3$), as shown by a 3-day delay in patent parasitemia. Five protected BALB/c animals (Table 2; experiment 1) were rechallenged, 1 and 2 months after immunization, respectively, with WT sporozoites and did not develop blood stage infections up to 2 weeks after challenge. C57BL6 mice were partially protected when immunized only twice with either RAS (33%) or *p36p*$^-$ sporozoites (25%) (Table 2).

The protective immune response induced by *p36p*$^-$ sporozoites as well as RAS sporozoites seemed to be parasite stage-specific, because BALB/c mice were not protected from a challenge with parasite-infected red blood cells and developed a normal blood stage infection comparable to the control group (Table 2).

Discussion

The lack of significant progress with (recombinant) subunit vaccines that contain only (parts of) single proteins of sporozoites has led to renewed interest in vaccines based on live, attenuated whole sporozoites (12). Recently, it has been shown that GAS, like RAS, produced by engineered inactivation of sporozoite-specific genes can induce protective immunity in the *P. berghei* rodent model of malaria (16, 17). Our studies demonstrate another example of GAS that is produced through the inactivation of the sporozoite-specific protein P36p and induces protective immunity. P36p is a member of the P48/45 family of surface proteins (21) that include a number of promising vaccine candidate antigens, such as P48/45 and P230 that are expressed on the surface of gametes (20, 22). P36p has no function in sporozoite motility and invasion of both salivary glands and hepatocytes, and instead plays an essential role during development of the liver trophozoite. *P36p*$^-$ parasites apparently fail to maintain a PV, arresting early during intrahepatocytic development; importantly, immunization with *p36p*$^-$ sporozoites induces a fully protective immune response against subsequent challenge with WT sporozoites. Interestingly, as has been shown for RAS, only a single immunizing dose of *p36p*$^-$ sporozoites is required to elicit a long-lasting protective immune response in BALB/c mice (40) and induces a protective immune response that is parasite stage specific (16, 17). As in the immunization studies performed with *uis3*$^-$ and *uis4*$^-$ GAS (16, 17) and RAS (40), three immunizing doses of *p36p*$^-$ sporozoites are required to completely protect C57BL6 mice for minimally 1 month. Furthermore, our results demonstrate that the choice of the host strain in studying the mechanisms of protective immunity induced by attenuated sporozoites is critical, as has been shown by earlier studies (40). Intriguingly, *p36p*$^-$ sporozoites, like *uis4*$^-$ GAS, albeit at a lower frequency, initiated a delayed blood stage infection in some mice, but only when *p36p*$^-$ sporozoites are inoculated i.v. The reasons for this are unclear and await further investigation. There is a close paralogue of *p36p*, *p36* that is also expressed in sporozoites (E. Lasonder, personal communication) and located directly adjacent to *p36p* in the parasite genome. Improved levels of protection may be achieved by creating a double *p36p*$^-$/*p36p*$^-$ parasite.

It is now possible to consider a comparative study of the intrahepatocytic development and the mechanism(s) of immunity invoked by different GAS and RAS. Like GAS, RAS sporozoites invade liver cells and transform into the rounded trophozoite stage, but do not enter the process of schizogony. However, trophozoite development of RAS arrests at a later stage and produces a visible PV compared to *p36p*$^-$ sporozoites. Moreover, RAS-infected hepa-

tocytes persist in culture longer than their GAS-infected counterparts, which, in the case of *p36p*$^-$ parasites, results from their failure to prevent the host cell from entering apoptosis. Although RAS populations provoke apoptosis to a greater degree than WT sporozoites, they do so to a significantly lower degree than *p36p*$^-$ sporozoites. We also determined *in vivo* the persistence of RAS- and *p36p*$^-$-infected hepatocytes in whole liver extracts of BALB/c mice by using real-time PCR for liver schizont detection (36). Our results confirm the significantly more rapid clearance of *p36p*$^-$ parasitized hepatocytes within 6 h after infection compared to RAS-infected hepatocytes as has been observed *in vitro* (data not shown). However, similar low numbers of RAS- and *p36p*$^-$-infected hepatocytes are observed 1 week after infection.

Immune responses against RAS are complex and involve both cell-mediated and humoral immunity (13–15). However, the differing biological characteristics of RAS and GAS described here suggest that the immune responses elicited by RAS and GAS could be different. For example, cell death by apoptosis was originally described to occur in the absence of inflammation. Interestingly, in the context of infection, apoptosis is being redefined based on a number of studies demonstrating that apoptotic death of host cells after pathogen infection can trigger powerful innate and adaptive immune responses. In fact, apoptosis-induced inflammation is actively being investigated as a way of enhancing vaccine function improving accessibility of the effector cells of the immune system to the site of infection (41, 42). Moreover, presentation of parasite antigens in the context of host cell apoptosis has recently been shown as an alternative mechanism involved in the induction of protective immunity by RAS (43). As apoptosis of *p36p*$^-$-infected hepatocytes was also detected *in vivo*, it might well be that host-cell apoptosis is associated with the ability of *p36p*$^-$ sporozoites and other GAS to induce protective immune responses and needs further investigation.

The mechanisms and (parasite) molecules involved in preventing host cell apoptosis by intracellular parasites including apicomplexans are being investigated (44, 45). Recently, it has been shown that *Plasmodium* parasites exploit the host molecule hepatocyte growth factor (HGF), secreted exclusively by hepatocytes damaged by parasite transit (32), to activate anti-apoptotic signaling pathways in the host cell through HGF/MET signaling (34). The ability of the *p36p*$^-$ sporozoites to invoke this pathway is currently unknown; however, such interactions might now be explored in greater detail. In particular, these studies will benefit from the use of a battery of GFP-expressing parasites available in our laboratory, including (radiation-attenuated) WT parasites expressing GFP constitutively (PbGFPcon) (23) and the *p36p*$^-$*:gfp* parasites reported here. These GFP-expressing lines will, for example, provide a means to purify by flow sorting cultured, infected hepatocytes containing aborted parasites allowing a detailed analysis of the transcriptome and proteome of both parasite and host cells and as a consequence shed more light on the parasite strategies used to influence host cell apoptosis.

Furthermore, our studies show that *p36p*$^-$ sporozoites are able to invade hepatocytes but seem to be unable to produce a mature PVM, which might be a critical step in the intracellular survival of the parasite. It might well be that the presence of a PVM is essential in escaping from host defense mechanisms (avoiding internal cellular surveillance by the hepatocyte). Indeed, if the parasite enters the host cell either without a PVM or with an incomplete PVM, infection may be readily detected by the host cell, and this may in turn trigger xenophagy, leading ultimately to an inflammatory apoptotic response (46). Therefore, the attenuated parasite may survive only as long as either the killing response of the host cell allows or the parasite can avoid or neutralize the host cell defense mechanisms.

P36p may be essential for intracellular survival/development through being involved in an essential preceding step affecting gene-expression through signaling; therefore, the absence of P36p results in the lack of the necessary parasite mediators that control parasite survival, formation and/or maintenance of an effective PV, and direct involvement in the regulation of the host cells apoptotic machinery. Interestingly, P36p is anticipated to be a GPI-anchored membrane protein and, therefore, may play a role in the interactions between the developing trophozoite and its host cell.

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Appendix II

Genetically attenuated P36p-deficient *Plasmodium berghei* sporozoites confer long-lasting protection and partial cross-species immunity

Running title: GAS whole-organism based experimental malaria vaccine

Bruno Douradinha[†1], Melissa R. van Dijk[†2], Ricardo Ataide[1], Geert-Jan van Gemert[3] Joanne Thompson[4], Dominique Mazier[5], Robert Sauerwein[3], Chris J. Janse[2], Andrew P. Waters[2], and Maria M. Mota[1,*]

[1]Unidade Malaria, Instituto de Medicina Molecular, Universidade de Lisboa, 1649-028, Lisboa, Portugal
[2]Department of Parasitology, Leiden University Medical Center, P.O. Box 9600, 2300 RC, Leiden, The Netherlands
[3]Department of Medical Microbiology, University Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[4]Institute of Immunology and Infection Research, Ashworth Laboratories, School of Biological Science, University of Edinburgh, The Kings Buildings, West Mains Road, Edinburgh EH9 3JT, United Kingdom
[5]Inserm U511, Immunobiologie Cellulaire et Moléculaire des Infections Parasitaires, Centre Hospitalo-Universitaire Pitié-Salpêtrière, Université Pierre and Marie Curie, F-75013 Paris, France

† B.D. and M. R. v. D. contributed equally to this work

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* Corresponding author: Mailing address: Unidade Malaria, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028, Lisboa, Portugal. Phone: 351 21 799 9509. Fax: 351 21 799 9504. E-mail: mmota@fm.ul.pt

Abstract

Immunization with *Plasmodium* radiation-attenuated sporozoites (RAS) can confer sterile protection against malaria in humans, monkeys and mice. More recently, genetically-attenuated sporozoites (GAS) inducing the same protective immunity in mice were generated by genetic modification of *P. berghei* parasites.

Our previous work with one of those GAS has demonstrated that sporozoites lacking the sporozoite specific protein Pb36p (*pb36p*-), induce a protective immune response in both C57BL6 and BALB/c mice, that lasted for up to 1 and 4 months, respectively. Here, we show that protection induced by *pb36p*- sporozoites can last at least up to 12 and 18 months, in C57BL6 and BALB/c mice, respectively (last time point tried for either strain). Protection is also achieved when *pb36p*-parasite numbers were reduced as low as 1,000 sporozoites per immunization or when different routes of immunization such as subcutaneous, intradermal or intramuscular were used.

Moreover, immunization with either RAS or *pb36p*- sporozoites also protects against subsequent challenge with a heterologous rodent *Plasmodium* species, *P. yoelii*. To our knowledge, this is the first report of attenuated malaria sporozoites inducing cross-species protection. These results reinforce the potential of using live attenuated sporozoites as a long-lasting and cross-species experimental vaccine against malaria.

Introduction

Malaria claims the lives of nearly 2 millions African children annually and constitutes a huge impairment in social and economical development of countries where it is endemic.

Malaria infection occurs when an infected anopheline mosquito bites a host and *Plasmodium* sporozoites reach the bloodstream, heading directly to the liver and invade the hepatocytes, their first obligatory target, for further development. Such process involves migration through several hepatocytes (Mota, 2001; Mota and Hafalla, 2002) and activation of signalling pathways such as HGF/MET (Carrolo, 2003), before establishing infection in a final hepatocyte with formation of a parasitophorous vacuole. Subsequently, they develop and mature into trophozoites, schizonts and finally into merozoites, which will be released to the bloodstream, initiating the symptomatic phase of infection.

The hepatic stage of a *Plasmodium* infection constitutes an appealing target for the development of an intervention strategy, where the aim is to act before the onset of clinical symptoms. In fact, up to present, the only demonstrable effective vaccine shown to confer a sterile and lasting protection both in mice (Nussenzweig, 1967) and in humans (Clyde, 1973) relies upon the inoculation of radiation-attenuated sporozoites (RAS), that are able to invade but not fully mature inside the hepatocyte. For these reasons, the development of a vaccine targeting the pre-erythrocytic stage of infection has drawn much attention in recent years. The leading vaccine candidate, RTS,S/AS02A, a subunit vaccine, containing parts of the *P. falciparum* circumsporozoite protein (CSP) fused to the hepatitis B virus surface antigen, is already in Phase IIb trials (both in adults and in children). However, to date, the vast majority has only short-lived protection, achieving low efficacy (reviewed in Hill, 2005). A vaccine that would induce long-lasting protection against malaria is highly desirable, since single doses would be able to confer immunity for an extensive period, being ideal for sub-Saharan, military personnel and travellers. So far, only *P. falciparum* RAS immunizations have conferred long-lasting protection to human volunteers against challenge with infectious sporozoites, lasting up to 10.5 months (Hoffman, 2002). Additionally, RAS immunizations present some concerning disadvantages, namely safety and strict control of radiation dose. When high doses are applied to parasites, loss

of protective immunity is observed, and low doses do not lead to impairment on development of parasites in the liver, remaining therefore infectious.

An additional major hurdle for developing a live-attenuated *Plasmodium* sporozoites vaccine is the production of adequate number of sporozoites (Luke, 2003). To overcome this obstacle, one must determine the minimum dose of attenuated parasites which can confer protection. In humans, it is estimated that the minimum dose of *P. falciparum* RAS to confer immunity would be approximately 10^4-10^5 sporozoites (Luke, 2003).

To date, public health measures state that all vaccines must be administered by one of the following routes: subcutaneous, intramuscular or intradermal (Luke, 2003). Therefore, a vaccine based on live attenuated parasites should also fulfil this requirement. A small proportion of mice immunized intramuscularly with *P. berghei* RAS were fully protected while the remaining had a delay on blood-stage parasitemia. In similar conditions, no protection was attained using subcutaneous immunization (Kramer, 1975). Studies in human volunteers need to be performed to validate the feasibility of these routes of a *P. falciparum* RAS based vaccine (Luke, 2003).

Recently, genetically-attenuated sporozoites (GAS) in *P. berghei*, which are also impaired in liver development but are capable of conferring immunity to mice against challenge with infectious sporozoites have been described (Mueller, 2005; Mueller and Camargo, 2005; van Dijk, 2005). One of those GAS suffered a genetic modification which made it unable to express Pb36p, a sporozoite's microneme protein that is excreted to surface during gliding (Ishino, 2005). P36p is a member of the 6-cys protein family, which comprises 10 members, some of them constituting potential vaccine candidates (van Dijk, 2001; Thompson, 2001). Here we show that protection induced by *pb36p*- sporozoites can last up to 18 months and that a low immunization dose as 1,000 *pb36p*- sporozoites is sufficient to confer protection. Furthermore, mice immunized intramuscularly, subcutaneously and intradermally with *pb36p*- sporozoites are found protected when challenged with infectious *P. berghei* sporozoites either intravenously or by mosquito bite. More surprisingly, this protection is not restricted to challenges with *P. berghei* homologous parasites. Both RAS and *pb36p*- sporozoites also protect mice against challenge with a heterologous rodent species, *P. yoelii*, with some mice even achieving sterile protection. To our best knowledge, this describes for the first time that experimental attenuated malaria sporozoite vaccines are capable of inducing cross-species protection. These findings have important implications regarding new strategies in developing a vaccine against malaria.

Material and Methods

Mice. BALB/c (H-2K^d) and C57BL6 (H-2K^b) were bred in the animal facility of Instituto Gulbenkian de Ciência (Oeiras, Portugal) or purchased to Harlan Ibèrica (Barcelona, Spain). For all experiments, 6-8 weeks old mice were use (except for long-lasting protection experiments, due to obvious reasons). All animal care and procedures were in accordance with European regulations.

Parasites. *P. berghei* parasites (clone 15cy1; ANKA strain) expressing GFP (PbGFPcon; Franke-Fayard, 2004), *pb36p- P. berghei* parasites (clone 1; ANKA strain) expressing GFP (*p36p-:gfp*; van Dijk 2005) and *P. yoelii* (265 BY strain) were maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and BALB/c mice. Sporozoites for immunizations/challenge studies were obtained by hand dissection of infected mosquitoes 18-21 days (*P. berghei*) or 14-17 days (*P. yoelii*) after mosquitoes had taken an infectious blood meal, as described elsewhere (Ozaki, 1984).

Immunization and Parasite Challenge Experiments. Mice were immunized with *pb36p-:gfp* sporozoites or PbGFPcon RAS (Orjih, 1982). According to the experiment performed, different immunization doses and administration routes (intravenous, intramuscular, subcutaneous or intradermal) were used. Immunization protocols are described further. Mice were challenged intravenously (i.v.) with 10,000 *P. berghei* sporozoites or *P. yoelii* (different doses of sporozoites, according to the experiment performed), at different time points (as illustrated in next section) or by *P. berghei* infected mosquitoes bite (6 highly infected mosquitoes, as confirmed subsequently, per mouse). Animals were monitored daily for blood stage parasitemia either by bloodsmears or by FACS analysis (when GFP parasites were available) 2-3 weeks after challenge, starting day 3. Naive animals were included in all experiments to verify infectivity of the sporozoite challenge dose.

Real-Time PCR. *P. yoelii* parasite burden in the liver was measured 40 h after challenge. Parasite-specific 18S rRNA was evaluated by a quantitative real-time reverse

transcription method, as described somewhere else (Bruña-Romero, 2001). Protection was defined as the percent of liver infection inhibition, and was estimated according to: $(1 - rRNA_{immunized} / rRNA_{naive})\times 100$, were $rRNA_{immunized}$ and $rRNA_{naive}$ represent the number of copies of parasite 18S rRNA in the livers of immunized and naïve challenged mice, respectively.

Statistical analysis. Student's t test was performed with the Prism version 4.03 software (GraphPad Software, San Diego, CA). *P* values of less than 0.05 were considered statistically significant.

RESULTS

pb36p- sporozoites induce long-lasting protection against continuous challenges with *P. berghei* sporozoites

BALB/c mice immunized with *pb36p*- sporozoites that were protected against challenge with wild-type (WT) *P. berghei* up to 4 months (120 days) (van Dijk, 2005) were re-challenged i.v with 10,000 WT parasites 6 months (180 days) post final immunization, and are fully protected (none has developed blood-stage parasites up to 3 weeks after challenge). In contrast, blood stage parasites could be observed in control mice on day 3.0 after challenge (Table 1, group 1). We next sought to test if this protection could last up to 1 year. The same group of protected BALB/c mice was again challenged i.v. with 20,000 WT sporozoites nearly 12 months (351 days) after immunization. A higher dose was used, since although age has no effect on lethality in *P. berghei* in mice, older rats are able to completely control the infection (Pierrot, 2003; Greenberg, 1953; Singer, 1955). All mice were fully protected and failed to develop blood stage parasites up to 3 weeks after the challenge, while blood stage parasites could be detected on day 3.5 in control animals (Table 1; group 1). Moreover, these mice were still fully protected when re-challenged i.v. with 20,000 WT *P. berghei* sporozoites 18 months after last boost, unlike matched naïve control mice (all positive on day 3.0 after challenge). The immune response elicited by *pb36p*- sporozoites was still able to fully protect BALB/c mice after 18 months (Table 1, group 1).

C57BL6 mice immunized i.v. with three doses of RAS or *pb36p*- were protected at least up to 1 month (30 days) after the last immunization (van Dijk, 2005). Like in BALB/c mice, we propose to determine the protective immune response persistence. After the

final immunization, these mice received i.v. challenges of 10,000 WT *P. berghei* parasites 2 and 3 months post final immunization and, of 20,000 WT sporozoites 6 months post final immunization. A complete absence of blood stage parasites up to 3 weeks post challenge was observed in 100% of the mice immunized with either RAS or *pb36p*- sporozoites (Table 1, group 2). *pb36p*- sporozoites were able to confer, like RAS-based immunizations, sterile protective immunity that lasts up to 6 months in C57BL6 mice. These mice were re-challenged, 12 months after last immunization, and 50% of *pb36p*- immunized mice were still fully protected (Table 1, group 2). Nevertheless, *pb36p*- immunized mice which developed parasitemia have a longer pre-patency period (4.5 days), when compared with naïve matched control (3.0 days).

BALB/c mice immunized with *pb36p*- sporozoites do not require continuous challenges to maintain protection up to 6 months

Previous immunization experiments were performed with subsequent multiple challenges within the same groups of mice. We asked whether the long-lasting protection observed was due to the constant strengthening of the immune response, elicited by the continuous presentation of antigens during infectious sporozoite challenge. To test this, RAS and *pb36p*- immunized mice were challenged i.v. with a single challenge performed after longer periods post immunization. Groups of BALB/c mice were immunized i.v. (50,000/20,000/20,000 weekly apart) of either RAS or *pb36p*- parasites, and challenged with 10,000 WT *P. berghei* sporozoites 2 or 3 months (60 or 90 days, respectively) or with 20,000 WT *P. berghei* sporozoites 6 months (180 days) after final immunization (Table 2). None of these immunized mice became ever positive for blood stage parasites whereas all control mice for each challenge experiment developed blood-stage parasitemias, as expected. These results show that full and long-lasting protection does not require continuous challenges to be maintained up to 6 months in BALB/c mice, when using a prime plus 2 boosts immunization regimen.

Furthermore, immunized mice which have shown to be protected 2 and 3 months after last immunization were also re-challenged with 20,000 infectious parasites 6 months (180 days) after last immunization (Table 1, groups 3-4). These mice were still protected and did not developed blood stage parasites, unlike their matched controls. These mice, as well as those which shown to be protected 6 months after final immunization, were challenged again 12 months subsequent to last immunization

(Table 1, groups 3-5). The results show that, after one year, protection starts waning as not all mice are fully protected. Still, pre-patency periods for immunized mice which became infected (Table 2, groups 3 and 5) is longer when compared with naïve control mice (6.0 and 5.0 days, respectively, vs. 3.0 days for naïve matched control).

Lower doses of *pb36p*- sporozoites confer protection against subsequent challenge

In any attenuated whole-organism immunizations the determination of the minimum number of pathogens required to confer protection is crucial for vaccine design. C57BL6 mice were immunized i.v. with RAS or *pb36p*- in three equal doses (with one week interval between them), of 10,000, 5,000 or 1,000 sporozoites. Control mice were also immunized i.v. with RAS or *pb36p*- sporozoites but with the previous regimen (50,000/20,000/20,000). Ten days after last immunization, all mice were challenged i.v. with 10,000 WT sporozoites. Mice immunized with three doses of 5,000 or 10,000 of RAS or *pb36p*- sporozoites were fully protected (as controls) against challenge with 10,000 WT sporozoites (Table 3). Matched naïve controls became blood-stage positive 3.2 days post challenge.

Three doses of 1,000 RAS sporozoites were not sufficient to protect mice against a challenge with 10,000 sporozoites. Nevertheless, they were partially protected as a delay in pre-patency was observed (6.2 days), when compared to naïve control infected mice (3.2 days). However, and contrary to RAS immunized mice, three doses of 1,000 *pb36p*- sporozoites confer full protection to mice posterior challenged with 10,000 *P. berghei* sporozoites. This difference between RAS and *pb36p*- immunizations is reproducible and observed in different independent experiments. The results suggest that *pb36p*- can confer protection with lower number of sporozoites than RAS.

Different routes of administration of *pb36p*- and RAS sporozoites partially protect against infection

Intravenous or mosquito bite as routes for sporozoite administration are not acceptable currently for human vaccination. Thus, we sought to determine whether *pb36p*- sporozoites would confer protection when administered by suitable routes. C57BL6 mice were immunized intramuscularly (i.m.), subcutaneously (s.c.) and intradermally (i.d) with *P. berghei* RAS or *pb36p*- sporozoites, using a prime plus 2 boots regimen (50,000/20,000/20,000, one week interval between them). Control mice were immunized i.v. using the same regimen. Ten days after last boost, mice were challenged

i.v with 10,000 WT *P. berghei* sporozoites. All mice immunized i.v with either RAS or *pb36p*- sporozoites were fully protected against challenge with infectious *P. berghei* sporozoites, as expected (Table 4). Some sterile protection was achieved (Table 4), in both i.m immunizations (20% and 13% for RAS and *pb36p*- immunized mice, respectively) and for RAS i.d. immunized mice (20%). The remaining immunized mice (both RAS and *pb36p*-), which became positive had a strong delay in pre-patency period, ranging from 4.5 to 5.3 days, while naïve mice developed blood-stage parasitemia 3.2 days after challenge (Table 4). Altogether, the results show that although other routes of administration are not as efficient as i.v., a partial protection is still achieved.

All challenges until now were performed by i.v. injection with 10,000 sporozoites that do not mimic the natural infections. Thus, we decide to perform *pb36p*- immunizations using the same administrations routes as before but followed by mosquito bite challenges. C57BL6 mice were immunized i.v., i.m., s.c. and i.d with *pb36p*- sporozoites (50,000/20,000/20,000, weekly apart) as above. Ten days later, *P. berghei* infected mosquitoes were allowed to feed on these mice, as well as in a control unimmunized group. Mice were checked daily for blood stage parasites up to two weeks post blood feed. As expected, mice immunized i.v. were fully protected, and naïve control mice became all positive. Mice immunized i.m., s.c. and i.d. reached much higher levels of sterile protection (83%, 80% and 75%, respectively) when compared to mice immunized likewise which suffer i.v. challenge.

_pb36p_- and RAS confer partial protection against challenge with _P. yoelii_ sporozoites

A vaccine that would confer protection to more than one *Plasmodium* species is highly desirable. We tested whether *pb36p*- or RAS could induce such cross-species protection. BALB/c mice were *pb36p*- immunized with 3 different regimens: (i) single immunization dose of sporozoites (50,000), (ii) prime-boost regimen (50,000/20,000, one week interval between them), and (iii) full immunization regimen (50,000/20,000/20,000, weekly apart). Cross-species protection was also assessed for RAS immunizations using the last 2 regimens. Three weeks after last boost, mice were challenged with 100, 1,000 or 10,000 *P. yoelii* sporozoites. Low numbers of *P. yoelii* sporozoites as 100 or 1,000 were also used, since this *Plasmodia* species is known to be

2,000× more infective in BALB/c mice than *P. berghei* (Khan, 1991). Naïve controls were challenged with the same number of *P. yoelii* sporozoites.

Fourty hours after infection, some mice that received a single immunization or prime-boost regimen were sacrificed and liver collected for parasite burden assess by quantitative RT-PCR (qRT-PCR). Prime-boost immunization with RAS reduces significantly infection in liver compared to naïve controls, achieving levels of inhibition of 99.9% (P<0.001) and 94.8% (P<0.05), against challenges of 100 and 1,000 *P. yoelii*, respectively (Fig. 1A and B). Similar *pb36p*- sporozoites immunization also show a significant higher level of *P. yoelii* infection inhibition when challenged with 100 and 1,000 *P. yoelii* (94.6% (P<0.001) and 88.6% (P<0.05), correspondingly) (Fig. 1A and 1B, respectively). On the other hand, although a single immunization with *pb36p*- sporozoites followed by challenge with 100 and 1,000 *P. yoelii* sporozoites leads to a reduction in liver infection (58.5% and 51.1%, respectively) this is not statistically significant (Fig. 1A and B)

Blood-stage parasitemia were followed daily up to 2 weeks post infection, for all immunization regimens mentioned. A single immunization with *pb36p*- sporozoites did not lead to significant lower maximum blood-stage parasitemias neither to a higher pre-patency period in mice challenged with either 100 or 1,000 *P. yoelii* sporozoites, when compared to naïve infected control (Fig. 2A and Table 5, groups 1 and 4). On other hand, mice which received an extra boost of *pb36p*- sporozoites have lower maximum parasitemias (Fig 2A), showing significant decreases, both regarding challenge with 100 and 1,000 *P. yoelii* sporozoites (P<0.05 for both) when compared to control infected mice. Additionally, sterile protection is attained in a proportion of mice that were challenged with 100 *P. yoelii* sporozoites (Table 5, group 2). The remaining immunized mice had a delay in pre-patency period, when compared to naïve control (Table 5, groups 2 and 5). All mice immunized likewise with RAS achieved sterile protection when challenged with 100 *P. yoelii* sporozoites (Fig. 2B and Table 5, group 2), never developing blood-stage parasitemia. Challenged with 1,000 *P. yoelii* sporozoites in RAS immunized mice only leads to partial protection, and mice which became blood stage positive have both a delay on parasitemia onset and significant lower levels of maximum parasitemia in the blood (P<0.05), compared to naïve control (Fig. 2B and Table 5, group 5).

pb36p- immunized mice using a prime plus 2 boosts regimen challenged with 100 *P. yoelii* sporozoites did not present a delay on blood stage parasitemia (Table 5, group 3)

when compared to naïve counterpart, although a marked inhibition in maximum parasitemia (Fig. 2A) is also observed ($P<0.05$). Mice immunized with RAS in an identical regimen and also challenged with 100 *P. yoelii* sporozoites, 75% of sterile protection was attained, and the only mouse which became positive had a higher pre-patency period (Table 5, group 3) presenting significantly low parasitemia ($P<0.01$, Fig. 2B) when compared to naïve control mice.

pb36p-and RAS prime plus 2 boosts immunizations also led to higher pre-patency periods, when compared to naïve control, regarding a challenge with 1,000 *P. yoelii* sporozoites (Table 5, group 6). Maximum parasitemia value achieved was also significantly lower for *pb36p*- ($P<0.05$, Fig. 2A). RAS likewise immunized mice reached 75% of sterile protection against a challenge of 1,000 *P. yoelii*, and the single mouse which developed blood stage parasites present a significant decrease in maximum parasitemia ($P<0.01$, Fig. 2B).

In mice challenged with 10,000 *P. yoelii* sporozoites both *pb36p*- and RAS prime plus 2 boosts immunized mice have lower pre-patency periods (Table 5, group 8), as well as significant lower values for maximum parasitemia ($P<0.01$ for both, Fig. 2).

Partial protection against challenge with *P. yoelii* is also observed in C57BL6 mice immunized with either *P. berghei* RAS or *pb36p*- sporozoites (regimen 50,000/20,000/20,000 weekly apart). As in BALB/c mice, blood-stage parasites were verified daily and immunized mice showed longer pre-patency periods (Table 5, group 7), reaching significant lower values for maximum parasitemia observed ($P <0.05$ for both RAS or *pb36p*-), when compared to naïve control mice (Fig. 3).

Altogether the results show that both types of *P. berghei* live-attenuated parasites can confer protection against challenge with another rodent *Plasmodia* species, *P. yoelii*, in two different mouse strains, BALB/c and C57BL6.

DISCUSSION

There are no doubts that novel intervention strategies are required to control malaria. The results presented in this report, together with our own and others previous work (Luke, 2003; Mueller, 2005; van Dijk, 2005), illustrates the potential of an attenuated whole organism vaccine approach.

The results presented in this report clearly show that at least for one of those genetically-attenuated sporozoites, obtained by deletion of *pb36p*, protection can be achieved for as long period of time as for RAS in 2 mouse strains. *pb36p*- sporozoites confer protection up to 12 and 18 months in C57BL6 and BALB/c, respectively, the last time tried to either strain. Mice live in average 18 months (Fox, 1984). Thus, experiments were stopped due to the low number of mice that reached the last time points. On the later time points, protection conferred by RAS was always stronger than by *pb36p*- sporozoites. Berenzon and colleagues have shown that presence of antigen is needed to maintain the persistence of intrahepatic memory T cells (Berenzon, 2003), and, therefore, immunity against the parasite. Since *pb36p*- sporozoites induce high rates of infected hepatocyte apoptosis (van Dijk, 2005) and this might mean that infected hepatocytes disappear faster from the liver. Whether this is responsible for some loss in memory in the later time points needs to be further investigated.

Similarly to RAS-induced protection, lower doses than those currently tested of *pb36p*- sporozoites are sufficient to confer sterile protection. Immunization of C57BL6 mice with 3 doses of 10,000 or 5,000 *pb36p*- sporozoites or RAS fully protects mice against challenge with 10,000 sporozoites. However, while 3 doses of 1,000 *pb36p*- sporozoites are able to confer fully protection to challenge with 10,000 sporozoites, similar RAS immunizations only confer partial protection, confirming previous reports for RAS (Jaffe, 1990). This suggests that lower doses of *pb36p*- sporozoites are required than those required for RAS sporozoites, to induce the same level of protection. We have shown previously that *pb36p*- has a higher clearance rate by apoptosis in the liver than RAS parasites (van Dijk, 2005). In addition, we have also proposed that apoptotic *Plasmodium* infected hepatocytes provide a source of parasite antigens for the initiation of the protective immune response (Leirião and Mota, 2005). Whether this explains the observed difference in protection remains to be elucidated.

All vaccines used as public health measures are administered intramuscularly, subcutaneously or intradermally (Luke, 2003). A vaccine based on live attenuated

sporozoites should also accomplish this requirement. Our results, as well as other studies for RAS (Kramer, 1975), clearly demonstrated that protection can be achieved administrating RAS and *pb36p*- sporozoites intramuscularly, being some mice fully protected. Protection is also achieved using subcutaneous route in both types of attenuated parasites, as clearly demonstrated by delay in patency period. This is not been observed in previous studies (Kramer, 1975), but different mouse strains and immunization regimens were used, which could explain these differences. Another GAS, *P. berghei uis3*-, contrastingly, when administered subcutaneously, confer full sterile protection against intravenous challenge with WT *P. berghei* sporozoites (Mueller, 2005), which leads to think its protection mechanisms differ greatly from those induced by RAS or *pb36p*- itself. To our knowledge, no studies regarding intradermal immunization with live attenuated *Plasmodium* sporozoites were described previously. Our results show that both RAS and *pb36p*- sporozoite confer some level of protection when administered by this route, with only a small proportion of RAS immunized mice being fully protected.

Mice immunized with *pb36p*- sporozoites through all these routes followed by mosquito bite challenge (the natural course of infection) have a higher level of sterile protection, around 80%, which supports the potential as an experimental vaccine against malaria of this particular GAS. Unfortunately, none of these routes confer the same level of sterile protection as immunizations performed intravenously, which is undoubtedly desirable. Although at the moment these routes do not lead to complete protection, the results are quite encouraging and promising, strengthening the potential of a human live attenuated parasites-based vaccine. Optimization of the conditions used (e.g., higher immunization doses) may increase (or even lead to full) protection, which ought to be further investigated.

Our results show for the first time the potential of attenuated malaria sporozoites to induce cross-species protection. We clearly demonstrated that immunization with *P. berghei* RAS and *pb36p*- sporozoites is able to partially inhibit the development of *P. yoelii* in both BALB/c and C57BL6 mice. Immunization with both RAS and *pb36p*- sporozoites lead to a significant decrease both in parasite liver burden and levels of blood parasitemia. In a proportion of the cases sterile protection was even achieved. However, significant levels of partial cross-species protection are only attained with, at least, one extra boost. These results confirm the potential of live-attenuated parasites as a multi-species vaccine against malaria. Cross-protection induced against one

Plasmodium species by a different one has already been observed for the blood-stages of infection both in mice (Legorreta-Herrera, 2004; McColm, 1983; Jarra, 1985) and in humans (Smith, 2001). Regarding *Plasmodium* liver stages of infection, there are no reports showing that immunization with attenuated parasites, including RAS, are able to elicit any type of cross-protection. Immunization with *P. falciparum* RAS did not confer protection against subsequent challenge with *P. vivax* sporozoites as reported only once in a single human volunteer (Hoffman, 2002). Our results show that the immune response elicited by both RAS and GAS *P. berghei p36p*- sporozoites also acts partially against another murine parasite, *P. yoelii*. Considering that BALB/c mice are 2,000 times more sensitive to *P. yoelii* than *P. berghei* sporozoites (Khan, 1991), the partial protection (completely sterile in some BALB/c mice) attained with *pb36p*- against *P. yoelii* sporozoites challenge is quite remarkable. *P. berghei* and *P. yoelii* have an average protein identity of 88.2% (Hall, 2005), which suggests that through apoptosis several conserved antigens common to *P. berghei* and *P. yoelii* would be presented to immune system, therefore eliciting an protective response strong enough to act against both parasite species. Since *P. berghei* and *P. chabaudi* have also a high average protein identity, 83.2% (Hall, 2005), one might speculate this *P. berghei* attenuated sporozoite induced immune response might also exert a protective effect against infection with *P. chabaudi* sporozoites, but that remains to be elucidated. Interestingly, a CD8 epitope present in CSP and known to be very important in RAS protection for P. yoelii is distinct in P. berghei and P. yoelii (Sano, 2001). Thus, other antigens with possibly common epitopes much be recognized. The crucial antigens and epitopes responsible for the cross-protection are an interesting subject of research. Optimized immunization protocols, i.e., using other immunization protocols and/or higher doses of RAS or *pb36p*- sporozoites per immunization, might lead to complete sterile protection against *P. yoelii* and other murine *Plasmodium* species, and require further investigation.

One can only speculate about possible immune mechanisms that may lead to the protection induced by *pb36p*- against both homologous and heterologous species. Unlike RAS, which immune response in mice is well studied and documented (Doolan, 2000), the features that lead to *pb36p*- induced protection are still unknown (van Dijk, 2005), as well as for other GAS parasites (Mueller, 2005; Mueller and Camargo, 2005).

Immunization strategies using *P. falciparum* RAS have been considered impossible due to technical, clinical and logistical hurdles: route of administration, large quantities of sporozoites needed and regulatory, potency and safety requirements (Luke, 2003).

However, some are pursuing this approach (Luke, 2003), which would be quite useful in a possible implementation of *P. falciparum* GAS immunization strategy. Orthologues of *p36p-*, and other GAS, in *P. falciparum* or *P. vivax* have already been described (Thompson, 2001; Kaiser, 2004; Mueller and Camargo, 2005), therefore being possible to perform this type of genetic modification in human parasites. GAS vaccine efficacy and safety could be improved by developing more complex GAS strains that lack multiple genes in a single parasite (Waters, 2005; Matuschewski, 2006). Each form of GAS must be evaluated for its immunization potential and the best single or combination one, taken forward to human trials.

Previously, we have shown the immunization potential of *p36p- P. berghei* (van Dijk, 2005). In this report, we show that potential can be extended to long-lasting immunity and cross-species protection against *P. yoelii*. Furthermore, protection can be achieved even with low immunization doses and using practicable routes of immunization commonly used as public health measures in humans. Understanding the mechanisms by which these deficient parasites induce immunity will no doubt provide ground-breaking relevant data for the development of a vaccine aimed for the pre-erythrocytic stage of Malaria. Those efforts may pave the way for new vaccination strategies.

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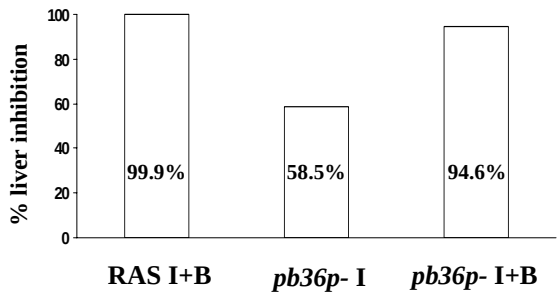
Figure 1

A


% liver inhibition
100
80
60
40
20
0
99.9%
58.5%
94.6%
RAS I+B
pb36p- I
pb36p- I+B

B

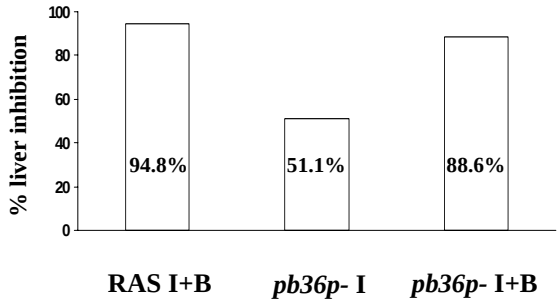

% liver inhibition
100
80
60
40
20
0
94.8%
51.1%
88.6%
RAS I+B
pb36p- I
pb36p- I+B

Figure 2

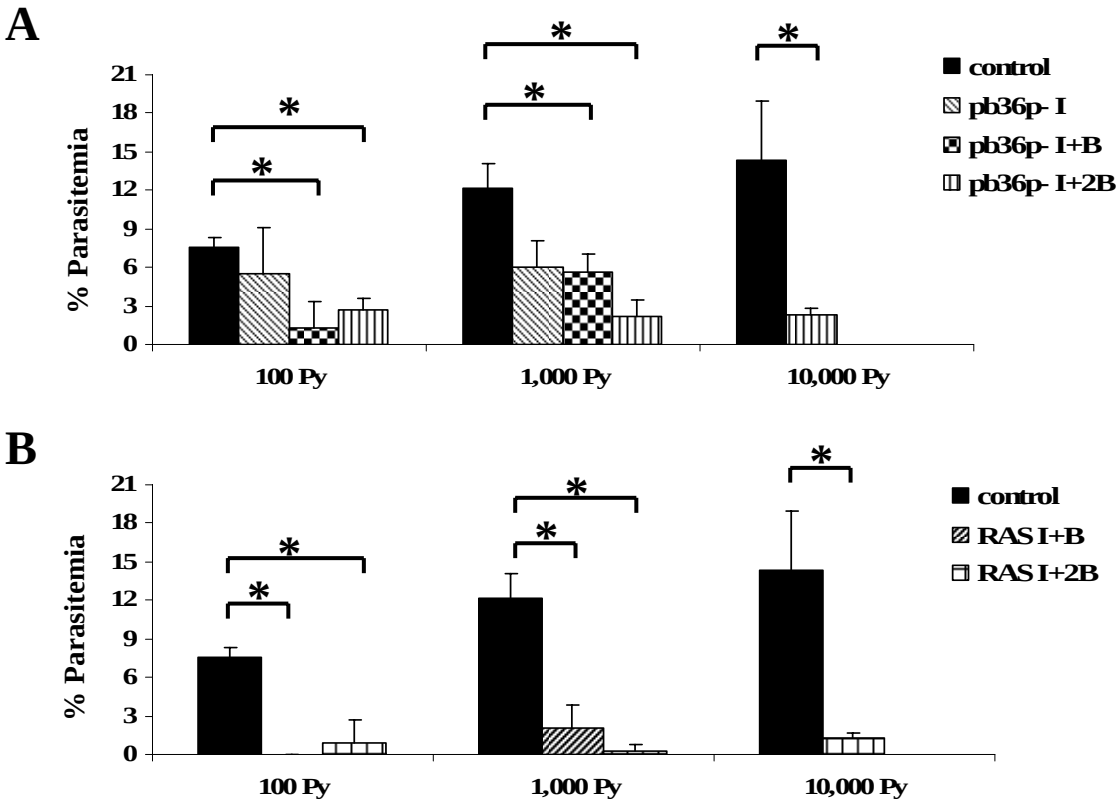

A
% Parasitemia
21
18
15
12
9
6
3
0
100 Py
1,000 Py
10,000 Py
control
pb36p- I
pb36p- I+B
pb36p- I+2B
*
B
% Parasitemia
21
18
15
12
9
6
3
0
100 Py
1,000 Py
10,000 Py
control
RAS I+B
RAS I+2B
*

Figure 3

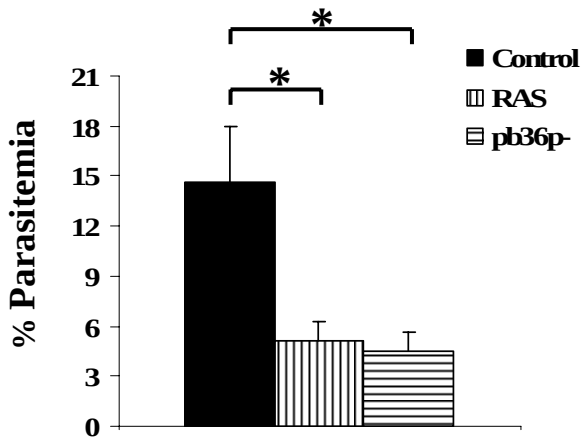


FIG 1. Level of inhibition of an infection with *P. yoelii* 265 BY sporozoites in mice immunized with *P. berghei* RAS and *pb36p*- sporozoites. BALB/c mice were immunized with RAS sporozoites (prime plus boost) or with *pb36p*- sporozoites (single immunization and prime plus boost) and challenged 3 weeks after last immunization with 100 (A) or 1,000 (B) *P. yoelii* sporozoites. Livers were collected 40 hours post challenge and parasite burden was assessed by Real Time PCR. Control mice have no inhibition in infection.

FIG. 2. Maximum blood-stage parasitemias levels achieved in *P. berghei pb36p*- and RAS immunized BALB/c mice which were subsequently challenged with *P. yoelii* 265 BY sporozoites. BALB/c mice were immunized with *pb36p*- sporozoites (single immunization, prime plus boost and prime plus 2 boosts) and with RAS sporozoites (prime plus boost and prime plus 2 boosts) and challenged 3 weeks after last immunization with 100, 1,000 or 10,000 *P. yoelii* sporozoites (only prime plus 2 boosts for the later). *pb36p*- (A) is able to decrease maximum blood-stage parasitemia after challenge with different doses of *P. yoelii* sporozoites in BALB/c mice, although at least two immunization doses are required to achieve significant reduction. RAS (B) equally induce diminution on maximum parasitemias observed in the same conditions, even achieving sterile protection in some cases. Parasitemias are expressed as the mean ± the standard deviation of individual animals. Data from two independent experiments are shown. * $P<0.05$, ** $P<0.1$

FIG. 3. Maximum blood-stage parasitemias levels achieved in *P. berghei* RAS and *pb36p*- immunized C57BL6 mice which were subsequently challenged with *P. yoelii* 265 BY sporozoites. C57BL6 mice were immunized with either RAS or *pb36p*- sporozoites (prime plus 2 boosts) and challenged 3 weeks after last immunization with 1,000 *P. yoelii* sporozoites. Both RAS and *pb36p*- are able to decrease maximum blood-stage parasitemia when challenged with *P. yoelii* sporozoites, attaining significant reduction. Parasitemias are expressed as the mean ± the standard deviation of individual animals. Data from two independent experiments are shown. * $P<0.05$

TABLE 1 – Immunization with *pb36p*- sporozoites lead to protection against subsequent multiple challenges

Group	Mouse Strain	Immunization[a], RAS/*pb36p* - × 1,000	Challenge[b] × 1,000	Challenge at ... days post last immunization	No. protected (no. challenged)		
					Control	RAS	*pb36p*-
1	BALB/c	50	10/20	180/351	0 (5)	ND	4 (4)
1	BALB/c	50	20	531	0 (5)	ND	3 (3)
2	C57BL6	50/20/20	10/10/20	60/90/180	0 (5)	5 (5)	4 (4)
2	C57BL6	50/20/20	20	365	0(5)	5 (5)	2 (4)
3	BALB/c	50/20/20	10/20	60/180	0 (5)	3 (3)	4 (4)
3	BALB/c	50/20/20	20	365	0 (5)	3 (3)	1 (2)
4	BALB/c	50/20/20	10/20	90/180	0 (5)	3 (3)	3 (3)
4	BALB/c	50/20/20	10/20	365	0 (5)	1 (1)	2 (2)
5	BALB/c	50/20/20	20	365	0 (5)	ND	1 (2)

[a] Groups of mice were immunized i.v. with one dose of RAS or *pb36p* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. ND, not determined

[b] Mice were challenged with WT *P. berghei* sporozoites, isolated from different mosquitoes batches. The pre-patent period was monitored by FACS analysis. All unimmunized mice become positive on day 3 or 4 after challenge.

TABLE 2 – Protection elicited by *pb36p*- sporozoites does not require subsequent multiple challenges to remain fully protective up to 6 months after last immunization in BALB/c mice

Immunization[a], RAS/*pb36* - × 1,000	Challenge[b], × 1,000	Challenge at ... days post last immunization	No. protected (no. challenged)		
			Control	RAS	*pb36p*-
50/20/20	10	60	0 (5)	3 (3)	4 (4)
50/20/20	10	90	0 (5)	3 (3)	3 (3)
50/20/20	20	180	0 (5)	4 (4)	4 (4)

[a] Groups of mice were immunized i.v. with one dose of RAS or *pb36p*- sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals

[b] Mice were challenged with WT *P. berghei* sporozoites, isolated from different mosquitoes batches. The pre-patent period was monitored by FACS analysis. All unimmunized mice become positive on day 3 or 4 after challenge.

TABLE 3 – Protection elicited by *pb36p*- sporozoites can be achieved even with low immunization doses in C57BL6 mice

Immunization[a], RAS/*pb36p*- × 1,000	**Pre-patency (days post infection)[b]**		**No. protected/no. challenged (%)**	
	RAS	***pb36p*-**	**RAS**	***pb36p*-**
50/20/20	-	-	5/5 (100%)	8/8 (100%)
10/10/10	-	-	5/5 (100%)	5/5 (100%)
5/5/5	-	-	5/5 (100%)	8/8 (100%)
1/1/1	6.2	-	0/5 (0%)	10/10 (100%)
None (Control)	3.2		0/8 (0%)	

[a] Groups of mice were immunized i.v. with RAS or *pb36p* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. Data from three independent experiments are shown.

[b] Mice were challenged with 10,000 WT *P. berghei* sporozoites, isolated from different mosquitoes batches. Pre-patency is defined as the time until the first appearance of blood-stage parasites, and it was monitored by FACS analysis.

TABLE 4 – *pb36p*- sporozoites administered intramuscularly, subcutaneously and intradermally are able to confer protection against intravenous challenge with infectious *P. berghei* sporozoites in C57BL6 mice

Type of immunization[a]	**Pre-patency (days post infection)[b]**		**No. protected/no. challenged i.v. (%)**		**No. protected/no. challenged m.b.[c] (%)**
	RAS	***pb36p*-**	**RAS**	***pb36p*-**	***pb36p*-**
intravenous	-	-	5/5 (100%)	5/5 (100%)	5/5 (100%)
intramuscular	4.5	5.3	1/5 (20%)	1/8 (13%)	5/6 (83%)
subcutaneous	4.8	5.0	0/5 (0%)	0/10 (0%)	8/10 (80%)
intradermal	4.5	4.8	1/5 (20%)	0/9 (0%)	6/8 (75%)
None (Control)	3.2		0/8 (0%)		0/8 (0%)

[a] Groups of mice were immunized as indicated with RAS or *pb36* - sporozoites isolated from different mosquitoes batches. Multiple immunizations with RAS or *pb36p*- sporozoites were performed with weekly intervals. Data from two independent experiments are shown.

[b] Mice were challenged with 10,000 WT *P. berghei* sporozoites, isolated from different mosquitoes batches. Pre-patency is defined as the time until the first appearance of blood-stage parasites, and it was monitored by FACS analysis. Data concerning pre-patency in this table only concerns i.v. challenge.

[c] m.b., mosquito bite challenge. Highly infected mosquitoes (50,000-80,000 salivary gland sporozoites per mosquito) were allowed to feed on mice during 10-15 min (5 mosquitoes per mouse)

TABLE 5 – Immunization with *P. berghei* RAS and *pb36p*- sporozoites lead to a delay on parasitemia onset in BALB/c and C57BL6 mice challenged with the heterologous species *P. yoelii*, and some even achieve sterile protection.

Groups	**Mouse Strain**	**Immunization × 1,000[a]**	**Challenge Dose[b] (*P. yoelii* sporozoites)**	**Pre-patency (days)**			**No. protected (no. challenged)**		
				pb36p-	**RAS**	**Control**	***pb36p-***	**RAS**	**Control**
1	BALB/c	50	100	5.0	ND	5.2	0 (3)	ND	0 (3)
2	BALB/c	50/20	100	7.0	-	5.2	1 (3)	3 (3)	0 (3)
3	BALB/c	50/20/20	100	5.0	6.0	5.2	0 (5)	3 (4)	0 (5)
4	BALB/c	50	1,000	4.0	ND	4.0	0 (3)	ND	0 (3)
5	BALB/c	50/20	1,000	4.7	5.5	4.0	0 (3)	1 (3)	0 (3)
6	BALB/c	50/20/20	1,000	5.2	7.0	4.0	0 (5)	3 (4)	0 (5)
7	C57BL6	50/20/20	1,000	4.8	5.5	4.0	0 (5)	0 (5)	0 (5)
8	BALB/c	50/20/20	10,000	4.6	5.0	4.0	0 (5)	0 (4)	0 (5)

[a] Groups of mice were immunized as described before with *P. berghei* RAS or *pb36p*- sporozoites. Data from two independent experiments are shown. ND, not determined.

[b] For all experiments described above, matched control mice groups immunized likewise with either RAS or *pb36p*- sporozoites from the same mosquitoes' batch and challenged with 10,000 homologous *P. berghei* sporozoites each. All failed to develop blood stage parasites confirming that these sporozoites were fully protective in homologous challenge in these conditions. All control naïve mice challenged equally with *P. berghei* sporozoites developed blood stage parasitemia, as expected.