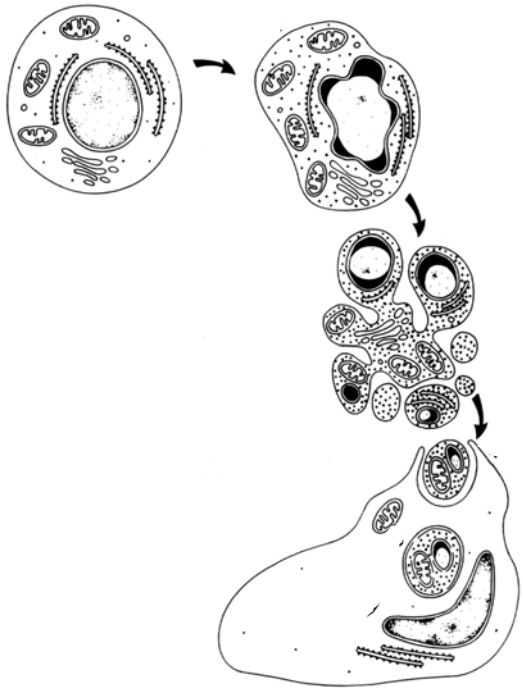
Plasmodium-Hepatocyte interactions: implications for protection against Malaria



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2006

UNIVERSIDADE NOVA DE LISBOA
Instituto de Higiene e Medicina Tropical

Plasmodium-Hepatocyte interactions: implications for protection against Malaria

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A dissertation submitted to obtain a Doctor of Philosophy degree in Biomedical Sciences, Parasitology speciality, by the Universidade Nova de Lisboa, Instituto de Higiene e Medicina Tropical.

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2006

Preface

This thesis assembles data obtained during my PhD research project developed at the New York University School of Medicine, Department of Medical and Molecular Parasitology; and at the Instituto Gulbenkian de Ciência, from August 2001 to July 2005. The work was supervised by Doctor Maria Manuel Mota and co-supervised by Doctor Ana Rodriguez. The financial support was provided by Fundação para a Ciência e Tecnologia with a PhD fellowship grant (SFRH/BD/3230/2000).

This thesis is structured in 7 chapters, which are preceded by a summary, both in Portuguese and English, outlining the aims, results and outcomes, of this Malaria research project. The first chapter places our work within the Malaria scientific field giving a background and significance of its input, and also specifies the objectives that we proposed to accomplish. A general introduction to Malaria ant its current world situation is presented in chapter two, together with a literature review of the late insights to liver stage biology and immunity, in addition to the present knowledge concerning apoptosis at host-pathogen interface. A description of the methods and materials employed to carry out the present work is done in chapter three. The results obtained throughout this research project are presented in the next three chapters from four to six. Each one is organized as follow: a short specific introduction to the particular subject, data observed and analyzed, and a discussion of those. Finally, chapter seven encloses an overall discussion and conclusion of the studies performed, together with an additional perspective of the recent highlights to what lies ahead within this scientific area of Malaria research. In Appendix are included the publications that derived from this project.

The data presented in this dissertation is the result of my own work and it is stated in the text whenever data or reagents produced by others as part of collaborations were used. This work has not been previously submitted for any degree at this or any university.

Cover Legend| A cell undergoing apoptosis with generation of apoptotic bodies that are engulfed by a phagocyte. (adapted from Kerr,1995)

Acknowledgements

Once I heard someone state that "Better than doing Science itself, is who you do it with!" This complies what I believe has been one of the greatest accomplishes of these last years, all of you that I had the privilege to live, work and learn from. Looking back I now realize that it really made all the difference and therefore I take this opportunity to acknowledge every one.

First I would like to thank Maria Manuel Mota for accepting me as student and giving me the opportunity to perform this research project. Your supervision, ideas, discussions, patience, friendship and your special endless optimism and enthusiasm towards Science (everything is always fantastic!), has contributed to the very rewarding experience that turn out to be these last years. I also would like to thank you for your efforts in providing all of us a top scientific environment to work. I learn a lot and I will be always grateful to you.

Several thanks to Ana Rodriguez for receiving me in your lab and accepting to co-supervise this project. Thank you for introducing me to the fascinating dendritic cell world and for showing me the beauty of cell biology. Your guidance, opinions, and discussions, contributed to make this life time experience.

To Professor Virgílio E. do Rosário and to the Centro de Malária e outras Doenças Tropicais, for all your support, patience with the administrative dealings, and for offering and persuading me into starting this PhD. Thank you also for accepting to be my university advisor and being part of my scientific committee. It was from you that I first hear about the importance of working within the malaria research field.

To Professor Dr Maria Amélia Grácio, for accepting me as a PhD student at the Instituto de Higiene e Medicina Tropical, giving me the opportunity to present and submit this research work for a doctoral degree. I also would like to acknowledge Dr Ruth Nussenzweig for welcoming me at the NYU Medical and Molecular Parasitology Department. Her sympathy and example of perseverance were extraordinary. To Dr Michael Parkhouse and Dr Sukalyan Chatterjee, for accepting to be part of my IGC committee and for the superb discussion of my work and an overall look at Science as a *big picture*.

To Sónia, my colleague and friend, your interest, work and dedication, contributed directly to the outcome of the HGF work presented in this thesis. Thank you so much for your precious help and endless hours at the microscope…without you I would never had finished this. Love your unique and exquisite sense of humour and your attitude (please do not ever change girl! Big kiss!).

To the rest of my past and present colleagues of the Malaria Cell Biology group, Dani, Laurita, Cris, Mar, Sa, Ana, Bruno, Susana, Marta, Miguel, Nuno, Silvia, Iana, Ricardo, Cristina, Carina and Ana Roberto; I am in debt to each one of you for all your help, support, enthusiasm, incentive, ideas, suggestions, discussions, patience and friendship. I sincerely think that we have *one of kind group* of people that is rare to find. I do not take you for granted, so therefore I will always cherish these years and hope to continue to do so with you near by. Mil obrigadinhos ppl!!!

For a liver stage malaria lab, the most precious and indispensable gift is our invertebrate friends. Thus, enormous big thanks is entitle to all of the fantastic people that produce, maintain and infect our superb mosquitoes. Without them this entire project would have not been possible. So here is my acknowledge to Ivette Caro-Aguilar, Jean Noonan (amazing Jamaica smile!), Claudia Canasto, Catarina Alves, Catarina Casimiro, Geert-Jan van Gemert and Robert W. Sauerwein. Many thanks also to Simona Corso and Silvia Giordano for establishing a great collaboration with our group, and especially for providing the HGF/MET cell lines and useful advices. To Susana Constantino for a precious technical help.

To Carlos (Latin music rules of course!), Jullius, Urvashi, Purnima, Kathy, Alexandre, Gen, Photini, Gerard, Jessica, Karine, and Kristine; a big thanks to every one for your help, technical assistance, friendship and for making the department a fun set to work. To my big apple Portuguese acquaintances: Margarida Carrolo (the unbeatable morning energy), it was great having you around, thanks for everything we shared; and Catarina Figueiredo, huge thanks for all your help, caring and for just being like you are!

To the Instituto Gulbenkian de Ciência and to its crowd, especially to the members of the Malaria groups and our neighbour groups in ala Gil Eanes, thank you for providing an exceptional environment to work and having fun doing it, and also for your permanent availability to help. As they say the penthouse is always the best (just teasing, lols)! But, of course in any rule there are exceptions, so a big thanks to the Stress and Transcription group, Célia, Filipa, Susaninha, Nuno, Marisa, and everyone else of course.

I would like to thank Carina, Miguel and Tiago for having the patience of reviewing this thesis and some of you that also gave your opinion at certain point of this work. To Fundação para a Ciência e Tecnologia, and to the PRAXIS XXI program for funding me with a PhD fellowship.

To Cristy, Michelle and Kathy, the crazy Latin girls that gave a whole new meaning to the Big Apple my life there without you…many thanks for your friendship. To Heloísa, for sharing her strength, sincerity and incredible *alto*

astral. Your presence and friendship was crucial at historic moments. Also to Amanda, Mike, Lulu, Aisha, David, Elena (simply a brit blast!), Damara (you really gave a new meaning to R&B groove girl), Carole, and Yvonne; thank you for the loads of fun and terrific moments.
To a fantastic Mary Jane, my scientific partner, for your constant presence and continuous motivation. To Dinora (the number one responsible for the beginning of this story) for all your help and support. To the rest of the gang, João, Cati, and Célia; many thanks for being my friends. To Beta, Carina, Filipa, Sandra, Carlos, Rafael and Zé, for your presence, support, incentive and sharing *quality time*. To Paula, our eternal conversations, emails, discussions, and your understanding were amazing. The music that we make together renovates my soul. I will always be grateful to you all!
To each and every one of my friends and colleagues that were not mention here, I deeply appreciate all your precious help that in someway allowed this thesis to be carry out, and for all the sympathy with which you have done it.
Finally, to my parents and sister, for their unconditional support, love, caring, comfort, values and sense of responsibility towards the others! I can never thank you enough! Muito Obrigada!

Sumário

A malária é uma das doenças infecciosas mais importantes a nível mundial, sendo anualmente responsável por mais de 1 milhão de mortes. O agente causador da doença é o parasita intracelular, denominado *Plasmodium*, que possui um ciclo de vida bastante complexo. A infecção tem início com a inoculação de esporozoítos através da picada de um mosquito fêmea *Anopheles*, o vector de transmissão da doença. Uma vez na corrente sanguínea, o esporozoíto migra até ao fígado onde infecta a célula hospedeira, o hepatócito. No fígado, o parasita replica-se e desenvolve-se até atingir o seu próximo estado de maturação – o merozoíto.

Dada esta complexidade, é natural que o *Plasmodium* provoque no hospedeiro uma variedade de mecanismos distintos, especialmente ao nível imunitário. Por isso, a resposta imune desenvolvida pelo hospedeiro, contra o parasita, é caracterizada como sendo complexa e específica em relação à espécie e ao estádio do mesmo.

De forma a adquirir uma resposta imune protectora contra a malária é necessário que o indivíduo seja infectado consecutivamente durante a vida. Mesmo assim o resultado obtido é apenas uma imunidade parcial contra o parasita.

Melhorias significativas têm sido registadas, no que diz respeito à compreensão dos mecanismos de protecção envolvidos na doença, assim como na identificação de novas moléculas que possam ser utilizadas no desenvolvimento de novas vacinas. No entanto, ainda não está disponível uma vacina que seja eficaz conferindo uma protecção total.

O uso de esporozoítos irradiados em imunizações induz uma protecção total contra a doença, que é mediada pela activação de linfócitos T $CD8^+$ específicos para antigénios do parasita. O início desta resposta é mediado por células dendríticas, embora a origem dos antigénios intervenientes seja ainda desconhecida.

Os esporozoítos irradiados conseguem infectar os hepatócitos. Contudo, não são capazes de progredir para a fase sanguínea da doença. Este desenvolvimento incompleto da fase hepática é uma característica fundamental para que ocorra imunidade. Embora alguns dos mecanismos protectores induzidos pela infecção com esporozoítos irradiados já tenham sido identificados é, ainda, necessário proceder a uma caracterização detalhada dos mesmos.

Sendo o fígado um local de extrema importância durante o ciclo de vida do parasita da malária, qualquer descoberta ao nível das interacções que se estabelecem entre o *Plasmodium* e o hepatócito, terá uma repercussão no melhoramento do processo de indução de uma resposta imune contra a doença.

Utilizando um modelo murino, demonstrou-se que os hepatócitos infectados com esporozoítos irradiados entram em apoptose logo após o início da infecção. Durante esta fase as células

dendríticas são recrutadas para o fígado, local onde fagocitam os corpos apoptóticos provenientes dos hepatóctios que entraram em morte celular. Uma vez que estas células são capazes de apresentar antigénios exógenos e ainda induzir o *priming* e activação das células T, os resultados por nós obtidos sugerem que os hepatócitos infectados apoptóticos são a fonte de antigénios do parasita utilizada pelas células dendríticas durante a iniciação de uma resposta imune contra a malária.

Durante o curso de uma infecção, a morte celular possui um papel fundamental no estabelecimento de uma resposta imune contra um agente patogénico. Os parasitas possuem a capacidade de modular esta resposta através da indução ou inibição da morte da célula hospedeira, de forma a possibilitar o seu desenvolvimento e sobrevivência.

Previamente, foi demonstrado que durante a migração dos esporozoítos através dos hepatócitos, as células atravessadas secretam um factor de crescimento específico, o HGF -"hepatocyte growth factor", que aumenta a susceptibilidade celular à infecção. Esta via de sinalização iniciada pelo HGF através do seu receptor, o MET, provoca uma série de efeitos em diversos tipos de células. Entre eles destaca-se a protecção contra a morte celular programada. Considerando tal facto, estudou-se qual o efeito desta protecção durante a infecção por *Plasmodium*, tendo como hipótese que a activação da via de sinalização do HGF/MET induziria uma protecção da apoptose nas células infectadas. Os resultados por nós obtidos confirmaram esta teoria. A inibição desta via de sinalização induziu um aumento na quantidade de morte celular observada.

Tendo em conta que, usualmente, a activação da sinalização do HGF ocorre segundo o sinal de transdução do PI3K/Akt, testou-se se o bloqueio desta via produzia algum efeito na infecção. De facto, os resultados observados indicam que esta via de sinalização é utilizada durante a infecção quando o HGF/MET é activado. Estas observações demonstram que a inibição da apoptose da célula hospedeira durante a infecção por *Plasmodium* é necessária para ocorrer doença.

Na parte final deste trabalho, procurou-se ainda identificar um gene do parasita responsável pela inibição da morte do hepatócito. Algumas observações preliminares levaram-nos a sugerir que a proteína HSP70 do parasita possa exercer uma função neste processo, daí que sugerimos que no futuro este envolvimento seja mais aprofundado.

Assim, os resultados apresentados nesta tese contribuem para um maior esclarecimento e compreensão das interacções que se estabelecem no fígado aquando da infecção por *Plasmodium*, e para um conhecimento mais alargado da relação entre o parasita e o hepatócito.

Palavras-chave: malária; apoptose; células dendríticas; infecção hepática; *Plasmodium* esporozoíto; resposta imune.

Abstract

Malaria is one of the most predominant infectious diseases worldwide, accounting for more than 1 million deaths annually. The intracellular parasite *Plasmodium* is the causative agent of malaria which undergoes a complicated life cycle. Infection is initiated by inoculation of sporozoites through mosquito bite, which journey to the liver where they must migrate and invade hepatocytes in order to replicate and mature.

Immunity to malaria is complex and is essentially both species and stage specific, thus a wide variety of distinct immune mechanisms are provoked by the parasite in the host.

The generation and maintenance of protective immune responses requires repeated infections over the lifetime of an individual and even though only partial immunity is achieved against the disease. Despite the significant advances in understanding mechanisms of protection and identifying new targets for vaccine design, an effective protection against malaria is still not available.

However, immunization with irradiated *Plasmodium* sporozoites induces antigen-specific $CD8^+$ T cells immune response that confers complete protection against malaria. The initiation of this response is mediated by dendritic cells, but the source of parasite antigens intervening in this response remains unknown. Irradiated sporozoites are capable of infecting hepatocytes but do not progress into blood stages forms. Both this incomplete liver development and the hepatic stage itself are indispensable steps for the outcome of a successful malaria protection. Although some protective mechanisms conferred by irradiated sporozoites have been identified, a thorough characterization is still needed.

The liver plays a key role in the life cycle of the malaria parasite and therefore insights into *Plasmodium*-hepatocyte interactions will have a promising effect in improving the process of triggering an immune response against the disease.

Using a rodent malaria model, we show that hepatocytes infected with irradiated *Plasmodium* sporozoites undergo apoptosis shortly after infection. In addition, after infection dendritic cells are recruited to the liver where they phagocytose apoptotic bodies derived from infected hepatocytes. Given that dendritic cells are capable of cross-presenting exogenous antigens and elicit the priming and activation of T cells, our results suggest that the apoptotic *Plasmodium* infected hepatocytes provide a source of parasite antigens for the initiation of the protective immune response against the disease.

Cell death plays a central role in the course of an infection helping establish an immune response against a pathogen. Furthermore, some parasites have the capacity to modulate this response by apoptosis induction or inhibition of the infected host cell, in order to survive and develop within the host.

Previously it was shown that wounding of hepatocytes by sporozoite migration induces the secretion of hepatocyte growth factor (HGF) by traversed cells, which renders neighbor hepatocytes susceptible to infection. The signaling initiated by HGF through its receptor MET has multifunctional effects on various cell types. Survival signals and protection of host cells is one of these features of HGF/MET signaling. The role of this protection on *Plasmodium* infected hepatocytes was also a subject of study in this thesis.

Therefore, we hypothesize that HGF/MET would induce in infected host cells protection from apoptosis, which in turn would lead to an increased infection. Our data confirms that HGF/MET signaling protects infected cells from apoptosis, since an increase in apoptosis of infected cells was observed when the signaling pathway was inhibited.

Given that HGF inhibits cell death primarily through the PI3-kinase/Akt signal transduction pathway, we tested if the infection susceptibility increase was impaired by inhibition of this pathway. In fact, inhibition of PI3-kinase completely abrogates the HGF effect on malaria infection. Taken together, these results implicate that the permissive effect of HGF for susceptibility to malaria infection is, at least in part, mediated by its anti-apoptotic signal. To our knowledge, these results demonstrate for the first time that active host's cell apoptosis inhibition during infection by *Plasmodium* is required for a successful infection.

Finally, an attempt at identifying a *Plasmodium* candidate gene responsible for the apoptosis inhibition of the host cell was carried out. Preliminary results evidence a promising role for *Plasmodium* heat shock protein 70 which broad function should be studied in the future.

In summary, data presented in this thesis contributes to a wider understanding of the events that occur in the liver during a malaria infection and expand our knowledge within the interactions established between the malaria parasite and its host.

Keywords: malaria; apoptosis; dendritic cells; hepatocyte infection; *Plasmodium* sporozoite; immune response.

Abbreviations

Ab	antibody
APC	antigen presenting cell
CS	circumsporozoite protein
CTL	cytotoxic T Lymphocyte
DAPI	4'6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbeco's modified eagles medium
EEF	exo-erythrocytic form
FCS	Foetal calf serum
FADD	Fas-associated death domain
GFP	green fluorescence protein
Hepa 1-6	mouse hepatoma cell line
HepG2	human hepatoma cell line
HGF	hepatocyte growth factor
HSP	heat shock protein
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
MAPK	mitogen-activated protein kinase
MET	tyrosine kinase receptor
MHC	major histocompatibility complex
NFkB	nuclear factor kappa B
NK	natural killer cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphoinositide 3-Kinase
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
Spz	Sporozoite
TNF	tumour necrosis factor
TRAP	thrombospodin-related adhesive protein

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Chapter One

Background, Significance & Objectives

1.1 Background and Significance

Malaria is one of the most prevalent and severe human infectious diseases in the world. Recently its incidence has increased and it is estimated more than 300 million episodes of acute illness occur in endemic countries and at least 1 million people die per year from this disease (WHO, 2003). With 41% of the world's population exposed to this threat, malaria also imposes an extreme burden onto affected populations as economy and development are deeply impaired resulting from the symptoms experienced by infected individuals. Applicable measures to control the disease vary according to each country's endemicity but several factors contribute to malaria resurgence such as drug-resistant parasite strains and insecticide-resistant mosquitoes (Greenwood *et al.*, 2005; WHO, 2003).

After decades of a relative lack of attention, new efforts are presently being made to address these challenges, such as new strategies that are being applied to the development of an effective vaccine (Moorthy *et al.*, 2004; Tongren *et al.*, 2004). Although in terms of public health vaccination has always been a priority, it still remains an elusive and complex research field. A comprehensive knowledge of the host-parasite interface would constitute a reinsured guarantee for attaining this goal successfully.

Malaria is caused by the intracellular Apicomplexan parasite *Plasmodium spp.*, which holds a complex life cycle involving different hosts and stages of infection. The first step in malaria infection is the invasion of the liver by *Plasmodium* sporozoites. These are the infective form of the parasite, transmitted by female anopheline mosquitoes during a blood meal. Inside hepatocytes, sporozoites replicate and develop into a merozoite state, a process that constitutes the hepatic stage of the parasite life cycle. Although, being an obligatory step towards the establishment of a successful malaria infection, this stage of *Plasmodium* life cycle is poorly understood. This parasite specificity towards the liver cells indicates that parasite-encoded surface proteins and host surface receptors are implicated in the process of invasion and development, playing a key role in the establishment of infection. However, the mechanisms, as well as the host and parasite molecules, at play during the course of infection in the mammalian host by malaria sporozoites are not entirely known (Gruner *et al.*, 2003; Miller *et al.*, 2002; Plebanski and Hill, 2000).

The full requirements for *Plasmodium* development inside hepatocytes are still unknown. The lack of an adequate *in vitro* system capable of delivering sufficient material has deeply impeded research within the study of host/parasite interactions. Currently, *in vitro* cultivation methods have been developed, and hepatoma cell lines are being used as *in vitro* models for *Plasmodium* infection to study the molecular and cellular basis of invasion mechanisms and intracellular development of the parasite. Nevertheless, infection yields remain very low and the limited number of infective

mosquitoes available impairs a swift progress in the understanding of malaria liver stage biology (Mota and Rodriguez, 2000; Calvo-Calle *et al.*, 1994; Hollingdale *et al.*, 1983b).

The availability of the genome sequence of both several *Plasmodium* species and their host provides a genetic tool that greatly increases the possibilities of research in this area (Cooke and Coppel, 2004).

The liver, besides being the place where amplification and molecular changes of *Plasmodium* parasites take place, is also a unique organ in what regards the host's immune responses (Knolle and Gerken, 2000). The antigenic pool generated within this organ is required for the induction and maintenance of a protective anti-malaria immune response. Thus, the hepatic stage may well hold the secret for understanding the parasite's preference for this organ within the mammalian host and, simultaneously, providing effective immune targets against the disease (Baldacci and Ménard, 2004; Frevert, 2004; Krzych *et al.*, 2000).

Unlike many other diseases in which a lifelong resistance to re-infection is induced, malaria causes only partial immunity after several years of recurring infections and illness. Nonetheless, a complete resistance to malaria can be achieved by vaccination with radiation-attenuated sporozoites in both mice and humans (Doolan and Hoffman, 2000; Weiss, 1990; Clyde *et al.*, 1975; Nussenzweig *et al.*, 1972; Nussenzweig *et al.*, 1967).

Irradiated sporozoites infect hepatocytes as normal sporozoites, but they do not reach a merozoite stage (Scheller *et al.*, 1995; Sigler *et al.*, 1984). Additionally, it is known that it is essential that a hepatic stage occurs during infection for the effectiveness of irradiated-sporozoite protection (Scheller and Azad, 1995). Although, until now this is the only vaccine that confers complete protection, the mechanism behind the process of the establishment of the immune response associated is still unclear (Doolan and Hoffman, 2000).

Intracellular pathogens have the capacity to modulate the host cell response and exploit its resources in order to develop and replicate. Parasites can manipulate host cell behavior, including immune modulation and regulation of apoptosis (James and Green, 2004). While infected cells are capable of initiating their own death, a process called apoptosis, which can be used by the organism as a defense mechanism against pathogens, inhibition of host cell apoptosis is frequently used by parasites as a strategy for survival (Heussler *et al.*, 2001; Luder *et al.*, 2001).

During the course of infection, apoptosis of infected host cells may either be induced by the host cell response or be a direct result of pathogen invasion. In both cases, apoptotic death results in the formation of apoptotic bodies. These apoptotic bodies are taken up by phagocytes that rapidly recognize and phagocytose them, eliminating the parasite together with the remains of the infected cell. The pathogen-derived antigens included in apoptotic bodies can be presented by dendritic cells in the context of both class I and class II molecules, which are recognized by $CD8^+$ and $CD4^+$ T

cells respectively, and activate naïve lymphocytes for the initiation of an immune response (Schaible *et al.*, 2003; Savill *et al.*, 2002; Rodriguez *et al.*, 1999; Albert *et al.*, 1998a).

Dendritic cells are key players in initiating immune responses because they are the only cell type that are able to prime naïve T cells efficiently as well as cross-present exogenous antigens (Banchereau *et al.*, 2000; Mellman *et al.*, 1998).

It has been shown that dendritic cells are able to induce *P. yoelii*-specific $CD8^+$ and $CD4^+$ T cells (Bruna-Romero and Rodriguez, 2001). Additionally, dendritic cells-depleted mice failed to induce cytotoxic T cell responses with a result in the loss of priming potential of dendritic cells during *P. yoelii* infection (Jung *et al.*, 2002).

Altogether, these observations suggest an important role of dendritic cells during the immune response initiated against liver stages. An extended comprehensive knowledge of these molecular and cellular events will provide promising immediate applications in the field of vaccine development.

1.2 Objectives

Hepatocyte infection by gamma-irradiated *Plasmodium* sporozoites is required to achieve complete protection against malaria. The basic trigger of this conferred immunity is still unknown. Conversely, apoptotic bodies derived from infected cells enclose a source of antigens that are processed by dendritic cells, which efficiently present pathogen antigens for the initiation of immune responses. The major goal of this project is to elucidate the role of hepatocyte apoptosis in the course of a malaria liver infection.

For this purpose, we focused on two main aspects. First, it was determined whether apoptotic death of hepatocytes infected with irradiated sporozoites would play a role in the initiation of an anti-malarial immune response. The immunological mechanisms that mediate this protection were studied, namely the origin of *Plasmodium* antigens, and the way in which its processing and presentation occur. Furthermore, the involvement of dendritic cells as key mediators of the protective immunity conferred by irradiated-sporozoites immunization was also examined.

Secondly, molecular aspects of parasite survival and development during the establishment of a successful liver infection were also addressed. The hypothesis of hepatocyte apoptosis modulation by the parasite was studied as well as the involvement of a host cell pathway.

Additionally, an attempt was made at identifying a *Plasmodium* gene product as a candidate responsible for the inhibition of apoptosis of hepatocytes. In this context, the role played by the parasite's heat shock protein 70 during liver infection was studied.

Chapter Two

GENERERAL INTRODUCTION

2.1 Malaria: historical aspects and current global picture

The malaria situation deteriorated during the 90's and recent estimates have shown an increase in numbers of morbidity and mortality as a result of overlooked prevention measures together with the widespread of resistance to drugs and insecticides. To overcome this situation new efforts are being undertaken with a focus on both cure and prevention of this disease. Attempts to address these challenges include the use of new technologies in research and the combination of different therapies in the field (Breman *et al.*, 2004; Nchinda, 1998).

The fight against this menace is currently being pursuit by an international partnership launched by the World Health Organization (WHO), the Roll Back Malaria, whose goal is to attain a 50% reduction of the burden of malaria by 2010. The impact of this achievement will reach far beyond the disease burden itself since it will implicate several factors and success will result in a global development for the countries at risk and the world's populations in general (WHO, 2003; Fig. 2.1).

Malaria is a parasitic disease caused by the intracellular pathogen *Plasmodium* and transmitted by a mosquito vector. In ancient times it was believed that the disease had its origin in the injurious air inhaled from swamps and marshes, thus the name 'mal aria' (bad air). In 1880, Lavern's discovery of the unicellular parasite P. *falciparum* in the blood of a French soldier put an end to this belief (Laveran, 1880). In 1897, the mode of transmission to humans was uncovered and attributed to the female *Anopheles* mosquito bite by Ronald Ross (Ross, 1897). The missing part of the malaria parasite's cycle, the exo-erythrocytic liver schizont**,** was only elucidated in 1948 by Shortt and Garnham, when malaria parasites where found developing in livers of sporozoite-infected monkeys and, subsequently, in livers of human volunteers infected by mosquitoes carrying *P. vivax* (Shortt and Garnham, 1948).

Presently, around 40% of the world's population is at risk of infection and more than a million people die each year, mostly children under five years of age and pregnant women. Symptoms appear within a week or two after transmission and consist of fever, nausea, vomiting, fatigue and headaches. They can progress into organ failure, coma and death, 90% of which occurs in tropical sub-Saharan African countries. Eradication of malaria from western European countries was achieved after the World War II when the anti-malarial drug chloroquine and the insecticide DDT became widely available (Hay *et al.*, 2004; Greenwood and Mutabingwa, 2002).

The search for a safe and effective vaccine against malaria has become a case of endless failures. Years of vaccine research have produced few hopeful candidates and although scientists are doubling research efforts, an effective vaccine is, at best, years away. In terms of epidemiology, human malaria is extremely complex, depending on transmission levels and acquired immunity. The relationship between the prevalence of severe malaria phenotypes and higher transmission rates is

not linear. In fact, exposure during early childhood could account for a lower risk of severity as protection is stimulated against subsequent attacks (Snow *et al.*, 1997).

There are four *Plasmodium* species that infect humans: *P. vivax*, *P. falciparum*, *P.malariae*, and *P. ovale*. The first two are the most common, with *P. falciparum* being the most deadly form of the parasite and responsible for the majority of the worst case scenarios of severe malaria infection. *P. vivax* accounts for the major cause of morbidity but is no longer a significant cause of mortality. A wide range of clinical symptoms, including fever, life threatening anemia, and coma in children and naïve adults characterize infection with *P. falciparum* (Greenwood *et al.*, 2005; Trigg and Kondrachine, 1998).

The present crisis is mainly due to resistance developed against drugs by *Plasmodium* parasites and against insecticides by *Anopheline* mosquitoes. Additionally, national transmission control programs have weakened and increased migration and tourism contribute to the current situation (Greenwood and Mutabingwa, 2002). Malaria's burden has an impact on the economical and political prospects of developing countries in which disease is endemic; therefore concerted funding for interventions and research priorities such as drug/vaccine development are being used to overcome this deteriorating situation (Sachs and Malaney, 2002).

In particular, vaccine development requires the achievement of several goals: induction of strong, durable and strain-transcending immune responses; identification of protective antigens for stage-specific immunity; and successful combination of candidate immunogens (Greenwood, 2005; Carvalho *et al.*, 2002).

In humans, the first report of protection induced by a vaccine occurred in 1973 when volunteers where being submitted to infected-gamma-irradiated mosquito bites (Hoffman *et al.*, 2002; Clyde *et al.*, 1973a). However, this approach was impracticable at a large-scale, and so a search for molecules-based vaccines was initiated. Throughout the last two decades, success in protection of animal experimental models raised the enthusiasm about a few candidate molecules, but these met with failure during human trials. More recently, efforts were translated in a pack of potential candidates that are currently under clinical assessment (Moorthy *et al.*, 2004; Moorthy and Hill, 2002).

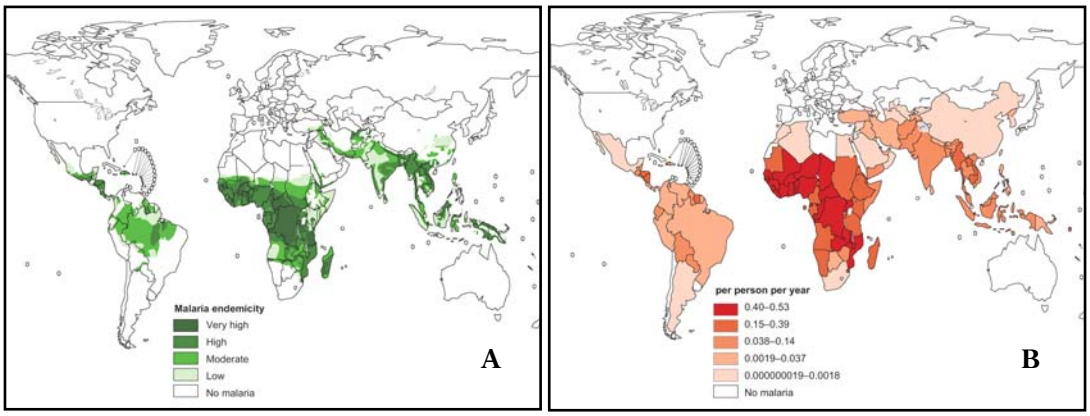


***Fig. 2.1*|Global distribution of malaria**. (A) World's malaria transmission risk in 2003 and (B) the estimated incidence of clinical malaria episodes caused by any *Plasmodium* species, resulting from local transmission, country level averages in 2004. (adapted from Roll Back Malaria partnership report, 2005)

2.2 *Plasmodium* life cycle

Plasmodium presents an extremely complex life cycle involving two hosts, a vertebrate host, and the invertebrate host, the female *Anopheles* mosquito vector. Interaction between them results in transmission and allows the infection to endure. Parasites enter the mammalian host through the bite of an infected female *Anopheles* mosquito during a blood meal. During such a meal, approximately five to twenty sporozoites will be injected into the host, which reach the liver within minutes (Vanderberg and Frevert, 2004; Ponnudurai *et al.*, 1991; Rosenberg *et al.*, 1990; Vanderberg, 1977). The sporozoites are deposited under the skin of the host, migrate into the bloodstream and aim directly to the liver, where they infect and develop inside hepatocytes (Matsuoka *et al.*, 2002; Sidjanski and Vanderberg, 1997; Shin *et al.*, 1982). This is known as the asexual exo-erythrocytic or hepatic stage of the parasite's life cycle. For human malaria, this stage lasts 5 to 7 days on average while in rodent malaria it lasts only 2 days (Meis and Verhave, 1988). This is also the asymptomatic stage of the disease. At the end of the hepatic stage, 10.000 to 30.000 merozoites per invading sporozoite will be released into the blood-stream from where they disseminate systemically. Each merozoite invades an erythrocyte and divides mitotically to form an erythrocytic schizont, containing up to 20 daughter merozoites. These merozoites can re-infect fresh erythrocytes, giving rise to a cyclical blood stage infection with a periodicity of 48-72 hours, depending on the *Plasmodium* species. This constitutes the asexual blood stage of a malaria infection. In this stage, disease symptoms occur and infected individuals can get sick. In order to complete the cycle, there are some merozoites that develop into sexual parasite stages, the male and female gametocytes, which will be taken up by mosquitoes during blood meals. Gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form that migrates into the mosquito hemocele

and develops into an oocyst form where sporozoites are developed (Miller *et al*., 2002; Gysin, 1998; Landau and Gautret, 1998). When fully matured, oocysts burst and release sporozoites which migrate into the mosquito's salivary glands, completing *Plasmodium*'s life cycle (Fig. 2.2).

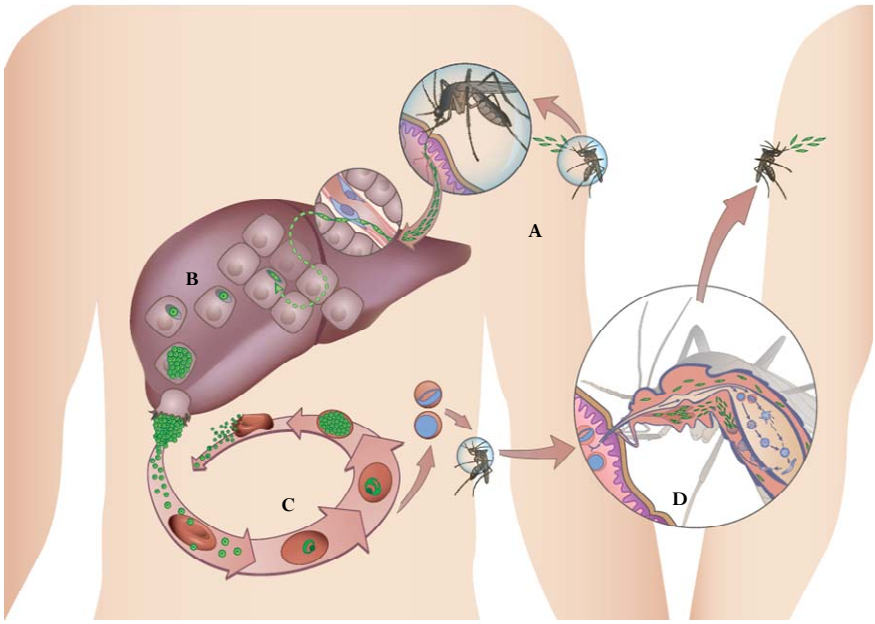


***Fig. 2.2|Plasmodium* life cycle**. When an infected female anopheline mosquito takes a blood meal, sporozoites enter the host bloodstream and travel through the circulation to the liver (A). Sporozoites invade hepatocytes and enter a phase of asexual reproduction in which they amplify their number thousands of times by the production of merozoites (B). The liver cell ruptures and the merozoites are released into the blood, attaching to and invading erythrocytes, beginning the erythrocytic cycle. Each merozoite invades an erythrocyte and replicates initiating a cycle that ends with the burst of the mature erythrocytic schizont and the release of new merozoites, which will infect new erythrocytes. Illness starts when the mature asexual erythrocytic schizont, ruptures (C). Other blood stages differentiate into male and female sexual stages parasites called gametocytes. These gametocytes enter a mosquito as it takes a blood meal. Sexual reproduction occurs in the mosquito midgut, and gametes fuse forming a motile zygote, the ookinet, that mature and migrates through the mosquito midgut developing into an oocyst, within which sporozoites develop. After being released, novel sporozoites travel to the salivary glands, making the mosquito infective (D).

2.3 Liver stage biology

The discovery of a mammalian malaria exo-erythrocytic stage was the last missing piece to be filled in the parasite's life cycle, when liver schizogony was described in African monkeys (Shortt and Garnham, 1948). Since no symptoms are associated with this parasite stage, little attention was given to the events that occur during sporozoite invasion of hepatocyte and the intrahepatic development. However, this scenario changed when in 1967, Nussenzweig reported that immunizing mice with radiation-attenuated *Plasmodium berghei* sporozoites protected them against challenge with fully infectious sporozoites (Nussenzweig *et al*, 1967). These rodent studies provided the impetus for human studies, and, during the 1970s, Clyde, Rieckmann and colleagues established that immunizing human volunteers with the bites of irradiated mosquitoes carrying *P. falciparum* sporozoites in their salivary glands could protect volunteers against challenge with fully infectious *P. falciparum*

sporozoites (Edelman *et al.*, 1993; Herrington *et al.*, 1991; Rieckmann, 1990; Rieckman *et al.*, 1979; Clyde *et al.*, 1975; Rieckman *et al.*, 1974; Clyde *et al.*, 1973 a, b)

Still until the mid-1980s, knowledge of the biology of malaria liver stage was restricted to the fact that these forms of the parasite could arrest in the liver and later be responsible for relapses during infections with certain *Plasmodium* species, the so-called uninucleated "hypnozoite" form (Cogswell *et al.*, 1991; Krotoski *et al.*, 1980).

Since then, experimental data from several laboratories began to elucidate the numerous steps taken by sporozoites from the starting point of invasion to their development inside the liver as well as the immune response that occurs during different immunization strategies with special emphasis for the immunization with attenuated parasites (Engwerda and Good, 2005; Baldacci and Menard, 2004).

The study of the cell biology of the *Plasmodium* liver stage is particularly difficult due to lack of a high infectious *in vitro* culture system, isolation and number of sporozoites needed. Still, the recent advance in direct live observation of parasites within the liver represents a useful resource, promising an outbreak of insights into this field (Frevert *et al.*, 2005; Hollingdale *et al.*, 1998; Calvo-Calle *et al.*, 1994; Hollingdale *et al.*, 1983b).

The reasons why *Plasmodium* has elected the liver and the hepatocyte as a first cellular home inside mammalian hosts are not fully elucidated. However, it is possible that the reason is related to the hepatocyte's highly complex metabolism, which is capable of fulfilling all parasite replication needs (Frevert *et al.*, 2004; Saliba and Kirk, 2001). Others support the idea that the immunologic characteristics of this organ allow parasites to survive and pursue infection, either by minimal immune responses that are thought to occur or by the induction of immune tolerance. In addition, hepatocytes are capable of expressing major histocompatibility complex (MHC) class I and class II, while erythrocytes are not (Crispe, 2003; Krzych *et al.*, 2000; Rajan, 1997). Another aspect to take into account is the morphology of the liver itself and the fact that hepatocytes are heterogeneous and allow easy access to venules and arteries separated by the space of Disse (Enomoto *et al.*, 2004; Wisse *et al.*, 1985).

2.3.1 From skin to liver

To reach the first and essential stop of its journey, the hepatocyte, *Plasmodium* sporozoites have to travel from the skin inoculation site to the liver. Being a highly vascularized organ, the skin is the perfect place for a mosquito blood meal, which usually last for 30s in *Anopheles* and occurs after a single probe of blood taken in small pools originated by capillary damage. Mosquito injections are accompanied by saliva, which has an anti-coagulant activity, facilitating blood digestion. In spite of the hundreds of sporozoites present in the mosquito's salivary glands only a small number are

transmitted during the bite of an infected mosquito (Matsuoka *et al.*, 2002; Ponnudurai *et al.*, 1991; Rosenbergh *et al.*, 1990; Vanderberg, 1977; Griffiths and Gordon, 1952; Boyd and Kitchen, 1939).

The migration of the sporozoite from the site of bite to the liver has been an issue under discussion. Previous studies with *P. yoelii*-infected mosquitoes allowed to feed in mice, provided evidence that mosquitoes deposit sporozoites in an avascular skin tissue area and, within 10 minutes post-inoculation, sporozoites could be found in the host's bloodstream, long enough to find a blood vessel (Sidjanski and Vanderberg, 1997). A recent study applying intravital observations of *P. berghei*-infected mice at the site of mosquito bite revealed that parasites migrate widely across the skin covering distances of many micrometers for several minutes before reaching circulation (Vanderberg and Frevert, 2004). Anti-sporozoite acquired immunity reduces motility speed and could interfere with skin crossing (Frevert *et al.*, 2005; Vanderberg and Frevert, 2004). Either because they find themselves in a new rough environment when they change from salivary glands to connective tissue or to prevent hostile encounters with the host immune system defenses, sporozoites have a short life span of approximately of 20 minutes to leave circulation in liver sinusoids and enter the parenchyma to infect hepatocytes.

The gliding motility presented by sporozoites is used during infections *in vivo* for their migration through the dermis, as was previously demonstrated *in vitro* (Vanderberg and Frevert, 2004; Vanderberg, 1974). *Plasmodium* gliding movement in host cells is characterized by trails of circumsporozoite (CS) protein that are left behind, similar to what occurs when they are placed in contact with artificial surfaces (Frevert *et al.*, 1998; Stewart and Vanderberg, 1991). The released CS protein is distributed throughout the cytoplasm of the cell and it has been proposed that is capable of inhibiting translation at the initiation step of protein synthesis, as it binds to RNA-associated binding sites on ribosomes (Frevert *et al.*, 1998; Hugel *et al.*, 1996). Nevertheless, mosquito-transmitted sporozoites are more infective than sporozoites injected intravenously, and are even capable of avoiding the antibody response mounted against them (Krettli and Dantas, 1999; Vaughan *et al.*, 1999; Beier *et al.*, 1991; Ponnudurai *et al.*, 1991).

2.3.2 Reaching the liver

The liver is organized in lobules formed by connective tissue with branches for the portal venule and the hepatic arteriole. It possesses a diverse population of cells such as specialized endothelial cells, Kupffer cells (liver resident macrophages) and stellate cells (fat-storing cells) (Enomoto *et al.*, 2004; Sinnis, 1996).

There are several interactions established between sporozoite proteins and the liver cells components. Malaria sporozoites possess an apical complex constituted by secretory organelles, unique to Apicomplexa parasites and essential for the invasion process. The major component of

the surface coat of *Plasmodium* sporozoites is the CS protein, which is responsible for the oocyst maturation and sporozoite morphogenesis (Kappe *et al.*, 2004; Nussenzweig and Nussenzweig, 1989). It is mainly stored in the micronemes (sporozoites secretory organelles) and is continuously exported to the cell surface and discarded at the parasite posterior pole (Thathy *et al.*, 2002; Stewart and Vanderberg, 1991; Posthuma *et al.*, 1989). It also possesses a glycosyl-phosphatidylinositol (GPI) sequence as anchor at the C-terminus of the CS protein, which also contains a species-specific central repeat region and two conserved motifs. Specific regions within these motifs are responsible for the linkage to the glycosaminoglycan of liver sulfated proteoglycans and sporozoite motility and invasion (Tewari *et al.*, 2002; Ying *et al.*, 1997).

Another protein present in the micronemes that is also expressed at the sporozoite membrane surface upon secretion induced by cell-contact is the thrombospodin-related adhesive protein (TRAP) (Bhanot *et al.*, 2003; Gantt *et al.*, 2000; Templeton and Kaslow, 1997). Besides being involved in sporozoite invasion of salivary glands and the hepatocyte, its vital role was assessed when *P. berghei* mutant TRAP parasites were shown to lose their gliding motility (Matuschewski *et al.*, 2002; Wengelnik *et al.*, 1999; Sultan *et al.*, 1997).

On the host side, the molecules that seem to play an important role during infection by allowing sporozoites to recognize and reach the liver are the proteoglycans. These molecules, which are highly sulfated and abundant, are involved in the molecular mechanism of sporozoite adhesion, where CS protein binds to hepatocyte membranes (Frevert *et al.*, 1993; Cerami *et al.*, 1992). Both CS and TRAP are capable of recognizing in particular the glycosaminoglycan chains (GAG) in the sulfated proteoglycans expressed by liver cells (Ying *et al.*, 1997; Robson *et al.*, 1995).

When entering the liver sinusoids either via the hepatic arteriole or the portal venule, sporozoites glide along the endothelial cell layer through the endothelia, interacting with extracellular matrix proteoglycans that protrude from the space of Disse until they encounter a Kupffer cell and recognize the proteoglycans expressed on its surface (Pradel *et al.*, 2002; Pradel and Frevert, 2001). Although previous work with *P. berghei* in rats suggested that these cells were responsible for parasite phagocytosis removing them from circulation, recently Kupffer cells were shown to be the liver portal entry to sporozoites from a set of experimental data based on *in vitro* models with *P. berghei* and *P. yoelii* sporozoite infections and Kupffer cells isolated from rat livers (Pradel and Frevert, 2001; Meis *et al.*, 1983). Moreover, the development of most exo-erythrocytic forms close to the liver portal venules, where the Kupffer cells are located, also suggests that the these cells could well function as access gates to the liver (Verhave *et al.*, 1985; Sleyster and Knook, 1982).

Thus, the current model proposes that sporozoites recognize and bind to proteoglycans expressed on the surface of Kupffer cells using their major surface proteins, CS protein and TRAP (Pinzon-Ortiz *et al.*, 2001; Cerami *et al.*, 1992). They actively invade, safely traverse and successfully exit

Kupffer cells protected by a non-fusogenic vacuole through the space of Disse (Frevert *et al.*, 2005; Meis *et al.*, 1983c).

2.3.3 Hepatocyte invasion

Hepatocyte infection occurs after sporozoites exit the space of Disse and gain access to the liver parenchyma. Sporozoites first migrate through several hepatocytes before invading a final one within a parasitophorous vacuole. The traversed cells have their plasma membrane wounded by the parasite and can either survive, undergoing a resealing process, or die (Mota *et al.*, 2001). Experimental evidence demonstrates that when sporozoites invade host cells without formation of a vacuole the parasites do not develop, meaning that migration is a feature that precedes infection (Mota *et al.*, 2001). This migration of parasites leaves behind wounded cells that could be needed to potentiate infection, which would be beneficial for the parasite's development within infected hepatocytes. On the other hand, one could also suppose that wounding would increase the generalized inflammation detectable shortly after inoculation (Khan and Vanderberg, 1992). This particular sporozoite feature can be observed either *in vitro* or *in vivo* in mice livers, and it is unspecific as is shown by the fact that a range of cell types are suitable for hosting *Plasmodium* migration (Mota *et al.*, 2001). Moreover, during parasites *in vivo* journey from the skin to the liver they encounter different tissues and probably migrate through them (Mota and Rodriguez, 2004).

The molecular mechanism is still not fully elucidated, but recent reports revealed a newly discovered sporozoite protein that plays a role in migration. The SPECT protein (sporozoite microneme protein essential for cell traversal), which is localized in sporozoites micronemes, is required for the parasite's migration *in vitro*. Yet, exo-erythrocytic development was maintained, and a lower infectivity was shown *in vivo*, suggesting that host cell migration is mandatory for sporozoite access into the liver parenchyma (Ishino *et al.*, 2004). Recently, another SPECT protein was described and a similar function was shown for a protein present in mosquito ookinete invasion into the midgut epithelium (Ishino *et al.*, 2005a; Kadota *et al.*, 2004).

The final invasion is accompanied by the secretion of TRAP and the parasite finds itself surrounded by a parasitophorous vacuole, inside which it replicates and develops (Silvie *et al.*, 2004; Mota *et al.*, 2001; Meis *et al.*, 1983b). Detailed information about how this event takes place is scarce, in part because of the low infectivity of sporozoites *in vitro*. By analogy with related parasites such as *Toxoplasma gondii*, it is believed that sporozoite internalization with formation of a parasitophorous vacuole occurs within a few seconds and is dependent on the plasma membrane-associated motor that also drives parasite gliding motility (Soldati and Meissner, 2004). In addition, there is an associated intense secretory activity in their apical end, which is either a constitutive exocytosis of

molecules from within the apical organelles, or a regulated exocytosis restricted to the formation of a cap (Mota and Rodriguez, 2004; Mota *et al.*, 2002).

Moreover, migration induces secretion of "hepatocyte growth factor" (HGF) that render the surroundings more adequate to parasite growth and are imperative in order to obtain a successful infection (Carrolo *et al.*, 2003). Experimental evidence has suggested that other host cell factors are involved in sporozoite infection in the liver, for example, the interaction of the CS protein with the low-density lipoprotein-related protein receptor or the requirement of CD81 tetraspanin for *P. falciparum* and *P. yoelii* sporozoites to invade hepatocytes (Silvie *et al.*, 2003; Shabkibaei and Frevert, 1996).

2.3.4 Intrahepatic development

Knowledge concerning parasite development inside hepatocytes is still very limited considering the large range of morphological characteristics that occurs during this intrahepatic development.

Hepatic schizogony, where parasites grow, maturate and replicate, lasts 2 days for rodent species and 5 to 7 days for primates, resulting in 10.000 to 30.000 of merozoites from each invading sporozoite (Meis *et al.*, 1985 a, b; Meis *et al.*, 1983 a, b). From seize a single generation, merozoites individualize in the cytoplasm, and then separate as islets within which membrane formation occurs before rupture into the bloodstream. An infective mosquito bite leads to the formation of around 12 exo-erythrocytic schizonts in a few days, reaching 100 μm in size, contrasting with blood stages that only enlarge up to 12 μm, replicating into 8 to 24 erythrocytic schizonts. Thus, the parasite's needs for membrane and nucleic acid synthesis during the liver stage are enormous, almost three times more than for blood stages schizogony (Hollingdale, 1985). Nutrients are obtained from the hepatocyte which as it harbors glycogen and serum protein factories, allowing the maturation process to progress ending in the release of merozoites that will invade erythrocytes (Frevert, 2004; Meis *et al.*, 1985 a, b).

However, sporozoites are able to undergo partial development to early exo-erythrocytic forms in the absence of host cells or other cell types, and preserve some morphological and molecular features presented by regular ones (Wang *et al.*, 2004; Kaiser *et al.*, 2003).

2.4 Immunity to malaria liver stage

When naturally exposed to malaria, humans gradually acquire immunity to the parasite, although repeated infections are required in order to maintain it. Naturally acquired immunity is characterized by being short-lived and strain-specific (Hviid, 2005; Day and Marsh, 1991). The reasons for such

scarce protection are not known. It is mediated by blood-stage specific antibodies and is partially T cell-based (Stevenson and Riley, 2004; Good and Doolan, 1999). But the contribution of each of the different stages to the general immunity-related protection remains poorly understood. During the blood stages it is clear that the host develops mechanisms either to neutralize the effects of parasite-released toxins or to kill the parasite or even to inhibit its replication. The reduced acquisition of malaria immunity in naturally exposed populations has also been explained by the fact that the parasite actively modulates the immune system of the host during blood stages, preventing the development of specific immune responses (Ocana-Morgner *et al.*, 2003; Urban *et al.*, 1999; Plebanski *et al.*, 1997). Concerning the liver, the fact that the gut is located close to the liver is one of the reasons why this organ has such a peculiar immunologic profile, predominantly tolerogenic when responding to foreign antigens. Liver dendritic cells, Kupffer cells and sinusoidal endothelia are mature antigen-presenting cells that undertake the task of maintaining this immune environment (Doherty and O'Farrelly, 2001; Knolle and Gerken, 2000; Lohse *et al.*, 1996). Together with anti-inflammatory cytokine secretion, the liver controls the inflammation induced by influx of bacteria and endotoxins from intestines, and reaches a state of portal vein tolerance. Antigen-specific activated $CD8^+$ T cells are eliminated by Fas-induced apoptosis considering the model of activation-induced cell death, thus accounting for liver tolerance (Crispe, 2003).

Nevertheless, naturally acquired protection observed in infected individuals and several experimental data records, raised hopes for the development and feasibility of an effective vaccine against this disease (Carvalho *et al.*, 2002).

In what regards the liver stage of infection, major hopes were raised based on the fact that sterile protective immunity was obtained both in mice, monkeys and humans; against *P. berghei*, *P. yoelli*, *P. knowlesi*, *P. falciparum* or *P. vivax* sporozoite challenge induced by immunization with radiation-attenuated sporozoites (Hoffman *et al.*, 2002; Gwadz *et al.*, 1979; Clyde, 1975; Clyde *et al.*, 1973 a, b; Nussenzweig *et al.*, 1969; 1967).

Protective mechanisms against the liver stage of malaria infection have been the aim of many studies, since knowledge concerning the type of immune responses induced towards the infected hepatocyte are still very limited. The advantages of inducing exo-erythrocytic stage immunity would not only be a decrease in mortality and disease transmission, but also a prevention of symptoms, since the parasite would be arrested before reaching the erythrocytic stage (Tdryk and Walther, 2005; Tsuji and Zavala, 2003).

2.4.1 Natural infection *versus* immunization

Mimicking features occurring in naturally infected individuals is the general aim of vaccines which, in turn, means that they must be capable of inducing antibodies and T cell responses, if possible to

more than one antigen. In particular, a greater magnitude in responses than the one achieved in individuals that have been infected, together with transcendence both in time and strains, would be desirable for the development of a vaccine against malaria. Administration of a cocktail of several antigens has been the prevailing hypothesis for many years now, but an expensive and complex product would be the likely result. Ideally, a vaccine against the exo-erythrocytic stage of the parasite should fulfill two roles: the induction of high titers of functional antibodies against sporozoites inoculated by the infectious mosquito, in order to stop them from entering the liver; the establishment of potent cytotoxic T lymphocyte immunogenicity against the liver stage to kill all infected hepatocytes while not harming the host (Hill, 2006; Doolan and Hoffman, 2000; Doolan and Hoffman, 1997).

In that respect, the discovery that immunization with radiation-attenuated sporozoites could lead to full protection paved the way to determine the full range of factors involved in this protection, namely *Plasmodium* antigens, initiation of host immune response and immune response types (Nardin *et al.*, 1999). Gamma-irradiated sporozoites infect the liver but are arrested there without pursing to blood stages. Later, it was also found that the targets of this protection were not only the sporozoite but mainly the infected hepatocyte (Scheller and Azad, 1995; Suhrbier *et al.*, 1990; Weiss, 1990; Hoffman *et al.*, 1989). In fact, sporozoites irradiated with a dose that did not allow them to reach the liver were not protective any more (Silvie *et al.*, 2002; Nussler *et al.*, 1989). Still, is not clear if this relies on the presentation of different and specific antigens or on the way that antigens are presented to the host immune system (Langhorne *et al.*, 2004; Krzych *et al.*, 2000).

2.4.2 Exo-erythrocytic antigens

Some sporozoite antigens are also expressed within the newly formed liver stage forms. In fact, a limited number have been already identified and characterized as targets for $CD8^+$ T cell responses. Among these are the CS protein and the TRAP protein (Khusmith *et al.*, 1994; Rogers *et al.*, 1992; Weiss *et al.*, 1992). Another protein that has also been shown to be a target for T cells is the *P. yoelii* hepatocyte exported protein 17 (PyHEP17), an homolog of the *P. falciparum* exported protein 1 (PfEXP) (Doolan *et al.*, 1996).

The first strictly specific malaria liver stage antigen discovered was the *P. falciparum* liver stage antigen 1 (PfLSA-1), which is expressed in the vacuole lumen and is solely expressed during the hepatic stage (Guerin-Marchand *et al.*, 1987). The LSA-3 antigen was characterized through antibody recognition in immunized and non-immunized individuals (Daubersies *et al.*, 2000; Connelly *et al.*, 1997). A few other parasite proteins appear at this stage of the parasite's life cycle, such as glutamine-rich protein and parasite heat shock protein 70 (Kumar *et al.*, 1993). More parasite

proteins are being identified through recent new genomic approaches, leading to an increase in the number of the antigenic repertoire known in malaria (Gruner *et al.*, 2003).

Recently, several *Plasmodium* genes, which are not expressed in the blood stages and some are also not expressed in the sporozoites, were shown to possess a vital role for parasite liver development (Ishino *et al.*, 2005b; Mueller *et al.*, 2005; van Dijk *et al.*, 2005; Kraiser *et al.*, 2003).

2.4.3 Initiation of host immune response

Naïve T cell priming by sporozoites can occur by specialized presenting cells, macrophages, dendritic cells, and B cells (Riley, 1999; Druilhe *et al.*, 1998; Pape *et al.*, 1997). There is evidence that sporozoites are able to avoid destruction by macrophages, from which they can exit and induce death (Vanderberg *et al.*, 1990; Seguin *et al.*, 1989; Danforth *et al.*, 1980). In fact, sporozoites are too large to be internalized by B cells, but released material might be taken up by specific B cells, thereby initiating activation of a T cell response (Link *et al.*, 1993; Stewart and Vanderberg, 1992). Although it has been shown that the infected liver cell can present parasite-derived peptides on its surface, expressed on MHC class I or class II molecules and recognized by $CD8^+$ and $CD4^+$ T cells, respectively, the hepatocyte does not normally express co-stimulatory molecules necessary for naïve T cell activation, which the high levels of Interleukin-10 (IL-10) present in the liver, may further delay (Renia *et al.*, 1993; Weiss *et al.*, 1993; Weiss, 1990; Hoffman *et al.*, 1989). Thus, the possibility that infected hepatocytes can activate naïve T cells is unlikely (Krzych *et al.*, 2000; Vanderberg *et al.*, 1993). Furthermore, the existence of a parasitophorous vacuole between the parasite and the host cell poses some questions. Trafficking of *Plasmodium* antigens inside the hepatocyte between the parasitophorous vacuole and the host cell membrane is still an unclear issue. This double biological membrane constitutes a barrier to the transport of malarial peptide epitopes by MHC molecules (Frevert, 2004; Gruner *et al.*, 2003; Mellman *et al.*, 1998).

2.4.4 Host immune responses after immunization

The protective mechanisms induced by immunization with irradiated sporozoites were extensively studied. Since the liver is an immune privileged site, the infected hepatocyte was for decades considered to be protected from the immune system and, therefore, immunity was thought to be anti-sporozoite only. However, later was shown that this immune response is mounted against the infected liver cell (Lau *et al.*, 2001; Hoffman and Doolan, 2000; Hoffman *et al.*, 1998).

The first line of effector mechanisms triggered by malaria infection is antibody response. CS protein is a target for the production of neutralizing antibodies that induce protection as conservation within each plasmodial species is very high (Hollingdale *et al.*, 1998; McCutchan *et al.*, 1996; Hollingdale *et al.*, 1984; Lockyer *et al.*, 1989; Santoro *et al.*, 1983). Antibodies against the CS protein protected mice

by either blocking sporozoite invasion or by killing sporozoites themselves, ultimately leading to immunity. This immune response mechanism against sporozoites is described in the literature for different parasite species: *P. berghei*, *P. yoelli* and *P. vivax* (Charoenvit *et al.*, 1991; Wirtz *et al.*, 1991; Weber *et al.*, 1987; Potocnjak *et al.*, 1980; Yoshida *et al.*, 1980). In rodent models there are examples of antibody production against single epitopes of different proteins, like the *P. yoelii* HEP17 that is capable of removing infected hepatocytes in culture (Charoenvit *et al.*, 1995). Hepatocyte invasion and development of *P. falciparum* was prevented when an anti-Pf-CS protein repeats monoclonal antibody was used *in vitro* (Mazier *et al.*, 1986; Hollingdale *et al.*, 1984). However, protection does not consistently correlate with anti-CS antibody levels in malaria-exposed individuals or immunized volunteers. Besides, no vaccine has been shown to induce a strong sterile immunity based on the induction of anti-CS antibodies alone (Herrington *et al.*, 1987; Hoffman *et al.*, 1987).

The role of T cells was first demonstrated when irradiated sporozoites conferred protection in mice, which were not capable of making antibodies (Chen *et al.*, 1977). Additionally, spleen cells or immune T cells from sporozoite-immunized mice could confer protection against malaria, when adoptive transfer was performed *in vivo* into naïve mice (Egan *et al.*, 1987; Verhave *et al.*, 1978). Protective immunity induced by irradiated sporozoites is completely dependent on the presence of $CD8^+$ T cells while a requirement for $CD4^+$ T cells has only been demonstrated in a few strains (Tsuji and Zavala, 2003; Doolan and Hoffman, 2000; Weiss *et al.*, 1993; Tsujii *et al.*, 1990).

$CD8^+$ T cells have been implicated as critical effector cells in this protection, thus being the core of several immunologic studies, which provided the following observations. Induction of IFN-γ is a direct consequence of the $CD8^+$ T cell activation, IFN-γ production precedes and initiates production of IL-12, and then IL-12 in turn induces IFN-γ production by APCs and/or natural killer cells (NK) in a positive feedback loop that represents an important amplifying mechanism. The IFN-γ then activates inducible nitric oxide (NO) synthase and induces the L-arginine-dependent NO pathway, subsequently eliminating the infected hepatocyte or the intrahepatic schizont (Morrot and Zavala, 2004b; Krzych *et al.*, 2000; Doolan and Hoffman, 1999; Sedegah *et al.*, 1994; Nussler *et al.*, 1993; Nussler *et al.*, 1991; Schofield *et al.*, 1987a). Nevertheless, irradiated sporozoite immunization induces distinct mechanisms of protection in different hosts (Doolan and Hoffman, 2000).

In vitro, $CD8^+$ T cells against a single epitope in the carboxyl terminus of the *P. yoelii* CS protein eliminated infected hepatocytes from culture in an antigen-specific, MHC-restricted manner (Weiss, 1990). Protection against infection in mice was also achieved by using $CD8^+$ T cells that are specific for an epitope of the CS protein either for *P. berghei* CS or *P. yoelii* CS, which could, in some cases, be abolished by *in vivo* treatment with anti-IFN-γ (Weiss *et al.*, 1992; Rodrigues *et al.*, 1991; Romero *et al.*, 1989). Besides CS protein, other clones against different proteins could protect mice such as TRAP,

which conferred protection in a $CD4^+$ T cell, IFN-γ dependent manner (Wang *et al.*, 1996; Khusmith *et al.*, 1994). $CD4^+$ T cells directed against a single epitope in the amino terminus of the *P. yoelli* CS protein eliminated infected hepatocytes from culture in an IFN-γ-independent manner (Renia *et al.*, 1993; Renia *et al.*, 1991). A heat-shock-like protein (PfHSP70) on the hepatocyte surface was a target for *in vitro* antibody-dependent cell-mediated cytotoxic mechanisms by liver non-parenchymal cells (Renia *et al.*, 1990).

Besides antibodies and T cells contributions to immunity, the effect and the role of the most important cytokines in malaria infection have also been studied. Regarding IFN-γ, a systemic administration of this cytokine is capable of partly protect against *P. berghei* and *P. cynomolgi* sporozoite challenge in mice and in monkeys, respectively (Ferreira *et al.*, 1986, Schofield *et al.*, 1987b; Maheshwari *et al.*, 1986). On the other hand, *in vivo* IFN-γ depletion leads to abrogation of irradiated *P. berghei* and *P. yoelii* sporozoites-induced immunity (Rodrigues *et al.*, 1991; Hoffman *et al.*, 1989; Weiss *et al.*, 1988; Schofield *et al.*, 1987a). When treated with IFN-γ, *Plasmodium spp.*-infected hepatocytes were eliminated from *in vitro* cultures. IFN-γ's activity was also shown to be nitric oxide-dependent (Mellouk *et al.*, 1994; Mellouk *et al.*, 1991; Ferreira *et al.*, 1986). Another important cytokine that is able to modulate hepatic stage development during malaria infection is tumour necrosis factor-α (TNF-α). Inhibition of development is obtained in *P. berghei*-infected HepG2 cells (Schofield *et al.*, 1987b). *In vivo* administration of murine recombinant TNF-α induced high protection in mice challenged with *P. yoelii* sporozoites (Nussler *et al.*, 1991). However, when purified cultures of primary hepatocytes infected with *P. berghei* or *P. yoelii* were used, no effect was observed (Mellouk *et al.*, 1991). Nevertheless, the addition of non-parenchymal cells to the hepatocytes restored TNF-α-induced parasite inhibition. Subsequently, it was shown that this was due to IL-6 secretion from non-parenchymal cells (Nussler *et al.*, 1991). Administration of recombinant interleukin-12 (IL-12) protects mice from sporozoite challenge with *P. yoelii* and *P. cynomolgi* in monkeys (Hoffman *et al.*, 1997; Sedegah *et al.*, 1994). Other reports have also mentioned the inhibitory effect of IL-1 on intrahepatic development of *P. yoelii* and *P. falciparum*, in this case when applied before sporozoite inoculation (Pied *et al.*, 1992; Mellouk *et al.*, 1987).

Still, doubts subsist regarding the differences induced by irradiated and live sporozoites especially in what concerns how antigen presentation occurs, which given the opposed outcome have necessarily to hold divergences. Within it for sure is the mediated switch from tolerogenic immune status to a local inflammatory one, accomplishing malaria exo-erythrocytic forms clearance (Stevenson and Riley, 2004).

Immunization with blood stage antigens, heat-killed, formalin-inactivated or lysed sporozoites, or even sporozoite antigens, did not lead to a level of protection similar to that achieved when

irradiated sporozoites were used (Plebanski and Hill, 2000). Instead, live sporozoites and chloroquine-treated mice immunizations, were capable of preventing erythrocyte infection (Belnoue *et al.*, 2004). These data provided further insights to the fact that immunity conferred by irradiated sporozoites is triggered by the infected hepatocyte as a first target and sporozoite liver development is required for it to occur. Moreover, live sporozoites have to target the liver for protection to occur. Additionally, sporozoites last 30 min or less in the mammalian host, so any immune response targeting this stage has to be activated and completed within minutes of infection. On the other hand, the liver stage is much more extended both in rodents, where parasite develops for 24h-48h, and in humans, where, depending on *Plasmodium* species, development can last for 5-14 days (Doolan and Hoffman, 2000).

How the knowledge obtained with study of the immunizations with irradiated sporozoites conciliates with the field observations is also crucial to fully understand the potential of the liver stages. T cells specific for both variant and conserved pre-erythrocytic malaria antigens are not found consistently in naturally exposed individuals and T cell responses are generally suppressed during the course of malaria infection (Good and Doolan, 1999; Hviid *et al.*, 1991; Ho *et al.*, 1986; Troye-Blomberg *et al.*, 1984).

A complete description of the mechanisms involved in this protection could drive adequate strategies for the development of a vaccine capable of conferring complete immunity (Hill, 2006).

2.5 Apoptosis at the host-pathogen interface

2.5.1 Cell death

In humans, approximately 10 billion cells die per day during its maintenance. The need of eliminating excess cells or potentially dangerous ones, protecting the organism from threats to its homeostasis and simultaneously allowing the control of cell numbers and tissue size, confers cell death an importance comparable to the cellular processes of cell division or migration. This is a highly complex molecular process of cellular destruction, and is defined as programmed cell death or apoptosis (Gavrilescu and Denkers, 2003).

Apoptosis is a genetically controlled biological event, characterized by a pattern of molecular and morphological changes that typically take place in multicellular organisms when they undergo death. It holds a major role in development, proliferation, maintenance, perpetuation of cellular integrity and tissue homeostasis. It plays a central role in the normal development and function of the immune system of higher vertebrates (Vaux and Strasser, 1996; Raff, 1992).

Such a powerful process has to be tightly regulated as abnormalities in the apoptotic progression commonly lead to pathology, including development of autoimmune diseases, ageing, cancer and neurodegenerative disorders (Strasser *et al.*, 1997; Thompson, 1995).

Over the years, apoptosis and its regulation became an essential area of research, allowing significant progress in the understanding of this cellular process. Programmed cell death was first described by Carl Vogt in 1842 and, since then, acquired several names. The term apoptosis was created in 1972 by Kerr and collaborators to describe the morphological appearance of the dying cells observed in various tissues and cell types (Kerr *et al.*, 1972). These dying cells presented and shared many morphological features distinct from the ones observed in cells undergoing necrotic cell death and suggestions were made that these belonged to a conserved, common and endogenous cell death program (Wyllie *et al.*, 1980).

Since those early days, the nematode *Caenorhadditis elegans* was used as a model to uncover the apoptotic machinery and its molecular components allowing the identification of several molecules involved in this process (Hengartner and Horvitz, 1994; Ellis and Horvitz, 1986). Mammalian orthologs were also described and it is now generally accepted that the apoptotic process involves a shared biochemical suicide program that exists in most cells and is turned on by a variety of normal developmental and pathogenic triggers (Fink and Cookson, 2005; James and Green, 2002).

This process of self-destruction is a direct consequence of the activation of a large number of conserved gene and protein families. Apoptosis differs from other types of cell death, such as necrosis, and is characterized by a unique range of morphological and biochemical features that are commonly present in a dying cell and which constitute the basis for the identification of this biological event (Hengartner, 2000). Nuclear chromatin condensation and fragmentation, vacuolization of the cytoplasm, cell shrinkage with organelle preservation, membrane blebbing, DNA degradation followed by detachment from the substrate and formation of membrane-bound apoptotic bodies, are features that determine apoptotic cell fate, a process that cannot be reversed once it has been initiated (Hacker, 2000). The lack of inflammatory response is also a hallmark of death by apoptosis as apoptotic bodies are rapidly engulfed by phagocytes without causing inflammation (Savill and Fadok, 2000).

Conversely, necrosis results from a physical injury and is not genetically controlled. Cellular swelling with disruption of organelles and rupture of the plasma membrane are marks that typify a non-apoptotic death. Thus, cells dying by apoptosis or necrosis present large differences in their appearance. Furthermore, necrosis contrary to apoptosis is usually associated with inflammation. Although apoptosis and necrosis are independent processes they can be related since an event that produces necrosis may trigger apoptosis in the surrounding tissue. On the other hand the induction of apoptosis under certain conditions could indirectly result in necrosis (Serhan and Savill, 2005).

A number of cysteine proteases that are specifically activated in apoptotic cells are responsible for the morphological changes observed. These death proteases are homologous to each other, and are part of a large protein family known as caspases (Thornberry and Lazebnik, 1998; Alnemri, 1997). They are highly conserved throughout evolution and can be found in organisms ranging from worms to mammals, including insects and nematodes. Caspases are synthesized as precursors that have almost no catalytic activity. When activated, they are known to be the central executioners of the apoptotic process and are capable of regulating their own activity. Caspase-mediated proteolytic reactions are very specific, recognizing and cleaving aspartic acid-containing recognition sequences (Thomberry *et al.*, 1997). When caspase-activity is abrogated, either by inhibitors or through mutations, prevention or reduction of cell death occurs (Hengarther, 2000).

2.5.2 Pathways of apoptosis

Apoptosis is triggered by a stimulus that is responsible for initiating the whole cascade process. Several types of stimuli are capable of initiating cell death by apoptosis. These include internal stimuli like death ligands binding to specific cell surface receptors, such as Fas or tumor necrosis factor receptor, and external stimuli like chemotherapeutic agents, irradiation, reactive oxygen species, and growth factors (Chipuk and Green, 2005). Transduction of these pro-apoptotic signals via different pathways results either in activation of caspases or in the release of cytochrome *c* from the mitochondria into the cytosol, leading to the formation of the apoptosome and to the activation of a caspase cascade, which ultimately results in cell death. Depending on whether the trigger for apoptosis is internal or external, two main pathways, the extrinsic (death receptors) and intrinsic (mitochondria) pathways, can be used by the organism (Hengartner, 2000).

The extrinsic pathway is initiated by the interaction of specific ligands with appropriate cell-surface receptors, like the Fas-FasL and the TNF-α, and TNF-α receptor (TNFR). These are receptor-ligand interactions in which both members are part of the death receptor families of proteins. When FasL binds to Fas at the cell surface, a death-inducing signaling complex (DISC) is formed. The DISC incorporates one or more adapter molecules like the Fas-adapter death domain (FADD), which bind the receptor on one side, and the pro-initiator caspase-8 or -10 on the other, through their death domains. Subsequently, activated caspase-8 cleaves and activates the effectors caspases -3, -6, -7 from their precursor state thereby completing the cascade. Caspase-8 can also cleave Bid, a pro-apoptotic cytosolic Bcl-2 family protein that, in its truncated form, translocates to the mitochondria allowing the bridge between pathways and eliciting the intrinsic course (Nagata, 1997).

The latter pathway has is centered in the mitochondrial changes that are limited to the breaking of the outer membrane integrity with the release of cytochrome *c* and other mitochondrial intramembrane components into the cytosol. Cytochrome *c* interacts with apoptosis-activating factor

(Apaf)-1 from the cytosol, inducing its oligomerization into a complex structure named apoptosome which also includes pro-caspase-9. Once activated, this structure is capable of activating the downstream effector caspases, leading to apoptosis through the mitochondrial pathway. At certain key points there are other molecules that allow this catalytic cascade to be regulated by either positive or negative feedbacks. The Bcl-2 family proteins, that include the anti-apoptotic Bcl-2, Bcl-xl, Bcl-w, Mcl-1 constitute an example of this. These proteins prevent apoptosis by protecting mitochondria from permeabilization and inhibiting the action of the pro-apoptotic Bcl-2 family members (Bax, Bak, Bad, Bid), which are responsible for inducing cytochrome *c* release from the mitochondria triggering the apoptotic cascade (Kroemer and Reed, 2000; Adams and Cory, 1998).

In addition to these two major pathways, there are other alternative routes to caspase activation. The release of perforin and granzymes by NK cells or antigen specific CTLs, which, through endocytosis and pore formation, give access to the cytosol where Bid and/or caspase-3 is activated and apoptosis is induced in target cells (Kojima *et al.*, 1994; Shi *et al.*, 1992; Odake *et al.*, 1991; Poe *et al.*, 1991) (Fig. 2.3).

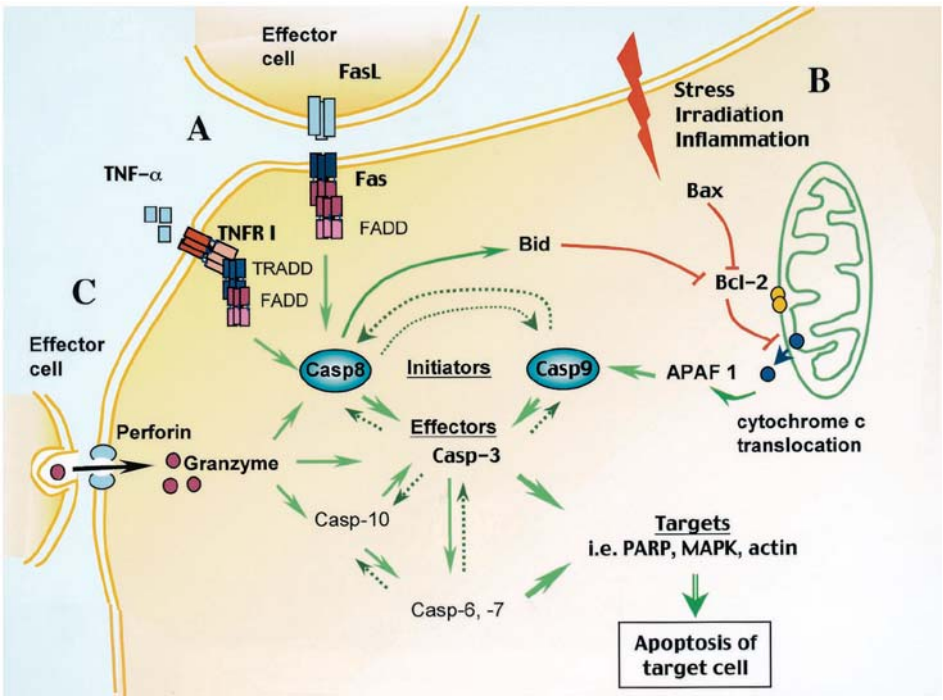


***Fig. 2.3*| Pathways of apoptosis.** (A) Extrinsic or death receptor-mediated pathway. Extracellular molecules (TNF-α and FasL) bind to TNFR family members (TNFRI and Fas, respectively). Recruitment of FADD to the receptors leads to Casp-8 activation, which in turn results in activation of Casp-3, Casp-6, and Casp-7. (B) Intrinsic or mitochondrial pathway. Stress, irradiation and inflammation act on mitochondrial through intermediary pro-apoptotic Bcl-2 family members such as Bax, resulting in blocking of the anti-apoptotic activity of Bcl-2 (red lines). As a result, cytochrome *c* translocates into the cytoplasm and activates Casp-9 through APAF-1. Casp-8 may also trigger the mitochondrial pathway through the activation of Bid, which like Bax, inhibits the anti-apoptotic activity of Bcl-2. (C) Cytotoxic cells introduce granzyme molecules into the target cell in a process mediated by multimerization of the perforin molecule. Granzymes cleave various substractes, including caspases, resulting in cell death. The dashed arrows indicate secondary effectors of activated caspases, and the green arrows indicate cleavage and activation. (adapted from Gavrilescu and Denkers, 2003)

2.5.3 Intracellular living

Intracellular organisms invade cells in order to exploit cellular resources and multiply, ensuring the perpetuation of the pathogen's life cycle. The relationship between a host and a pathogen has a dynamic nature that has evolved to enable coexistence between the two entities. Pathogens use different strategies to make use of the host's resources that determine the fate of the interaction established between host and pathogen. A successful infection results in a considerable cellular reorganization with clear advantages for the infectious agent. Therefore, obligatory intracellular parasitism requires a close regulation of crucial events like a successful entry, intracellular replication and exit from the host cell (Alonso and Garcia-del Portillo, 2004; Nyalwidhe *et al.*, 2003; Kahn et al., 2002).

Pathogens encounter different environments along their way, which lead to changes in microorganism responses through signal transduction pathways which have to adapt to the surrounding conditions. This lifestyle demands a subversion and exploitation of the host cell at various levels, from using host resources to evading the host's defense system (Ploegh, 1998). Probably the most obvious potential outcome of host-pathogen interactions is the death of host cells (James and Green, 2002). It has long been known that this can result from an infection, either as a direct effect of the pathogen or through pathogen-produced products. Pathogen-induced cell death seems like a simple infection outcome, but undergoing research has demonstrated that is rather complicated as it may occur by a variety of complex mechanisms. When a cell detects that has been invaded and is fated to die, it is capable of accelerating the dying process in order to arrest parasite growth, limiting the damage to the host organism and thereby protecting the uninfected cells and ultimately preventing infection (Luder *et al.*, 2001).

Pathogens have developed mechanisms to prevent host cell death by disabling the host's apoptotic machinery and thereby promoting its own survival and replication. Descriptions of such behaviors can be found in the literature for several pathogens like viruses, bacteria, parasites, and fungi, where modulation includes prevention of apoptosis in parasitized host cells and also promotes the death of immune cells that are targeted to them (Hasnain *et al.*, 2003; DosReis and Barcinski, 2001).

Thus, uncovering mechanisms responsible for pathogenesis is critically dependent on elucidating factors required by a pathogen to kill or preserve the host's life. Such knowledge may ultimately contribute towards the discovery of novel therapeutic targets with potential use in various diseases (Fink and Cookson, 2005).

2.5.4 Modulation of apoptosis and parasite survival/death

Upon infection there is a complex interaction of parasite proteins with cellular host proteins originating a variety of responses from both of them. Phagocytosis of the pathogen, release of

cytokines, secretion of toxins, production of reactive oxygen species, are common outcomes of this type of relationship. Many examples of strategies used by viruses and bacteria preventing the death of the host cell through either of the apoptotic pathways have been reported (Gao and Kwaik, 2000; Granville *et al.*, 1998; Liles, 1997).

Analogous mechanisms have been proposed for protozoa including receptor-mediated apoptotic triggers and stress-mediated apoptosis stimuli, suggesting that a close interaction at different check points is established with the apoptotic machinery of the host cell. Therefore, the abrogation of host cell apoptosis often turns out to be beneficial for the pathogen and to result in a successful invasion (Heussler *et al.*, 2001).

While in many cases the mechanisms for resistance to apoptosis are still unknown, in others there appear to be a variety of ways in which the pathogen ensures cellular survival. These include production of molecules that resemble and mimic Bcl-2 protein, the expression of inhibitors for death receptor signaling pathway, the generation of caspase inhibitors and interference with nuclear factor kB (NFkB) pathway. So, several studies point out to parasite-produced molecules that are capable of directly activating apoptotic pathways in the target cells (Gavrilescu and Denkers, 2003; Hasnain *et al.*, 2003).

Among these are examples of parasitic infections conferring protection to induced-apoptosis in infected-host cells. Decreased caspase activation and reduced levels of poly (ADP-ribose) polymerase expression were described in *Toxoplasma*-infected cells (Goebel *et al.*, 2001; Nash *et al.*, 1998). On the other hand, cell viability in the absence of growth factors was shown to be induced when *Leishmania*-infected macrophages soluble mediators were released (Moore *et al.*, 1994).

Both induction and inhibition mechanisms were described in *Trypanosoma cruzi*-infected cells, depending on the stimulus elicited. Hence, an apoptotic pathway can be induced during infection by radiation and chemicals and when a different pathway, like Fas or TNF-α, was triggered, host cells became protected and resistant to death (Nakajima-Shimada *et al.*, 2000; Nunes *et al.*, 1998; Lopes *et al.*, 1995). In addition, *Trypanosoma* is able to produce a *trans*-sialidase that can interact with the host cell PI3K-Akt pathway to promote survival in the peripheral nervous system and also seems to be responsible for cell depletion in the thymus during infection (Mucci *et al.*, 2002; Chen *et al.*, 2001; Chuenkova *et al.*, 2001).

Theileria is another parasitic pathogen that is able to subvert the host for its own benefit, making use of remarkable approaches, resembling a cancer cell. This parasite places a huge pressure on the host cell by taking control of both the cell cycle and the apoptosis process through several mechanisms that involve the activaction of transcription factors like AP-1, ATF-2, which are implicated in JNK and MAPK pathways (Heussler *et al.*, 1999; Romashkova and Makarov, 1999; Palmer *et al.*, 1997). NFkB signaling pathway is normally associated with transcription of pro-inflammatory mediators

and resistance to infection. However, it can also induce expression of several anti-apoptotic proteins, including IAPs and c-FLIP, which blocks caspase-8 activity leading to survival (Barkett and Gilmore, 1999). *Theileria*-infected cells show an activated phenotype as the inhibitor molecule (IkB) is degraded by phosphorylation, maintaining the nuclear translocation leading to immortalized-infected cells. Consequently, these modulations of the biology of *Theileria*-infected cells lead to death-resistance to both extrinsic and intrinsic apoptotic triggers (Kuenzi *et al.*, 2003; Heussler *et al.*, 2002; Heussler *et al.*, 1999).

Other examples of mitochondrial pathway induced-death are also found in the literature, although not directly involving the infected cell. During acute *T. gondii* infection, $CD4^+$ and $CD8^+$ T cells accumulate in the brain and express high mRNA levels of Bax and Bad, while in chronic infection T lymphocyte expression of pro-apoptotic molecules is downregulated and anti-apoptotic levels of Bcl-2 and Bcl-xl are increased (Schluter *et al.*, 2001; Khan *et al.*, 1996).

Similar processes occur with *P. berghei* lethal infection (a model for rodent cerebral malaria), where increased levels of Bax, Bcl-2, p53, and cytoplasmic cytochrome *c* in the brain were measured. Furthermore, alterations on mitochondria morphology were detected, suggesting that the dysfunction of this organelle may play a role in cerebral malaria phenotypes, although it is not clear which apoptotic pathway was followed (Kumar and Babu, 2002).

For extracellular parasites the induction of apoptosis occurs in cells that constitute their diet or appear along their pathway of invasion. *Entamoeba histolytica*-infected cells undergo apoptosis through a cell lectin-binding surface contact mechanism while evidence shows helminths parasites targeting for host cells mobilized to attack them using also Fas-FasL interaction (Lopez-Briones *et al.*, 2003; Chen *et al.*, 2002; Jenson *et al.*, 2002; Kuroda *et al.*, 2002; Rumbley *et al.*, 2001; Huston *et al.*, 2000).

Similar events occur in a virus-host relationship. Upon viral infection many cells undergo apoptosis, thereby reducing the viral load. Therefore, interfering with host cell apoptosis would lead to replication and spreading of progeny. For this purpose, strategic points in the apoptotic pathways are targets for viral proteins that evolved to be able to inhibit or delay the host's protective actions by targeting cells of the immune system or the host cell itself (Granville *et al.*, 1998; Ploegh, 1998). The anti-apoptotic members of the Bcl-2 family, the inactivation of tumor suppressor p53 and caspases inhibition are examples of targets for this modulation (Tabakin-Fix *et al* ., 2005; Li *et al.*, 2002; Yang *et al.*, 2002; Bertin *et al.*, 1996; Shen and Shenk, 1995). In latent viral infections, the host protein translation machinery is controlled by the virus. Initiation of a viral infection leads to the shutting down of the host's protein synthesis, which in turn leads to a decline of metabolic processes and apoptosis induction (Clemens, 2005).

2.5.5 Modulation of apoptosis and immunity

Cell-specific apoptosis contributes to the regulation of pathogen-induced immune responses (Luder *et al.*, 2001). As the Fas-FasL signaling pathway is specifically involved in apoptosis, disruption of the Fas-FasL pathways can be directly related to an alteration in apoptosis steady-state (Dockrell, 2003). Studies with knockout mice infected with different protozoa showed that FasL-triggered apoptosis plays a role in resistance to infection as the lack of function of this molecules induced higher levels of parasitemia and lesions that the mice failed to resolve (Lopes *et al.*, 1999; Conceicao-Silva *et al.*, 1998). However, it is not known if this outcome results from infected cell lysis or prevention of uncontrolled inflammatory reactions at the lesion site.

Production of FasL, which, through interaction with its receptor Fas will initiate the apoptosis cascade, for instance in T- and B-cells, leading to lymphocytopenia, occurs in *Plasmodium*, *T. gondii* and *Leishmania* (Eidsmo *et al.*, 2002; Nishikawa *et al.*, 2002; Wipasa *et al.*, 2001; Matsumoto *et al.*, 2000).

When ovalbumin- or parasite-specific T cells were adoptively transferred into *P. yoelii*-infected T cell-deficient nude mice, only the *P. yoelii*-specific T cells underwent apoptosis and demonstrated signs of increased Fas expression. These data suggest a mechanism to eliminate anti-*Plasmodium* T-cell effectors. Moreover, in an acute blood stage infection with *P. chabaudi*, 60% of the spleen cells express the Fas molecule (Wipasa *et al.*, 2001; Helmby *et al.*, 2000). In human malaria, infected individuals express high levels of apoptotic markers in peripheral blood mononuclear cells, which *in vitro* are sensitive to apoptosis induction and have elevated soluble Fas levels in serum. Thus, this apoptotic pathway may also play a role in infection. In addition, FasL levels have been shown to decrease with malaria chemotherapy (Kemp *et al.*, 2002).

CTLs employ perforin and granzyme molecules to directly trigger apoptosis in infected target cells. Several intracellular protozoans elicit MHC class I-restricted $CD8^+$ CTL activity; these parasites include *Toxoplasma gondii*, *Plasmodium spp.*, *Trypanosoma cruzi* and *Leishmania major* (Bonelo *et al.*, 2000; da Conceicao-Silva *et al.*, 1994; Grazzinelli *et al.*, 1993; Nickell *et al.*, 1993; Rodrigues *et al.*, 1991). However, it seems that perforin-mediated CTL activity does not protect the host, as perforin-knockout mice were resistant to infection with *T. gondii* and *T. cruzi* (Nickell and Sharma, 2000; Denkers *et al.*, 1997).

Reports concerning immunity-related apoptosis describe additional immunologic factors that possess a significant role in the outcome phenotype. Pro-inflammatory cytokines are able to mediate apoptosis process during pathogenic infections. However, it is difficult to determine the biological significance of apoptosis driven by mediators such as IFN-γ, IL-12, NO and TNF-α; because these molecules are, in addition, involved in resistance to pathogens and are needed for host survival (Denkers, 2003; Martins *et al.*, 1999; Grazzinelli *et al.*, 1998; Silva *et al.*, 1998).

Chapter Three

MATERIALS and METHODS

3.1 Cells

Two hepatoma cell lines were used for *in vitro* culture experiments: a human hepatoma cell line, HepG2 (ATCC, HB8065) and a mouse hepatoma cell line Hepa 1-6 (ATCC, CRL-1830) (Darlington *et al.*, 1980). Both cell lines are efficiently infected by rodent malaria parasites which are capable of undergoing complete development (Mota and Rodriguez, 2000; Hollingdale *et al.*, 1983 a,b). This constitutes the *in vitro* model system used to reproduce a malaria liver stage infection during the experimental studies performed.

3.2 Parasites, mosquitoes and mice

Plasmodium yoelii (17XNL, non-lethal strain) or *Plasmodium berghei* (NK65 or ANKA strains) sporozoites were obtained from infected mosquitoes and were used either for *in vitro* or *in vivo* experiments. Parasites were maintained by alternate cyclic passages in mosquitoes and mice (Vanderberg and Gwadz, 1980).

Additionally, *Anopheles stephensi* infected mosquitoes were also obtained from the Centro de Malária e outras Doenças Tropicais (Lisboa, Portugal) and the University Medical Center St. Radbound (Nijmegen, The Netherlands), and further maintained in our departments under adequate conditions (Benedict, 1997). BALB/c or C57/BL6 mouse strains aged between 6-8 weeks were purchased from Taconic Farms (Germantown, New York, US) or Instituto Gulbenkian de Ciência (Oeiras, Portugal). Animals were bred and maintained in a pathogen-free animal facility.

3.3 Sporozoite isolation and purification

Female *Anopheles stephensi* mosquitoes were fed on infected *Plasmodium spp.* mice. Sporozoites were obtained by dissection from their salivary glands at days 18-21 post feeding. Dissections of mosquito salivary glands were performed in RPMI 1640 medium (Gibco) containing 1% mouse serum (Gibco). The glands were mechanically disrupted and homogenized to free the parasites. The debris was pelleted after spinning at 20*g* for 5 min at 4ºC. Sporozoites were then collected, counted and maintained on ice until use (adapted from Ozaki *et al.*, 1984). Sporozoites infectivity was dependent on the parasite strain in use. The number of sporozoites per infected mosquito was determined using a hemacytometer (Neubauer chamber). Irradiated sporozoites were submitted to gamma-source radiation equivalent to 20 Krad dose (^{137}Cs source). Heat-killed sporozoites were submitted to a heat shock treatment of 56º C for 15-30 min.

3.4 *In vitro* infections

One day prior to infection, 2×10^5 Hepa 1-6 (or HepG2 cells) were harvested in 24-well plates over a glass coversilp in complete Dulbecco's MEM medium (DMEM; Sigma) supplemented with 10%

heat-inactivated fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2mM glutamine and grown at 37ºC with 5% CO_2.

P. yoelii sporozoites (10^5/well) or *P. berghei* sporozoites (3×10^4/well) were added to the monolayers of Hepa 1-6 or HepG2 cells respectively, centrifuged for 5 min at 1800*g* and incubated at 37ºC with 5% CO_2. At different time points after infection, cells were washed twice with Phosphate Buffered Saline (PBS) and fixed in 4% Paraformaldehyde (PFA) for 20 min at room temperature (RT) (Fig. 3.1).

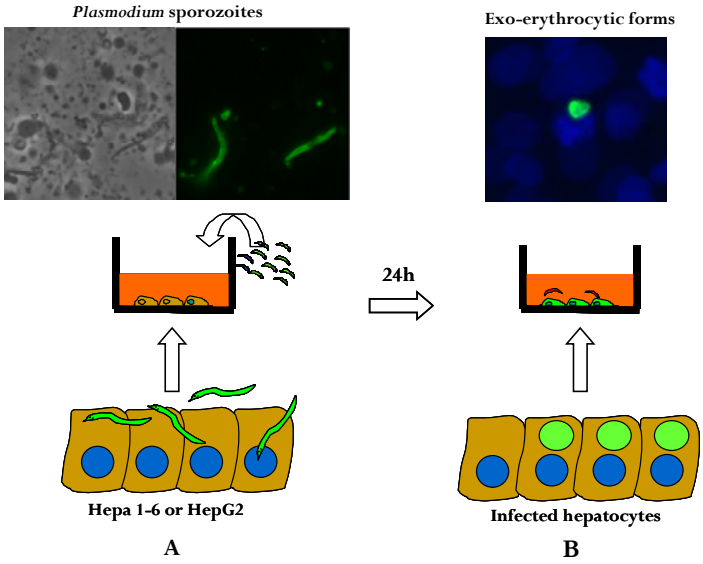


***Fig. 3.1*|Schematic representation of the *in vitro* sporozoite infection system**. Hepatoma cells were seeded in 24-well plates over glass coverslips and allowed to grow overnight at 37ºC in a 5% CO_2 atmosphere. On the next day sporozoites are added to the cells and allowed to develop into exo-erythrocytic forms (EEFs) for 24h (A). The number of EEFs in a coverslip was counted under a fluorescence microscope (*Plasmodium* exo-erythrocytic form, green; nucleus, blue) (B).

3.5 Immunofluorescence assays

After fixation with PFA, hepatoma cells were washed with PBS and incubated for at least 1h in a protein blocking solution (3% Bovine Serum Albumin, 100mM Glycine, 10% Goat serum; Sigma) to avoid unspecific reaction, containing 0.1% Saponin (Sigma) for permeabilization. Cells were then kept for 45 min at RT with the primary antibody against parasite heat shock protein 70 (HSP70) (Tsuji *et al.*, 1994). After washing twice with PBS, cells were incubated for 45 min at RT with secondary anti-mouse IgG fluorescein (FITC)-conjugated antibody (Sigma) diluted in blocking solution. Hepatoma cells were then washed twice in PBS and incubated with 4'6-diamidino-2-phenylindole (DAPI, Sigma) diluted in PBS during 1 min for staining the nuclei. Afterwards, cells were washed 3 times with PBS and the coverslip was mounted on a slide with mounting medium Mowiol (Calbiochem) and observed in a fluorescence microscope (Leica DM LB2). All the immunofluorescence assays (IFA) were performed following the procedure described above using the antibodies relevant for each assay as listed in Table 3.1.

Table 3.1 | Antibodies used in immunofluorescence stainings.

Designation	*Staining feature*	*Origin*
NYSI	*P. yoelii* CS protein	(Charoenvit *et al.*, 1987)
Anti- Mouse IgG FITC or R-PE	Mouse IgG	Sigma
2E6	*P. yoelii* HSP70	(Tsuji *et al.*, 1994)
Anti-NFkB p65 (relA)	NFkB	Santa Cruz Biotecnology
Anti-albumin	Mouse albumin	Cappel / ICN Irvine
PE-Anti-CD11c	Dendritic cell	Pharmingen
FITC-Anti-Mac3	Macrophages	Pharmingen
Anti-Rabbit IgG FITC or Texas Red	Rabbit IgG	Jackson BioLabs
Anti-Active Caspase-3	p18 fragment of caspase-3	Promega

3.6 Cell treatment during sporozoite infection

The study of the effect of different treatments during the course of *in vitro Plasmodium* sporozoites infection was carried out according to the effect under study. Therefore, (i) hepatocyte growth factor (HGF) (500 ng/ml, Calbiochem) was added to cells 1h prior infection; (ii) carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) (20μM, Promega) was also added to cells 1h prior infection; (iii) the agonist antibodies DN-30 and DO-24 (1μg/ml) (Prat *et al.*,1998) were added to cells 90 min post infection; (iv) and LY294002 (25μM, Sigma) and PD98059 (30μM, Sigma) were added to cells 1h prior infection and followed by washes with PBS before addition of *Plasmodium* sporozoites. Reagents were diluted in serum-free DMEM, which was used under the same conditions as the control.

3.7 Apoptosis induction assays

For the apoptosis induction assays, Hepa1-6 or HepG2 cells were treated with Tumor Necrosis Factor- α (TNF-α, 10ng/ml, R&D Systems) and Cycloheximide (CHX, 10μg/ml, Sigma) or exposed to UV light in order to induce apoptosis 18h after sporozoite infection. Heat killed parasites (30min; 56ºC) were used as control. Six hours later cells were fixed and stained for parasite detection and host cell apoptosis quantification.

3.8 Detection of apoptosis

Apoptotic cells were detected and quantified by different methods based on features that a cell commonly displays during the apoptotic cascade process (Chipuk and Green, 2005).

The TUNEL assay using *In Situ* Cell Death Detection reagent (Roche) was performed according to the manufacturer instructions in order to examine DNA breaks in cells undergoing apoptosis. Briefly, after apoptosis induction cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing with PBS, cells were incubated with the reaction mixture for 60 min at 37°C. Stained cells were mounted with Mowiol and analyzed under a fluorescence microscope (Leica DM LB2).

Apoptosis was also detected by immunofluorescence assays (see section 3.5). Cells were stained with anti-NFkB p65 (relA) (Santa Cruz) antibody to observe the translocation of the transcription factor NFkB from the cytoplasm to the nucleus of the cell (Barkett and Gilmore, 1999), and caspase activity was measured with anti-active-caspase-3 antibody (Promega). Both NFkB translocation and caspase activity are characteristic features of apoptosis (Thornberry and Lazebnik, 1998).

Simultaneously, all cells were incubated with DAPI for nuclear morphology assessment (see section 3.5).

3.9 Dendritic cell recruitment to the liver

Groups of 3 mice were infected by mosquito bite (each anesthetized mouse was in contact with 50 *P. yoelii*-infected mosquitoes for 20 min) or by intravenous (i.v.) administration with 10^5 *P. yoelii* sporozoites in a total volume of 100 µl (gamma-irradiated or non-irradiated) or with salivary glands of uninfected mosquitoes as control. At different times after infection, livers were collected and frozen in Tissue-Tek (Sakura), and 15 histological sections (10 µm) from each mouse liver were examined with PE-anti-CD11c mouse antibody (Pharmigen) for detection and quantification of dendritic cells.

3.10 *In vivo* infection, isolation and staining of liver mononuclear cells

BALB/c female mice of 6-8 weeks of age were infected with *P. yoelii* gamma-irradiated (20 Krad), non-irradiated, or heat-killed (15 min; 56ºC) sporozoites by i.v. injection (1×10^6 sporozoites/mouse). Six hours after infection the liver of each animal was collected, manually homogenized, and passed through a cell strainer in DMEM medium. The cell suspension was washed and resuspended in a 35% Percoll (Pharmacia Biotech) gradient solution and centrifuged (500*g*; 10 min). The mononuclear-cell pellet was resuspended in 1ml erythrocyte lysis buffer (Cappel) for 1 min and washed 3 times with PBS. Isolated cells were counted and placed for 1h on poly-L-lysine coated coverslips (~1.5×10^6 cells/liver were obtained). After fixation with 2% PFA, mononuclear cells were stained with PE-anti-CD11c and FITC-anti-Mac3 antibodies (Pharmigen) for dendritic cell and macrophage labeling, respectively. Parasites were stained with anti-*P. yoelii* HSP70 monoclonal

antibody (2E6). Cells were also stained with anti-albumin (Cappel/ICN Irvine) and anti-active caspase-3 (Promega). Quantification was performed with a fluorescence microscope.

3.11 Detection and quantification of *in vivo Plasmodium* sporozoite infection using Reverse Transcription and Real-Time PCR

Forty hours post infection, livers of mice inoculated with 50.000 *Plasmodium* sporozoites by i.v. injection were mechanically homogenized (Tissue Tearer Blader) in 4 ml denaturing solution (4M Guanidium Thiocyanate, 25 mM Sodium Citrate pH 7, 0.5% N-Laurosyl-sarcosine, 0.1% β-mercaptoethanol; Sigma) and processed for RNA isolation using RNeasy Mini kit (Qiagen) according to the protocol supplied by the manufacturer. Total RNA (1 μg) was reverse-transcribed to cDNA by using a first strand cDNA synthesis kit (Roche) in a 20 μl reaction volume on a PTC 100 programmable Thermal Controller (MJ Research, Inc).

A 4 μl sample of the resulting product was used for real-time PCR amplification of *P. berghei* 18S rRNA sequence using SYBRGreen I (Light cycler-FastStart DNA master SYBRGreen I, Roche) as fluorogenic probe and specific primers (5'-AAGCATTAAATAAAGCGAATACATCCTTAC-3' and 5'-GGAGATTGGTTTTGACGTTTATGTG-3'). Amplification was performed in Roche LightCycler Quantitative Realtime PCR system (Roche) with the following temperature profile: 95ºC for 10 min, and 40 cycles of denaturation at 95ºC for 15 sec, annealing at 60ºC for 5 sec and extension at 72ºC for 20 sec. Control plasmids with standard concentrations of 18S rRNA gene were used as described elsewhere (Bruña-Romero *et al.*, 2001). The amount of parasite 18S rRNA molecules detected in each sample is represented as the number of plasmid-equivalent in comparison with a standard curve generated with plasmid DNA (Bruña-Romero *et al.*, 2001).

3.12 Inhibition of PI3-kinase pathway during *Plasmodium* infection

LY294002 (2.5 mg, PI3-kinase inhibitor, Calbiochem) in DMSO was injected intraperitonealy (i.p.). The control group was injected with the same volume of DMSO. Thirty minutes later, mice were infected by i.v. injection with *P. berghei* ($5x10^4$) sporozoites. Real-time PCR was used for quantification of parasite load (see section 3.11).

3.13 Analysis of AKT expression during *Plasmodium* infection

Mice were injected with LY294002 or DMSO (control) 30 min prior to infection with *P. berghei* sporozoites. Three hours after infection, livers were collected and cell lysates were separated by sodium dodecyl sulfate-palyacrylamide gel electrophoresis (SDS-PAGE) and probed with polyclonal anti-phospho-Akt (phospho-Ser-473) and total Akt antibodies (Cell Signalling). Detection was

performed with the enhanced chemiluminescence system (ECL; Amersham International) following the manufacturer's instructions.

3.14 Immunopreciptitation and Western blot analysis

Cells were lysed on ice in EB buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) and in the presence of a cocktail mixture of protease and phosphatase inhibitors. Immunoprecipitation was performed according to standard protocols and with the appropriate antibodies (Celis *et al.*, 1994). When amounts of the same protein in different samples were compared, the total protein in the lysates was determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol.

Immunoprecipitated or total lysate proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then probed with specific antibodies and binding was detected by Western blotting, which was performed according to standard methods. Final detection was performed with the enhanced chemiluminescence system (ECL; Amersham International) following the supplier's instructions.

3.15 MET down-modulation by siRNA in hepatoma cell line

MET expression was down-modulated in HepG2 cells by transduction with the lentiviral vector PCCLsin.PPT.hPGK.GFP.Wpre, engineered to carry either the required sequence for synthesizing MET-targeted siRNA or a control siRNA, under the transcriptional control of the H1 promoter. siRNAs were produced as previously described (Pennacchietti *et al.*, 2003). Two different siRNAs against Met were used as well as for the control siRNA.

The sequences used were the follwing: for MET (5'-GATCCCCGTCATAGGAAGAGGGCATTTTCAAGAGAAATGCCCTCTTCCTATGACTTTT TGGAAA-3' and 5'-AGCTTTTCCAAAAAGTCATAGGAAGAGGGCA TTTCTCTTGAAAATGCCCTCTTCCTATGACGGG-3') and for the control (5'-GATCCCCCTCATAGGAAGACCCCATTTTCAAGAGAAATGGTGGTCTTCCTATGACTTT TTGGAAA-3' and 5'-AGCTTTTCCAAAAACTCATAGGAAGACCCCAT TTCTCTTGAAAATGGGGTCTTCCTATGAGGGG-3').

Lentivirus production and infection of HepG2 cells were performed as previously described (Vigna and Naldini, 2000). Briefly, lentiviruses were produced by transient transfection of 293T cells. Transfections (200nM of each oligo) were done with Oligofectamine reagent (Invitrogen) follwing supplier's instructions. HepG2 cells were transduced by overnight incubation with lentiviruses collected from 24 h supernatants, in Iscove supplemented medium (Gibco) with 8 μg/mL Polybrene (Sigma). Selection was done with G418 (1 μg/ml; Sigma) and the resistant cells were pooled and

assayed for the expression of MET protein by Western blot analysis, as follows. After selection, cells were kept in culture for 72h and then extracted with boiling Laemmli buffer (Laemmli, 1970). Equal amounts of proteins evaluated by BCA method (Pierce) were loaded in each lane. Western blots were probed with antibodies against human MET (C12; Santa Cruz Biotechnology) and standard protocols used (Vigna and Naldini, 2000).

3.16 Assembly of DN MET-GFP hepatoma cell line

For virus production, Met cDNA was subcloned in pLXSN plasmid. Dominant-negative Met was produced by fusing the extracellular and transmembrane portions of Met with GFP sequence in the pEGFP-N1 vector. A *Kpn*I site was introduced at position 2962 of the Met coding sequence by PCR (forward oligonucleotide: 5'-GGAGCACAATAACAGGTGTTG-3; reverse oligonucleotide: 5'-TTCCGGTACCCAATCTTTAATTTGCTTTCTCTTTTTC-3'). The sequences coding for the extracellular and transmembrane portions of Met were subcloned as a *Sac*I–*Kpn*I fragment into the pEGFP-N1 vector. This chimeric construct was then sequenced and subcloned in the lentiviral vector p156RRLsin-PPT-hCMV-MCS-pre. The transfections were performed by the calcium phosphate and DEAE dextran methods using 2-5 μg of each construct (Giordano *et al.*, 2002; Vigna and Naldini, 2000).

In vitro infection of hepatocytes was done by incubating the cells overnight in the presence of virus-containing supernatant. Efficiency of infection was evaluated using GFP fluorescence and infected hepatoma cell lines expressing the Met-GFP construct were sorted by FACS to select positive cells. A control cell line was made with equal amounts of viral particles containing GFP alone (Carrolo *et al.*, 2003; Giordano *et al.*, 2002; Vigna and Naldini, 2000).

3.17 Isolation of plasmid DNA

All plasmid DNA were isolated either on a small scale (minipreps) using the QIAprep spin Kit (Qiagen) or on a large scale (maxipreps) with the Plasmid Maxi Kit (Qiagen). In both procedures the manufacturer's protocols were followed.

3.18 DNA restriction enzyme digestion

The digestion of plasmid DNA was performed according with the dilution advised by the supplier using suitable volumes of the buffer specific for each enzyme used, according to standard procedures (Sambrook and Russell, 2001).

3.19 DNA Electrophoresis

DNA samples smaller than 20Kbp were resolved in 1-3% (w/v) agarose gels in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) using conventional submarine methods and apparatus.

3.20 DNA Extraction from agarose gels

DNA samples were extracted from agarose gels using a QIAquick gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

3.21 Cloning PCR products

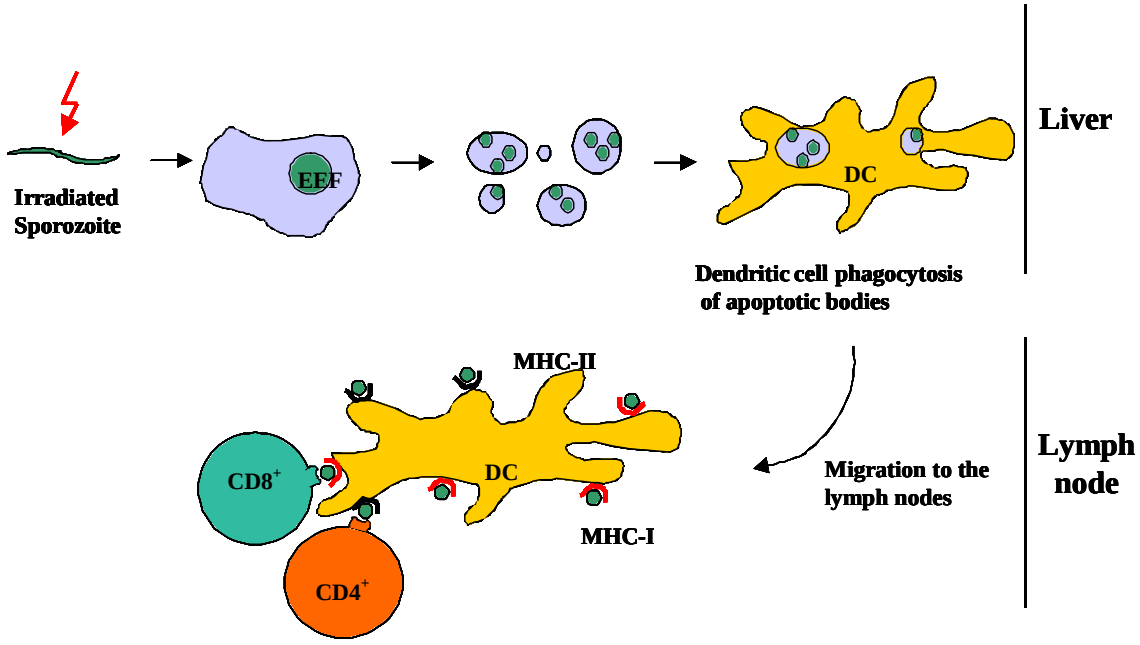
PCR products larger than 3 Kb were cloned into pCR 2.1-TOPO (Invitrogen) vector and transformed into TOP10 One Shot cells following the manufacturer's instructions.

3.22 HSP 70 construct

The DNA sequence of interest (*P. yoelii* HSP70) was amplified by PCR using specific primers (forward 5'-CCGCTCGAGATGGCTAACGCAAAAGCATCAAAGCCA-3 and reverse 5'-ATAAGAATGCGGCCGCTTAATCAACTTCTTCAACAGTTGGTCC-3). Results were analyzed in 1% (w/v) agarose gel in TE buffer and PCR products cloned in PCR 2.1 TOPO vector (Invitrogen). The fragments were subsequently digested with the appropriate restriction enzymes and subcloned into a mammalian expression vector, pCI-neo (Promega) (see section 3.18).

3.23 *In vitro* transient transfection system

Plasmids were introduced into hepatocytes using a cationic polymer transfection reagent, jetPEI-Gal (PolyPlus). Transfection was performed according to the manufacturer's protocol. For stable transfection the selection was performed using G418 sulfate (Cellgro) as the limiting reagent.



Chapter Four

RESULTS I

Role of dendritic cells during liver stage malaria and the initiation of an immune response

***Legend*|Model of anti-malaria immune responses induced by irradiated sporozoites.** Protective immune responses to malaria can be induced in humans and mice by injection of irradiated sporozoites. These infect hepatocytes as well as non irradiated sporozoites do, but they are not capable to develop into merozoites. The decrease observed in the number of infected hepatocytes suggests that these cells are dying during this period of development. Our working hypothesis predicts that the apoptotic bodies originate from infected hepatocytes by irradiated sporozoites are phagocytosed by dendritic cells. After migration to the lymph nodes, DCs have the ability to present *Plasmodium* antigens included in these apoptotic bodies and activate T lymphocytes, initiating an immune response against malaria.

4.1 Introduction

A large variety of strategies are being followed in the quest for a vaccine against Malaria. Complete protection against the disease is based on attempts to exploit any existing checkpoints throughout the complex life cycle of the *Plasmodium* parasite. Although several infection-blocking candidates, aiming at specific stages of the cycle, are under scrutiny, none of them are able to induce protection and long term immunity comparable to the levels achieved with irradiated-attenuated sporozoites (Greenwood, 2005).

As mentioned before, full protection to malaria is only achieved in mice, monkeys and humans by immunization with irradiated-attenuated *Plasmodium* sporozoites (Doolan and Hoffman, 2000; Chatterjee *et al.*, 1996; Nussenzweig *et al.*, 1967).

Protection was at first attributed to the antibody response mounted against *Plasmodium* circumsporozoite protein (CS), mainly because these antibodies were capable of neutralizing sporozoite infectivity and high levels of anti-CS led to protection against a sporozoite challenge in humans (Mazier *et al.*, 1988; Herrington *et al.*, 1987). Currently, it is well established that the sporozoite-infected hepatocyte is the main target of this response generated by irradiated sporozoites and that the strong cytotoxic T lymphocyte (CTL) activity parasite-specific elicited has a pivotal role in immunity (Hoffman *et al.*, 1989; Weiss *et al.*, 1988; Schofield *et al.*, 1987 a, b). Taken together with the acquisition of effective antimalarial immunity in endemic populations, these data provide reasons to hope that a malaria vaccine conferring protective immunity is feasible (Carvalho *et al.*, 2002). Thus, revealing the mechanism behind the initiation of the immune response generated with this immunization is of extreme importance to the development of vaccine delivery systems that can induce such protection (Stevenson and Riley, 2004).

Irradiated sporozoites fail to establish a blood-stage infection. They are capable of invading the liver and infect hepatocytes as normal sporozoites do, but then growth is abrogated when a trophozoite stage is reached and schizogony is aborted (Ngonseu *et al.*, 1998; Chatterjee *et al.*, 1996; Scheller *et al.*, 1995; Sigler *et al.*, 1984). Although this is a short term intrahepatocytic development, the profile of liver antigens expressed is enough for inducing immunity (Suhrbier *et al.*, 1990). In fact, the development inside the hepatocytes appears to be a requisite for protection since infected hepatocytes were able to induce partial protection once grafted in the spleen after infectious sporozoite challenge, while inactivated sporozoites and their extracts where shown to be incapable of inducing any effect (Scheller and Azad, 1995; Renia *et al.*, 1994; Mazier *et al.*, 1988; Alger and Harant, 1976).

Hepatocytes are the most abundant cells in the liver and the only cell population that can sustain complete *Plasmodium* exo-erythrocytic form (EEF) development (Meis *et al.*, 1983 a, b). Thus, besides being important for the parasite's survival, the liver has a crucial role on the anti-*Plasmodium*

immunity establishment and maintenance as it harbours the source of parasite antigens that initiate an immune response in the host, the infected hepatocyte (Krzych *et al.*, 2000; Scheller and Azad, 1995).

This irradiated sporozoites immune response has been characterized as being dependent of $CD8^+$ T cells as they recognize an epitope in the CS protein and, upon passive transfer of these cytotoxic T cell clones, a high degree of protection was obtained (Romero *et al.*, 1989). On the other hand, *in vivo* depletion of these T cells decreases drastically the protective immunity against live sporozoite challenge (Weiss *et al.*, 1988; Schofield *et al.*, 1987a).

The target of these malaria specific $CD8^+$ T cells is the infected hepatocyte (Hoffman and Doolan, 2000; Suhrbier *et al.*, 1990; Hoffman *et al.*, 1989). Since T cells only recognize antigens after they have been processed and presented on cell surfaces along with major histocompatibility complex (MHC) molecules, $CD8^+$ T cells would exclusively be activated by parasite-derived epitopes in association with MHC class I molecules, given that lymphocytes are antigen recognition-restricted concerning MHC molecules (Townsend and Bodmer, 1989).

As the hepatocyte is the only cell type that can bear parasite development and is able to express MHC class I molecules on its surface, it is only when the hepatic stage occurs that this type of immunity is established (Doolan and Hoffman, 2000; Suhrbier *et al.*, 1990). However, in order to have a $CD8^+$ T cell response it is required to have efficient priming of naïve T cells that will be responsible for the initiation of the immune response itself. In subsequent infections, a stimulation of memory T cells resident in the liver also has to occur (Morrot and Zavala, 2004b; Chatterjee *et al.*, 1999; Riley, 1999).

$CD8^+$ T lymphocyte responses are primed by MHC class I-associated peptides usually generated from endogenous proteins in the cytosol of professional antigen-presenting cells (APC), the dendritic cells. They are known to be highly efficient modulators of the immune system uniquely specialized in initiating T cell immunity either *in vitro* or *in vivo* (Larsson *et al.*, 2001; Banchereau and Steinman, 1998). During malaria infection, no dendritic cells are infected by *Plasmodium* sporozoites as they can only replicate in hepatocytes. However, specific $CD8^+$ T cells reactive to *Plasmodium* antigens were found in irradiated sporozoite-immunized hosts, suggesting that parasite antigens are transferred to APCs (Krzych *et al.*, 2000; Rodrigues *et al.*, 1991; Hoffman *et al.*, 1989; Romero *et al.*, 1989; Weiss *et al.*, 1988). This phenomenon in which dendritic cells can also present exogenous antigens associated with MHC class I molecules generating $CD8^+$ T cell epitopes has been denominated as "cross-presentation" (Heath and Carborne, 2001). In addition, recent data demonstrated that dendritic cells are required for induction of this specific anti-malaria $CD8^+$ T cells response after immunization with irradiated sporozoites as *in vivo* depletion of these APCs led to the abrogation of *Plasmodium* specific $CD8^+$ T priming (Jung *et al.*, 2002).

Infection is often accompanied by the apoptotic death of the infected host cell, which normally leads to the formation of apoptotic bodies that carry pathogen antigens within. Several studies have now demonstrated that cross-presentation of antigens in apoptotic bodies is an alternative way used by dendritic cells of cross-priming cytotoxic immune responses against intracellular pathogens which do not infect directly professional APCs (Winau *et al.*, 2005; Schaible *et al.*, 2003; Yrlid and Wick, 2000; Albert *et al.*, 1998b). This mechanism of antigen presentation via uptake of apoptotic infected cells has turned out to be a tool through which immunity is modulated (Albert, 2004; Fonteneau *et al.*, 2002). Recently, it has been proposed that apoptosis of host cells contributes to immunity induced by irradiated-parasite vaccines. Irradiation is a potent inducer of apoptosis and for decades irradiated-attenuate parasites have been used as live vaccines which in case of *Plasmodium* resulted in the achievement of full protection against the disease (James and Green, 2004; Hoffman *et al.*, 2002). The data presented in this chapter elucidates one mechanism behind the protection reached with *Plasmodium* irradiated-sporozoites immunization, highlighting one source of parasite antigen that mediates the immune response. Evidence showed that hepatocytes infected with irradiated *Plasmodium* sporozoites undergo apoptosis shortly after infection. In addition, infection with irradiated sporozoites induces the recruitment of dendritic cells to the liver of the host, where they phagocytose the apoptotic infected hepatocytes. Moreover, it is proposed that these apoptotic *Plasmodium*-infected hepatocytes constitute a source of parasite antigens responsible for the initiation of a protective anti-malarial immune response by dendritic cells.

4.2 Results

4.2.1 Irradiated *Plasmodium* sporozoites induce infected hepatocytes apoptosis *in vitro*

Although it is a fact known for some years now that irradiated sporozoites induce protection, the mechanism behind this effect is still not understood. Given the outstanding results concerning the immune responses obtained with irradiated sporozoites, its importance in the development of a hepatic stage vaccine is a clear requirement. The scrutiny of how this immunity is established is the focus of intense research efforts and an essential task for the control of the malaria parasite (Krzych and Schwenk, 2005).

Since the first description of the malaria exo-erythrocytic stage that attempts were made to establish EEF cultures *in vitro* (Hollingdale *et al.*, 1983 a, b). Several studies were carried out, using different *in vitro* methods and parasite strains, pursuing a culture system where the parasite fully develops into mature infectious EEFs (Mota and Rodriguez, 2000; Calvo-Calle *et al.*, 1994).

It is known that even high doses of radiation do not affect hepatocyte's invasion by sporozoites *in vitro* (Nussler *et al.*, 1989). It was also described previously that at early time points in culture the EEFs from both irradiated and non-irradiated sporozoites are indistinguishable. However, at time points 1-2 days post-infection, a pronounced decrease in the number of irradiated EEFs was observed (Suhrbier *et al.*, 1990; Suhrbier *et al.*, 1987; Hollingdale, 1985; Sigler *et al.*, 1984).

In order to study the survival of hepatocytes infected with irradiated *Plasmodium* sporozoites at earlier times after infection, a murine hepatoma cell line, Hepa 1-6 (or a human equivalent, HepG2), was incubated with either irradiated or non-irradiated *P. yoelii* (or *P. berghei*) sporozoites and infected cells were counted at different time points post-infection. Infected cultures were stained with an antibody against HSP70, a protein that is highly expressed in developing parasites inside hepatocytes but not in sporozoites (Kumar *et al.*, 1993).

The result revealed that the number of EEFs found in culture for both non-irradiated and irradiated sporozoites decreases with the course of time and this reduction is more marked in Hepa 1-6 hepatoma cells infected with *P. yoelii* irradiated sporozoites (squares, Fig. 4.1) rather than when non-irradiated sporozoites were used (circles; Fig. 4.1). This decrease starts occurring shortly after infection and in the 24h-48h time frame the number of EEFs in hepatocytes infected with non-irradiated sporozoites stabilizes while it decreases steeply in those infected with irradiated ones.

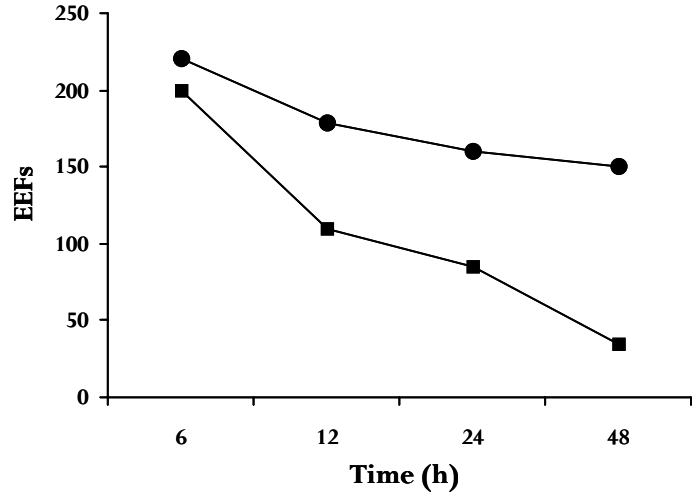


***Fig. 4.1*|Time-course of an *in vitro* infection with non-irradiated or irradiated *P. yoelii* sporozoites.** Hepa 1-6 cells were infected with non-irradiated (circles) or irradiated *P.yoelii* sporozoites (squares) during different incubation periods. The cells were fixed and EEFs were stained with anti-HSP70 antibody for quantification under a fluorescence microscope.

The reason for this decrease in the number of infected cells has been attributed to the degeneration of intracellular parasites shortly after invasion (Suhrbier *et al.*, 1990; Sigler *et al.*, 1984). We tested whether this was the case or whether, alternatively, hepatocytes infected with irradiated sporozoites were dying by apoptosis of the infected cells. To evaluate this hypothesis, infected cells were examined for nuclear morphology and activation of NFkB p65 (relA), a transcription factor that is translocated to the nucleus after initiation of certain apoptotic pathways (Barkett and Gilmore, 1999).

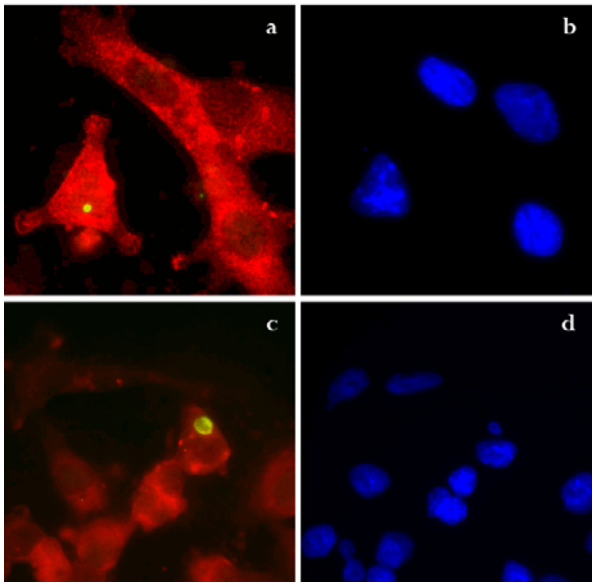


***Fig. 4.2*|Irradiated *P. yoelii* sporozoites lead to the apoptosis of the infected hepatocyte.** Hepa 1-6 cells were incubated with *P. yoelii* irradiated (a,b) or non-irradiated (c,d) P. *yoelii* sporozoites. Cells were fixed after 6h and stained with anti-*Py*HSP70 Ab to detect EEFs (green), anti-NFkB to observe the transcription factor translocation from the cytoplasm to the nucleus of the cell (red) and DAPI to evaluate nuclear morphology (blue).

Both the staining with DAPI (blue; Fig. 4.2), showing the irregular nuclear shape, and the translocation of the transcription factor NFkB (red; Fig. 4.2) from the cytoplasm to the nucleus, constitute evidence of apoptosis in cells infected with irradiated sporozoites (Fig. 4.2 a,b), while infection with non-irradiated sporozoites results in no signs of apoptotic cells (Fig. 4.2 c,d). Apoptotic cells were quantified for infection with each type of sporozoite and the results are shown below (Fig. 4.3). Apoptosis level is higher in cells infected with irradiated sporozoites in both methods used for detection.

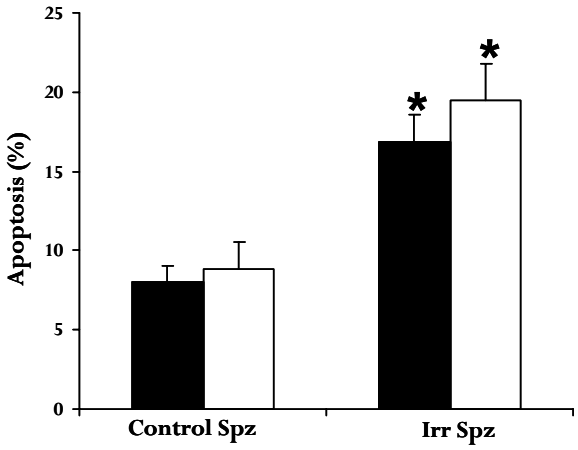


***Fig. 4.3*|Quantification of apoptosis at early time points after infection.** Hepa 1-6 cells were incubated with irradiated or non-irradiated *P. yoelii* sporozoites. Apoptosis was monitored by assessment of nuclear morphology (black bars) and nuclear translocation of NFkB p65 (white bars). Error bars represent the standard deviation of triplicate samples. Asterisks (*) indicate a significant difference between control and irradiated sporozoites ($P<0.05$).

It could also be observed that EEFs formed by irradiated sporozoites are much smaller than those formed by non-irradiated ones (Fig. 4.2). It has previously been reported that although irradiation leaves the sporozoites capable of invading and transforming into trophozoites, development is compromised resulting in a smaller EEF size. The rapid growth and replication observed in normal

sporozoite infections is limited when sporozoite irradiation takes place (Scheller *et al.*, 1995; Nussler *et al.*, 1989; Sigler *et al.*, 1984). Comparing parasite strains and host cell types, it is evident that the pair formed by host cell/parasite is of extreme importance for achieving normal rates of development when *in vitro* cultures of hepatoma cells are used and could also account for differences in EEFs size (Scheller *et al.*, 1994; Hollingdale *et al.*, 1983a).

Apoptotic cell death is mediated by molecular pathways that culminate in the activation of a family of cysteine proteases denominated caspases. These proteases orchestrate the dismantling and clearance of the dying cell. Caspase-3 plays a central role in the apoptosis cascade and its activation is a conserved feature of the apoptotic process (Chipuk and Green, 2005; Thornberry and Lazebnik, 1998). Therefore, apoptosis in hepatocytes infected with irradiated sporozoites was also confirmed by detection of caspase-3 activation (Fig. 4.4). The high degree of caspase-3 activation (red; Fig 4.4) seen in cells infected with irradiated spororozoites (green; Fig. 4.4) coincides with apoptotic cells carrying condensed chromatin observed with DAPI staining in blue.

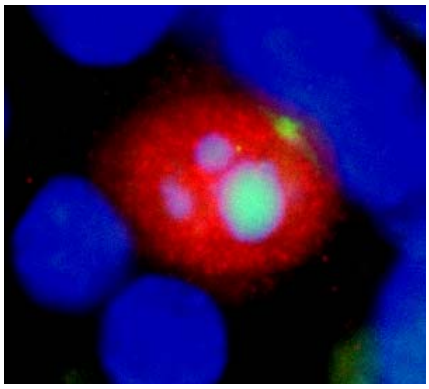


***Fig. 4.4*|Apoptotic cell infected with irradiated *P. yoelii* sporozoites.** Hepa 1-6 cells were incubated with *P. yoelii* irradiated sporozoites. 6h post-infection cells were fixed and stained with anti-*Py*HSP70 Ab to detect EEFs (green), anti-active-caspase-3 (red) and DAPI to observe nuclear morphology (blue).

Parasite irradiation probably reduces the ability of *de novo* synthesis of parasite molecules responsible for inhibition of apoptosis of the infected cell, eventually leading to the abrogation of infection and cell death. However, a lower but significant amount of apoptosis also occurs during a non-irradiated sporozoite infection, suggesting that, in some cases or at certain level, the normal parasite cannot prevent the apoptosis of their host. This observation raises some questions about whether this phenomenon could play a role during the establishment of infection and, specifically, in generating immunity.

4.2.2 The role of dendritic cells during *P. yoelii* infection

Dendritic cells are antigen-presenting cells, capable of initiating and modulating immune responses. In their immature form, they traffic from blood into peripheral tissues, which they scan like sentinels looking for antigens that they can capture and process. They subsequently migrate to draining lymph

nodes, where they are converted into mature and fully activated cells and present antigens to resting lymphocytes (Figdor *et al.*, 2004; Banchereau and Steinman, 1998).

4.2.2.1 Recruitment of dendritic cells to the liver after malaria infection

In order to determine if dendritic cells are implicated in the process of establishing a liver-stage immune response, *in vivo* assessment of whether dendritic cells were present in the liver during infection was done.

Livers of mice injected with irradiated and non-irradiated *P. yoelii* sporozoites were collected at different time points post-injection. Histological sections stained with antibody against mouse myeloid dendritic cell marker, anti-CD11c, constitute evidence of the presence of dendritic cells in the liver shortly after infection (Fig. 4.5).

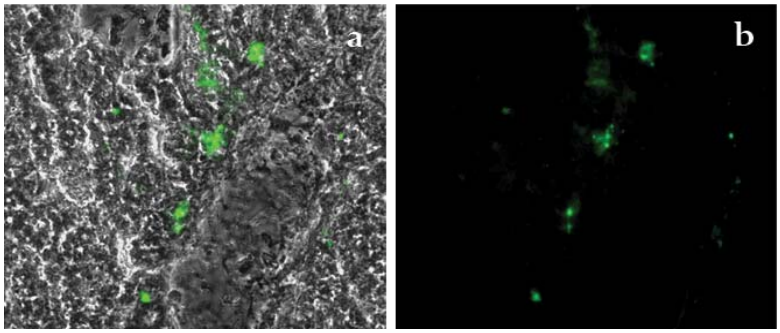


***Fig. 4.5*|Dendritic cells are present in the liver after infection with *Plasmodium* sporozoites.** Histological liver sections of mice infected with malaria by mosquito bite were stained with antibodies against mouse dendritic cell marker anti-CD11c (green)(a, transmitted light overlay; b). There is evidence of dendritic cell presence near a blood vessel.

The quantification of these histological sections showed that during malaria infection the number of dendritic cells increases in the liver (Fig. 4.6a). Similar results were obtained when mice were subjected to bites from infected mosquitoes (Fig. 4.6b). In both cases an increased recruitment of dendritic cells early after infection was observed, which was independent of sporozoite irradiation and infection route used. However, when the recruitment was induced by bites from infected mosquitoes, a lower amount of dendritic cells was detected, probably because of the smaller numbers of sporozoites injected by mosquito bite (Medica and Sinnis, 2005; Vaughan *et al.*, 1999; Vanderberg, 1977).

The recruitment of these antigen presenting cells in the liver indicates that they might play a specific role during *Plasmodium* infections. The increase in the number of dendritic cells in the liver during malaria infection would allow these cells to rapidly capture parasite antigens. However, in order to be able to present those antigens, these cells have to receive a maturation signal and become fully activated. The source for these signals may have various origins. Among them are the pathogen itself

or inflammatory cytokines (Guermonprez *et al.*, 2002; Medzhitov and Janeway, 2000; Albert *et al.*, 1998b). The fact that *Plasmodium* sporozoites migrate through several hepatocytes in the liver before choosing one to infect, and, in this process, disrupt plasma membranes, causing cell wounding and necrotic death, could account for the origin of this maturation signal (Mota *et al.*, 2001; Meis *et al.*, 1985a). In fact, wounded and dying cells induce local inflammatory responses that can be responsible for mediating the dendritic cell recruitment to the tissues and, consequently cause maturation (Shi *et al.*, 2000; Dieu-Nosjean *et al.*, 1999). It was also described that *Plasmodium* hepatic stages lead to specific inflammatory responses with recruitment of mononuclear cells (Khan and Vanderberg, 1992). In addition, the release of uric acid from dying cells would, likewise, provide additional maturation signals for dendritic cells (Shi *et al.*, 2003).

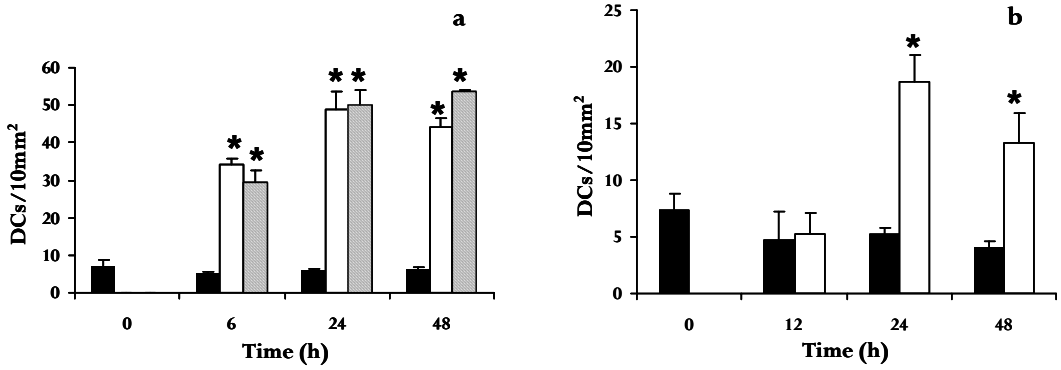


***Fig. 4.6*|Dendritic cells are recruited to the liver after infection with *P. yoelii* sporozoites.** Quantification of dendritic cells in histological sections of mice livers by anti-CD11c staining. (a) Dendritic cell number in livers of non-infected mice (black bars) and in mice injected with non-irradiated (white bars) or irradiated (striped bars) *P. yoelii* sporozoites, at different times post-infection. (b) Dendritic cell number in livers of mice bitten by 50 uninfected (black bars) or infected (white bars) mosquitoes obtained at different times post feeding. Error bars represent the standard deviation of triplicate histological samples, each composed of ≥5 liver sections to cover 10 mm^2. Asterisks (*) indicate a significant difference between the sample and the control ($P<0.02$).

4.2.2.2 *Plasmodium* antigens are phagocytosed by dendritic cells and macrophages

As shown above infected hepatocytes undergo apoptosis during infection. In this process, they originate apoptotic bodies, which have to be cleared from the liver. Dendritic cells and macrophages are professional antigen-presenting phagocytes capable of large-scale phagocytosis of apoptotic cells and clearance of microbial pathogens (Savill *et al.*, 2002; Rubartelli *et al.*, 1997). To determine whether apoptotic hepatocytes containing *P. yoelii* antigens are phagocytosed by antigen presenting cells in the liver, we isolated non-parenchymal mononuclear cells from the livers of mice immunized with irradiated sporozoites at different times after injection. *P. yoelli* antigen-carrying dendritic cells and macrophages were found at 6h post-infection, demonstrating the uptake of *Plasmodium* antigens by these phagocytic cells (Fig. 4.7).

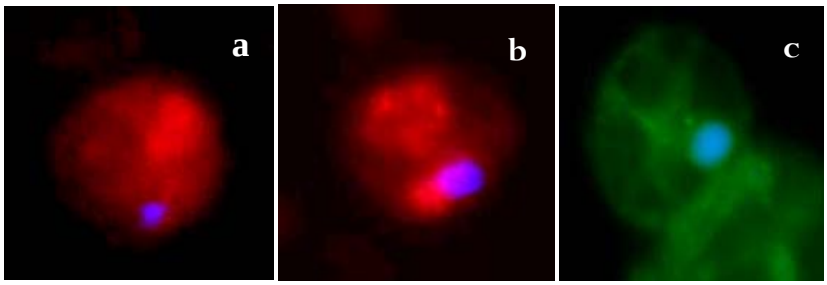


***Fig. 4.7*|Phagocytosis of apoptotic hepatocytes containing *Plasmodium* antigens by dendritic cells and macrophages.** Liver non-parenchymal mononuclear cells obtained 6h after injection of mice with irradiated *P. yoelii* sporozoites and stained with different antibodies: dendritic cell (anti-CD11c, red; a and b), macrophage (anti-Mac3, green; c) and *P. yoelii* HSP70 (blue; a, b, c). *Plasmodium* antigens were found inside both phagocytic cells type.

An average of 3 macrophages and 2 dendritic cells in each PyHSP70 vacuole-containing liver were found in 3 different experiments. The number of dendritic cells containing these vesicles is probably higher in the mouse, but after dendritic cells have internalized and processed the apoptotic bodies, they leave peripheral organs and migrate to the lymph nodes, subsequently to having received an activation signal. As a result, the time that they remain in the liver is very short. Therefore, the quantification obtained 6h after immunization might, in fact, correspond to what would be expected, because it corresponds to the number of phagocytic cells that were still in the liver and have not yet migrated to the lymph node. Thus, the time frame for detection of phagocytic cells carrying parasite antigens is very short and restricted as the time gap between these two events is of approximately 1 h (Sallusto and Lanzavecchia, 2000).

The HSP70-positive vesicles found inside macrophages and dendritic cells must be derived from infected hepatocytes, since this protein is not expressed in sporozoites and appears only in infected hepatocytes, approximately 6 h post-infection (Kumar *et al.*, 1993).

In order to evaluate the origin of the phagocytosed *Plasmodium* antigens, phagocytic cells were also stained for mouse albumin, a common protein known to be found in the cytosol of hepatocytes (Doweiko and Nompleggi, 1991). The results demonstrated that albumin is present in the phagocytosed vesicles, confirming that infected hepatocytes, and not dead sporozoites, are the source of uptaken antigens, corresponding to the colocalization observed between the parasite and albumin (Fig. 4.8). As a control, non-parenchymal liver mononuclear cells were isolated from non-infected control mice and processed likewise. No *P. yoelii* HSP70 or albumin staining was found in these cells.

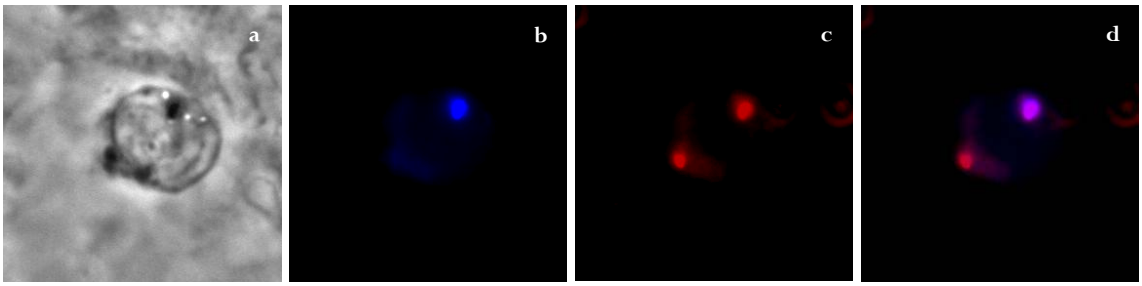


***Fig. 4.8*|Hepatocyte proteins are found in *P. yoelii* phagocytosed vesicles.** Liver non-parenchymal mononuclear cells obtained 6h after injection of mice with irradiated *P. yoelii* sporozoites were stained with anti-*P. yoelii* HSP70 (blue; b) and anti-albumin (red; c) antibodies. *Plasmodium* vesicles colocalized with albumin, a protein abundant in the liver (b and c overlay; d). Transmitted light view (a). Cells isolated from livers of non-infected control mice did not present any albumin staining.

To confirm if these HSP70-positive vesicles were derived from apoptotic cells, phagocytic cells were stained with anti-active-capase-3 antibody. Phagocytes showed evidence that, indeed, there is activation of caspase-3 in the *Plasmodium* antigen-containing vesicles implicating that dendritic cells phagocytose apoptotic infected hepatocytes during malaria infections *in vivo* (Fig. 4.9). Non-infected control mice did not exhibit any positive staining for capase-3 activation.

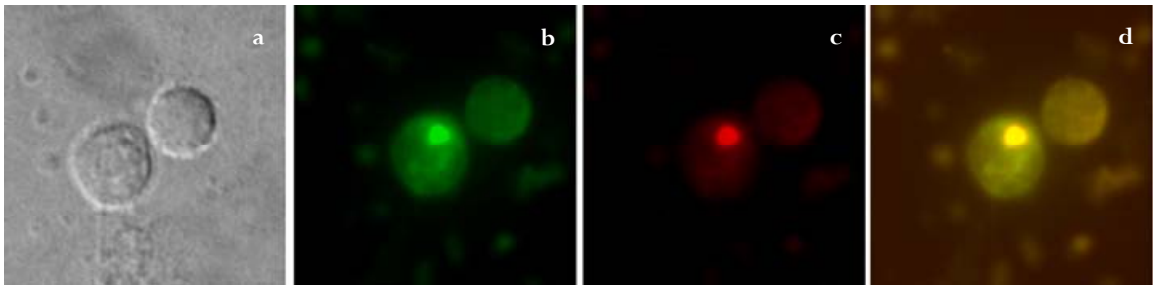


***Fig. 4.9*|Caspase-3 is active in *P. yoelii* phagocytosed apoptotic bodies.** Liver non-parenchymal mononuclear cells obtained 6h after injection of mice with irradiated *P. yoelii* sporozoites were stained with anti-*P. yoelii* HSP70 (green; b) and anti-active-caspase-3 (red; c) antibodies. *Plasmodium* vesicles colocalized with activated caspase-3 indicating an apoptotic origin (b and c overlay; d). Transmitted light view (a). Cells isolated from livers of non-infected control mice did not present any caspase-3 activation.

When liver non-parenchymal mononuclear cells were analyzed 12 h post-infection, no cells were found containing *P. yoelii* HSP70, suggesting that infected hepatocyte death and clearance occurs early during *in vivo* infections with irradiated sporozoites. This is consistent with the observation that *Plasmodium* sporozoite infection and initiation of the immune response occurs rapidly, since activation of specific anti-*P. yoelii* $CD8^+$ T cells is detected as soon as 8 h after inoculation of sporozoites (Hafalla *et al.*, 2003; Hafalla *et al.*, 2002; Sano *et al.*, 2001).

4.2.2.3 Establishment of a cross-presentation assay

To initiate immune responses, dendritic cells need to activate naïve T lymphocytes into cytotoxic T lymphocytes by taking up, processing and degrading antigens into peptides which can then be presented on MHC class I molecules. Dendritic cells can present antigens acquired either by internalization or synthesized in their cytosol. When pathogens do not infect these professional antigen-presentation cells, antigens are acquired via an exogenous route, transported into the cytosol and presented by MHC class I molecules through a cross-presentation process (Gil-Torregrosa *et al.*, 2004; Albert *et al.*, 1998 a, b).

Since *Plasmodium*-infected apoptotic hepatocytes are phagocytosed by dendritic cells and apoptotic cells constitute an efficient exogenous source for antigens, able to induce a malaria immune response, a cross-presentation assay capable of proving that dendritic cells are the ones responsible for this cross-presentation, after the antigen uptake provided by the apoptotic infected hepatocytes, was necessary.

Several attempts were made pursing this aim but the results obtained were either inconclusive and inconsistent or irreproducible between different experiments for an array of different reasons. For example, the total cell number present in a liver (10^9) and the small proportion of these that were potentially infected and antigen-providing was a huge disadvantage on setting up such an assay. Therefore, the positive results obtained constitute preliminary data only and are not shown here. Nevertheless, the approaches followed in this process are described next.

Plasmodium circumsporozoite protein construct

A genetic approach was undertaken to evaluate if dendritic cells are capable of cross-presenting *Plasmodium* antigens derived from apoptotic bodies. A plasmid construct was made using the *P. yoelii* CS protein (kindly supplied by Dr. Oscar Bruña-Romero). Hepa 1-6 cells were transfected with this construct and apoptosis was induced by UV light. The apoptotic bodies generated in the process were incubated with dendritic cells for 6h to allow phagocytosis. These cells were then injected into mice and an ELISPOT assay was performed nine days post injection for determination of activated $CD8^+$ T cells specific for *Plasmodium* CS protein through the quantification of IFN-γ production (Carvalho *et al.*, 2001).

$CD8^{\pm}$ T cell clone specific for *Plasmodium* CS

Another approach that was carried out was based on the use of an immunologic tool for detection of *P. yoelii*-specific antigen presentation. To observe if dendritic cells were able to activate T cells after phagocytosing the parasite antigens in the liver, mice were injected with irradiated and heat-killed sporozoites (used as negative control). Liver cells were isolated and placed together with the $CD8^+$ T cell clone (Y26) specific for an epitope from *P. yoelii* CS protein (Rodrigues *et al.*, 1991).

After an incubation period of 24h the T cells were stained with anti-IFN-γ antibody (Molecular Probes) to analyze T cell activation. Although this would not prove that dendritic cells mediate cross-presentation of *Plasmodium* antigens *in vivo*, it could have suggested that they had the capacity to do so.

CD11c^{+} depleted transgenic mouse

A novel diphteria toxin-based system that allows the inducible, short-term ablation of dendritic cells *in vivo* was lately reported (Jung *et al.*, 2002). The loss of T cell activation using this system would implicate dendritic cells in the process of presenting *Plasmodium* antigens to T cells.

To determine whether liver dendritic cells were the ones responsible for the observed T cell activation, transgenic mice expressing the diphtheria toxin receptor under the control of CD11c^{+} promoter were used. These mice can be depleted of CD11c^{+} dendritic cells by a diphtheria toxin injection 24h prior malaria infection. Irradiated and non-irradiated sporozoites were injected and livers were removed 6h later. Liver mononuclear cells were incubated with the T cell clone specific for an epitope from *P. yoelii* CS protein (Y26) and activation was detected by IFN-γ expression 24h later.

Further antigen presentation assays will have to be performed to establish and confirm the preliminary data already obtained with these approaches. The search for a more accurate cross-presentation assay making use of the technology that is becoming available in this area of research will continue.

4.3 Discussion

It has been over 30 years since the first successful human malaria vaccine trial (Clyde *et al.*, 1973a). Bites of hundreds of irradiated infected mosquitoes where then used to immunize volunteers, and established that protection against malaria could also be achieved in humans as had been shown for mice by Nussenweig and colleagues (Nussenzweig *et al.*, 1967). However, such approach was dismissed for large-scale vaccinations due to safety and logistics problems.

Nevertheless, it is important to understand the mechanisms behind irradiated sporozoite-induced protection, as their efficiency has not yet been replicated by other forms of human anti-malaria vaccines. In fact, consecutive drawbacks have been reported when subunit vaccine strategies were applied, although very promising candidates are presently on trial (Hill, 2006; Stoute *et al.*, 1997). Thus, whole parasite approaches are still being considered, either by pursuing a doable protocol with

irradiated sporozoite immunization or by making use of genetically modified parasites that are being shown to be capable of inducing protection in mice (Mueller *et al.*, 2005; Luke and Hoffman, 2003). Protection was attributed to a continuous supply of antigen provided by the intracellular hepatic parasites that remained in the liver for extended periods of time (Scheller and Azad, 1995). Furthermore, it is known that irradiated sporozoite-induced immunity is mediated by $CD8^+$ T cells and dendritic cells, although the mechanisms underneath antigen presentation to T cells remain unclear, since the low number of *Plasmodium*-infected hepatocytes in the liver has prevented the development of direct observation of this process (Morrot and Zavala, 2004b; Jung *et al.*, 2002; Bruña-Romero and Rodriguez, 2001; Weiss *et al.*, 1988).

The results presented in this chapter show that in the *P. yoelii* experimental malaria model, there is an increase in the apoptotic death of hepatocytes infected with irradiated sporozoites when compared with normal sporozoite infection. The apoptotic bodies originated from infected hepatocytes were shown to be phagocytosed by both dendritic cells and macrophages present in the liver, implying that presentation from these cells would be responsible for the protection induced by irradiated sporozoites. However, it was previously established that only dendritic cells are required for activation of *Plasmodium*-specific $CD8^+$ T cells *in vivo* (Jung *et al.*, 2002). Dendritic cells are professional antigen-presenting cells with the unique ability to capture exogenous antigens within the phagosome and to present them on MHC class I molecules to antigen-specific $CD8^+$ T cells inducing its activation (Guermonprez *et al.*, 2003; Houde *et al.*, 2003).

The low number of vesicles containing material derived from apoptotic *Plasmodium*-infected hepatocytes detected 6h post-sporozoite inoculation is explained by the restricted time frame within dendritic cells migration. These cells take approximately 1h to leave the liver and travel to the lymph nodes, after antigen uptake (Sallusto and Lanzavecchia, 2000). Thus, at 6h post-immunization we could only find the cells that had phagocytosed parasite material but that had not yet left the liver. Therefore, we could not exclude the possibility that more cells acquire antigens from apoptotic hepatocytes during *Plasmodium* infection. Still, low numbers of dendritic cells that migrated to the lymph nodes could activate T cells and, therefore, initiate efficient T cells responses (Martin-Fontecha *et al.*, 2003). In addition, protective $CD8^+$ T cells are generated within the first 8h post irradiated sporozoite-infection, suggesting that only the antigens delivered before this time contribute to immunity (Hafalla *et al.*, 2002).

Apoptotic hepatocytes containing *Plasmodium* antigens provide an optimal source of antigens for dendritic cells cross-presentation, as was observed for viral antigens presentation originated from virus-induced apoptosis (Albert *et al.*, 1998a). Besides, apoptotic hepatocytes possess the appropriate size, which ranges approximately between 1 and 10 μm, to be internalized by phagocytosis. A cross-presentation mechanism would also provide an explanation for the fact that liver infection is a

requisite for protection against this stage of the parasite's life cycle, as well as for the activation of $CD8^+$ T cells by an obligate intracellular parasite that can develop only inside hepatocytes, which are non-professional antigen-presenting cells. Moreover, the inflammatory environment induced during sporozoite infection of the liver, mediated either by Kupffer cells or by wounded hepatocytes traversed during sporozoite migration, may also favour the recruitment of large numbers of dendritic cells and provide maturation signals for them (Frevert, 2004; Mota *et al.*, 2002; Khan and Vanderberg, 1992).

Apoptotic cells originated as a consequence of a physiological cell death that occurs during growth or development do not release maturation signals nor induce immunity during clearance (Gallucci *et al.*, 1999). On the other hand, apoptotic cells expressing HSPs are very effective stimulators of dendritic cells and T cell activators (Masse *et al.*, 2004; Feng *et al.*, 2003). Indeed, this could be applied to hepatocytes infected with irradiated sporozoites, since besides being stressed cells they also express *Plasmodium* HSP70.

Invasion of hepatocytes is essential for protection to occur. The decrease observed in the number of host cells infected with normal sporozoites 48h after infection suggests that normal sporozoites may also use this mechanism to generate $CD8^+$ T cells and protective immune responses that are observed when blood-stage infection is inhibited (Belnoue *et al.*, 2004; Ocana-Morgner *et al.*, 2003; Orjih *et al.*, 1982; Alger and Harant, 1976; Spitalny and Nussenzweig, 1972).

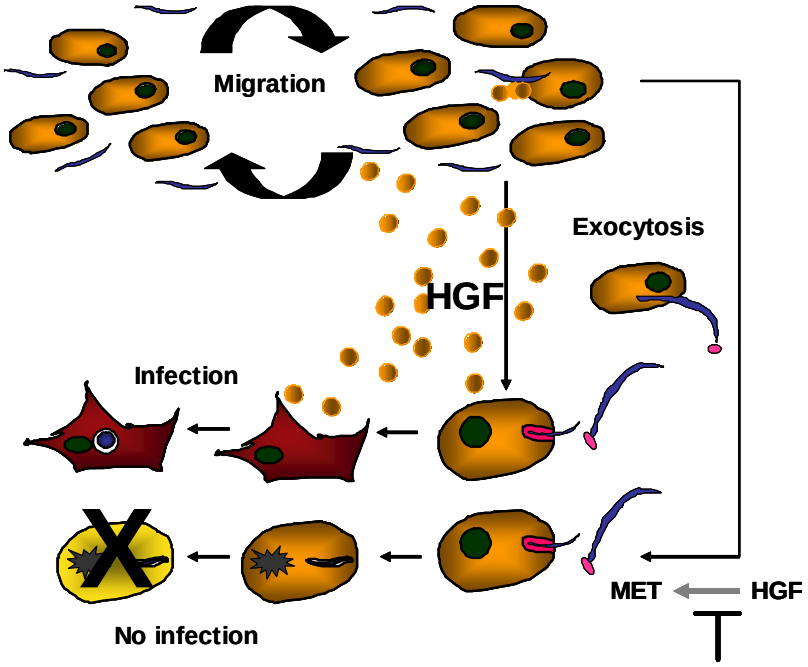
The apoptotic pathway that determines hepatocyte death during infection is not yet fully understood. However, it seems likely that the mitochondrial pathway would be used instead of the death receptor-mediated or the granzyme/perforin pathway. The fact that our results show activation of caspase-3 does not exclude or confirm any of these routes since this enzyme is a final player of the apoptosis cascade (Hengartner, 2000). Additionally, since the immune response to liver stage parasites occurs in either perforin-deficient or Fas-deficient mice, it is unlikely that these pathways are involved (Doolan and Hoffman, 2000).

Dendritic cells use virus-infected dead or dying cells as exogenous sources of antigens for presentation on MHC class I and class II molecules to initiate T cell responses. This pathway is thought to be critical for the development of effective antiviral immunity *in vivo* (Albert, 2004; Fonteneau *et al.*, 2002; Norbury *et al.*, 2002; Albert *et al.*, 1998a).

Recently, dendritic cell presentation of *Mycobacterium tuberculosis* antigens derived from apoptotic infected macrophages has been described as an alternative pathway of T cell activation (Schaible *et al.*, 2003). Like *Plasmodium* sporozoites in hepatocytes, *Mycobaterium* is secluded in an intracellular compartment during infection, which suggests that presentation of antigens from apoptotic infected cells may represent a common mechanism of immune activation for vesicle-contained pathogens (Winau *et al.*, 2005; Winau *et al.*, 2004).

Experimental live vaccines based on irradiated parasites have been used as a model for protection in malaria and schistosomiasis (Bergquist *et al.*, 2002; Nussenzweig and Long, 1994). It is possible that irradiation of these parasites blocks the ability to neutralize the apoptotic machinery of host cells allowing them to resume their natural tendency to undergo apoptosis upon being infected (James and Green, 2004).

Even though the existence of cross-presentation has been proposed, it had not yet been shown for malaria infection (White *et al.*, 1996). Nevertheless, further work is required in order to demonstrate that phagocytosis of apoptotic infected host cells results in efficient presentation of parasite antigens to T cells.



Chapter Five

RESULTS II

Anti-Apoptotic HGF/MET signalling in malaria infection

***Legend*|Model of hepatocyte infection by *Plasmodium* sporozoites.** Sporozoites traverse several hepatocytes, disrupting their plasma membranes. This induces the activation of sporozoite exocytosis and the secretion of HGF from hepatocytes. Within 2 to 4h, HGF binds to its receptor MET, in neighbouring hepatocytes, transducing signals that lead to actin cytoskeleton reorganization and inhibition of infected host cell apoptosis. As a result, parasite development occurs successufully.

5.1 Introduction

Once they reach the liver, *Plasmodium* sporozoites can invade hepatocytes in two distinct ways: either by disrupting their plasma membrane or by inducing the formation of a parasitophorous vacuole around themselves. The first mode of entry allows the parasite to migrate through hepatocytes, experiencing a direct contact with the host cytoplasm and is followed by rapid exit of the sporozoites using the same mechanism. Thus, sporozoites traverse several hepatocytes before invading a final one where a vacuole is formed by invagination of the host plasma membrane and liver infection starts (Mota *et al.*, 2001; Mota and Rodriguez, 2001). This feature of *Plasmodium*'s invasion process is essential since parasites become activated during the migration process, inducing exocytosis of apical organelles, a requirement for vacuole formation when the final hepatocyte is infected (Mota *et al.*, 2002). Most of the sporozoite-traversed hepatocytes survive by resealing their plasma membrane and avoid death, but for as long as rupture persists, growth factors and other proteins stored in the cytosol are released into the extracellular environment. Moreover, after resealing, wounded cells express and secrete growth factors, like the hepatocyte growth factor (HGF), which render neighbouring hepatocytes more susceptible to infection (Carrolo *et al.*, 2003).

HGF, also known as scatter factor, was first described as a potent mitogen for hepatocytes during liver regeneration as well as motility factor inducing "scattering activity" in polarized epithelia cells (Bussolino *et al.*, 1992; Bottaro *et al.*, 1991; Stoker *et al.*, 1987; Nakamura *et al.*, 1986). This glycoprotein is secreted as a biologically inert precursor (pro-HGF) that, under specific conditions, such as tissue damage, and through a proteolytic digestion becomes a bioactive heterodimer mature HGF form (Zarnegar and Michalopoulos, 1995).

The receptor for HGF was identified as a c-met proto-oncogene product encoding a receptor tyrosine kinase (Bottaro *et al.*, 1991; Naldini *et al.*, 1991; Park *et al.*, 1986; Cooper *et al.*, 1984).

The MET receptor has two main structural characteristics: a unique multifunctional docking site through which recruitment of all the downstream signalling molecules occurs, and one single large adaptor protein with multiple substrates, known as Gab1, that mediate most of the complex cellular responses subsequent to activation (Birchmeier *et al.*, 2003; Furge *et al.*, 2000). Like its ligand, MET is also a heterodimer with an entirely extracellular alpha chain and a polypeptide chain with an intracellular tyrosine kinase domain. The stimulation of MET results in a series of biological and biochemical effects within the cell, leading to scattering, proliferation and growth, enhanced cell motility, angiogenesis, survival and the invasion of extracellular matrices (Trusolino and Comoglio, 2002).

MET expression occurs in the normal epithelium of almost every tissue, although other cell types such as endothelial cells, hematopoietic cells, microglial cells, neurons, and a variety of tumor cell

lines also express this receptor (Zarnegar and Michalopoulos, 1995). Alterations in the HGF/MET signalling have emerged as crucial features of many human malignancies suggesting that interfering with activation of this pathway might hold therapeutic value. Thus, several strategies have been developed in order to manipulate the transduction signals involved, either by constitutive activation or inhibition to different degrees (Corso *et al.*, 2005).

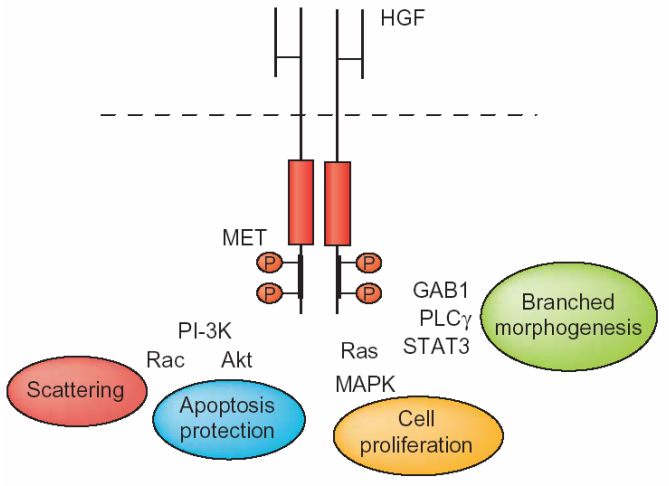


***Fig. 5.1*|Schematic representation of the different pathways that are activated by HGF/MET signalling.** Upon HGF stimulation, two receptor molecules dimerize and cross-phosphorylate each other, thereby creating binding sites for signalling molecules within the cell. From the activation of each transducer results an array of biological activities that are here summarized (adapted from Mota *et al.*, 2004).

HGF/MET signalling enhances *Plasmodium* liver infection and is required not for invasion but rather at early stages of parasite development within the host cell. *In vivo* inactivation of MET inhibits *P. berghei* liver infection, confirming the relevance of this mechanism for infection. Furthermore, MET signals induce the reorganization of host cell's actin cytoskeleton which is believed to have a contribution for the final outcome of *Plasmodium* sporozoite infection (Mota and Rodriguez, 2004; Carrolo *et al.*, 2003).

A host-pathogen interaction triggers a variety of responses that force the pathogen to develop strategies to manipulate the host. As already mention, direct, as well as indirect, effects of pathogens and their products modulate host cell death (Luder *et al.*, 2001). The induction of apoptosis upon infection results from a complex interaction of parasite proteins with cellular host proteins. Infected cells constitute a threat to the host and therefore are normally destroyed. On the other hand, abrogation of host cell apoptosis is often beneficial for the pathogens as they are able to exploit host resources in order to satisfy their needs and extending their life inside the host, which outcomes in a successful invasion (Hasnain *et al.*, 2003).

Inhibition of apoptosis by bacteria and other intracellular parasites such as *Leishmania* and *Toxoplasma* is well documented (Heussler *et al.*, 2001; Gao and Kwaik, 2000; Nash *et al.*, 1998; Moore and Matlashewski, 1994). However, the genes involved in this process have not yet been identified (Payne *et al.*, 2003).

Although the mechanism through which the HGF/MET axis elicits inhibition of apoptosis is not completely understood, there is no doubt that HGF is a strong promoter of cell survival, since this biological response was observed in liver development through gene ablation or overexpression studies in transgenic mice (Amicone *et al.*, 1997; Maina *et al.*, 1996; Schmidt *et al.*, 1995). In addition, the anti-apoptotic effect of HGF has also been reported on various cell types, from epithelia to tumors (Xiau *et al.*, 2001; Bardelli *et al.*, 1999; Fan *et al.*, 1998).

In this chapter, an additional role is proposed for HGF in the maintenance of *Plasmodium* infection: protection from apoptosis. The results showed that apoptosis inhibition on infected cells is mediated by HGF/MET signalling via the PI3K pathway and allows parasite persistence within the liver until full development is reached.

5.2 Results

5.2.1 Sporozoite infection protects cells from apoptosis

To determine whether *Plasmodium* infected cells were protected from apoptosis, HepG2 cells were incubated with *P. berghei* sporozoites (live or heat-killed, as control) and treated 18h later with TNF and Cycloheximide (CHX) to induce apoptosis. Six hours post-induction, apoptosis was quantified using different methods to evaluate distinct apoptosis features. Infected cells are more protected from apoptosis than non-infected ones, as shown when activation of caspase-3 is measured (red; Fig. 5.2) and nuclear morphology analyzed after staining with DAPI (blue; Fig. 5.2). Apoptosis was also quantified by TUNEL assay for assessment of DNA breaks, a common apoptotic mark (striped bars; Fig. 5.3). Similar data were obtained using a different cell type (Hepa 1-6 cells) and a different inductor of apoptosis (UV light; Fig. 5.3 b). In this case a higher level of protection than the one observed when infected cells were induced with TNF and CHX was observed. Taken together, these data constitute evidence that *P. berghei* sporozoite infection protects host cells from apoptosis.

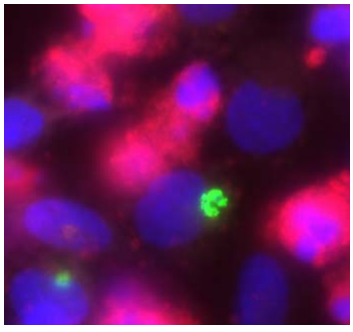


***Fig. 5.2 | Plasmodium* infection protects from death.** A host cell infected with *P. berghei* sporozoites not showing signs of apoptosis and surrounded by apoptotic non-infected cells, as detected by anti-PyHSP70 (green), DAPI (blue) and anti-active-caspase-3 antibody (red)

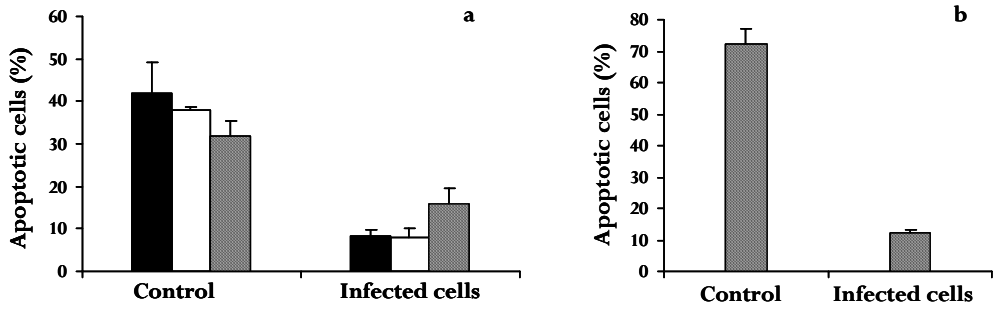


Fig. 5.3 | **Infected cells are more resistant to apoptosis.** HepG2 cells were incubated with *P. berghei* sporozoites (heat-killed sporozoites were used as control) and treated 18h later with TNF and Cicloheximide to induce apoptosis. (a) Apoptosis was quantified 6h post-induction by nuclear morphology following DAPI staining (black bars), active caspase-3 (white bars) and TUNEL detection (strip bars). (b) Hepa 1-6 cells were incubated with *P. berghei* sporozoites (heat-killed sporozoites were used as control) and exposed to UV light 18h post-infection. After 6h, apoptotic cells were counted by TUNEL detection. (a, b) Infected cells were stained with anti-PyHSP70 antibody to detect EEFs in culture.

5.2.2 HGF/MET signalling enhances infection and confers protection from apoptosis

Several cytokines and growth factors are capable of inducing different cell mechanisms and essential cell functions such as proliferation, differentiation, chemotaxis and survival. One of these molecules is the hepatocyte growth factor (HGF) (Trusolino and Comoglio, 2002). Binding of HGF to its receptor c-Met induces the activation of a tyrosine kinase-dependent cascade of events that results in phosphorylation of a series of residues and leads to the recruitment of intracellular signaling molecules which trigger a range of biological responses within the cell (Mota *et al.*, 2004). The presence of this growth factor during a malaria infection enhances the infection outcome and is mediated by HGF-induced MET activation (Carrolo *et al.*, 2003).

Taking this into account, it was hypothesized that the increase in the infection is caused by HGF-induced protection from apoptosis. To test this possibility, first, the HGF effect on *Plasmodium*-sporozoite infected cells was determined. In order to do so, HepG2 cells were treated with HGF (500 ng/ml) 1h prior to infection with *P. berghei* sporozoites. Control cells did not undergo HGF treatment. After 6h, cells were stained for parasite detection (anti-HSP70 antibody) and apoptosis quantification by nuclear morphology (DAPI) of infected hepatocytes. The results showed that the presence of HGF leads to an increase in infection and to a reduction in the number of apoptotic infected cells, suggesting that HGF-induced MET activation is protecting the host cell from death (Fig. 5.4).

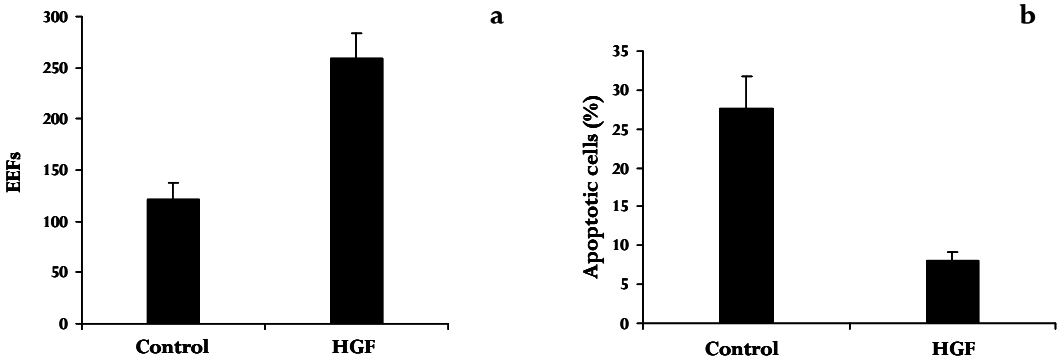


***Fig. 5.4*|HGF-induced MET activation enhances infection and protects infected cells from apoptosis.** (a, b) HepG2 cells were treated with HGF for 1h, and incubated with *P. berghei* sporozoites. Hepatocytes were fixed after 24h (infection, a) or 6h (apoptosis, b), and stained with anti-HSP70 antibody (to visualize the parasite) and with DAPI (nuclear morphology) to quantify apoptosis.

In addition, two monoclonal antibodies, DO-24 and DO-30, that act on the extracellular domain of the MET receptor were used to assess the same question with a different approach. These agonist monoclonal antibodies have been described to have diverse effects on cells and to activate various pathways (Prat *et al.*, 1998). While DO-24 is a full agonist, capable of inducing a strong response from the receptor and trigger what is known as the complete invasive growth program, including cell survival, the DO-30 antibody has a partial effect, causing a weak activation of MET and inducing motility only (Prat *et al.*, 1998).

To evaluate the effect of these agonists on infection, HepG2 cells were infected with *P. berghei* sporozoites 2h after addition of the antibodies. The number of EEFs was quantified by immunofluorescence staining with anti-HSP70 antibody for parasite detection. Results show that both antibodies lead to an increase in infection, although to different extents. Apoptosis quantification either by nuclear morphology and caspase-3 activity revealed a correlation between the increment in infection and a reduction of infected hepatocyte death (Fig. 5.5). The full agonist DO-24 had a stronger effect than the partial agonist DN-30. The caspase-3 activity is involved in late stage apoptosis justifying why this percentage is lower although showing the same trend (Thromberry and Lazebnick, 1998). These results demonstrate that signaling through HGF/MET pathway during a malaria infection is directly implicated in the protection from apoptosis of the infected host cell.

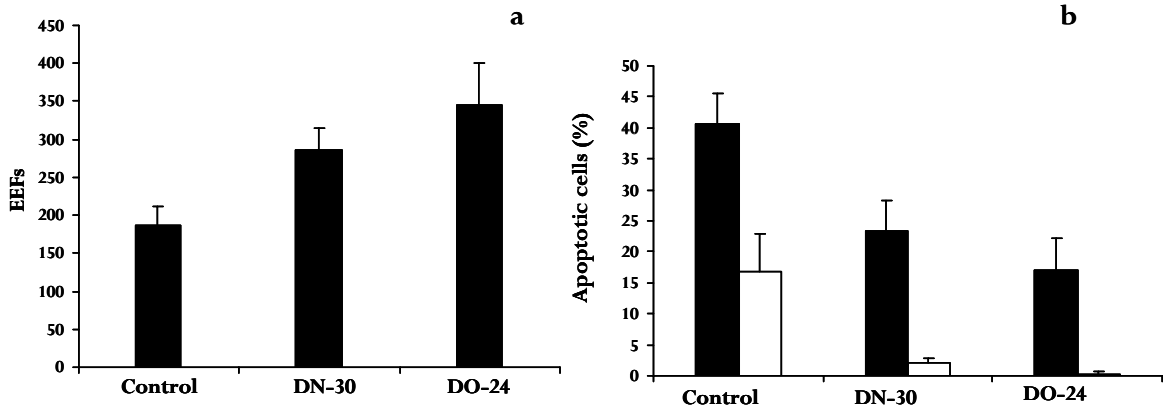


***Fig. 5.5*|HGF agonists induce infection and host cell protection.** HepG2 cells were pre-treated for 2h with the agonist anti-MET monoclonal antibodies DN-30 or DO-24 (1μg/ml) and afterwards infected with *P. berghei* sporozoites. (a) The number of infected cells (EEFs) was quantified 24h post infection by counting anti-*Plasmodium*-HSP70 positive cells. (b) Apoptosis was quantified 6h post infection by DAPI (black bars) and caspase-3 activity (white bars). Untreated-infected cells were used as control.

In order to analyze the behavior of infected cells in the presence of an apoptosis inhibitor, we used Z-VAD-FMK, a cell permeable broad-spectrum caspase inhibitor that binds covalently to active caspases in living cells, is non-cytotoxic and has been shown to inhibit apoptosis randomly (Chipuk and Green, 2005; Al-Olayan *et al.*, 2002). HepG2 cells were treated with Z-VAD-FMK (20μM) or with HGF (500 ng/ml) 1h prior to infection with *P. berghei* sporozoites. The chemically induced inhibition of apoptosis led to a higher infection than that observed either with HGF or with untreated cells, lending further support to the previous results (Fig. 5.6).

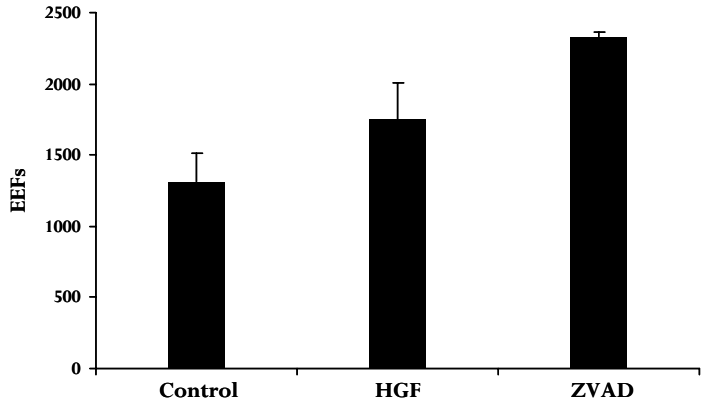


***Fig. 5.6*|Inhibition of caspase activity increases infection.** 1h prior to infection with *P. berghei* sporozoites, HepG2 cells were treated either with Z-VAD-FMK (20μM) or with HGF (500 ng/ml). The number of infected cells (EEFs) was determined 24h post infection after staining with anti-HSP70 antibody. As control, untreated infected cells were quantified.

5.2.3 MET inactivation leads to apoptosis during infection

The HGF-mediated signaling cascade occurs through a tyrosine kinase receptor, c-Met. Its activity is essential for the progress of the downstream catalytic processes. In order to determine the effect of MET signaling impairment on infected hepatocytes, a stable transfected HepG2 MET-dominant-negative-form cell line (DN MET-GFP) was used. For this purpose, a lentivirus expressing a

chimeric construct containing the extracellular and transmembrane domains of *met* fused to *gfp* sequences was transfected into HepG2 cells. The product of this construct is expressed at the plasma membrane and binds to HGF but it is unable to transduce signals into the cell since it lacks the kinase domain and the tyrosines acting as docking sites for intracellular substrates. This chimeric receptor behaves as a dominant negative, because it dimerizes with endogenous MET and consequently prevents its activation (Carrolo *et al.*, 2003; Giordano *et al.*, 2002; Vigna and Giordano, 2000). Because cells transfected with this lentivirus become fluorescent, it is possible to determine the infection level of single-transduced cells 24h after infection.

As previously mentioned, the activation of the MET receptor is essential for the infection of hepatocytes by *Plasmodium* sporozoites (Carrolo *et al.*, 2003). The impairment of the signal abolishes completely the infection 24h post-transduction, as no individual cells transduced with MET-GFP were found to be infected with *Plasmodium* (Fig. 5.7a). The number of apoptotic cells correlates with MET inactivation as in these cells apoptosis increased to 68% while control cells (GFP-transduced only) show a 21% level of death, as determined by observation of nuclear morphology 6h post-infection following DAPI staining clearly shows apoptosis features in MET-GFP transduced cells (Fig. 5.7b; Fig. 5.8). These results strongly suggest that MET signaling is required for host cell apoptosis inhibition and in that way for host cell survival and infection success through an anti-apoptotic mechanism.

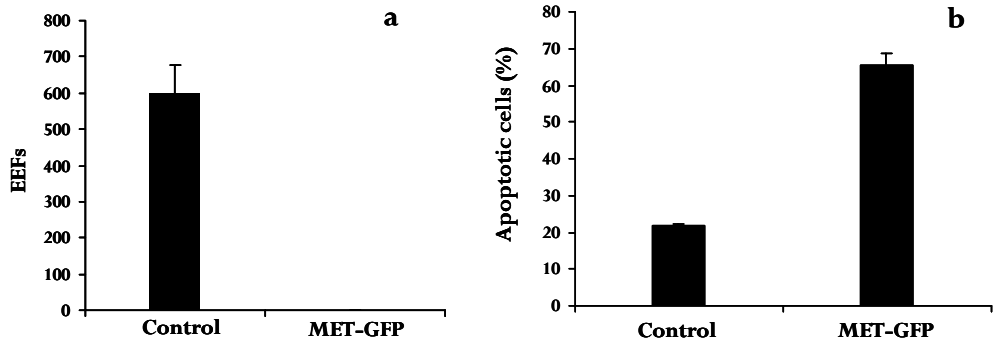


***Fig. 5.7*|MET inactivation leads to early apoptosis of infected cells.** HepG2 cells were transduced with a lentivirus expressing MET-GFP or GPF alone (control). The dominant negative hepatocyte MET mutant cell and control lines were infected with *P. berghei* sporozoites. 24h post-infection the number of EEFs was determined (a) and 6h post-infection apoptosis was quantified by nuclear morphology observation (b). All $P \leq 0{,}001$.

It is likely that all infected cells undergo apoptosis in the absence of a functional MET receptor within 24h, as no infected cells were found at this time point. No increase in apoptosis of non-infected transduced cells was observed.

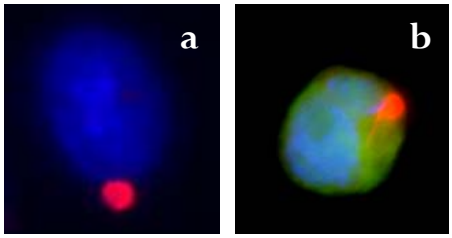


Fig. 5.8|**Dominant negative MET-GFP infected cell undergoing apoptosis.** (a) HepG2 cell 6h after infection with *P. berghei* sporozoites (red, anti-HSP70) with a normal nuclear morphology (blue, DAPI). (b) Infected cell expressing MET-GFP (green) 6h post-infection with a parasite starting to develop (red, anti-HSP70) and exhibiting apoptosis features detected by nuclear morphology with DAPI (blue).

5.2.4 MET down-regulation leads to apoptosis during infection

As shown above the impairment of HGF/MET signaling using a dominant negative form of MET leads to higher levels of apoptosis and promotes the early death of the infected cell. To confirm these results we also abrogated MET expression using RNA interference. HepG2 cells were transduced with a lentivirus vector expressing a short interfering RNA (siRNA) oligonucleotide targeted at MET. A control sequence was used that did not cause any reduction on MET expression as detected by Western blot analysis (Fig. 5.9).

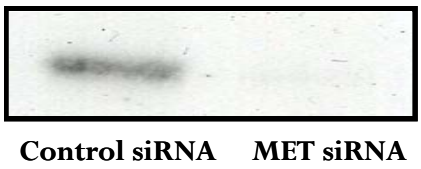


Fig. 5.9|**MET expression on short interfering RNA cell line.** Western blot analysis of MET expression in HepG2 cells transduced with lentiviruses expressing control siRNA oligonucleotide (left lane) or MET siRNA oligonucleotide (right lane) to knockdown met.

The down-modulation of MET activity showed similar results as the ones obtained for the dominant negative cell line, that is, an inverse correlation between a decrease in infection (Fig. 5.10a) and an increase in apoptosis of infected cells (Fig. 5.10b). This evidence confirms the relation between hepatocyte survival and HGF/MET activation, indicating that its anti-apoptotic activity is essential during *Plasmodium* infection.

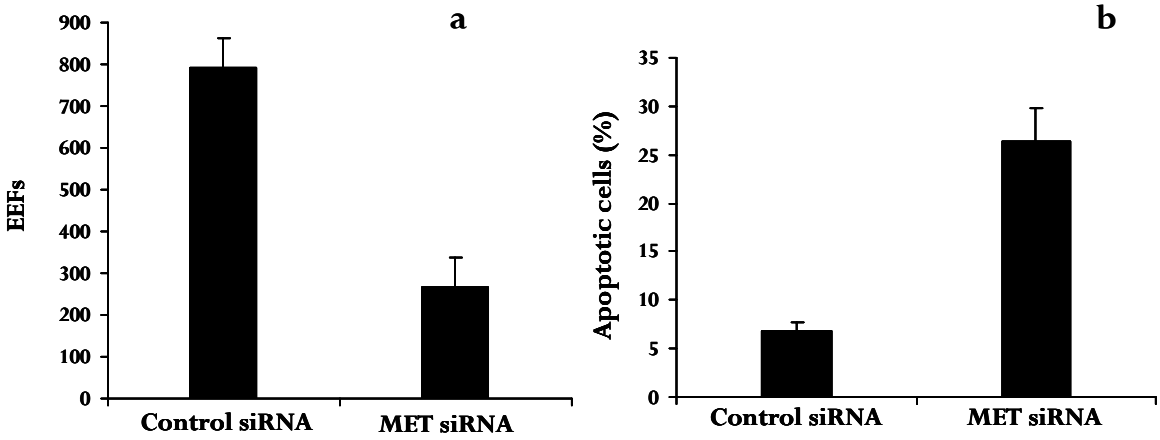


Fig. 5.10|**Down-modulation of HGF/MET signaling leads to early apoptosis of infected cells.** Lentivirus engineered to express MET-specific siRNA oligonucleotides were used to transduce HepG2 cells. Hepatocytes transfected with a siRNA specific for the *met* gene were infected with *P. berghei* sporozoites. As control, hepatocytes were transfected with a siRNA of an unrelated sequence. (a) Infection was quantified 24h post-sporozoite addition by counting the number of EEFs and (b) 6h post-infection apoptosis was quantified by nuclear morphology observation with DAPI staining. All $P \leq 0{,}001$.

5.2.5 Anti-apoptotic effect of HGF/MET signalling is mediated via activation of PI3K pathway

In a signalling cascade, a series of subsequently biological events take part in the process of reaching a target or accomplishing a certain level of activation or induction. In HGF cascade the survival pathway is mediated by a phosphoinositide 3-Kinase (PI3K)/Akt, whose activity directs the cell to a death inhibition state (Webster and Anwer, 2001; Xiau *et al.*, 2001).

To determine if this pathway is implicated in malaria infection, HepG2 cells were treated with a specific PI3K inhibitor (LY294002) 1h prior infection with *P. berghei* sporozoites. Additionally, a mitogen-activated protein kinase (MAPK) inhibitor (PD98059) was used to evaluate if this pathway was involved in protection from apoptosis. An anti-apoptotic role has also been described for MAPK pathway, since once activated by HGF, it was capable of rescuing cells from apoptosis, although to much smaller extent (Xiau *et al.*, 2001).

As shown below in Figure 5.11 when cells are treated with either of these inhibitors there is a significant decrease in infection. This effect is stronger when the PI3K activity is inhibited. When apoptosis was quantified after 6h on the same infection experiment, a significant increase was detected in the apoptotic cells number, only when cells were pretreated with the PI3K inhibitor. These results show that, although both PI3K and MAPK pathways seem to be involved in *Plasmodium* infection, only PI3K/Akt via appears to be important for host cell protection from apoptosis.

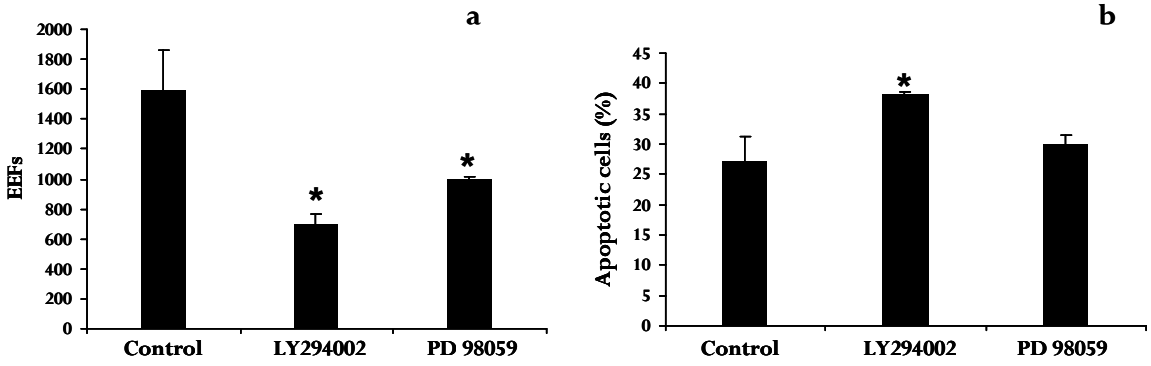


***Fig. 5.11*|Inhibition of PI3K/AKT pathway *in vitro* leads to a decrease in infection.** HepG2 cells were pretreated with LY294002 (25μM, PI3K inhibitor) and with PD98059 (30μM, MAPK inhibitor) for 1h. Inhibitors were washed before infection with *P. berghei* sporozoites. Control cells were incubated with DMSO. (a) 24h post-infection cells were fixed, stained and EEFs were counted ($P_{LY} \leq 0{,}001$; $P_{PD} = 0{,}003$). (b) 6h post-infection cells were fixed and apoptotic cells quantified by DAPI staining ($P_{LY} = 0{,}002$; $P_{PD} = 0{,}09$).

5.2.6 Inhibition of PI3K pathway decreases *Plasmodium* infection *in vivo*

In vivo assays with PI3K inhibitor have previously been performed, demonstrating its activity in mouse models (Hu *et al.*, 2002; Semba *et al.*, 2002). To determine if the effect observed *in vitro*, could also be extrapolated to a natural infection *in vivo*, mice were injected with 2.5 mg of LY294002, 30 minutes prior to sporozoites infection. Livers (from 4 animals in each experimental group) were collected 40h later and the parasite load was determined by real-time RT-PCR with primers specific for *P. berghei*. Data obtained reveal a significant reduction in liver infection observed in animals pretreated with the PI3K inhibitor (Fig. 5.12a). A Western blot analysis was also performed to confirm whether the inhibitory treatment applied was targeting the PI3K pathway. As shown below the phosphorylation of Akt was reduced in the treated animals. Since this molecule is located downstream of PI3K, its decreased phosphorylation state expresses the inhibition level obtained (Fig. 5.12b).

Taken together, the *in vitro* and *in vivo* results reveal that the anti-apoptotic effect exerted by HGF/MET signalling through activation of the PI3K/Akt pathway is crucial for the success of a malaria infection.

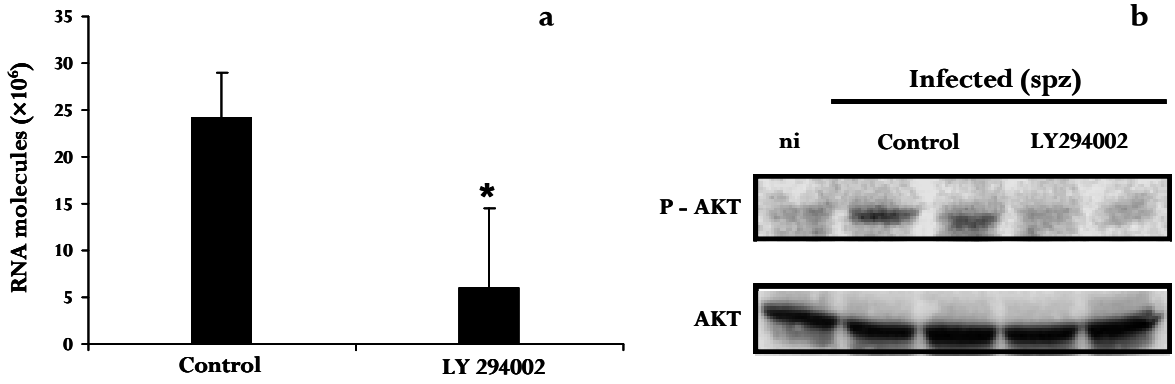


***Fig. 5.12|In vivo* inhibition of PI3K/AKT pathway during infection.** (a) Mice were injected with LY294002 or DMSO (control), 30 min prior *i.v.* injection of *P. berghei* sporozoites. Livers were removed and real time RT-PCR was performed 40h post-infection with parasite specific primers to quantify infection (P≤ 0,005). (b) Mice were injected with LY294002 or DMSO, 30 min prior to infection with *P. berghei* sporozoites. Livers were extracted 3h post-infection and phospho-Ser473-Akt and total Akt were quantified (ni, non-infected).

5.3 Discussion

Plasmodium infection is initiated by the inoculation of sporozoites in the skin of the host, from where they migrate towards the liver, which is the first obligatory step crucial for parasite development and success of a malaria infection. Inside hepatocytes, sporozoites migrate through several hepatocytes

by breaching their plasma membrane before infecting a final one surrounded by a parasitophorous vacuole where the intrahepatic form of the parasite grows and multiplies (Mota *et al.*, 2001).

Although during this period there is an extensive parasite multiplication in the liver each one giving rise to 10-30 thousand new parasites in 2-10 days, depending on the parasite species not much is known about its requirements and strategies used to survive and to accomplish a successful development (Baldacci and Menard, 2004).

It is known that intracellular pathogens have developed powerful strategies to manipulate host cell functions. Many of them have evolved to exploit the host cell machinery involved in normal cell proliferation, development and cell death to their own advantage (Sibley, 2004; Heussler *et al.*, 2001). Obligatory life inside a host cell directs the pathogen to induce host cell survival or to prevent its death in order to prolong the parasite's life and ensure its full development. Thus, inhibition of apoptosis constitutes a significant advantage for a parasite. Such events have been reported for several pathogens, like bacteria and other intracellular parasites, as *Leishmania* and *Toxoplasma* (Gao and Abu Kwaik, 2000; Nash *et al.*, 1998; Moore and Matlashewski, 1994).

The results presented in this chapter show that *P. berghei* sporozoites are no exception and require an active process of inhibition of host cell apoptosis in order to reach the next infective stage. However, *Plasmodium* sporozoites seem to use a unique feature since they take advantage of a host molecule not secreted by the infected cell but by neighboring ones, previously traversed by the parasite, instead. Whether this is the only mechanism used by the parasite to avoid host cell apoptosis remains unknown.

Our results show that PI3-kinase activation is important for the survival of the infected cell. The anti-apoptotic effect mediated by HGF uses this signal transduction pathway. However, we cannot exclude that the infection inhibition observed both *in vitro* and *in vivo* by the specific PI3-kinase inhibitor could be due to PI3-kinase mediated survival signals other than those generated through HGF/MET signaling.

While sporozoites migrate through cells by breaching hepatocyte plasma membrane, HGF is secreted (Carrolo *et al.*, 2003). This secretion of a factor by the host cell leads to an increase in the number of *Plasmodium*-infected cells. In fact, results showed that infection was dependent on HGF receptor MET activation, and HGF/MET signalling was not required for invasion but rather for early parasite development. In the liver, hepatocytes do not normally express HGF which is produced by non-parenchymal cells, but, the MET receptor is selectively expressed by hepatocytes (Ishikawa *et al.*, 2001).

Since HGF-MET signalling confers resistance to death and infection was higher under its stimulus it can be suggested that these events were correlated (Fan *et al.*, 1998; Bardelli *et al.*, 1996). Thus, we now report this additional role for HGF in the maintenance of *Plasmodium* infection, which is the

prevention of infected host cell apoptosis, a crucial requirement to elicit the full development of the parasite. Moreover, in infected cells a higher level of apoptosis was observed when HGF/MET signalling or PI3K/Akt pathway were inhibited, suggesting that these two pathways are connected.

The earlier work already mentioned also showed that one of the causes of HGF requirement for hepatic infection was the remodeling of the host cell actin cytoskeleton (Carrolo *et al.*, 2003). In fact, it was observed that pretreatment of host cells with HGF abrogates the negative effect that cytochalasin-D has on infection. This strongly suggests that HGF/MET signaling effect on infection involves the host cell actin reorganization.

Upon HGF stimulation, MET mediates the coordination of multiple cellular processes. Thus, it is likely that many of these processes might play a role during the development of *Plasmodium* sporozoites inside cells. It is also likely that apoptosis protection and actin reorganization are linked. In fact, studies in bacteria have demonstrated that several bacteria interfere with the fate of host cells through their activity on Rho GTPases, also favouring survival or death depending on their needs (Fiorentini *et al.*, 2003).

It has also been reported that hepatitis patients, which express high levels of HGF, are more susceptible to severe malaria. A requirement for MET signalling might provide an explanation for this observation (Thursz *et al.*, 1995; Naoumov and Eddleston, 1994).

Recently, a protein called SPECT (sporozoite microneme protein essential for cell traversal) was characterized in one of the secretory organs of sporozoites, the micronemes (Ishino *et al.*, 2004). Transgenic parasites lacking this protein do not migrate through cells *in vitro* and show a strong reduction of infectivity *in vivo*, indicating that migration through host cells is required in order to establish a successful infection in the host. However, these results appear to contradict our hypothesis that migration through cells leads to HGF secretion required for infection, as SPECT mutants are able to infect host cells *in vitro* without previous migration through cells. The reason for this apparent discrepancy may be found in the particular characteristics of the *in vitro* cell system used for infection. The hepatoma cell line constitutively produces HGF and other factors that induce a basal level of activation of MET receptor (Conrotto *et al.*, 2004). This is different from the conditions that sporozoites encounter *in vivo*, since in resting conditions primary hepatocytes do not express HGF and MET is not activated (Matsumoto and Nakamura, 1991). These constitutive levels of HGF are probably enough to support sporozoite infection at a basal level and therefore the additional HGF induced by migration would not be required.

The outcome of our studies on the anti-apoptotic role of HGF signalling, help understand why signalling via HGF/MET is essential for an efficient malaria infection to occur. Further studies on parasite requirements inside hepatocytes to achieve successful development, may provide effective strategies for early intervention.

Chapter Six

RESULTS III

Does PyHSP70 inhibit infected hepatocyte apoptosis?

6.1 Introduction

Heat shock proteins are highly evolutionarily conserved molecular chaperones that have a wide range of functions within the cell. They are classified into several families, according to their molecular size. When cells are exposed to stress, such as temperature increase, hypoxia, and intoxication with heavy metals, the biosynthesis of these proteins is engaged in order to help preserve normal cell functions and to facilitate adaptation to environmental changes (Lindquist, 1992).

From bacteria to mammalian cells, these protein families ensure proper folding and function of other proteins, allowing them to acquire the correct shape and preventing or reverting stress-induced protein misfolding (Schlesinger, 1990). Initially, it was thought that these proteins were effective only when high temperature was used as a stress inducer, as was described in salivary glands of *Drosophila* in 1962 (Ritossa, 1962). However, these molecules are not only expressed under stress conditions but also possess important functions during common physiological processes (Bukau and Horwich, 1998; Langer *et al.*, 1992). Moreover, the exposure of cells to damage induces signals that are able to mediate cell death or, alternatively, survival pathways that allow cells to overcome damage. Therefore, heat shock proteins can act at different sites in the apoptotic cascade, inhibiting death and promoting survival (Fig. 6.1) (Beere, 2004).

Among these families of proteins is the heat shock protein 70 (HSP70) family. This family constitutes one of the most abundant and conserved group of chaperones and its members are implicated in a large variety of activities within the cell, such as assembly and disassembly of newly synthesized protein complexes, refolding of denaturated proteins and protein translocation and degradation (Young *et al.*, 2004; Hartl, 1996; Craig *et al.*, 1993). Localized in different cellular compartments, they are present in mammalian cells, yeasts, plants, bacteria, and parasites (Mayer and Bukau, 1998; Lindquist and Craig, 1988). Typically, HSP70 proteins have three domains: an N-terminal ATPase domain, a substrate-binding domain and a C-terminal conserved region containing a peptide sequence motif, EEVD. At the molecular level, they recognize unstable peptides to which they bind in an ATP-dependent process. In addition, their binding to exposed hydrophobic regions, facilitates the folding of nascent proteins into a tertiary structure (Mayer *et al.*, 2000; Blatch and Lassle, 1999).

In a variety of infections, HSPs are synthesized by both the host and the pathogen and their role in the host-pathogen relationship is very important and diverse. As a host response to stress, these molecules contribute to its defence from toxic stimuli triggered by pathogen invasion. Conversely, pathogen's HSPs help to escape the host defence system and perpetuate infection (Hisaeda and Himeno, 1997; Polla, 1991).

Virulent strains of some protozoa express higher amounts of HSPs as compared to low virulent strains. During *Toxoplasma gondii* infection, expression of HSP70 is induced in virulent strains, a fact that seems to provide protection for these strains since parasites are able to persist as tachyzoites without the requirement for the encystations observed in avirulent strains (Dobbin *et al.*, 2002; Lyons and Johnson, 1995).

HSPs are also responsible for the pathogen's adaptation to the host environment. Parasites that possess a biphasic cycle involving two different hosts have to adapt to the temperature changes that occur between the vector and the mammalian environments. The response to this temperature shift has been shown to involve the production of HSP70, both in *Leishmania major* and in *Trypanosoma brucei*, which allows the parasite to differentiate into proliferative forms (Polla, 1991; Shapira *et al.*, 1988; van der Ploeg *et al.*, 1985).

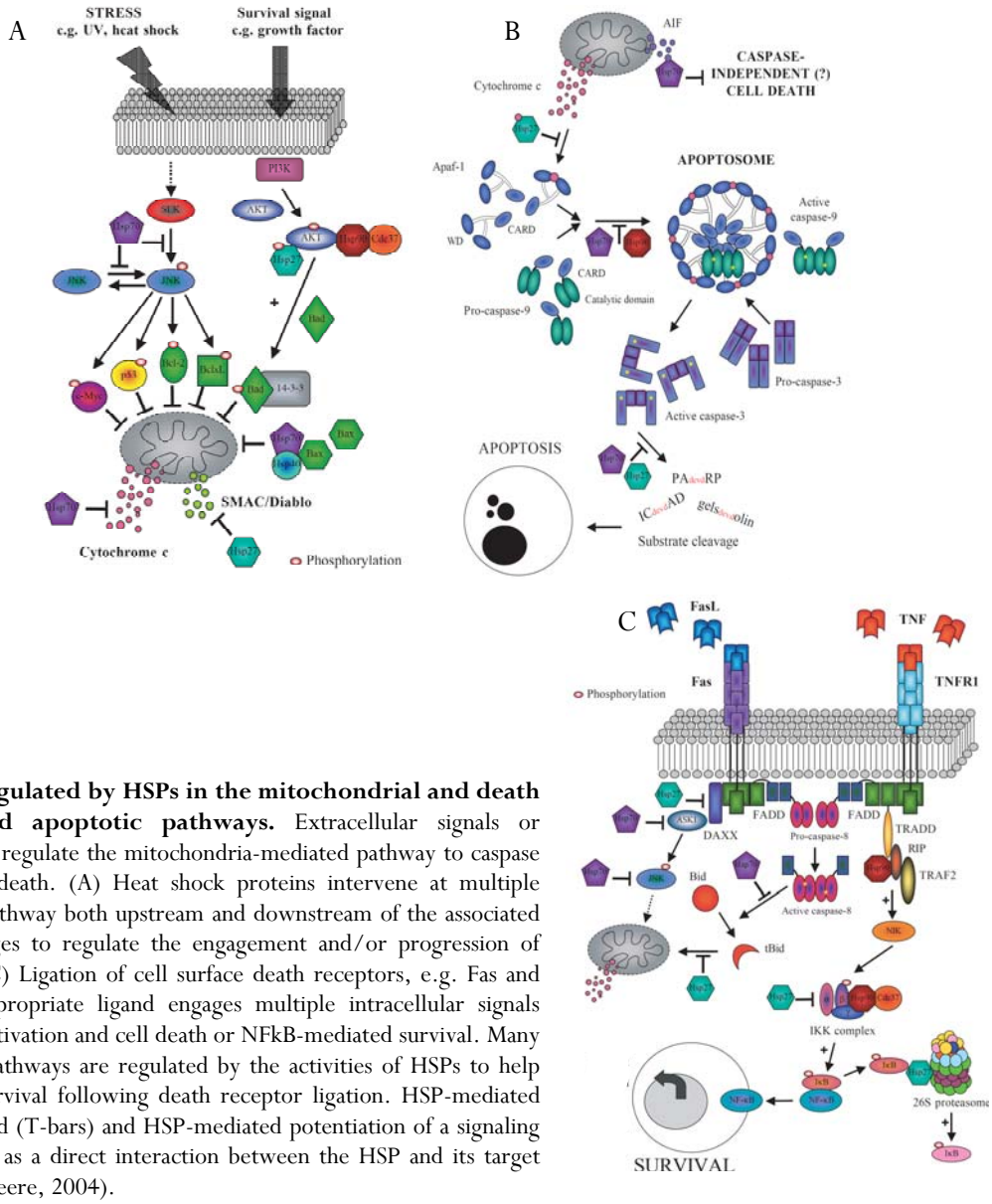


Fig. 6.1 **| Events regulated by HSPs in the mitochondrial and death receptor-mediated apoptotic pathways.** Extracellular signals or stresses converge to regulate the mitochondria-mediated pathway to caspase activation and cell death. (A) Heat shock proteins intervene at multiple points within this pathway both upstream and downstream of the associated mitochondrial changes to regulate the engagement and/or progression of apoptotic events. (C) Ligation of cell surface death receptors, e.g. Fas and TNFR1, by the appropriate ligand engages multiple intracellular signals leading to caspase activation and cell death or NFkB-mediated survival. Many elements of these pathways are regulated by the activities of HSPs to help maintain cellular survival following death receptor ligation. HSP-mediated inhibition is indicated (T-bars) and HSP-mediated potentiation of a signaling pathway is depicted as a direct interaction between the HSP and its target (+) (adapted from Beere, 2004).

HSPs of malaria parasites were cloned and shown to be extremely similar to homologous mammalian ones (Syin and Goldman, 1996; Bonnefoy *et al.*, 1994; Su and Wellems, 1994; Kumar *et al.*, 1991). Within the different *Plasmodium* species several homologs of HSP70 were identified, like genes encoded by *P. chabaudi* and *P. cynomolgi* that present a 97% of sequence homology, indicating a close relation between these molecules (Kumar and Zheng, 1998; Eckert *et al.*, 1992; Mattei *et al.*, 1989; Sheppard *et al.*, 1989).

P. berghei (PbHSP70) and *P. falciparum* (PfHSP70) HSP70s are expressed in the blood and liver stages of these parasites, but not in sporozoites (Tsuji *et al.*, 1994; Kumar *et al.*, 1993; Bianco *et al.*, 1986; Renia *et al.*, 1990).

Additional studies have shown that during a natural infection, *Plasmodium* stress proteins are targets of the host immune system. Immune responses to malarial HSPs have been demonstrated in patients with malaria (Behr *et al.*, 1992; Kumar *et al.*, 1993; Bianco *et al.*, 1986). PfHSP70 is in fact a target to antibody-dependent response and is recognized by T cells from infected individuals (Behr *et al.*, 1992; Kumar *et al.*, 1993). Like mammalian HSP70s, the *P. falciparum* protein has the EEVD characteristic motif at its C-terminus. The presence of an additional motif (GGMP repeats) is thought to be responsible for inducing this immune response in the host, since this motif is only observed in proteins of parasitic origin (Kumar and Zheng, 1998).

Similar to other parasites, *P. falciparum* showed a marked increase in HSP70 expression when submitted to higher temperatures. This heat shock response was shown to be transient since once the stress was removed the induction was repressed. Such behaviour could indicate a protective function during malaria fever episodes, resulting in the *in vivo* survival of the parasite (Biswas and Sharma, 1994; Joshi *et al.*, 1992; Kumar *et al.*, 1991).

However, this cytoprotection of the malaria parasite through its action as a molecular chaperone requires further studies, since the biochemical and chaperone properties of PfHSP70 are not totally understood. Due to an extremely A/T-rich genome and the usage of codons that are rarely used in *Eschericia coli*, it has been difficult to overexpress *Plasmodium* proteins (Matambo *et al.*, 2004; Baca and Hol, 2000). Recently, further studies were performed to address the activity of PfHSP70 *in vivo* and its role as a chaperone. Data demonstrated the presence of a basal ATPase activity which was thermo-inducible and shown to be cytoprotective (Shonhai *et al.*, 2005).

Other families of heat shock proteins are also present in malaria parasites. HSP90, the most abundant chaperone in mammalian cells, plays an essential role in the folding of proteins that act on cell cycle regulation and signal transduction. The PyHSP90 is differently expressed throughout the life cycle, highly on blood asexual stages and very scarcely on gametocytes (Zhang *et al.*, 1999). PfHSP90 was shown to be essential for parasite viability in blood stages, since treatment with

geldanamycin, a highly specific inhibitor of HSP90, was able to abrogate parasite growth (Banumathy *et al.*, 2003; Kumar *et al.*, 2003).

Overall, the induction of HSPs upon cellular exposure to stress is clearly related to the involvement of these proteins in inducing survival and allowing cells to tolerate and recover from damage (Beere, 2005). Such evidence has also been implicated in tumour resistance to chemotherapeutic strategies and carcinogenesis (Creagh *et al.*, 2000).

As mentioned in chapter II of this thesis, several records show evidence that cell death is a conserved mechanism throughout evolution and is also present in parasitic protozoa (Deponte and Becker, 2004). Moreover, *Plasmodium* infection does lead to host cell death inhibition. However, the plasmodial proteins responsible for this inhibitory function are still unknown. Therefore, like other pathogens, *Plasmodium* must carry anti-apoptotic genes with homology to cellular regulators with similar functions or genes not directly coding for anti-apoptotic proteins, but able to modulate the expression of cellular apoptosis-regulators genes in hepatocytes. Consequently a search for homologues of known apoptotic inhibitors within *Plasmodium* genome databases could uncover possible candidates with this ability in the parasite. Furthermore, members of these families are present in a wide range of organisms and all contain defined and conserved motifs (Hurd and Carter, 2004).

This chapter represents a pilot attempt to study the function of PyHSP70 as a possible candidate responsible for the inhibition of apoptosis in infected hepatocytes. The preliminary results presented here support this hypothesis. Hepatoma cells transfected with a *Plasmodium* HSP70 gene are protected against apoptosis, suggesting that *Plasmodium* HSPs may account for parasite survival during liver stage infection.

6.2 Results

6.2.1 PyHSP70 expression protects hepatocytes from death

As previously shown, *Plasmodium* sporozoites are capable of protecting the hepatocyte from undergoing apoptosis by inhibiting host cell death (see chapter V).

To evaluate whether a *Plasmodium* HSP70 has any role during liver infection, apoptosis of hepatocytes transfected with a construct expressing PyHSP70 was induced by UV light exposure. Results showed a decrease in apoptosis in the presence of HSP70, while cells transfected with a green fluorescence protein (gfp) construct or another irrelevant gene (control), presented much higher levels of cell death (Fig. 6.2).

The inhibition of apoptosis induced by PyHSP70 overexpression in hepatocytes suggests that this protein could have a protective function during liver infection, preventing hepatocytes from undergoing apoptosis.

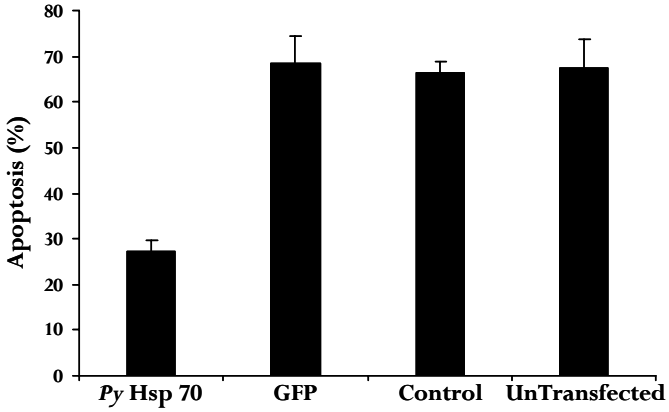


***Fig. 6.2 | P. yoelii* HSP70 protects hepatocytes from apoptosis.** Hepa 1-6 cells were transfected with different DNA plasmid constructs expressing *P. yoelii* HSP70 (PyHSP70), green fluorescence protein (gfp), an irrelevant gene (control). 24h post-transfection apoptosis was induced by UV light. After 6h, cells were stained with DAPI and apoptotic cells were quantified.

In order to establish a stable cell line expressing this protein that would allow a broader application of this tool to further studies, an attempt was made to assemble a construct with a mammalian vector that would additionally provide the possibility to control gene expression by induction through a Zeocin resistance gene (Invitrogen). After selection the results obtained were not satisfactory since the expected appropriate expression was not achieved. A likely explanation for these observations is the already known difficulty inherent to the cloning of *Plasmodium* proteins due to its A/T–rich genome that confers problems to protein expression experiments.

6.2.2 PyHSP70 knock out parasite

To analyze and validate the actual role of HSP70 in apoptosis inhibition, it is essential that functional studies are made using parasites in which expression of this protein has been knocked out. The first step to achieve this goal, is to produce a construct that will block protein expression, which can be accomplished by cloning only a fraction of the gene sequence. This experiment is still under progress and a final conclusion was not yet possible.

6.3 Discussion

Many pathogens like schistosomes, leishmania, trypanosomes and malaria plasmodia, have to face sudden temperature shifts during transmission from vector to the host. The increase in temperature imposes a heat shock to parasites, which leads to the induction of HSPs. Moreover, the host-parasite interface is apoptosis dependent since this process can be used by the host as a defence mechanism

or by the parasite to perpetuate infection (Polla, 1991). Therefore, the existence of mechanisms able to induce or inhibit apoptosis is essential within this dual relationship and the balance between them dictates host cell fate. Furthermore, in order to control pathogenesis and regulate homeostatic development, it seems intuitive that all species should harbour cellular protective mechanisms and conversely members of the apoptotic machinery ready to induce cell death. Several characteristic features of apoptosis have already been described for protozoan parasites indicating that they possess cellular mechanism of cell death similar to that of multicellular eukaryotes (Arnoult *et al.*, 2002). For *P. berghei*, caspase-like activity was detected in the cytoplasm of ookinetes along with apoptotic morphological and biochemical features (Al-Olayan *et al.*, 2002). Therefore, together with the existence of apoptotic inducers within *Plasmodium*, there should also be apoptosis inhibitors.

The presence of HSP70 in *Plasmodium* species makes it an obvious candidate for this task. In addition, one can expect that more cellular processes will be discovered that depend on the chaperone activity of HSP70. Its abundance and antigenicity, which triggers potent immune responses, makes it a vaccine candidate worth investigating.

Chapter Seven

Final Discussion & Conclusion

7.1 Final Discussion and Conclusion

When an intracellular parasite depends on a host in order to complete its life cycle, a close interaction must be established between the host and the parasite. Confined inside a host cell the parasite ensures a source of nutrients and, at the same time, a location where it can avert the host's immune system. Throughout infection, the nature of this relationship is dictated by the different parasite-specific requirements along its life cycle. However, host-parasite interactions also reflect the balance between the pathogen virulence mechanisms and host defences, which exert a major effect in the final outcome of infection as well.

For malaria parasites, this relationship is highly complex and diverse, in accord with the demands of the life cycle of *Plasmodium*. Since the liver is the first target of *Plasmodium* parasites and this site of infection is obligatory for disease progression, it is essential to reveal and comprehend the basic biological interactions established between the parasite and its host cell, the hepatocyte (Lau *et al.*, 2001; Shin *et al.*, 1982).

For a long time this was a hard task due to the limited experimental accessibility of the models and molecular tools available. However, these difficulties are being overcome and the possibilities of research in this area are increasing greatly, as a consequence of the development of new technologies and genetic tools (Amino *et al.*, 2005; Frevert *et al.*, 2005; Gardiner *et al.*, 2005). Nevertheless, there has always been the problem of obtaining enough quantities of sporozoites that would allow liver infection to be mimicked *in vitro* (Luke and Hoffman, 2003). Together with the low infectivity rates that are obtained experimentally, there is a rather remarkable poor knowledge of the mechanisms involved in invasion and intracellular development used by the parasite (Kappe *et al.*, 2004; Druilhe *et al.*, 1998).

Usually, it is considered that host manipulation is a strategy used by intracellular parasites. How parasites manage to evade host defences has always been a hub under a continuous survey. Antigenic variation, resistance to immunological attacks and escape to safe compartments are common strategies used (Zambrano-Villa *et al.*, 2002). Moreover, it is becoming common knowledge that different parasites make use of similar strategies to subvert host signalling pathways in their own benefit, such as activating some in order to exploit them for invasion and development or inhibiting others in order to prevent hostile responses (Kahn *et al.*, 2002).

During its permanence in the liver, sporozoites undergo a process of replication and development that exploits resources provided by the hepatocyte. In addition, as is the case with any intracellular pathogen, parasites develop ways of controlling host cell behaviour towards increasing their own survival and inducing changes in the host that can ultimately elicit effects in immune modulation and regulation of apoptosis (James and Green, 2004; Hasnain *et al.*, 2003). For *Plasmodium* parasites both of these effects happen during infection but none of them have been yet completely elucidated.

Aiming at clarifying some essential features within *Plasmodium*-hepatocyte interactions, the main goal of the project presented here was to determine the role of hepatocyte apoptosis in the inhibition of development of exo-erythrocytic forms and in the initiation of protective immune responses to malaria by dendritic cells.

Plasmodium liver stages are considered to be a singular phase of the parasite life cycle in terms of immunity. Besides constituting a crucial step for its survival, they are also imperative for the induction, establishment and maintenance of an effector anti-parasitic immune response (Doolan and Hoffman, 2000). Although characterized as multi-factorial, this protection is essentially dependent on *Plasmodium* antigen-specific T cells, in particular effector and memory $CD8^+$ T cells, which are responsible for the elimination of the infected hepatocyte and, in addition, are indispensable for enhancing the efficacy of a long lasting immunity against malaria (Morrot and Zavala, 2004a).

Natural recurrent infections cause only partial immunity, which is mediated by antibodies directed to the blood-stage of the parasite (Hviid, 2005; Day and Marsh, 1991). Only irradiated sporozoite immunization leads to complete resistance or sterile immunity in both mice and humans after repeated immunizations (Hoffman *et al*., 2002). These attenuated parasites infect hepatocytes in a similar way compared to normal ones. However, they fail to complete a full infection, presumably because their DNA was damaged upon radiation and protein synthesis was affected (Suhrbier *et al*., 1990). Nevertheless, protection is obtained when live attenuated sporozoites reach the liver while inactivated sporozoites or their extracts have consistently failed to achieve the same goal. Different studies also support the hypothesis that there is a requirement for intra-hepatic development for protection to be induced, as removal of liver parasites by primaquine treatment abolishes protection (Krzych *et al*., 2000; Scheller and Azad, 1995; Bates *et al*., 1990).

Discovered more than 30 years ago the feasibility of an anti-malaria vaccine through these irradiated sporozoites immunizations proved that protection against *Plasmodia* was possible. Why this is so is still under debate. Yet, a broad use of this type of vaccine was considered an impossible approach to undertake, because of the impossibility of obtaining high numbers of sporozoites, as they cannot be cultured *in vitro* (Luke and Hoffman, 2003). Thus, reproducing this induced immunity became an alternative approach. Consequently, to understanding the mechanisms occurring in the liver either during a natural infection or in model systems of protective immunity such as irradiated sporozoites immunizations will facilitate the exploitation of these responses to a expedite progress in vaccine development.

Sporozoites preferentially infect the liver, where immune responses tend to be biased towards immunological tolerance (Crispe, 2003). This unique feature may contribute to the success of *Plasmodium* infection of this organ, since it allows the parasite to develop and promotes conditions

that seem to fit the parasite's needs. Besides this, several hypotheses were proposed to explain why attenuated sporozoites are so efficient. Options are based on sporozoite activity after irradiation, its interaction with the hepatocyte, the subsequent initiation of the immune response and its persistence within the hepatocyte. As a result there is a high level of IFN-γ production when challenge is induced in immunized mice and humans, suggesting that an enhanced efficiency in T cell priming events takes place. Gamma-irradiation induces a partial delay in the maturation of the parasite, leading to its extended permanence in the liver, and generating a depot of antigen, which seems necessary for the establishment of a local antigen-specific protective immunity (Krzych *et al.*, 2000). However, different systems such as viral infections present an independency between antigen persistence and the maintenance of memory $CD8^+$ T cells responses (Wherry *et al.*, 2004; Mueller *et al.*, 2002).

Nevertheless, in endemic areas, naturally exposed individuals do not acquire long term protection, indicating a persistent lack of memory. This issue is still not fully understood and remains a rather controversial subject. Yet a few causes are appointed by some authors that could explain such evidence. The tolerant environment that dominates on the liver is a key feature, as is the sequestration of the liver-stage antigens within hepatocytes, and the relatively short duration of the liver infection (Struik and Riley, 2004). Polymorphisms at the regions recognized by the $CD8^+$ T cells induce an antagonistic effect that interferes with priming and survival of memory T cells, an event designated by altered peptide ligand, which is appointed as another explanation for lack of memory (Plebanski *et al.*, 1999; 1997). Furthermore, the mixture of different *Plasmodium* strains in endemic areas can aggravate the dynamic interactions of variant-specific T cell responses resulting in immune interference (Zimmerman *et al.*, 2004; Bruce and Day, 2002).

The initiation of a specific immune response against a disease requires the activation of T cells by professional antigen-presenting cells (Mellman *et al.*, 1998). In some biological systems the identity of these cells is still to be determined. However, in most cases, dendritic cells are the ones that efficiently activate naïve T cells because of their unique ability to induce primary immune responses through priming (Sher *et al.*, 2003; Bruña-Romero and Rodriguez, 2001).

During pathogenic infections, apoptosis of infected cells could either be caused by the host's immune response or be a direct effect induced by the pathogen (Gavrilescu and Denkers, 2003). But, in any case, the result is the formation of apoptotic bodies that are loaded with antigens derived from the pathogen. In order to reduce the risk of an inflammatory response these apoptotic cells have to be removed (Savill and Fadok, 2000). Phagocytes, such as macrophages and immature dendritic cells, have the ability to capture these vesicles and present enclosed antigens to activate T lymphocytes (Albert *et al.*, 1998 a, b). However, only dendritic cells have the capacity of presenting

such antigens in both MHC class I and II contexts, making use of a cross-presentation mechanism, and consequently inducing the priming of naïve T lymphocytes (Guermonprez *et al.*, 2003).

Immunization with irradiated sporozoites induces specific $CD8^+$ T cells against *Plasmodium* antigens which are involved in mounting protection against the disease (Rodrigues *et al.*, 1991; Hoffman *et al.*, 1989; Romero *et al.*, 1989; Weiss *et al.*, 1988). Moreover, a large number of pathogens do not infect or replicate within professional antigen presenting cells, but still their antigens are presented by MHC class I molecules since activated-specific cytotoxic T lymphocytes are found *in vivo*. For this activation to occur there is the requirement of a restricted-class I presentation (Gil-Torregrosa *et al.*, 2004). *Plasmodium* sporozoites fit in this category since they are only able to fully develop in hepatocytes. Consequently, the activation of specific anti-malaria T lymphocytes observed during irradiated sporozoite infection must occur through cross-presentation of parasite antigens performed by antigen presenting cells, the dendritic cells.

Several examples of transfer and presentation of cellular antigens can be found in the literature and this hypothesis has been already demonstrated for viral proteins, tumour antigens and protein-coated antigens (Albert, 2004; Shen and Rock, 2004; Fonteneau *et al.*, 2002; Sigal *et al.*, 1999; Huang *et al.*, 1994). Because of the immunological relevance of $CD8^+$ T cell activation, cross-presentation should undergo some sort of control in terms of specificity and efficiency. These two main characteristics can be ensured by receptor-mediated processes. Although both macrophages and dendritic cells are skilled cells in terms of antigen phagocytosis, in respect to cross-presentation and T cell priming, macrophages are known to be poor stimulators of naïve T cells *in vitro*. The higher efficiency of dendritic cells in the priming of naïve T cells is, at least in part, mediated by the presence of co-stimulatory molecules that allow longer and more efficient interactions with T cells (Heath *et al.*, 2004).

The experimental *in vitro* observation that a decrease in the number of infected hepatocytes in culture occurs with time led to the basis of our working hypothesis (Silvie *et al.*, 2002; Suhrbier *et al.*, 1990; Sigler *et al.*, 1984). Could apoptotic bodies generated from *Plasmodium* infected hepatocytes infected with irradiated sporozoites be phagocytosed in the liver by dendritic cells? And if so, could these cells mediate cytotoxic T lymphocyte activation by cross-presentation?

Thus, in the first part of this project, we accessed our hypothesis by determining whether infected hepatocytes were undergoing apoptotic death and therefore decreasing in number during the course of infection. Apoptosis was detected in infected hepatocytes which were then shown to be phagocytosed by both macrophages and dendritic cells present in the liver. An increased recruitment of dendritic cells was observed during *in vivo* infection which, together with the apoptotic markers and hepatic origin evidenced by phagocytosed vesicles, implicates dendritic cells in the uptake of *Plasmodium* antigens present in apoptotic hepatocytes.

This model links two key processes in the control of infection: apoptosis of infected cells and antigen uptake by antigen presenting cells. Data obtained strongly associate these events, which could provide a mechanism for the initiation of immune responses against malaria.

During the course of our studies, Jung and colleagues showed that during malaria infection there is a dendritic cell dependency in order to obtain a $CD8^+$ T cell immune response. This strongly suggests that priming takes place *via* cross-presentation of antigens originated from infected hepatocytes provided by dendritic cells. Making use of a conditional knock out mouse that can induce ablation of $CD11c^+$ dendritic cells, the essential requirement of these cells to elicit a $CD8^+$ T cell response to malaria was revealed. However, in this study, the interface of these cells with sporozoites or infected hepatocytes was not analyzed (Jung *et al.*, 2002).

To initiate the process of antigen presentation to T cells, dendritic cells must be able to phagocytose *Plasmodium* antigens. Thus, size is an important feature in this process. Either the whole sporozoite or CS protein that is shed from the parasite surface could be phagocytosed by antigen presenting cells (Hugel *et al.*, 1996). Yet, phagocytosis *per se* is not capable of explaining why for instance heat killed sporozoites do not induce T cell responses and why irradiated sporozoites are a better immunogen (Alger and Harant, 1976; Spitalny and Nussenzweig, 1972). Although there is a visible difference in apoptosis during irradiated *versus* non-irradiated sporozoite infections, the lower amount of apoptotic hepatocytes detected in non-irradiated sporozoite infections indicates that in some cases, normal sporozoites are not capable of inhibiting their host death. Such evidence could hold an effect in immunity and should be further analyzed.

As already mentioned, dendritic cells need to mature in order to achieve effective T cell activation. Since it is known that apoptotic bodies are unable to induce dendritic cell maturation, sporozoites must be responsible for this action (Albert, 2004; Liu *et al.*, 2002; Gallucci *et al.*, 1999; Albert *et al.*, 1998b). Furthermore, wounded hepatocytes generated during sporozoite migration could lead to dendritic cells maturation due to the pro-inflammatory environment induced and the release of maturation signals (Frevert, 2004; Khan and Vanderberg, 1992). Endogenous adjuvant activity was detected in the cytoplasm of cells undergoing cellular injury or tumor death. The resulting components can stimulate immune responses since they constitute a danger signal to the immune system (Shi *et al.*, 2003; Shi and Rock, 2002; Shi *et al.*, 2000).

During malaria liver stages, functional *in vivo* antigen presentation seems to be limited by time. After irradiated sporozoites immunization, the priming of naïve T cells is very fast reaching a maximum within the first 8h (Hafalla *et al.*, 2002). Moreover, antigen-specific activated $CD8^+$ T cells are inhibited as early as 24 to 48h after priming (Hafalla *et al.*, 2003; Mercado *et al.*, 2000). Extrapolating to a natural infection, the rate of exposure and priming of T cell should be dependent on the biting by infectious mosquitoes. However, very low frequencies of T cells against liver stage antigens are

observed. Therefore, this means that, when the threshold is reached, the $CD8^+$ T cells specific for sporozoite-derived antigens will be preserved regardless of the inoculation rates (Hafalla *et al.*, 2002).
In addition, the discrepancy between irradiated sporozoites and normal sporozoites in inducing immunity can be also supported by the subsequent occurrence of a blood stage infection in the second and not in the first, since a blood stage infection could lead to an immuno-suppression of $CD8^+$ T cell function against the initial liver stages (Ocana-Morgner *et al.*, 2003).
In the context of the variety of the cellular populations that are present in the liver the full role of Kupffer cells remains an unanswered question. Recent studies regarding their involvement and immune status during infection revealed that these cells could function as antigen-presenting cells, whose activity is much higher in challenged irradiated sporozoite-immunized mice than in normal sporozoite-infected mice. In addition, an enhanced expression of MHC classes I and II and costimulatory molecules was also a difference observed between these two types of Kupffer cells. Further analysis should still be carried out in order to understand the contribution of these cells during protection conferred by irradiated sporozoite immunizations. However, the absence of an increased expression of class I on Kupffer cells originated from immunized mice in response to *in vitro* normal sporozoite challenge suggests that other responses from different primed liver cells *in vivo* may occur (Steers *et al.*, 2005).
Recently, a number of studies using different epitopes from the *Plasmodium* CS protein as a way to pulse dendritic cells or access the induction of MHC class I-restricted antigen-specific T cells, have validated and confirmed the role of dendritic cells as skilled to process and present sporozoite antigens. Moreover, the priming events are clearly different between normal or irradiated parasites, where the latter induce higher levels of IFN-γ production (Plebanski *et al.*, 2005).
Furthermore, it was shown that the successful synthetic peptide vaccine trial that induced high antigen-specific T cell responses in volunteers is likely to be due to dendritic cell cross-presentation, which requires the proteosome and the MHC class I pathway for processing and the endosomal compartment for the presentation (Prato *et al.*, 2005).
Analyzing the extremely complex interaction between *Plasmodium* sporozoites and hepatocytes, it becomes clear that several other molecules must play significant roles during liver infection, either from the parasite or the host sides. New findings are elucidating the recognition and invasion process of the hepatocyte by the parasite. These processes appear to involve other sporozoite proteins besides CS and TRAP, such as microneme proteins essential for cell traversal, other proteins involved in sporozoite motility or even proteins essential for the recognition of surface receptors on host cells (Ishino *et al.*, 2005 a, b; Ishino *et al.*, 2004; Khater *et al.*, 2004).
In this thesis, we have also addressed the host's input to this interplay. Cellular death can be either induced or inhibited (Vaux and Strasser, 1996). Both events constitute opposite mechanisms that

carry different benefits to each one of the parts involved. On the host side, apoptosis is seen as a defence mechanism because infection is eliminated. On the other hand, for the parasite, the inhibition of host cell apoptosis has huge advantages as it allows the pathogen to prolong its life within the host cell, thereby perpetuating infection. Examples of such inhibitors can be found among different intracellular parasites like *Leishmania* and *Toxoplasma* (Sinai *et al.*, 2004; Luder *et al.*, 2001). Such evidence is also present within viral infections, where a few inhibitors have been characterized in detail (Hasnain *et al.*, 2003). Moreover, parasites find ways of imitating the modulation of apoptotic pathways that occur in normal cells. All these data suggest that *Plasmodium* might engage in similar strategies during liver infection, but little is known regarding this issue.

Thus, our study's starting point was to examine how sporozoite infection related to these observations and in order to achieve that understanding, we analyzed the outcome of induced apoptosis on infected hepatocytes. Our results confirmed that *Plasmodium* infection protects host hepatocytes from apoptosis during liver stage malaria. Such behaviour was expected since similar observations were already made for other intracellular parasites like *Toxoplasma* and *Leishmania* (Nash *et a*l., 1998; Moore and Matlashewski, 1994).

Previous work has shown that, during *Plasmodium* infection, sporozoites migrate through several hepatocytes before establishing in a definitive one (Mota *et al.*, 2001). By breaching their plasma membranes, the parasite leaves a trail of wounded cells which secrete a host molecule, HGF, to the surrounding environment. The secreted HGF and the activation of its receptor c-Met, makes host cells more susceptible to malaria infection. Furthermore, sporozoite infection is dependent on this activation and induced stimuli, which facilitates parasite development (Carrolo *et al.*, 2003).

HGF is responsible for activating a vast cascade of signaling pathways within cells, including inhibition of apoptosis (Birchmeier *et al.*, 2003). Therefore, we hypothesized that, during liver infection, HGF/MET signaling could play a role in the previously observed anti-apoptotic effect. Data obtained confirmed our hypothesis and provided further evidence that infection-induced protection is mediated by the PI3K/AKT signalling pathway. Previously, numerous studies had already shown that this survival cascade is usually constitutively activated in several types of cancer cells and is currently being targeted as therapeutic approach (Kim *et al.*, 2005).

More recently, it was demonstrated that *P. berghei* inhibition of hepatocyte apoptosis also occurs during the late phase of liver infection. Resistance to induced apoptosis of infected cells was shown to be increased 2 days after infection, independent of the HGF/MET signalling pathway (van de Sand *et al.*, 2005). Our results concerning apoptosis inhibition at the early phase of the parasite development are in agreement with the observation that HGF/MET signalling protracts for several hours only. Additionally, a new interest has arisen concerning a different but highly plausible occurrence is to know how the parasite inhibits pro-apoptotic signals instead (van de Sand *et al.*,

2005). Our data implicate and reinforce the inhibition of apoptosis as a crucial strategy through which *Plasmodium* controls the survival outcome of the hepatocytes.

The HGF/MET complex has been as well extensively studied in cancer research, and constitutes an attractive target for use in therapy since several approaches have been described that can be employed to interfere with this pathway. However, the practical results of an application of such strategies remain unclear, mainly due to the lack of appropriate delivery systems (Corso *et al.*, 2005).

An extrapolation of these procedures to malaria infection could lead to an interesting progress in terms of preventive strategies, since HGF/MET usage as a targeting candidate for drug development would hold an effect in liver infection. In addition, such a target could avoid the emergence of drug resistance since there is no direct interaction with the pathogen itself.

At the end of our studies, we attempted to identify a *Plasmodium* gene responsible for infection-induced hepatocyte survival. It is known that cell death is a highly conserved process throughout evolution (Vaux *et al.*, 1994; Wyllie *et al.*, 1980). Therefore, parasites themselves should encode proteins that may be responsible for interfering with the regular function of this cellular process. In fact, there are reports of viruses that carry genetic information for anti-apoptotic genes, namely homologues to cellular regulators (Clarke *et al.*, 2005; Barry and McFadden, 1998). Finding *Plasmodium* homologues of a known apoptosis inhibitor would open future possibilities for functional studies using knock out parasites. Additionally, gene targeting knock out experiences could lead to the elimination of this putative apoptosis inhibitor gene. The availability of the *Plasmodium* genome sequence facilitates the identification of prospective candidates that might be involved in liver infection, which may hold a huge potential as novel targets in disease control (Duffy *et al.*, 2005; Cooper and Carucci, 2004).

Likewise, heat shock proteins (HSPs) belong to a group of molecular chaperones that are also highly conserved (Lindquist and Craig, 1988). These proteins are specialized in rescuing cells from damage when they are exposed to stress, and their mode of action includes ensuring cellular survival. Therefore, HSPs commonly act as anti-apoptotic chaperones being themselves checkpoints along the apoptosis pathways with the ability of shutting these pathways off (Beere, 2005).

HSP70 is one of the most abundant protein families that is present in several organisms including parasites (Dobbin *et al.*, 2002; Rico *et al.*, 1999; Levy *et al.*, 1992). *Plasmodium* HSP70 is expressed among different parasite species and has a high degree of immunogenicity which makes it a target of immune responses during infection (Zhang *et al.*, 2001; Behr *et al.*, 1992).

All together and since *P. yoelii* HSP70 (PyHSP70) fitted in these profiles we decided to examine its function as an apoptosis inhibitor candidate. Our preliminary results suggest that the PyHSP70 could influence the infection process, as this protein has the ability of inhibiting hepatocyte apoptosis and is, therefore, worth considering and exploring in a future project. This plausible

identification of a *Plasmodium* gene responsible for apoptosis inhibition of hepatocytes does not exclude the possibility of more and different genes being implicated in this process, which justifies the continuing study and search to find more candidates.

7.2 Perspectives

The work presented in this thesis provides immediate contribution to the understanding of the cellular and molecular mechanisms involved in some aspects of *Plasmodium*-hepatocyte interactions. Crucial steps in malaria infection take place inside hepatocytes which are related not only to parasite development but also with the immune responses developed against the disease.

One of the greatest challenges facing infectious diseases research at the present time is to learn how to arm the host immune system strong enough to defeat disease. At the moment vaccine development interests are focused in finding *Plasmodium* molecules that can confer a long-lasting protection against liver and subsequent blood stage infection. The use of a single protein in a vaccine has consistently led to failure, thus progress must also be applied to the development of whole attenuated parasite vaccine. Besides irradiation other options are now being exposed such as the use of genetically engineered inactivated parasites and combinations of various vaccination regimens (Good, 2005; Todryk and Walther, 2005).

Due to the latest examples and the enlightening on different infection models, some authors do agree that dendritic cells should be targeted in order to obtain an effective malaria vaccine application (Bertholet *et al.*, 2005; Blachere *et al.*, 2005; Plebanski *et al.*, 2005; Sher *et al.*, 2003; Norbury *et al.*, 2002). The selection of the right *Plasmodium* antigen as well as the adjuvant that would target an innate response involving dendritic cells could be much more protective. Therefore, in the future it should be considered the interaction maintained by *Plasmodium* with these antigen presenting cells, and also the direct relation that is observed between these and the $CD8^+$ T cell interactions (Engwerda and Good, 2005).

In addition, remaining questions address a few issues like the migration of parasite-specific T cell to the infected liver, their elimination and the development of memory against the parasite. It is still very important to engage a thorough characterization of the important antigens relevant to vaccine development and theirs respective induced-immunity (Krzych and Schwenk, 2005).

Within technical support new tools are becoming available in particular in the imaging field, which will contribute to enhance the accuracy of our knowledge specially and if possible at a more physiological environment closer to what occurs *in vivo*. A new input is also being applied to the search for alternative methods concerning detection of immune responses (Amino *et al.*, 2005; van Baalen *et al.*, 2005).

Plasmodium antigens presented in the context of host cell apoptosis are an alternative mechanism involved in the induction of protective immunity by irradiated sporozoites. Our findings are being reinforced by out coming results. Clearly the consequences of apoptosis are being redefined specially at the immunological level. Certainly with all the evidence raising from different infection models it would be very misleading to view apoptotic death as single simplistic event. An opposite outlook is taking this common cellular process to a level of importance that for sure will worth to continuous studying (Restifo, 2000).

In the future, and making use of both parasite and host transcriptome and proteome, an extensive study will help to reveal more parasite strategies used to influence host cell apoptosis during infection, either by activating anti-apoptotic mechanisms or by inhibiting pro-apoptotic ones (Cooper and Carucci, 2004).

As previously mentioned, the targeting of malaria parasites both through vaccine or anti-malaria drugs is mostly performed using components of the parasite. Those are involved in the host-parasite interactions and are essential for survival and development or on the other hand involved in disease pathology. Another strategy that could be undertaken is to act on and exploit the host molecules that interact with the parasite components or molecules produced by it. In this context, HGF/MET signalling should be consider a drug target candidate against malaria since an intervention based on the tools available should lead to a successful prevention of infection.

Presently, in the new post-genomic era is essential that a clear view of the elements that are involved either in basic understanding of *Plasmodium* species biology or in applied work aimed at control and prevention of the disease is achieved. Taking a step forward within research would leads us to the application of systems-biology to malaria, since an integration of several data sets would raised the chances of being successful both in vaccine and drug development against the disease (Young and Winzeler, 2005).

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APPENDIX

MAJOR ARTICLE

Apoptotic *Plasmodium*-Infected Hepatocytes Provide Antigens to Liver Dendritic Cells

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(See the editorial commentary by James, on pages 1573–5.)

Malaria starts with infection of the host liver by *Plasmodium* sporozoites. Inoculation with radiation-attenuated *Plasmodium* sporozoites induces complete protection against malaria. Protection is mediated by dendritic cells (DCs) and $CD8^+$ T cells, but the source of parasite antigens mediating this response remains unclear. Here, we show that hepatocytes infected with irradiated *Plasmodium* sporozoites undergo apoptosis shortly after infection. Infection with irradiated sporozoites induces the recruitment of DCs to the liver, where they phagocytose apoptotic infected hepatocytes containing parasite antigens. We propose that apoptotic *Plasmodium*-infected hepatocytes provide a source of parasite antigens for the initiation of the protective immune response.

Complete protection against malaria in humans and mice can be obtained by inoculation with irradiated *Plasmodium* sporozoites that induce specific $CD8^+$ T cells [1]. Irradiated sporozoites infect hepatocytes in vivo, as normal sporozoites do; however, irradiated sporozoites do not progress further to blood-stage infections and, therefore, do not induce malaria-associated pathology [2]. During sporozoite infection, no "professional" antigen-presenting cells (i.e., cells that may stimulate not only memory T cells but also naive T cells) are infected by *Plasmodium* sporozoites, since they replicate only in hepatocytes. Nevertheless, specific $CD8^+$ T cells reactive to *Plasmodium* antigens are found in irradiated sporozoite–immunized hosts [3, 4], suggesting that parasite antigens are transferred to professional antigen-presenting cells [5]. Dendritic cells (DCs) are able to present exogenous antigens associated with major histocompatibility complex (MHC) class I molecules and activate $CD8^+$ T cells, a process called "cross-presentation" [6]. Interestingly, DCs are required for induction of specific antimalaria $CD8^+$ T cells after vaccination with irradiated sporozoites [7].

Intrahepatocytic development of irradiated sporozoites appears to be required for induction of protection, since infected hepatocytes and their extracts have been shown to induce significant protection [8], whereas inactivated sporozoites and their extracts have consistently failed to achieve the same goal [9, 10]. That hepatocyte infection is required for induction of a cytotoxic response in the host suggests that the source of *Plasmodium* antigens for the initiation of an immune response is not individual sporozoites but infected cells [11].

During pathogenic infections, apoptosis of infected cells results in the formation of apoptotic bodies loaded with pathogen antigens. Cross-presentation of antigens in apoptotic bodies phagocytosed by DCs has been proposed as a mechanism of antigen presentation in cytotoxic immune responses against intracellular pathogens [12, 13]. It has also been proposed that apoptosis of host cells contributes to immunity induced by irradiated-parasite vaccines [14]. Here, we show that apoptotic infected hepatocytes are phagocytosed by DCs in the liver of the host, providing a source of *Plasmodium* antigens for the initiation of antimalaria immune responses.

Received 27 September 2004; accepted 18 November 2004; electronically published 4 April 2005.

Presented in part: 43rd Meeting of American Society for Cell Biology, San Francisco, CA, 13–17 December 2003.

Financial support: National Institutes of Health (grant R01 AI049432 to A.R.); Fundaçao para a Ciência e Tecnologia, Portugal (support to M.M.M. and Ph.D. fellowship SFRH/BD/3230/2000 to P.L.).

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The Journal of Infectious Diseases 2005; 191:1576–81

0022-1899/2005/19110-0002$15.00

MATERIALS AND METHODS

Host cells, parasites, and mice. Hepa 1-6 (ATCC CRL-1830), a hepatoma cell line derived from a C57L/J mouse that is efficiently infected by rodent malaria parasites, was used for in vitro hepatocyte cultures. *Plasmodium yoelii* (cell line 17X NL) and *Plasmodium berghei* (parasite line NK65) sporozoites were isolated from salivary glands of infected *Anopheles stephensi* mosquitoes. Sporozoites were irradiated with a γ source (20 krad). BALB/c mice were purchased from Taconic.

In vitro infections. One day before infection, 2×10^5 Hepa 1-6 cells/mL were plated in 24-well plates (Costar) over glass coverslips in Dulbecco's MEM (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C with 5% CO_2. A total of 1×10^5 sporozoites were added to Hepa 1-6 cell coverslips on plates, centrifuged for 5 min at 1800 *g*, and incubated for different durations. Cells were washed twice with PBS and fixed in 4% paraformaldehyde.

In vivo infection, isolation, and staining of liver mononuclear cells. Two mice per group were infected with *P. yoelii* γ-irradiated, nonirradiated, or heat-killed (15 min; 56°C) sporozoites by intravenous (iv) injection (1×10^6 sporozoites/mouse). Six hours after injection, livers were removed and mechanically homogenized. The cell suspension was washed and resuspended in a 35% Percoll (Pharmacia Biotech) gradient solution and centrifuged at 500 *g* for 10 min. The mononuclear-cell pellet obtained was resuspended in 1 mL of erythrocyte lysis buffer for 1 min and washed. Isolated cells were counted and placed for 1 h on poly-L-lysine coverslips ($\sim 1.5 \times 10^6$ cells/liver were obtained). After fixation, cells were incubated with phycoerythrin (PE)–anti-CD11c and fluorescein isiothiocyanate (FITC)–anti-Mac3 (Pharmigen) for staining of DCs and macrophages, respectively. Parasites were stained with anti-Hsp70 monoclonal antibody (2E6). Cells were also stained with anti-albumin (Cappel/ICN Irvine) and anti–activated caspase-3 (Promega).

Detection of apoptosis. Apoptotic cells were detected and quantified by staining with anti–NF-κB p65 (relA) (Santa Cruz Biotechnology), for examination of the translocation of NF-κB from the cytoplasm to the nucleus of the cell, or with anti–activated caspase-3 (Promega). All cells were also incubated with 4′6′-diamidino-2-phenylindole (DAPI) for nuclear staining, to allow morphological detection of apoptosis.

DC recruitment to the liver. Groups consisting of 3 mice were infected by mosquito bite (each anesthetized mouse was in contact with 50 *P. yoelii*–infected mosquitoes for 20 min) or by iv injection with 1×10^5 *P. yoelii* sporozoites (γ-irradiated or nonirradiated) or with salivary glands of uninfected mosquitoes as a control. At different times after infection, livers were removed and frozen, and 15 histological sections from each mouse were examined with PE–anti-CD11c (Pharmigen) for detection and quantification of DCs.

RESULTS

During in vitro infection of hepatocytes by *Plasmodium* sporozoites, a decrease in the number of developing parasites is observed 2–3 days after infection. This decrease is more pronounced for irradiated sporozoites than for nonirradiated ones [2, 15]. To study the survival of hepatocytes infected with irradiated *Plasmodium* sporozoites at earlier times after infection, we stained *Plasmodium*-infected hepatocyte cultures with an anti-Hsp70 antibody, a molecule that is highly expressed in developing parasites inside hepatocytes but not in sporozoites [16]. We found that the number of Hepa 1-6 hepatoma cells infected with *P. yoelii* and *P. berghei* (data not shown) irradiated sporozoites was lower than the number infected with nonirradiated sporozoites and that the number of infected cells had already decreased shortly after infection (figure 1*A*).

The decrease in the number of infected hepatocytes has been attributed to degeneration of intracellular parasites [2]. We wanted to determine whether the reduction in the number of infected hepatocytes is due to hepatocyte apoptotic death triggered by degenerated parasites. Apoptosis in infected hepatocyte cultures was first monitored by assessment of nuclear morphology and activation of NF-κB p65 (relA), a transcription factor that is translocated to the nucleus early after initiation of certain apoptotic pathways [18] (figure 1*B*). We observed that hepatocytes infected with irradiated sporozoites undergo apoptosis 6 h after infection (figure 1*C*). Apoptosis in hepatocytes infected with irradiated sporozoites was also confirmed by detection of caspase-3 activation (figure 1*D*). Irradiation probably inhibits the synthesis of parasite factors that are necessary for the prevention of apoptosis of infected hepatocytes, resulting in an abortive infection and cell death. However, a lower but significant amount of apoptosis was also found in hepatocytes infected with normal sporozoites, suggesting that, in some cases, normal sporozoite infection cannot prevent the apoptosis of host cells.

In response to inflammatory signals, immature DCs are recruited from the blood into peripheral tissues, where they efficiently phagocytose foreign antigens. To determine whether liver infection by *P. yoelii* sporozoites induces the recruitment of DCs, livers of mice injected with irradiated or nonirradiated sporozoites were obtained at different times after injection. Histological sections showed a recruitment of DCs early after infection (figure 2*A*). When mice were subjected to bites from infected mosquitoes, a similar effect was observed (figure 2*B*). The recruitment of DCs induced by bites from infected mosquitoes is of a lower magnitude, probably because of the smaller numbers of sporozoites injected by mosquito bite [19].

Increased numbers of DCs in the liver during malaria infection would allow rapid capture of parasite antigens. However, to activate DCs for antigen presentation, these cells need to receive a maturation signal, which can be provided by the

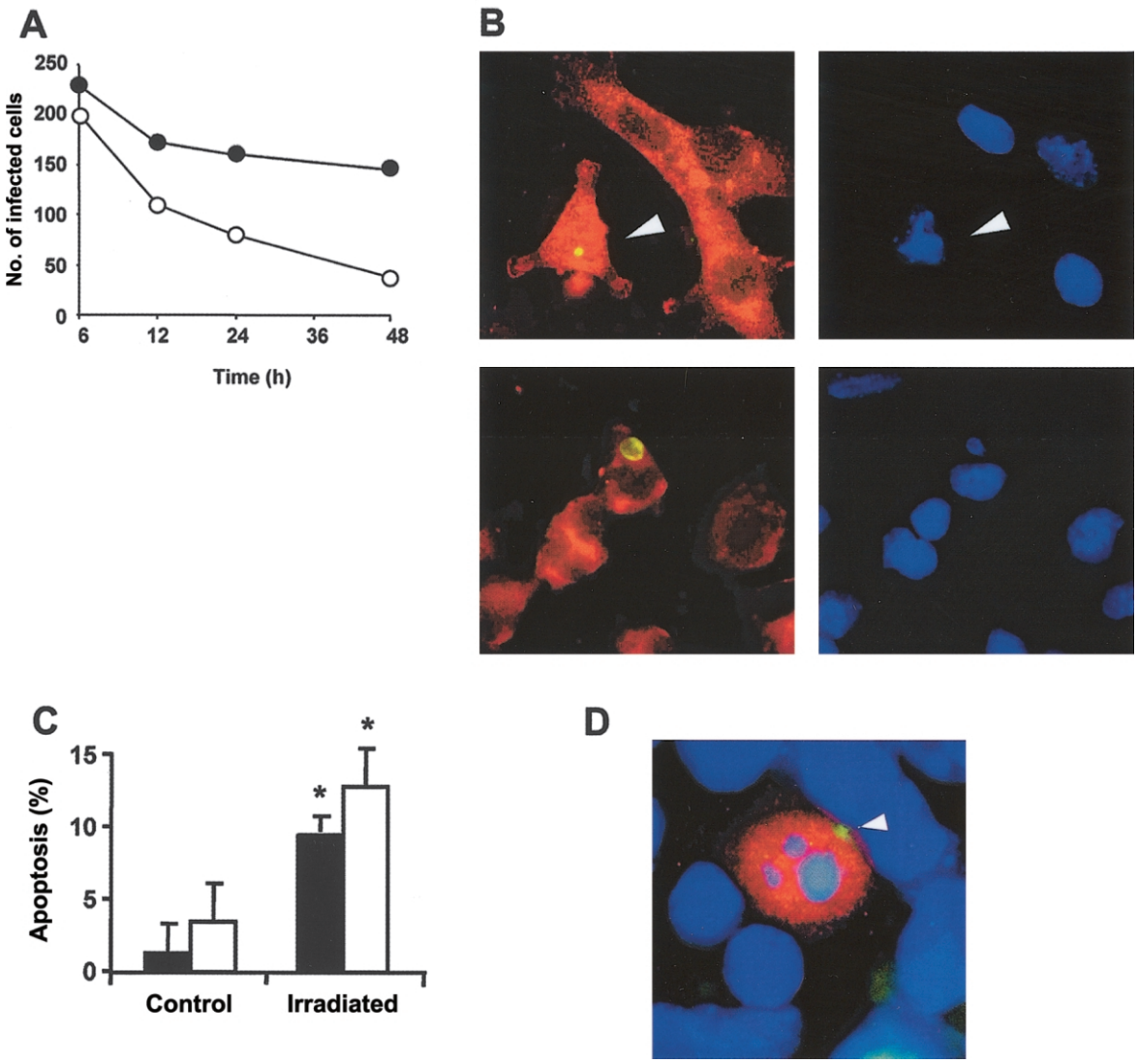

Figure 1. Infected hepatocyte apoptosis. *A,* Infection of Hepa 1-6 cells by irradiated *(white circles)* or nonirradiated *(black circles) Plasmodium yoelii* sporozoites, quantified at different times after infection. Infection was quantified by counting the no. of infected cells with developing parasites in duplicated samples. Variation between duplicates was <8%. Shown are the results of 1 experiment that was representative of 3. *B,* Hepa 1-6 cells were incubated with *P. yoelii* irradiated *(upper panels)* or control *(lower panels)* sporozoites for 6 h. Intracellular parasites were stained with anti-Hsp70 antibody *(green).* The size of the parasite vesicle is smaller in irradiated parasites [17]. Apoptosis was monitored by assessment of nuclear morphology (4′6′-diamidino-2-phenylindole [DAPI] staining, *blue*) and nuclear translocation of NF-κB p65 *(red)* [18]. *C,* Quantification of apoptotic infected cells by nuclear morphology *(black bars)* and NF-κB translocation *(white bars).* Error bars represent the SD of triplicate samples. Asterisks (*) indicate a significant difference between control and irradiated sporozoites ($P < .05$). *D,* Apoptosis was also detected by anti–activated caspase-3 staining *(red)* in infected cells containing intracellular parasites (*green,* marked by an arrowhead).

pathogen itself or by inflammatory lymphokines [20]. Before infection, *Plasmodium* sporozoites migrate through several hepatocytes in the liver, disrupting their plasma membranes and causing cell wounding and necrotic death [21, 22]. Wounded and dying cells induce local inflammatory responses that mediate the recruitment of DCs to tissues [23]. In fact, *Plasmodium* liver infection induces an inflammatory response and the recruitment of mononuclear cells [24]. Release of uric acid from dying cells would provide an additional maturation signal for DCs [25].

Apoptotic cells are rapidly phagocytosed in the body by macrophages and DCs [26]. To determine whether apoptotic hepatocytes containing *P. yoelii* antigens are phagocytosed by antigen-presenting cells in the liver, we isolated nonparenchymal mononuclear cells from the livers of mice immunized with irradiated sporozoites, at different times after injection. We

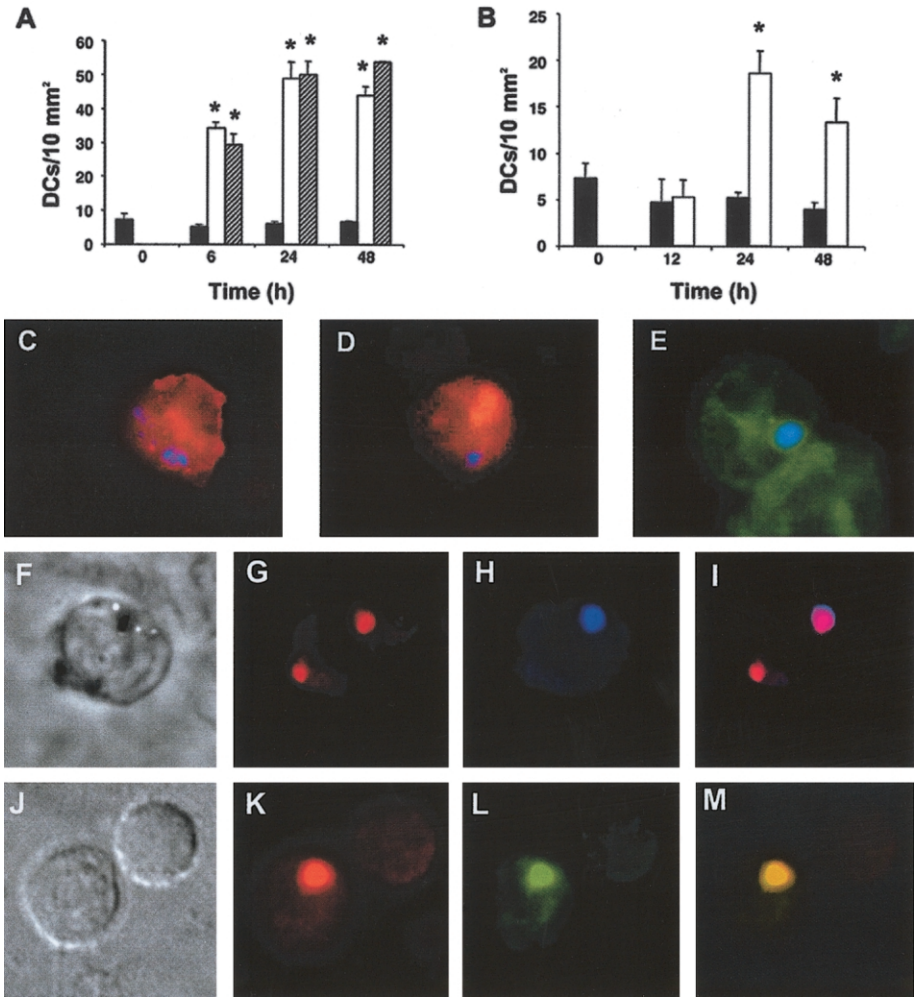

Figure 2. Phagocytosis of apoptotic hepatocytes containing *Plasmodium* antigens by dendritic cells (DCs) and macrophages in the liver. *A* and *B,* Quantification of DCs in histological sections of mouse livers. *A,* DCs in uninfected mice *(black bars)* and in mice after different times of infection with *P. yoelii* nonirradiated *(white bars)* or irradiated *(striped bars)* sporozoites. *B,* DCs in mice bitten by 50 uninfected *(black bars)* or infected *(white bars)* mosquitoes. Error bars represent the SD of triplicate histological samples, each composed of ⩾5 liver sections to cover 10 mm^2. Asterisks (*) indicate a significant difference between the sample and the control at time 0 h ($P < .02$). *C–M,* Liver nonparenchymal mononuclear cells, obtained 6 h after injection of mice with *P. yoelii* irradiated sporozoites and stained with different markers: DC marker (CD11c, *red*), macrophage marker (mac-3, *green*), and *Plasmodium* Hsp70 *(blue).* DCs (*C* and *D*) and macrophages *(E)* were found to contain vesicles positive for Hsp70. *F–I,* Albumin/Hsp70 staining, in the same microscope field showing a cell in transmitted light *(F),* staining for albumin *(red, G), Plasmodium* Hsp70 *(blue, H),* and an overlay of albumin and Hsp70 staining *(I). J–M,* Apoptosis/*Plasmodium* staining, in the same microscope field showing cells in transmitted light *(J),* staining for activated caspase-3 *(red, K), Plasmodium* Hsp70 *(green, L),* and an overlay of caspase-3 and Hsp70 *(M).*

found that, 6 h after injection, macrophages and DCs in the liver carry vesicles that contain *P. yoelii* Hsp70 (figure 2*C*–2*E*). In 3 separate experiments, we found an average of 3 macrophages and 2 DCs in each liver containing Hsp70-positive vesicles. The number of DCs containing these vesicles is probably higher in the mouse, but DCs rapidly leave peripheral organs and migrate to the lymph nodes after receiving activation signals. The Hsp70-positive vesicles found inside macrophages and DCs must be derived from infected hepatocytes, since this protein is not expressed in sporozoites and appears only in infected hepatocytes ~6 h after infection [16]. In addition, we found that *P. yoelii* Hsp70-containing vesicles also contain mouse albumin, a protein present in the cytosol of hepatocytes (figure 2*F*–2*I*), confirming that these vesicles are derived from infected hepatocytes. No *P. yoelii* Hsp70 or albumin staining was present in nonparenchymal liver mononuclear cells isolated from noninfected control mice. To confirm that these Hsp70-positive vesicles were derived from apoptotic cells, we stained cells with anti–activated caspase-3. We found that vesicles positive for *Plasmodium* Hsp70 were also positive for activated caspase-3

(figure 2*J*–2*M*), confirming that DCs phagocytose apoptotic infected hepatocytes during malaria infections in vivo.

When liver nonparenchymal mononuclear cells were analyzed 12 h after infection, no cells were found containing *P. yoelii* Hsp70, which suggests that infected hepatocyte death and clearance occurs early during in vivo infections with irradiated sporozoites. This is consistent with the observation that *Plasmodium* sporozoite infection and initiation of the immune response occurs rapidly, since activation of specific anti–*P. yoelii* $CD8^+$ T cells is detected as soon as 8 h after inoculation of sporozoites [27].

DISCUSSION

Protection against malaria can be achieved by immunization with irradiated sporozoites [28]. This discovery raised expectations for the development of a malaria vaccine; however, because of the lack of an in vitro system for the generation of large numbers of sporozoites, broad use of an irradiated sporozoite vaccine against malaria is not considered feasible. It is important to understand the mechanisms underlying protection induced by irradiated sporozoites, since this efficient protection has not yet been replicated by other forms of human antimalaria vaccines. $CD8^+$ T cells and DCs mediate immunity induced by irradiated sporozoites [4, 7], but the mechanisms involved in antigen presentation to T cells remain unclear, since the low number of *Plasmodium*-infected hepatocytes in the liver has prevented the development of direct antigen-presentation studies.

We found that both macrophages and DCs phagocytose infected hepatocyte apoptotic bodies but that, in *Plasmodium* liver infection, only DCs are required for activation of $CD8^+$ T cells in vivo [7]. DC phagocytosis triggers cross-presentation of antigens within the phagosome and results in efficient $CD8^+$ T cell activation [29, 30]. Apoptotic hepatocytes containing *Plasmodium* antigens provide an optimal source of antigens for DC cross-presentation, since they are of the appropriate size (1–10 μm) to be internalized by phagocytosis. This mechanism would also explain the requirement for liver infection to achieve protection against this stage of the parasite, as well as the activation of $CD8^+$ T cells by an obligate intracellular parasite that can develop only inside hepatocytes, which are nonprofessional antigen-presenting cells. The inflammatory environment induced during sporozoite infection in the liver [24] will also favor this process, by inducing the recruitment of large numbers of DCs and providing maturation signals for these cells.

DCs can acquire virus-infected dead or dying cells as exogenous sources of antigens for presentation on MHC class I and II molecules to initiate T cell responses [12]. This pathway is thought to be critical for the development of effective antiviral immunity in vivo [13]. Recently, DC presentation of *Mycobacterium tuberculosis* antigens derived from apoptotic infected macrophages has been described as an alternative pathway of T cell activation [31]. Like *Plasmodium* sporozoites in hepatocytes, *Mycobacterium* is secluded in an intracellular compartment during infection, which suggests that presentation of antigens from apoptotic infected cells may represent a common mechanism of immune activation for vesicle-contained pathogens.

Experimental live vaccines based on irradiated parasites have been used as a model for protection in malaria [1] and schistosomiasis [32]. It is possible that irradiation of these parasites blocks their ability to neutralize the apoptotic machinery of host cells, allowing them to resume their natural tendency to undergo apoptosis upon being infected. Further work is required to demonstrate that phagocytosis of apoptotic infected host cells results in efficient presentation of parasite antigens to T cells.

Acknowledgment

We thank V. E. do Rosário (Centro de Malaria e outras Doenças Tropicais).

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Cellular Microbiology (2005) **7**(4), 603–609 doi:10.1111/j.1462-5822.2005.00490.x

HGF/MET signalling protects *Plasmodium*-infected host cells from apoptosis

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Summary

***Plasmodium*, the causative agent of malaria, migrates through several hepatocytes before initiating a malaria infection. We have previously shown that this process induces the secretion of hepatocyte growth factor (HGF) by traversed cells, which renders neighbour hepatocytes susceptible to infection. The signalling initiated by HGF through its receptor MET has multifunctional effects on various cell types. Our results reveal a major role for apoptosis protection of host cells by HGF/MET signalling on the host susceptibility to infection. Inhibition of HGF/MET signalling induces a specific increase in apoptosis of infected cells leading to a great reduction on infection. Since HGF/MET signalling is capable of protecting cells from apoptosis by using both PI3-kinase/Akt and, to a lesser extent, MAPK pathways, we determined the impact of these pathways on *Plasmodium* sporozoite infection. Although inhibition of either of these pathways leads to a reduction in infection, inhibition of PI3-kinase/Akt pathway caused a stronger effect, which correlated with a higher level of apoptosis in infected host cells. Altogether, the results show that the HGF/MET signalling requirement for infection is mediated by its anti-apoptotic signal effects. These results demonstrate for the first time that active inhibition of apoptosis in host cell during infection by *Plasmodium* is required for a successful infection.**

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Introduction

Liver infection is the first obligatory step of a *Plasmodium* infection. Consequently, it is essential that we understand which hepatocyte–*Plasmodium* interactions are necessary for establishment and success of infection. After *Plasmodium* sporozoites are injected into the mammalian host by infected mosquitoes, they migrate to the liver. There, sporozoites traverse the cytosol of several hepatocytes before invading a final one by forming a parasitophorous vacuole (Mota *et al*., 2001). We have shown that during this process of traversing cells, *Plasmodium* sporozoites not only become activated (Mota *et al*., 2002) but also induce traversed cells to secrete hepatocyte growth factor (HGF), which signals through its receptor MET on neighbouring cells, rendering them more susceptible to infection (Carrolo *et al*., 2003).

Exploitation of host cell resources is a well known survival strategy of intracellular pathogens (Muller and Rudel, 2001). Apoptosis can be used by the host cell as a defence mechanism against intracellular pathogens, as it is beneficial for the organism to eliminate infected cells rather than to preserve them and risk spreading of the pathogen. Conversely, inhibition of host cell apoptosis is advantageous for the pathogen to prolong life in the infected cell in order to complete its development. Studies have focused primarily on viral infections, where several inhibitors of host cell apoptosis have been characterized in detail (Blaho, 2003). Generally, inhibitors are homologues of cellular proteins used in normal cells for the modulation of apoptotic pathways. Inhibition of apoptosis by several bacteria and intracellular parasites like *Leishmania* and *Toxoplasma* has also been documented (Moore and Matlashewski, 1994; Nash *et al*., 1998). However, the genes involved in this process have not been identified yet (Payne *et al*., 2003).

Hepatocyte growth factor (HGF) stimulation elicits through MET a wide spectrum of biological responses such as motogenesis, mitogenesis and protection from apoptosis. HGF has strong anti-apoptotic effects in a series of different cells (Fan *et al*., 1998; Bardelli *et al*., 1999; Xiao *et al*., 2001). In this study, we sought to determine the role of apoptosis protection mediated by

HGF/MET signalling during *Plasmodium* infection. Our results show a crucial role for the anti-apoptotic signal mediated by HGF/MET on the maintenance of a malaria infection.

Results

Infected cells are protected from apoptosis

To determine whether infected cells were protected from apoptosis, HepG2 cells were incubated with *Plasmodium berghei* sporozoites (live or heat-killed, as control) and treated 18 h later with TNF and Cycloheximide to induce apoptosis. Six hours later apoptosis was quantified using different methods: (i) nuclear morphology detected by 4,6-diamidino-2-phenylindole (DAPI): control = 42.0 ± 7.1; infected cells = 8.3 ± 1.5 (Fig. 1B); (ii) active caspase-3: control = 37.9 ± 0.8; infected cells = 7.9 ± 2.4; (iii) TUNEL: control = 31.8 ± 3.8; infected cells = 15.9 ± 3.7.

Similar results were obtained using a different cell type (Hepa1-6 cells) and a different inductor of apoptosis (UV irradiation) (TUNEL: control = 72.3 ± 5.1; infected cells = 12.1 ± 1.5). These results show that *P. berghei* infection protects host cells from apoptosis.

MET activation enhances P. berghei *sporozoite infection and protects infected cells from apoptosis*

We have previously reported that HGF-induced increase on malaria infection is mediated through its receptor MET (Carrolo *et al*., 2003). Since HGF-induced MET activation protects different cell types from apoptosis (Fan *et al*., 1998; Bardelli *et al*., 1999; Xiao *et al*., 2001), we hypothesized that HGF/MET signalling increases infection by protecting host cells from apoptosis. Pretreatment of HepG2 cells with HGF leads to an increment in infection that is accompanied by an increase in apoptosis protection of *P. berghei*-infected cells (Fig. 2A and B). Similar results were obtained using staining for active caspase-3 (control = 15.7 ± 1.5; HGF = 6.9 ± 1.5).

Previous work has described two monoclonal antibodies (DO-24 and DN-30) directed against the extracellular domain of MET (Prat *et al*., 1998). These are agonist mAbs but while DO-24 is a full agonist and can induce a strong receptor activation and trigger the complete invasive growth programme, DN-30 is a partial agonist that can only weakly activate MET (Prat *et al*., 1998). Both mAbs enhanced the level of infection, although at distinct levels (Fig. 2C). The increment caused by the full agonist DO-24 mAb was higher than that induced by the partial agonist DN-30 (Fig. 2C). Apoptosis of infected cells was reduced proportionally to increased infection level, as measured by nuclear morphology detected by DAPI (Fig. 2D) and active caspase-3 (control = 16.8 ± 6.0; DN-30 = 2.2 ± 0.6; DO-24 = 0.3 ± 0.4). Similar results were also observed in Hepa1-6 cells (data not shown). Taken together, these results show that the increase on hepatic infection induced by the HGF/MET signalling correlates with the apoptosis protection conferred by this ligand–receptor system to the infected cells.

MET inactivation leads to early apoptosis of infected cells

Hepatocyte growth factor activation of its receptor MET is a prerequisite for the infection of hepatocytes with *Plasmodium* sporozoites (Carrolo *et al*., 2003). The above-described findings show that increase on *Plasmodium* sporozoite infection caused by MET activation correlates with an increased level of host cell protection from apoptosis. Thus, we hypothesized that MET requirement for infection could be dependent on its anti-apoptotic role in infected host cells. HepG2 cells were transduced with a lentivirus expressing a chimeric construct containing the extracellular and transmembrane domains of *met* fused to *gfp* sequences. The product of this construct is expressed

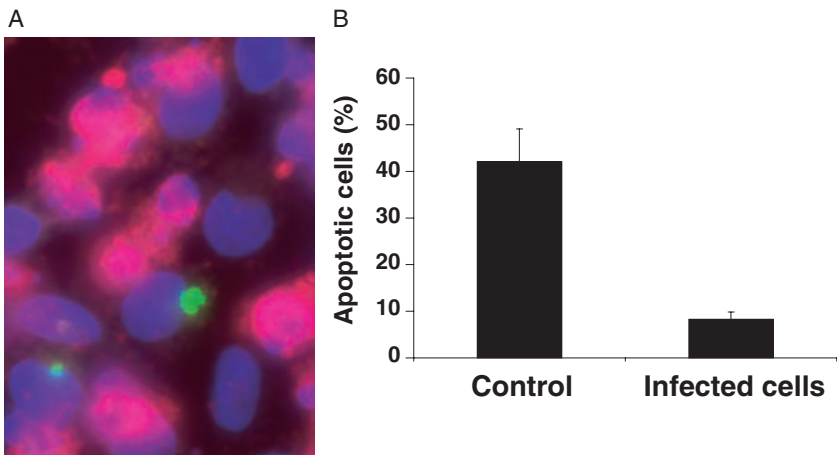

Fig. 1. Infected cells are more resistant to apoptosis. HepG2 cells were incubated with *P. berghei* sporozoites and treated 18 h later with TNF and Cycloheximide to induce apoptosis.
A. Visualization of an infected cell with *P. berghei* (green) not showing apoptotic signs and surrounded by apoptotic non-infected cells, as detected by DAPI in blue and anti-active caspase-3 in red.
B. Apoptosis quantification by nuclear morphology detected by DAPI of HepG2 cells infected with *P. berghei* sporozoites (live or heat-killed, as control) 6 h after apoptosis induction with TNF and Cycloheximide.

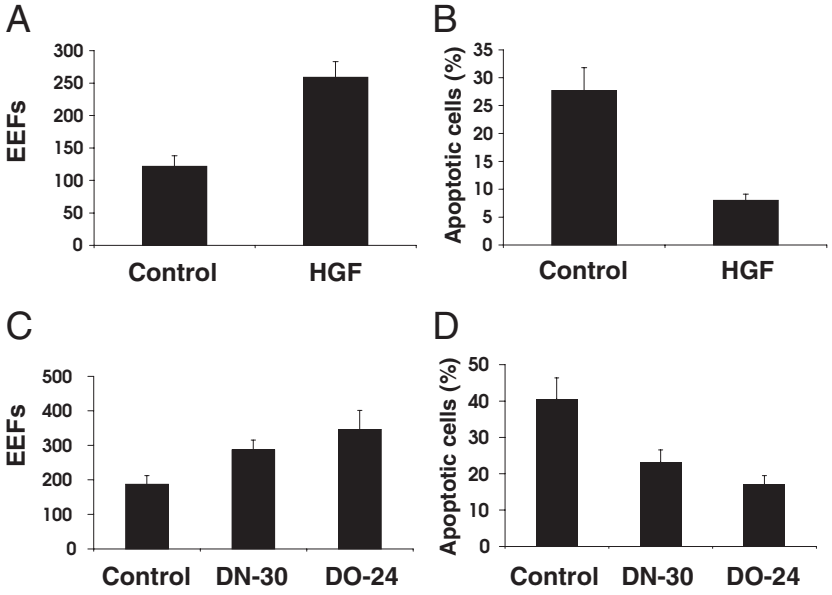

Fig. 2. Increased infection correlates with MET activation and protection of infected cells from apoptosis.
A, B. HepG2 cells were incubated or not (control) with HGF 1 h prior to infection with *P. berghei* sporozoites. (A) Infection was quantified 24 h post infection by counting the number of infected cells (EEFs). (B) Apoptosis was quantified 6 h post infection by nuclear morphology by DAPI staining.
C, D. HepG2 cells were incubated with the agonist anti-MET monoclonal antibodies DN-30 or DO-24 followed by addition of *P. berghei* sporozoites. The same procedure (as in A, B) was followed for quantification of infection and apoptosis.

at the plasma membrane and binds HGF but it is unable to transduce signals into the cell since it lacks the kinase domain and the tyrosines acting as docking sites for intracellular substrates. This chimeric receptor thus behaves as a dominant interfering protein since it dimerizes with endogenous MET and prevents its activation (Giordano *et al*., 2002). As control, HepG2 cells were transduced with a lentivirus expressing GFP only. Since cells transduced with these lentivirus (control or MET-GFP) become fluorescent, we were able to determine the infection level of single-transduced cells 24 h after infection (Fig. 3B). As already reported (Carrolo *et al*., 2003), no individual cells transduced with MET-GFP were found infected with *Plasmodium* 24 h later (Fig. 3B). The results show that MET inactivation correlates with a significant increase of apoptosis in infected cells (21% in control and 68% in infected MET-GFP transduced cells), as observed by nuclear morphology 6 h post infection detected by DAPI (Fig. 3A and C). Presumably, all infected cells undergo apoptosis in the absence of a functional MET receptor within 24 h, as no infected cells are found at this time (Fig. 3B). No increase in apoptosis of non-infected transduced cells was found.

To definitively assess the role of MET anti-apoptotic activity we abrogated MET expression by interference RNA. Lentivirus engineered to express MET-specific siRNA oligos were used to transduce HepG2 cells. Expression of MET siRNA, but not of a control sequence, caused a reduction of MET expression, as detected by Western blot (Fig. 3D). As expected, the *P. berghei* sporozoite infection rate of these cells was severely decreased as compared with cells infected with lentivirus expressing the control siRNA (Fig. 3E). Moreover, sporozoite infection in MET siRNA expressing cells was inversely proportional to the level of apoptosis, as measured by nuclear morphology detected by DAPI 6 h after infection (Fig. 3F). Altogether, these results show that MET-dependency for *Plasmodium* sporozoite infection is highly dependent on its anti-apoptotic activity.

HGF/MET signalling protects infected host cells via activation of PI3-kinase pathway

In several cellular models, HGF has a strong anti-apoptotic activity mediated by PI3-kinase/Akt signal transduction pathway (Webster and Anwer, 2001; Xiao *et al*., 2001), since it is almost completely inhibited by the specific PI3-kinase inhibitor LY294002. An additional anti-apoptotic role is played by MAPK, although the protective effect mediated by this pathway is less marked than that of PI3-kinase/Akt (Xiao *et al*., 2001). Thus, we sought to determine the role of each of these pathways on hepatocyte infection by *P. berghei* sporozoites. The results show that inhibition of either PI3-kinase/Akt or MAPK pathways (by LY294002 and PD98059 respectively) induced a decrease in infection. However, the decrease was more marked with LY294002 (Fig. 4A). When we quantified apoptosis on the same infection experiment (6 h post infection), we detected a significant increase in the level of apoptosis only in cells treated with the PI3-kinase/Akt inhibitor LY294002, as detected by DAPI (Fig. 4B) or active caspase-3 (control = 8.5 ± 2.1; LY294002 = 13.5 ± 3.5; PD = 21.5 ± 10.0). These results show that, although both PI3-kinase and MAPK pathways play a role in infection, only the PI3-kinase/Akt pathway seems to play a critical role in *Plasmodium* infection by protecting host cells from apoptosis.

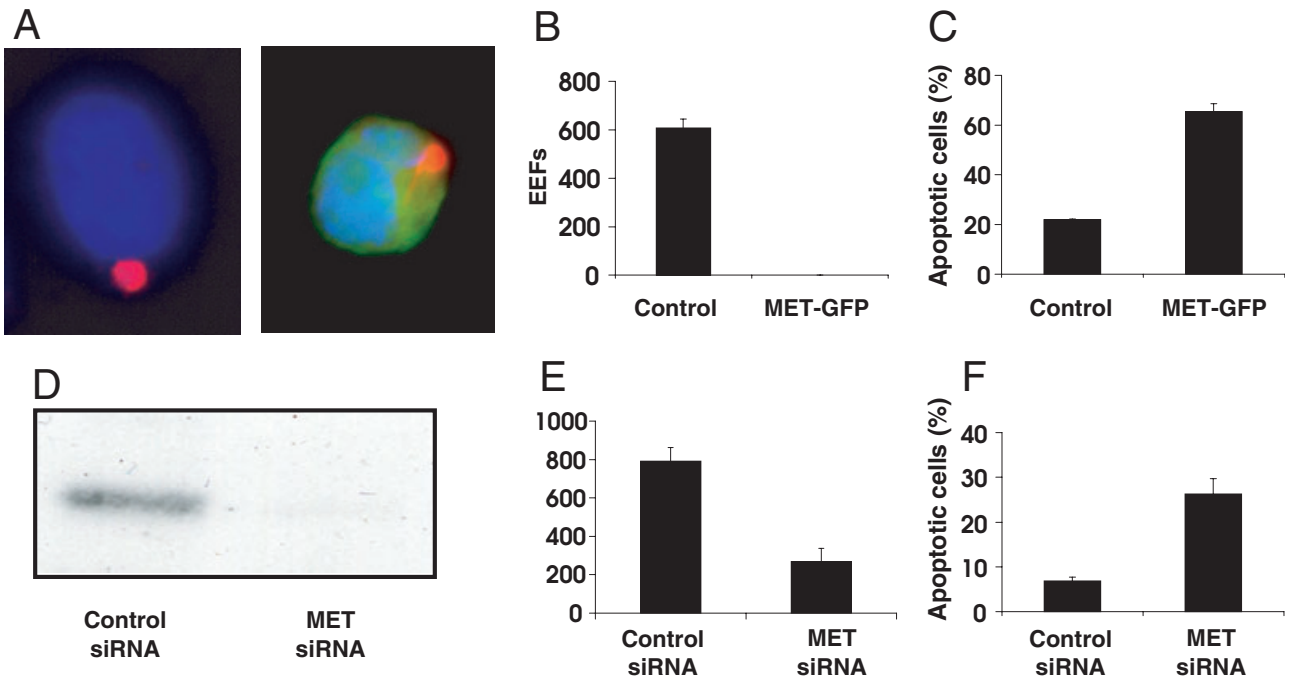

Fig. 3. MET inactivation leads to early apoptosis of infected cells.
A, B, C. HepG2 cells were transduced with lentivirus expressing MET-GFP or GFP alone (control). Cells were then seeded on glass coverslips 24 h prior to addition of *P. berghei* sporozoites. (A) Right panel represents an infected cell expressing MET-GFP (green) 6 h after infection with a parasite starting to develop (red) and showing signs of apoptosis as indicated by the nuclear morphology stained by DAPI (blue). Left panel represents an infected cell not expressing MET-GFP and not showing apoptotic features. (B) Infection was quantified 24 h post infection by counting the number of infected cells (EEFs). (C) Apoptosis was quantified 6 h post infection by nuclear morphology by DAPI staining.
D, E, F. HepG2 cells were transduced with lentivirus expressing MET siRNA oligos or an unrelated oligo (control) (D) Western blot analysis of MET in HepG2 cells transduced with lentiviruses expressing control oligos (left) or MET siRNA oligos (right) to knockdown *met*. (E, F) Infection and apoptosis were quantified as before. All *P*-values ≤ 0.001.

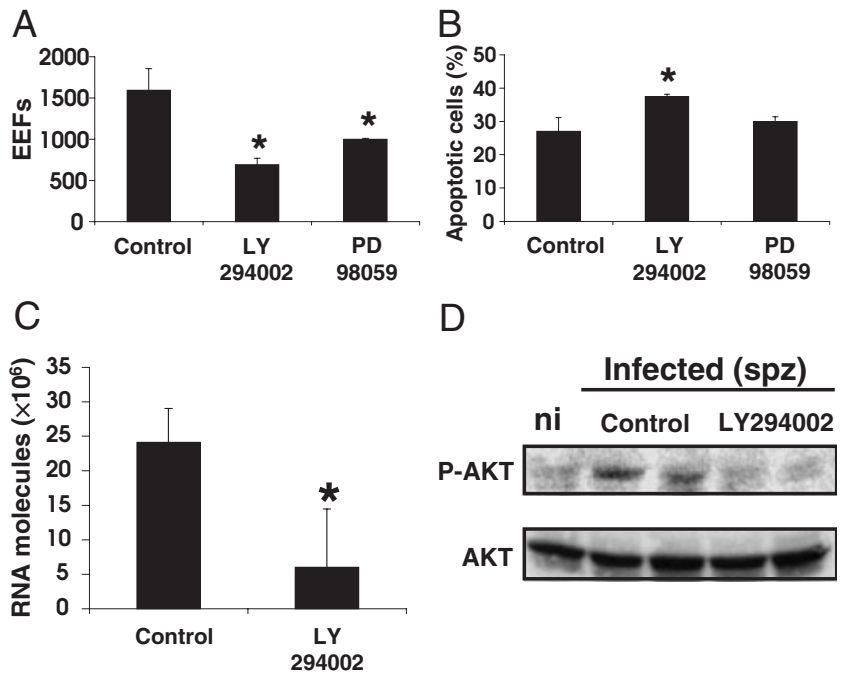

Fig. 4. Inhibition of PI3-kinase pathway leads to a decrease in infection both *in vitro* and *in vivo*.
A, B. HepG2 cells were pretreated with LY294002 or PD98059 for 1 h followed by washes and infection with *P. berghei* sporozoites. Control cells were not incubated with either LY294002 or PD98059. (A) Infection was quantified 24 h post infection by counting the number of infected cells (EEFs) (*$P_{LY} \leq 0.001$; $P_{PD} = 0.003$). (B) Apoptosis was quantified 6 h post infection by nuclear morphology by DAPI staining (*$P_{LY} = 0.002$; $P_{PD} = 0.09$).
C. Mice were injected or not (control) with LY294002 30 min prior to infection with *P. berghei* sporozoites. Livers from both groups were removed 40 h post infection and real-time RT-PCR performed with parasite-specific primers to quantify liver infection (*$P < 0.005$).
D. Mice were injected or not (control) with LY294002 or DMSO, 30 min prior to infection with *P. berghe*i sporozoites. Livers were extracted 3 h post infection and phospho-Ser473-Akt and total Akt were quantified. ni, not infected.

Inhibition of PI3-kinase pathway decreases Plasmodium *infection* in vivo

To determine whether the above-described finding observed *in vitro* could be extrapolated to a natural infection *in vivo*, we treated mice with the PI3-kinase inhibitor LY294002, that is also active in *in vivo* models (Hu *et al*., 2002; Semba *et al*., 2002). The results show that *in vivo* inhibition of the PI3-kinase pathway led to a reduced liver infection by *Plasmodium* sporozoites (Fig. 4C). Western blot analysis was also performed to confirm that the inhibitory treatment was targeting the PI3-kinase pathway. As shown in Fig. 4D, the phosphorylation of AKT was reduced in LY294002-treated animals. Taken together, our *in vitro* and *in vivo* results reveal that the anti-apoptotic effect exerted by HGF/MET signalling through activation of the PI3-Kinase/Akt pathway is crucial for the success of a malaria infection.

Discussion

On entering their host, *Plasmodium* sporozoites migrate directly to the liver. Once there, they migrate through several hepatocytes by breaching their plasma membranes before infecting a final one surrounded by a parasitophorous vacuole where the intrahepatic form of the parasite grows and multiplies (Mota *et al*., 2001). Although during this period there is an extensive parasite multiplication (each parasite gives rise to 10–30 thousand new parasites in 2–10 days, depending on the parasite species), not much is known about its requirements and the strategies used to survive and to accomplish a successful development. It is known that intracellular pathogens have developed powerful abilities to manipulate host cell functions that benefit the pathogen (Sibley, 2004). In particular, it seems that many pathogens have evolved to exploit host cell machinery involved in normal cell proliferation, development and cell death (apoptosis) to their own advantage (Blaho, 2003). The results presented in this report show that *P. berghei* sporozoites are not an exception and require an active process of inhibition of host cell apoptosis in order to reach the next infective stage. However, *Plasmodium* sporozoites seem to use a unique feature since they take advantage of a host molecule not secreted by the infected cell but by neighbouring ones, previously traversed by the parasite. Whether this is the solely mechanism used by the parasite to avoid host cell apoptosis remains unknown.

Our results also show that PI3-kinase activation is important for the survival of the infected cell. The anti-apoptotic effect mediated by HGF uses this signal transduction pathway. However, we cannot exclude that the infection inhibition observed both *in vitro* and *in vivo* by the specific PI3-kinase inhibitor could be due to PI3-kinase mediated survival signals other than those generated through HGF/MET signalling.

We have previously shown that HGF and its receptor are required for a malaria hepatic infection and that host cell actin cytoskeleton remodelling is one of the causes of such requirement (Carrolo *et al*., 2003). In fact, we have observed that pretreatment of host cells with HGF abrogates the negative effect that cytochalasin-D has on infection. This strongly suggests that HGF/MET signalling effect on infection involves the host cell actin cytoskeleton reorganization (Carrolo *et al*., 2003). We now report that part of HGF/MET activity on infection is also due to prevention of infected host cell apoptosis, a crucial requirement to elicit the full development of the parasite. Upon HGF stimulation, MET mediates the coordinated execution of multiple cellular processes. Thus, it is likely that many of these processes might play a role during the development of *Plasmodium* sporozoites inside cells. It is also likely that apoptosis protection and actin reorganization are linked. In fact, studies in bacteria have demonstrated that several bacteria interfere with the fate of host cells through their activity on Rho GTPases, also favouring survival or death depending on their needs (Fiorentini *et al*., 2003).

Recently, a protein called SPECT (sporozoite microneme protein essential for cell traversal) was characterized in one of the secretory organs of sporozoites, the micronemes (Ishino *et al*., 2004). Transgenic parasites lacking this protein do not migrate through cells *in vitro* and show a strong reduction of infectivity *in vivo*, indicating that migration through host cells is required to establish a successful infection in the host. However, these results appear to contradict our hypothesis that migration through cells leads to HGF secretion required for infection, as they are able to infect host cells *in vitro* without previous migration through cells. Therefore, they would not induce host cells to produce HGF and infection would be inhibited. The reason for this apparent discrepancy may be found in the particular characteristics of the *in vitro* cell system used for infection. The hepatoma cell line (HepG2 cells) produces constitutively HGF and other factors that induce a basal level of activation of MET receptor (Conrotto *et al*., 2004). This is different from the conditions that sporozoites encounter *in vivo*, as in resting conditions primary hepatocytes do not express HGF and MET is not activated (Matsumoto and Nakamura, 1991). These constitutive levels of HGF are probably enough to support sporozoite infection at a basal level and therefore the additional HGF induced by migration would not be required.

Hepatocyte infection by *Plasmodium* sporozoites begins by traversing several hepatocytes disrupting their plasma membranes. This process induces the activation of sporozoite exocytosis (Mota *et al*., 2002) and the secretion of HGF from hepatocytes (Carrolo *et al*., 2003). HGF

binds to its receptor (MET) in neighbour hepatocytes, and activates signal transduction pathways that make them susceptible for infection (Carrolo *et al.*, 2003). Inhibition of HGF/MET signalling leads to an increase of infected host cell apoptosis during early stages of intrahepatic parasite development, thus decreasing the success of infection.

Development of *Plasmodium* sporozoites inside hepatocytes is the first obligatory step in a mammalian host and it is decisive for the success of a malaria infection. Thus, the study of parasite requirements to achieve the next infective stage is an effective strategy for any form of early intervention.

Experimental procedures

Cells and parasites

HepG2 or Hepa1-6 cells were maintained in DMEM 10% FCS, 1% penicillin/streptomycin and 1 mM glutamine. *P. berghei* ANKA sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands.

Sporozoite infection

Plasmodium berghei sporozoites (3×10^4) were added to monolayers of 2×10^5 HepG2 or Hepa1-6 cells for 6 or 24 h before fixation and staining with anti-EEF mAb (2E6) (Tsuji *et al.*, 1994), followed by anti-mouse secondary antibodies. Infection was quantified by counting the number of infected cells (EEFs, exoerythrocytic forms) per coverslip. The study of the effect of different reagents on infection were performed in distinct ways: (i) HGF (500 ng ml^{-1}) was added to cells 1 h prior to infection; (ii) The agonist antibodies DN-30 and DO-24 (1 μg ml^{-1}) were added to cells 90 min post infection; and (iii) LY294002 (25 μM) and PD98059 (30 μM) were added to cells 1 h prior to infection and followed by washes before addition of *P. berghei* sporozoites.

Apoptosis induction and detection assays

For the apoptosis induction assays, HepG2 or Hepa1-6 cells were treated with TNF (10 ng ml^{-1}, R&D Systems) and Cycloheximide (10 μg ml^{-1}, Sigma) or with UV stimuli, in order to induce apoptosis, 18 h post infection with *P. berghei* sporozoites. Heat-killed parasites (30 min at 56°C) were used as control. Six hours later cells were fixed and stained for parasite detection and apoptosis quantification. Three distinct fluorescent methods for apoptosis detection were used, based on different features of apoptosis: (i) TUNEL (Roche), which detects DNA breaks; (ii) nuclear morphology by DAPI staining; (iii) active caspase-3 detection (Promega). During infections with *P. berghei* sporozoites, apoptosis was detected 6 h post infection. The effect of different reagents on infected cell apoptosis was performed as described above.

Hepatocyte cell lines transfection and MET modulation

Vectors collected from 24 h supernatants of transfected Phoenix cells were used to infect HepG2 cells. Infected cells were selected with G418 (1 μg ml^{-1}) and the resistant cells were pooled and examined by Western blot.

MET expression was down-modulated in HepG2 cells by transduction with the lentiviral vector PCCLsin.PPT.hPGK.GFP.Wpre, used to express siRNA for MET or control siRNA (under the transcriptional control of the H1 promoter). The oligonucleotides used were as follow: for MET, 5′- GATCCCCGTCATAGGAAGA GGGCATTTTCAAGAGAAATGCCCTCTTCCTATGACTTTTTGG AAA-3′ and 5′-AGCTTTTCCAAAAAGTCATAGGAAGAGGGCAT TTCTCTTGAAAATGCCCTCTTCCTATGACGGG-3′; for the control 5′-GATCCCCCTCATAGGAAGACCCCATTTTCAAGAGAAA TGGTGGTCTTCCTATGACTTTTTGGAAA-3′ and 5′-AGCTTTT CCAAAAACTCATAGGAAGACCCCATTTCTCTTGAAAATGGGG TCTTCCTATGAGGGG-3′. Lentivirus production and infection of HepG2 cells were performed as previously described (Vigna and Naldini, 2000).

Quantification of in vivo *infection by real-time PCR and Akt Western blot*

Mice were injected intraperitonealy with 2.5 mg of LY294002 (Calbiochem) in DMSO. The control group was injected with the same volume of DMSO. Thirty minutes later, mice were infected by intravenous injection with *P. berghei* (5×10^4) sporozoites. Real-time PCR using primers specific for *P. berghei* 18S rRNA (5′-AAGCATTAAATAAAGCGAATACATCCTTAC-3′ and 5′-GGAG ATTGGTTTTGACGTTTATGTG-3′) was used for quantification of parasite load in the livers of mice 40 h after challenge, according to the method developed to *P. yoelii* infections (Bruna-Romero *et al.*, 2001).

Mice were injected or not (control) with LY294002 or DMSO 30 min prior to infection with *P. berghei* sporozoites. 3 h post infection, livers were extracted and SDS-PAGE gels of cell lysates were probed with polyclonal antiphospho-Akt (phospho-Ser-473) and total Akt antibodies (Cell Signalling). Detection was performed by using enhanced chemiluminescence (Amersham International).

Acknowledgements

We thank António Jacinto for critically reviewing the manuscript, Susana Constantino for a precious technical help and Virgílio E. do Rosário for all his support. Support was provided by Fundação para a Ciência e Tecnologia (Project POCTI/MGI/44517/2003) and National Institutes of Health (grants AI49432 and AI 53698). P. Leirião (SFRH/BD/3230/2000), S.S. Albuquerque (SFRH/BD/9635/2002) and M.M. Mota (SFRH/BPD/3510/2000) are supported by Fundação para a Ciência e Tecnologia.

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