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INMUNIDAD A LA MALARIA LETAL EN MODELOS MURINOS : ADQUISICIÓN ESPONTÁNEA O MEDIADA POR TRATAMIENTO QUIMIOTERAPÉUTICO.
IMMUNITY TO TETHAL MALARIA IN MURINE MODELS : NATURAL AND CHEMOTHERAPY-MEDIATED ACQUISITION

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
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Inmunidad a la malaria letal en modelos murinos: adquisición espontánea o mediada por tratamiento quimioterapéutico

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Immunity to lethal malaria in murine models: natural and chemotherapy-mediated acquisition

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DOCTORAL THESIS

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CERTIFICAN:

Que la tesis doctoral titulada: **"Inmunidad a la malaria letal en modelos murinos: adquisición espontánea o mediada por tratamiento quimioterapéutico"** que presenta Isabel González Azcárate, licenciada en Biología por la Universidad Complutense de Madrid, ha sido realizada bajo su dirección, en el Departamento de Bioquímica y Biología Molecular IV de la Facultad de Veterinaria de la Universidad Complutense de Madrid y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con **mención europea**.

De acuerdo con la normativa vigente, firmamos el presente certificado, autorizando su presentación como directores de la mencionada tesis doctoral.

En Madrid, a 25 Abril de 2013

D. José Manuel Bautista Santa Cruz Dña. Patricia Marín García

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Ac/Ab	Anticuerpo	Antibody
TCA/ ACT	Terapias combinadas basadas en artemisinina	Artemisinin-based combination therapy
Ag	Antígeno	Antigen
AMA-1	Antígeno de la membrana apical 1	Apical membrane antigen 1
ARNt/tRNA	ARN de transferencia	Transfer RNA
ARS	Aminoacil ARNt sintetasa	Aminoacyl-tRNA synthetases
ASC	Células secretoras de anticuerpo	Antibody-secreting cells
CCDA/ ADCC	Citotoxicidad celular dependiente de Acs	Antibody-Dependent Cell-Mediated Cytotoxicity
CGs	Centros germinales	Germinal center
Chloroquine-30	Cloroquina a dosis de 30 mg^{-1} kg^{-1} day^{-1}	chloroquine at 30 mg^{-1} kg^{-1} day^{-1} dose
CSP	Circunsporozoíto	Circunsporozoite
DCs/CDs	Células dendríticas	Dendrític cells
ED	(Ratones de) muerte prematura	Early deceased (mice)
ELISA	Ensayo inmunoenzimático	Enzyme-linked immunosorbent assay
FO	Folicular	Follicular
FSC-SSC	Distribución relativa al tamaño y granularidad	Forward scatter-side scatter gate
G6PD	Glucosa-6-fosfato deshidrogenasa	Glucose-6-phosphate dehydrogenase
h	horas	hours
HRP	Peroxidasa de rábano	Horseradish peroxidase
i.p.	Vía intraperitoneal	Intraperitoneal route
IC_{50}	Concentración inhibitoria 50	Inhibitory concentration 50
IFN-γ	Interferón gamma	Interferon gamma
Ig	inmunoglobulina	Immunoglobulin
IL	Interleuquina	Interleukin
iRBCs	Eritrocitos infectados	Infected red blood cells
iv	Vía intravenosa	Intravenous route
LD	(Ratones de) muerte tardía	Late deceased (mice)
MHC	Complejo principal de histocompatibildad	Major histocompatibility complex
MSP	Proteína de superficie del merozoíto	Merozoite surface protein

NKs	Células citotóxicas naturales	Natural killer
NKT	Células T citotóxicas naturales	Natural killer T cells
NO	Oxido nítrico	Nitric oxide
OMS/WHO	Organización Mundial de la Salud	World Health Organization
P.	*Plasmodium*	*Plasmodium*
PAGE	Electroforesis en gel de poliacrilamida	Polyacrylamide gel electrophoresis
PB	Sangre periférica	Peripheral blood
PCR	Reacción en cadena de la polimerasa	Polymerase chain reaction
PDR/RDT	Pruebas de diagnóstico rápido	Rapid diagnostic tests
pi	Postinfección	Post-infection
PyL	*Plasmodium yoelii yoelii* 17XL	*Plasmodium yoelii yoelii* 17XL
RBC	Eritrocito	Red blood cell
RT	Temperatura ambiental	Room temperature
S	Superviviente	Surviving
SDS	Dodecilsulfato sódico	Sodium dodecyl sulfate
SEM	Error estándar	Standard error
T1	Transitoria 1	Transitional 1
T2	Transitoria 2	Transitional 2
TBM	Tetrametilbenzidina	Tetramethyl benzidine
TCR	Receptor de antígeno de células T	T cell receptor
TGF	Factor de crecimiento transformante	Transforming growth factor
TIP/IPT	Tratamiento intermitente Preventivo	Intermittent Preventive treatment
TLR	Receptores de tipo Toll	Toll like receptor
TNF	Factor de necrosis tumoral	tumor necrosis factor
Treg	Célula T reguladora	T regulatory cell
γδT	Células T con TCR de tipo γδ	T cells expressing γδ TCR

1. SUMMARY

1.1. Introduction

Malaria is one of the most important global health problems and, together with tuberculosis and HIV/AIDS, is one of the three infectious diseases that has the highest impact worldwide in terms of morbidity, mortality and socioeconomic consequences (WHO 2013). In countries where malaria is endemic, about 216 million cases were estimated in 2010 causing nearly 655,000 deaths, mostly in children under 5 years old and pregnant women (WHO 2012). Five *Plasmodium* parasites infect humans: *P. vivax, P. malarie, P. ovale, P. falciparum* y *P. knowlesi* (Miller *et al.* 1994), but the vast majority of cases occur in Africa, where the bulk of the infections and deaths are caused by *P. falciparum* (WHO 2012).

The incomplete immune response to malaria, together with the lack of a licensed vaccine and a the spread of drug resistant parasites hinder malaria control and turn the malaria disease into a major public health problem (WHO 2012). There is a global strategy for accelerating the development of effective malaria vaccines, which define two main steps for vaccine improvement: a vaccine achieving 50% protection against severe disease and death by 2015, and a vaccine that could prevent 80% of the clinical malaria episodes by 2025 (MVI-PATH 2013). Of the multiple approaches that have been pursued, the RTS,S/AS01 vaccine candidate represents the most developed and clinically validated malaria vaccine formulation, which has an efficacy against clinical malaria measured at 30-50% in the field (Agnandji *et al.* 2011; Agnandji *et al.* 2012).

At present, artemisinin-based combination therapy (ACT) is recommended for the treatment of *P. falciparum* malaria. Artemisinin derivatives, which include dihydroartemisinin, artesunate and artemether, are combined with drugs such as lumefantrine, mefloquine, amodiaquine and sulfadoxine/ pyrimethamine. Unfortunately, confirmation of artemisinin-resistance in Cambodia and Thailand is giving cause for concern as resistance could either spread or emerge spontaneously elsewhere (WHO 2012).

Thus, new molecular targets are needed to treat malaria. Inhibitors of aminoacyl-tRNA synthetases (ARS), essential enzymes for cell viability, have been validated as antimalarial compounds (Schimmel *et al.* 1998; Hurdle *et al.* 2005; Istvan *et al.* 2011; Hoepfner *et al.* 2012). Mupirocin is an inhibitor of isoleucyl tRNA synthetase (Hughes and Mellows 1978) while borrelidin is an inhibitor of prokaryotic threonil tRNA synthetase (Hutter *et al.* 1966) and yeast cyclin-dependent kinase Cdc28/Cln2 (Tsuchiya *et al.* 2001) as well as an activator of eukaryotic caspase-3 and caspase-8 (Kawamura *et al.* 2003). Several pharmacological activities have been reported for borrelidin: antibiotic (Berger *et al.* 1949), angiogenesis inhibitor (Wakabayashi *et al.* 1997), anti-metastatic (Funahashi *et al.* 1999), antimitotic (Tsuchiya *et al.* 2001), antiviral (Dickinson *et al.* 1965), herbicidal and insecticidal (Dorgerloh *et al.* 1988) and antitumoral (Habibi *et al.* 2012). Both antibiotics, borrelidin and mupirocin, have

antimalarial activity against *P. falciparum in vitro* (Otoguro *et al.* 2003; Istvan *et al.* 2011), although the mupirocin produced a delayed-death on the parasite and the borrelidin promotes an immediate parasite growth arrest (Jackson *et al.* 2012). Borrelidin has also antimalarial *in vivo* activity against *P. berghei* and *P. yoelii* ssp. during the first 4 days post-infection of primary contact (Otoguro *et al.* 2003).

As long as there are not efficient antimalarial treatments that are affordable, accessible and appropriate for use in all malaria endemic areas, the immune response is often the most valuable line of defense against malaria. The mechanisms that lead to a given outcome in malaria patients are thought to be influenced by host factors as age, genetics, gender, prevalence of common RBC polymorphisms and helminth coinfection; and immune evasion strategies employed by the parasite which include a complex life cycle, an intracellular location of the parasite in humans or the high diversity of exposed antigens (Hisaeda *et al.* 2005; Schofield and Grau 2005; Akpogheneta *et al.* 2008; Doolan *et al.* 2009; Mackinnon and Marsh 2010).

Natural acquired immunity against *P. falciparum* can be progressively acquired only after years of repeated infection in adults, but generally not in pregnant women, infants or young children, and does not persist over long periods of time (Doolan *et al.* 2009). Information on human immune responses against malaria has been mainly provided from peripheral blood sampling since it is the only readily accessible source of cells of both the innate and acquired immune system. Effective immune responses against *P. falciparum* seem to require T CD8 cells during the hepatic stage of the parasite, and innate mechanisms followed by secretion of antibodies during blood malaria stages (Stevenson and Riley 2004).

Although erythrocytic stages induce potent innate immune responses, sporozoites, immature liver stages and gametocytes induce little, if any, inflammation (Liehl and Mota 2012). Thus, the innate immune response is the first barrier to blood-stage parasites, which caused all the clinical symptoms associated with malaria. The production of pro-inflammatory cytokines as tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ) are consider key actors in the initial control of the parasitemia (Urban *et al.* 2005; Good and Engwerda 2011), but they need to be limited by anti-inflammatory interleukin (IL) 10 or transforming growth factor β (TGF-β) to avoid tissue damage. Among the innate cells, macrophages have a very remarkable role against malaria parasites through mechanisms such as infected erythrocyte (iRBC) phagocytosis (Serghides *et al.* 2003), production of nitric oxide which inhibits parasite growth (Stevenson and Riley 2004), production of TNF-α (Bouharoun-Tayoun *et al.* 1995) or antigen presentation to T CD4 cells (Serghides *et al.* 2003). Natural killer cells (NKs) fight against malaria parasites through the production of IFN-γ and degranulation (Artavanis-Tsakonas and Riley 2002; Bottger *et al.* 2012) while dendritic cells (DCs) are the main antigen presenting cells to T CD4 cells (Wykes *et al.* 2007) and their cytokine production can modulate innate and adaptive responses (Coquerelle and Moser 2010). However, during malaria infection, DCs maturation and functioning can be impaired during blood-stage malaria depending on the *Plasmodium* strain, the severity of the

infection, and the patient population (Stevenson *et al.* 2011). The activation of T regulatory cells, a minority T CD4 subpopulation, has also a role during the innate response, but it seems to be correlated to enhanced blood-stage parasite growth that is facilitated by suppression of proinflammatory cytokine responses (Walther *et al.* 2005).

Animal malaria models have demonstrated that CD4 T cells are an essential part of protective immunity (von der Weid and Langhorne 1993; van der Heyde *et al.* 1996; Langhorne *et al.* 1998). CD4 T cells fight against the progression of blood-stage parasitemia by producing inflammatory cytokines which activate other cell types such as macrophages and helping B cell activation to produce antibodies (Abs) (Good and Engwerda 2011). Besides, protection after RTS,S/AS01 vaccination has been correlated with numbers of CD4 T cells producing either TNF or IL-2 (Olotu *et al.* 2011).

The key role of B cells in controlling malaria infections has been clearly revealed in rodent models lacking these cells, which are not able to eliminate *P. yoelii* (Weinbaum *et al.* 1976) and *P. chabaudi* infections (von der Weid *et al.* 1996). Moreover, the protective role of induced Abs against malaria infection is supported by the transfer of immune serum into infected non-immune humans as an efficient treatment strategy (Cohen *et al.* 1961); that also is efficient in mouse models (Jayawardena *et al.* 1978). Specific Abs protect by different means like blocking merozoite invasion, cooperating with monocytes, NKs or DCs to inhibiting intraerythrocyte parasite development, or by inhibiting cytoadherence of iRBCs (Perlmann and Troye-Blomberg 2002). RTS,S vaccine has generally induced good antibody responses, but there is a large overlap in antibody titer between protected and unprotected vaccinees (Kester *et al.* 2009), what is maybe responsible for the fast decline of protection. The immunity to human malaria is associated with the production of cytophilic immunoglobulins (IgG1, IgG3), that cooperate with cellular immune response (Aribot *et al.* 1996), and with the recognition of a diverse repertoire of antigens to eventually defeat most variants encountered in the wild (Kinyanjui *et al.* 2004). At the same time, malaria infection gives rise to strongly elevated blood concentrations of non-malaria-specific Abs whose implication in innate response is still unknown (Perlmann and Troye-Blomberg 2002).

Induction of antimalarial immunity by prophylactic interventions would eventually help to control malaria disease (Achtman *et al.* 2005; Friesen *et al.* 2010). Following this goal, the experimental inoculations of very small doses of intact sporozoites in volunteers alongside a chloroquine regime have shown to confer higher and longer levels of protection than vaccination with radiation-attenuated sporozoites (Hoffman *et al.* 2002; Roestenberg *et al.* 2009; Roestenberg *et al.* 2011). Similarly, inoculation of low doses of *P. falciparum*-iRBCs followed by early treatment with drugs confer protection against the erythrocytic stage of malaria by a strong cell-mediated immune response, in the absence of detectable parasite-specific antibodies (Pombo *et al.* 2002). These studies highlight the potential of combining the inoculation of parasites with antimalarial drug regimes to favor a native

exposure of antigens for the development of long-term protective immunity (Sauerwein *et al.* 2010; Borrmann and Matuschewski 2011).

To gain a better insight into protective immunity and pathological processes of malaria *in vivo*, various murine models have been developed with parasites isolated from African wild rodents (Li *et al.* 2001). These models are relatively simple to maintain and reproduce different features of the malaria infection and immunological responses upon infection. Moreover, the mouse immune system is well characterized and intervention studies of a nature that is not permissible in humans may be performed (Taylor-Robinson 1995). Most bioassays have used inbred mouse strains infected with parasites such as *P. berghei, P. vinckei*, *P. yoelii* and *P. chabaudi.* The combination of mouse and *Plasmodium* strains determines the virulence of the infection. For example, *P. yoelii yoelii* lines 17XL (*PyL*) and YM are consider to cause uniformly lethal infections, whereas infections with lines 17XNL and 265 are completely resolved after an initial parasitaemia (Li *et al.* 2001; Singh *et al.* 2002). Consequently, to date little evidence has been compiled on natural resistance to *PyL* parasites. Only two studies, in our knowledge, have explored circulating B and T cells response. In the first, 5% of BALB/c naïve mice resolved a *P. yoelii nigeriensis* infection but do not develop immune memory (Singh *et al.* 2000). In the second, a protective role of nitric oxide in *PyL*- infected DBA/2 mice, which are resistant to the infection was inferred (Wang *et al.* 2009). Besides, most rodent malaria studies have examined lymphoid organs, rather than circulating PB cells as in humans, because of the large quantity of cells available in these organs.

1.2. Objectives

The specific aims of this project were the following:

1. To examine the activity of the antibiotic borrelidin against *P. falciparum in vitro.*
2. To evaluate *in vivo* the effects of the borrelidin and mupirocin treatments against the *P. yoelii yoelii* 17XL infection in a standard malaria model in mice.
3. To study the humoral response developed upon borrelidin treatment of malaria-infected mice
4. To study the cellular and humoral immune response against a primary *P. yoelii yoelii* 17XL infection in the ICR mice strain.
5. To analyse the immune memory developed against this infection.

1.3. Results from experimental work 1

In vivo antimalarial activity of borrelidin and mupirocin against lethal *P. yoelii* 17XL infection

Drugs were administered in mice the first four days following a primary contact with *PyL* infection. Borrelidin (0.25 mg/kg/day) was the only antibiotic successful at curing lethal malaria infection in mice comparable to the positive control of chloroquine 30 mg kg^{-1} day^{-1} (chloroquine-30). In contrast, individuals treated with mupirocin (2.5 mg kg^{-1} day^{-1}) or low chloroquine dosage (1 mg kg^{-1} day^{-1}) were unable to cure from malaria. After treatments withdrawal, borrelidin-treated mice showed higher parasitemia values tan chloroquine-30 treated mice. Analysis of parasite stages distribution in the iRBCs from control mice and mupirocin-treated mice showed a high proportion of ring-stage parasites during the first 2 days of increasing parasitemia while mature forms predominated the last 2 days before death. The borrelidin-treated mice showed a significant dominance of trophozoite-stage parasites at day 3 post-infection (pi) which persisted as the most abundant form until day 10 pi.

Parasite stages distribution in borrelidin treated *P. falciparum* cultures

Cultures of *P. falciparum* strain Dd2 were exposed to borrelidin and chloroquine for 48 hours (h) and then cultured along 4 subsequent life cycles. The concentration used was 20-fold the corresponding IC_{50} values of borrelidin and chloroquine for *P. falciparum* Dd2. We observed a reduced parasite growth after 48 h treatment with borrelidin and chloroquine compared to control culture. These remaining parasites treated with borrelidin, with a high percentage of ring forms, struggled throughout the 4 following days to recover their viability, which was only partially regained after 240 h.

Long-term immunity and humoral response in cured mice

Borrelidin- and chloroquine-30-treated mice were re-infected on day 75 pi. All the borrelidin-treated animals showed long-term full protection against the second lethal infection, but one chloroquine-30 treated mouse, corresponding to the only one that during the primary infection did not show microscopic parasitaemia, was unable to control re-infection. Borrelidin and chloroquine-30 cured mice from this re-infection were challenged again on day 340 pi, and all of them showed full protection. The low specific IgG levels detected in sera from borrelidin-treated mice after the first infection underwent a significant increase after the second challenge, and these levels persisted after the third antigenic challenge. Similar results were obtained in chloroquine-30 group. In borrelidin-treated animals the antibody avidity, defined as the strength with which an antibody binds to an antigen, increased significantly after the second infection. From the second to third challenge, a small but significant decrease was detected. A similar trend was observed in chloroquine-30 group.

Immunoblot analysis of total *P. yoelii* proteins was performed using the sera from different infection time-points. The profile of immunodetected parasite proteins in cured mice after both borrelidin and chloroquine-30 treatments revealed a progressive increase in specific IgG levels. Moreover, IgG antibodies recognized an ever-wider range of parasite antigens as the number of re-infections increased. In contrast, a reduced variety of antigenic proteins were recognized with the serum from the chloroquine-30 treated mouse that died after secondary infection. The images obtained from immunofluorescence microscopy showed that specific IgGs from the sera of borrelidin and chloroquine-30 cured mice from day 85 pi preferentially bind to late parasite stages on blood smears of iRBCs.

Curative properties of borrelidin against lethal blood-stage malaria

To study the curative drug effects a 4 days drug-treatment of mice starting when mice showed around 10% of parasitemia (day 3 pi) was carried out. Borrelidin was slower at decreasing parasitemia rates than chloroquine and the infection caused the death of 25% of treated mice. All survivor mice successfully overcame a re-infection on day 135 pi.

1.4. Results from experimental work 2

Primary *P. yoelii 17XL* infection leads to three malaria infection profiles in ICR mice

The intraperitoneal (ip) infection with $2x10^7$ *PyL* in ICR outbred mice resulted into three different infection profiles according to their parasitemia and survival kinetics. A 20% of mice spontaneously resolved the infection and were designated as surviving mice (S). A 60% were described as early deceased mice (ED), showed rapid-onset fulminating parasitemia and died before day 8 pi. The rest of deceased mice designated as late deceased mice (LD) underwent a slow increase in parasitemia, similar to that of S mice, but followed fatal outcome around day 11 pi. The slope of parasitemia growth was significantly different between ED and LD mice. After their recovery, S mice were reinfected on days 60 and 420 pi and 100% of them survived both reinfections. Anemia was detected in all groups of animals. In both LD and S mice, infection induced an increase in leukocytes in blood.

White blood cell populations in blood of ICR mice during *PyL* infection

Blood leukocytes were analysed by flow cytometry along the infection in the three groups of ICR mice. ED mice showed the highest changes in innate cells, activated monocytes (Mac-3^+ MHC II^+) and DCs ($CD11c^+$ MHC II^+), which increased in blood at day 6 pi. In contrast they showed a reduction in the number and proportion of CD8 T cells and unchanged CD4 T cell levels. S mice $CD4^+$ and $CD8^+$ T cells percentages decreased from day 9 pi onwards, but total numbers were elevated at the end infection. LD

mice showed a similar trend to that observed in S mice. Activated leukocytes ($CD44^+$), as well as activated T $CD4^+$ and $CD8^+$ populations, markedly augmented in S mice at the end of infection. The frequencies and numbers of $CD4^+$ $CD25^+$ cells, with a documented suppressor activity, were early increased in mice with fatal malaria while in S mice, only an increase in cell numbers was detected at the end of the 1st infection.

The maturation of B cells was explored through the expression of surface IgM and IgD. All subtypes of B cells except transitional 2 (T2) (IgM^{hi} IgD^{hi}) cells were detected in all mice and B-1b cells (IgM^{hi} IgD^{low} $CD11b^+$ $CD5^-$) were detected at very low percentages. Transitional 1 (T1) B cell numbers increase was highest in mice with the worse prognosis while those of isotype-switched B cell (IgM^- IgD^-) were highest in S mice at the end of infection. Mature cells (IgM^{low} IgD^{int}) presented minor changes.

Antigen (Ag) presenting B cells ($B220^+$ MHC II^+) significant decreased in S mice from day 14 pi. We could distinguish two populations of $B220^+$ MHC II^+ cells according to the B220 levels: $B220^{high}$ MHC II^+ (B^{high}) and $B220^{low}$ MHC II^+ (B^{low}). The proportion of B^{high}/ B^{low} cells in healthy mice was around 9:1 among total activated B cells. However, malaria infection promoted a rise in B^{low} cells and drop in B^{high} cells in all mice from day 3 or 6 pi. The expression of IgM and IgD in $B220^{low}$ and $B220^{high}$ cells in PB revealed that $B220^{high}$ were mainly mature cells (IgM^{low} IgD^+ $CD5^-$) whereas $B220^{low}$ were ~50% B-1 and T1 B cells (IgD^- IgM^+ $CD5^-$) and ~50% IgD^- IgM^- $CD5^-$ cells.

We compared serum cytokine profiles during the 1st wk of infection in S and ED mice by protein microarrays. At 3 days pi, S mice secreted higher levels of hematopoietic IL-3, the Th2 cytokine IL-4, and the Th1 cytokines IFN-γ and IL-2 than ED mice. Conversely at 7 days pi, ED mice showed higher levels of most of the markers.

Humoral response in ICR during *PyL* infections

While serum IgM levels peaked in the first infection, IgG Ab production started to increase after the 2nd wk of infection and peaked after the 2nd challenge in S animals. Among the serum *PyL*-specific IgG isotypes, IgG2b were most abundant in S and LD mice in the 1st infection. The 2nd challenge promoted a rapid expansion in IgG2b, IgG2a and IgG1 and the appearance of IgG3 Abs, which were always the least abundant subclass during all infections. Third *PyL* challenge, induced again the Ab production. IgG antibodies (Abs) recognized a wide range of parasite Ag detected by immunoblot. Finally, a 40% of BALB/c mice were protected from *PyL* infection after passive transfer of serum from S mice obtained on the 2nd infection.

1.5. Results from experimental work 3

B cell subpopulations changed in the spleen and peritoneal cavity across different infection fates

ICR mice were inoculated with $2x10^7$ *PyL*-iRBCs and classified by days 3 and 6 pi as low or high parasitemic depending on their detectable blood parasitemia, of 7 or 60% respectively. Mice with low parasitemia at day 6 pi were allowed to recover from infection and survivors were examined at day 500 pi. Splenocytes only increased significantly in high parasitized animals, but absolute B cell number increased in all infected mice. Survival animals also showed a significant increase in their spleen cellularity and B cell number 500 days after the infection. Although only switched B cells (IgM^- IgD^-) increased frequency in the spleen of mice with high parasitaemia at day 3 and 6 pi, absolute number of all B cell subsets, T1 (IgM^{hi} IgD^{low}), T2 (IgM^{hi} IgD^{hi}) and mature cells (IgM^{low} IgD^{int}), were significantly expanded at day 3 and 6 pi in these animals. Low parasitized mice showed significant increase in mature and switched cell numbers. Mice which survived the infection showed an augmented number of mature B cells in the spleen at day 500 pi.

Analysis of follicular (FO) cells ($CD21^{low}$ $CD23^+$) revealed a frequency decrease at day 6 pi, but an increase in absolute numbers in all infected mice from day 3 pi. Marginal zone (MZ) B cells number ($CD21^+$ $CD23^-$) only increased in mice suffering from high parasitemia. FO B cells numbers maintained enlarged levels after 500 days of the infection.

Frequencies and numbers of B-1 B cell in spleen (IgM^+ $CD23^-$ $CD43^+$) (Berland and Wortis 2002; Baumgarth 2010)showed an increase in the frequency on day 6 pi and in cell numbers in both days 3 and 6 pi in high parasitemic animals. Surviving mice showed the increased numbers of B-1 cells by day 500 pi. On the other hand PerC cellularity increased in mice with high parasitemia at day 3 pi. The infection did not interfered in the B-1:B-2 cells proportion, but mice with high parasitemia showed a significant increase in the absolute numbers of both B-2 ($B220^+$ IgM^- IgD^+ $CD5^-$) and B-1 (IgM^+ IgD^-) cells.

***PyL*-specific memory B cells remained in the spleen 500 days after a single infection**

PyL-specific MBCs were investigated by determining *PyL* specific IgG in the supernatant from cultures of stimulated MBCs. Splenocytes from mice which had cleared a primary infection on day 20 pi showed the presence of MBCs at day 500 pi.

1.6. Conclusions

The results obtained in the present doctoral thesis show that the ICR outbreed mouse strain is a valuable animal model to examine the development of different profiles of experimental malaria infections. The development of this new *in vivo* animal model for malaria research allows to study and compare the immunological response associated to different clinical outcomes. The surveillance of cell changes in peripheral blood during the infection shows that white cell populations are differently modulated in relation to the severity of the infection and outcome. Inthe first days of the experimental infection, a rapid increase in circulating $CD4^+CD25^+$ cells and immature B cells followed by a dramatic rise in activated innate cells and a small increase in class-switched B cells are characteristic features of the worst prognosis group. On same days, the mice surviving the infection show a controlled production of cytokines and mostly unchanged circulating innate cell kinetics. Splenomegaly, and switched, mature and B-1 B cells proportions in the spleen were differently modulated by the parasitemia profiles in ICR mice. Cells from peritoneal cavity were only increased ar day 3 pi in high parasitemic mice. From the second week of infection, an increase in circulating activated T cells and class-switched B cells together with the generation of a long-term protective humoral response stand out in the blood immune response in surviving mice. Moreover, the maintenance of memory B cells long time after a primary infection in surviving ICR mice demonstrates that one infection is enough to create a memory response.

On the other hand, our results provide new insights into the potential use of borrelidin as antimalarial drug and contribute to validate threonyl-tRNA synthetase as a target for prophylaxis or therapy against malaria. In addition we show that a low borrelidin dose regime has parasite-stage specific growth inhibition and leads to develop a robust long-term protective immune response in 100% of treated animals.

2. INTRODUCCIÓN

2.1. El problema de la malaria humana

La malaria, término que se acuño a partir de la expresión "mal aire" porque el padecimiento se asociaba con frecuencia a personas que vivían cerca de zonas pantanosas o malolientes, o también conocida como paludismo (del latín *palus, -ūdis*, "laguna") es la enfermedad parasitaria que produce una mayor mortalidad, morbilidad, e impacto socioeconómico en la población humana (WHO 2012). La Organización Mundial de la Salud (OMS) estima que en 2010 hubo 219 millones de casos de malaria que causaron aproximadamente 660.000 muertes, principalmente de niños menores de 5 años, en el África subsahariana (WHO 2012), aunque un análisis reciente indica que el número de fallecimientos excedieron de los 1.2 millones en 2010 (Umbers *et al.* 2011; Murray *et al.* 2012). Alrededor de un 80% de los casos de malaria ocurrieron en 17 países y aproximadamente el 90% de muertes en África subsahariana (WHO 2013) (Figura 1).

Actualmente, la malaria es endémica en 79 países localizados mayoritariamente en las regiones tropicales y subtropicales del planeta (WHO 2012). Estas zonas son visitadas por más de 125 millones de viajeros cada año, de los cuales se estima que enferman de malaria hasta unos 30.000 (Capdevila and Icart 2010). En áreas de baja tasa de transmisión de malaria casi todas las personas expuestas tienen un riesgo sustancial de enfermar; en cambio, en áreas endémicas el riesgo se ve restringido a turistas, niños y mujeres embarazadas. Aunque hay excepciones en pacientes con mucha anemia, y pueda no cumplirse en aquellos donde haya muchos parásitos secuestrados en el tejido, se ha propuesto la densidad de parásitos en sangre como marcador de morbilidad y mortalidad de la malaria (Doolan *et al.* 2009).

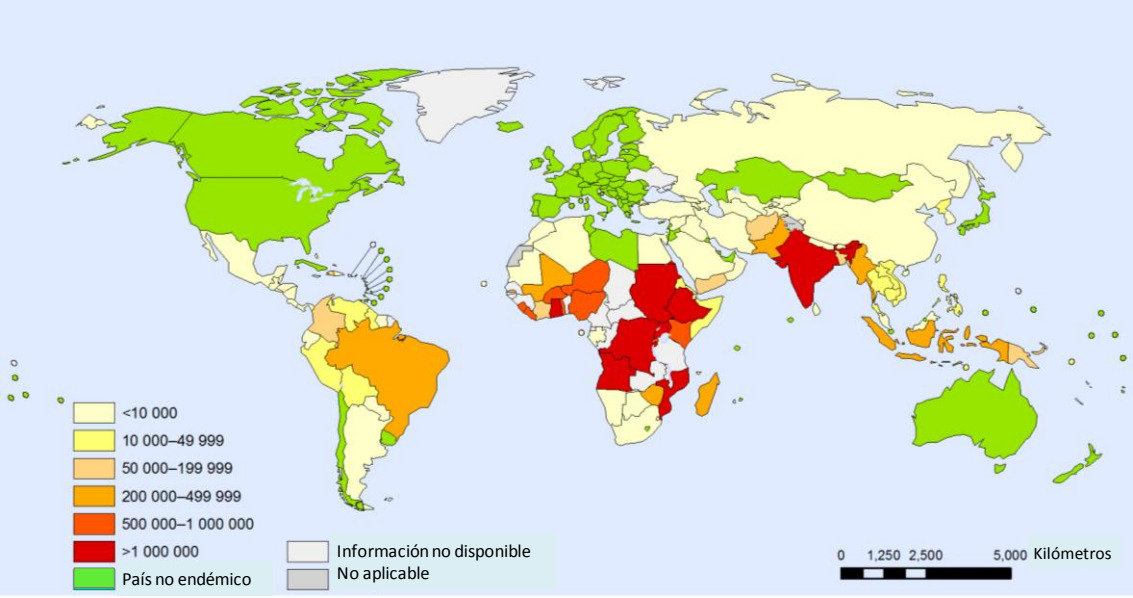

Figura 1. Casos confirmados de malaria en el mundo en el año 2010 (WHO 2012).

En mamíferos, reptiles y pájaros, el agente etiológico causante de esta enfermedad es un parásito protozoario del género *Plasmodium,* identificado por primera vez por Alphonse Laveran (Laveran 1880), que se transmite mediante la picadura de mosquitos hembra del género *Anopheles* (orden *Diptera*, familia *Culicidae*) previamente infectados, tal y como descubrieron Ronald Ross (Ross 1899) y Giovanni Batista Grassi (Grassi *et al.* 1899) para la malaria aviar y humana respectivamente. Ocasionalmente, también puede transmitirse por transfusión sanguínea, trasplante de órganos o congénitamente de la madre al feto (Menendez and Mayor 2007; Enweronu-Laryea *et al.* 2013). Estos parásitos eucariotas pertenecen al filo *Apicomplexa* y se caracterizan porque las formas que invaden las células hospedadoras presentan el denominado complejo apical para poder penetrar en ellas (Gállego Berenguer 2007).

De las más de 100 especies conocidas del género Plasmodium, solamente cinco son capaces de producir la enfermedad en humanos: *P. vivax, P. malarie, P. ovale, P. falciparum* y *P. knowlesi* (Miller *et al.* 1994). Esta última, usualmente atribuida a la infección de macacos, se ha descubierto recientemente en el sudeste asiático como especie patógena para humanos y puede ser transmitida por *Anopheles leucosphyrus* (Singh *et al.* 2004; Collins 2012). Dentro de cada especie de parásito coexisten, además, diferentes cepas o clones que son el resultado de polimorfismos alélicos en ciertos *loci* de proteínas que dan lugar a distintas formas antigénicas de una misma proteína (Kemp *et al.* 1990; Kyes *et al.* 2001).

P. falciparum es la especie más letal y la responsable de alrededor del 80% de los casos de malaria en el África subsahariana (Lim *et al.* 2005). *P. vivax,* responsable de 80 a 300 millones de casos clínicos estimados de malaria anuales (Mueller *et al.* 2009), es la especie más común en Oriente Medio, Asia y el oeste del Pacífico, mientras que en África sólo supone el 10% de los casos. *P. malariae* se encuentra distribuido mayoritariamente en África tropical, Sri Lanka, Nueva Guinea, y en regiones del sur de América (Collins and Jeffery 2007), mientras que *P. ovale* se distribuye en el África subsahariana y las islas del Pacífico oeste (Collins and Jeffery 2005). Finalmente, *P. knowlesi* se localiza en zonas boscosas de Asia sudoriental y es, hasta la fecha, el parásito que afecta menos frecuentemente al hombre (WHO 2013).

La malaria ha sido una enfermedad endémica en todos los continentes hasta principios del siglo XX; no se erradicó de Norteamérica, Europa y Australia hasta 1959, con el uso de insecticidas. En España fue erradicada oficialmente en el año 1964 (Astasio 2002). Esta patología es responsable tanto de un sufrimiento humano incalculable como de un elevado coste sanitario y económico, lo cual contribuye a incrementar el ciclo de pobreza-enfermedad en el que se encuentran muchos de los países afectados (Sachs and Malaney 2002).

Tabla 1. Puntos esenciales sobre la malaria. Resumen de la información contenida en el *Informe mundial sobre el paludismo* 2011 y 2012 (WHO 2011; WHO 2012).

Datos globales

- Hubo 660.000 millones de muertes por malaria y entre 219 millones de casos en el año 2011.
- El 81% de los casos de malaria se produjeron en la región africana y el 13% correspondía al sudeste asiático en el año 2010.
- El 91% de los casos de malaria se deben a *P. falciparum* en el año 2010.
- El 91% de las muertes por malaria se registraron en África subsahariana en el año 2010.
- Globalmente, alrededor del 86% de muertes de dio en niños.
- En 2011 hubo al menos 104 países del mundo endémicos en malaria, 45 de los cuales están en el continente africano.

Control vectorial, diagnóstico y tratamiento

- El porcentaje de hogares que contaron con al menos una mosquitera impregnada de insecticida creció del 3% en 2000 al 53% en 2011 en el África subsahariana. El 96% de las personas que cuenta con una mosquitera la utiliza habitualmente.
- Un 5% de la población global en riesgo y un 11% de la población en el África subsahariana en riesgo estuvo protegida por fumigación intradomiciliaria en 2011.
- Se registró resistencia a los piretroides en 27 países del África subsahariana en el año 2010.
- En 2011, 84 países y territorios adoptaron las terapias combinadas basadas en artemisinina (TCA) recomendadas por la OMS como tratamiento para el paludismo por *P. falciparum*; pero en 25 países se seguía permitiendo la comercialización de monoterapias orales a base de artemisinina, rechazadas por la OMS.
- El porcentaje de mujeres embarazadas que recibieron dos dosis de tratamiento preventivo intermitente (TPI) durante el embarazo osciló entre el 4% y el 68% en África.
- Ningún país ha adoptado aún el TPI para los bebés como medida sanitaria de ámbito nacional desde que se recomendó en 2009.
- El número de pruebas de diagnóstico rápido (PDR) suministradas por los fabricantes pasó de 45 millones en 2008 a 88 millones en 2010.
- Se han descubierto indicios de resistencia a las artemisininas en cuatro países de la subregión del Gran Mekong: Camboya, Myanmar, Tailandia y Vietnam.

Datos económicos

- La malaria endémica provoca una reducción del 1,3% de la tasa de crecimiento económico de un país a corto plazo y una reducción de más del 50% del producto nacional bruto a largo plazo.
- Los precios actuales de las PDR y las TCA (por ej. arteméter-lumefantrina) son de alrededor de 0,50 dólares y 1,40 dólares respectivamente.
- En programas de gran cobertura el coste por persona protegida por fumigación intradomiciliaria es de 2,62 dólares y por redes mosquiteras tratadas es solo de 1,39 dólares.
- La financiación internacional para el control del paludismo en los países endémicos ha incrementado desde menos de 100 millones de dólares en 2000 hasta 1.700 millones en el año 2010.
- En 2009, los programas contra el paludismo representaron aproximadamente el 8% de la ayuda oficial al desarrollo para la salud y la población.

Impacto y tendencias en el control del paludismo

- Entre el año 2000 y 2010, el número de casos confirmados de paludismo disminuyó en más del 50% en 35 de los 53 países con transmisión continua.
- Los índices de mortalidad por paludismo han disminuido en un 25% entre el año 2000 y 2010.
- En 2013 la financiación internacional destinada al control del paludismo será como máximo de 2.000 millones de dólares, pero se cree que disminuirá a 1.500 millones de dólares en 2015.

El mosquito determina las características de la transmisión. La transmisión de la malaria depende tanto del momento del día, los *Anopheles* pican por la noche, como del medio ambiente. Estos vectores necesitan zonas acuáticas durante sus fases de huevo, larva y pupa, por lo que la transmisión de la infección está también relacionada con el clima y la geografía. Los brotes de la infección habitualmente coinciden con las estaciones lluviosas de regiones con clima tropical, ya que las temperaturas superiores a 16ºC facilitan la rápida multiplicación del parásito y del vector (Grover-Kopec *et al.* 2006).

2.2. El ciclo de vida del *Plasmodium*

El ciclo de vida del parásito *Plasmodium* es muy complejo y similar en todas las especies. Consta de dos fases bien diferenciadas: una fase sexual (esporogonia) que tiene lugar en la hembra del mosquito *Anopheles*, que es el vector y hospedador definitivo, y una fase asexual que se desarrolla en un animal vertebrado, el hospedador intermediario, e incluye una etapa exoeritrocítica (abarca la esquizogonia exoeritrocítica en el hígado) y otra intraeritrocítica (esquizogonia intraeritrocítica) (Frederich *et al.* 2002) (Figura 2).

En mamíferos, el ciclo comienza con la fase exoeritrocítica cuando es infectado a través de la picadura de una hembra del mosquito *Anopheles*, que porta el *Plasmodium* en el estado de esporozoíto (células haploides del protozoo) en las glándulas salivares y lo inocula en la dermis y en el torrente sanguíneo (Boyd and Kitchen 1939) (Ponnudurai *et al.* 1991). Aunque puede variar, se estima que el número de esporozoítos depositados es generalmente menor de 100 (Rosenberg *et al.* 1990; Ponnudurai *et al.* 1991; Vanderberg and Frevert 2004). Los esporozoítos inoculados pueden permanecer en el tejido dermal, donde incluso una pequeña porción puede replicarse y sobrevivir durante semanas (Gueirard *et al.* 2010), o bien entrar en el torrente sanguíneo y migrar al interior de las células parenquimales del hígado en aproximadamente 2 minutos (Shin *et al.* 1982; Sidjanski and Vanderberg 1997) o entrar en los vasos linfáticos y dirigirse hacia los ganglios regionales (Amino *et al.* 2006). Algunos estudios han mostrado que esporozoítos de diversas especies de *Plasmodium* son capaces de atravesar células epiteliales y fibroblastos (Mota *et al.* 2001); incluso se ha descrito la infección y migración de los parásitos en el interior de diversos leucocitos como células dendríticas (CDs) (Wykes *et al.* 2011) o macrófagos (Vanderberg *et al.* 1990; Landau *et al.* 1999).

Una vez en el hígado, cada esporozoíto se aloja en un hepatocito donde forma una vacuola parasitófora, convirtiéndose así en un parásito intracelular (Mota *et al.* 2002; Ishino *et al.* 2004). Comienza entonces una multiplicación asexual en el hígado, llamada esquizogonia exoeritrocítica, que permite la maduración del esporozoíto hasta la formación de un esquizonte hepático (5-15 días postinfección, según la especie) que contiene miles de merozoítos (10.000 para *P. falciparum* y hasta 30.000 para *P. vivax*) (Warrell 2002). En el caso de *P. vivax* y *P. ovale* algunos esporozoítos pueden pasar

por una fase de latencia (hipnozoíto) que permite una permanencia durante meses en el hígado antes de iniciar la división asexual (Krotoski *et al.* 1982; Frevert and Nardin 2005). El hepatocito infectado se rompe y los merosomas, paquetes de cientos de merozoítos rodeados por la membrana del hospedador, se dirigen a los capilares pulmonares, donde en un periodo de 48-72 horas liberan los merozoítos que contienen en su interior.

Los merozoítos invaden finalmente los glóbulos rojos, en cuyo interior se desarrolla el ciclo asexual del parásito (fase de esquizogonia intraeritrocítica). Esta fase intraeritrocítica es la causante de la patología clínica asociada a la malaria (Schofield and Grau 2005). La invasión del eritrocito comienza cuando el merozoíto interacciona con su membrana provocando la fusión entre ambas células y, en aproximadamente 30 segundos (Gilson and Crabb 2009), el merozoíto penetra en el glóbulo rojo, abandona su capa de protección y desarrolla una vacuola parasitófora a partir de la membrana eritrocitaria en la cual el parásito evoluciona en distintos estadios (Fujioka and Aikawa 2002; Eksi and Williamson 2011). La forma más joven recibe el nombre de anillo que crece para convertirse en trofozoíto (Warrell 2002; Cowman and Crabb 2006). Este trofozoíto es metabólicamente muy activo y madura mediante la ingestión del citoplasma del eritrocito produciendo la desaparición paulatina de las vacuolas y la aparición del pigmento malárico hemozoína, que es el resultado de la degradación proteolítica de la hemoglobina. Además, comienza a dividir su cromatina de manera asexuada, dando lugar a una forma más madura denominada esquizonte que posee entre 16 y 32 merozoítos, según la especie de *Plasmodium* (Winzeler 2006). Tras 48 horas desde la invasión del eritrocito por *P. falciparum, P. ovale* o *P. vivax*, y 72 horas (h) por *P. malariae*, se liberan a la sangre nuevos merozoítos, junto con los productos metabólicos del parásito, que son los responsables de las crisis de fiebre cíclicas típicas de la malaria.

Aunque la mayoría de los merozoítos en el vertebrado comenzarán un nuevo ciclo intraeritrocítico, algunos se diferencian en formas sexuales inmaduras llamadas gametocitos en el interior del eritrocito (Frederich *et al.* 2002). Cuando una hembra del mosquito *Anopheles* pica a un individuo infectado por *Plasmodium* ingiriere la sangre parasitada con los gametocitos y éstos comienzan la reproducción sexual (o esporogonia) (Matuschewski 2006). El gametocito femenino abandona el eritrocito y se transforma en un macrogameto sin sufrir ninguna división, mientras que el microgameto masculino sufre tres divisiones mitóticas y libera microgametos flagelados, que se mueven a través de la sangre ingerida en busca de un macrogameto. El desarrollo de estos gametos se ve favorecido debido a una disminución de la temperatura y a la presencia de ácido xanturénico en el estómago del mosquito (Matuschewski 2006). La fecundación de un gameto femenino por el masculino origina el cigoto, la única forma diploide del ciclo parasitario. Transcurridos 12-24 h, el cigoto se alarga, y se convierte en una forma móvil denominada ooquineto (Matuschewski 2006). El ooquineto atraviesa el intestino del mosquito y se enquista en la pared exterior, formando un ooquiste que crece y, mediante división celular por meiosis, forma esporozoítos (Vernick and Waters 2004; Garcia *et al.* 2006). Entre 7 y

15 días postinfección, dependiendo de la especie de *Plasmodium* y de la temperatura ambiental, un solo ooquiste habrá formado más de 10.000 esporozoítos que poseen un complejo apical (Frederich *et al.* 2002). El ciclo se cierra cuando finaliza la maduración del ooquiste y libera los esporozoítos que alcanzan, a través del hemocele, las glándulas salivales desde donde serán inoculadas a un nuevo hospedador vertebrado (Matuschewski 2006).

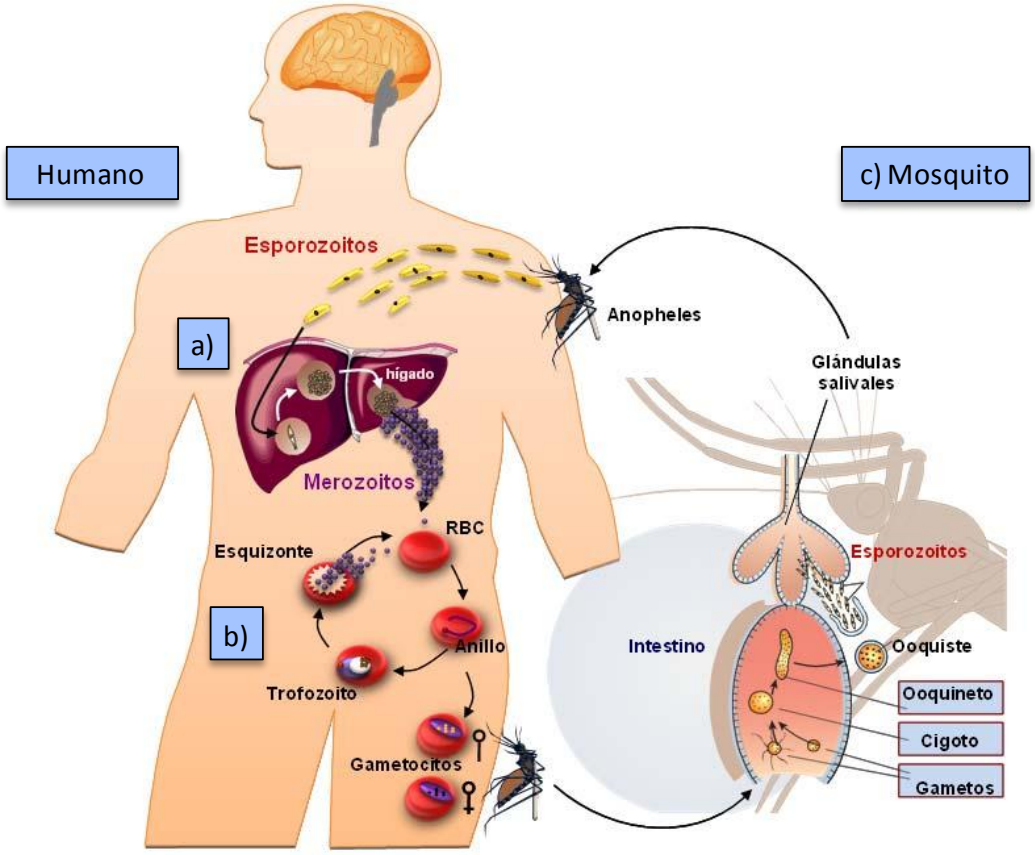

Figura 2. **Ciclo de vida de *Plasmodium* spp. en el humano.** a) Ciclo exoeritrocítico: Un mosquito anofelino inocula los esporozoítos en el humano, y son arrastrados por la sangre al hígado donde invaden los hepatocitos y se desarrollan a merozoítos. b) Ciclo intraeritrocítico: Los merozoítos liberados al torrente sanguíneo invaden nuevos glóbulos rojos formándose un anillo que se transforma en trofozoíto. Posteriormente madura a esquizonte, cuya ruptura libera de nuevo merozoítos al torrente sanguíneo. Algunos merozoítos que invaden los eritrocitos pueden madurar a gametocitos que pueden ser ingeridos de nuevo por otro mosquito. c) Ciclo esporogónico: Los gametocitos maduran a macrogametos y microgametos flagelados que, tras la fecundación, producen un ooquineto móvil que atraviesa la pared gástrica para formar un ooquiste que liberará miles de esporozoítos infectivos (Cowman and Crabb 2006).

2.3. Sintomatología clínica de la malaria humana

La malaria tiene un amplio abanico de manifestaciones clínicas dependiendo tanto del hospedador como de la cepa de *Plasmodium*, pero generalmente se define como una enfermedad febril con un periodo de incubación de al menos 7 días (WHO 2013).

Los síntomas de la enfermedad aparecen durante la fase intraeritrocítica del parásito. Los cuadros clínicos más graves de malaria están causados por *P. falciparum* y pueden incluir fiebre, escalofríos, calambres musculares, debilidad, vómitos, diarrea, dolor abdominal y anemia; además, si la enfermedad se agrava puede generar delirio, acidosis metabólica, anemia severa, malaria cerebral y afección de múltiples órganos, seguido finalmente por el coma y la muerte (Miller *et al.* 2002; Schofield and Grau 2005). En áreas endémicas, la forma más grave de *P. falciparum* es la malaria cerebral que es la causante de alrededor del 80% de las muertes acaecidas por esta patología infecciosa (Lou *et al.* 2001; Armah *et al.* 2007). La malaria causada por el resto de especies de *Plasmodium* causan gran morbilidad, pero muy rara vez provocan casos graves.

Los niños menores de 5 años, las mujeres embarazadas y los viajeros provenientes de zonas no endémicas son los más susceptibles de presentar las formas graves de malaria y sufrir sus complicaciones. A menudo, los síntomas iniciales de una malaria no complicada no son fáciles de reconocer debido a que nos son patognomónicos, lo que provoca una peligrosa desatención de la enfermedad, además de un incorrecto tratamiento y seguimiento de la misma.

2.4. Estrategias de control de la malaria

El problema de la malaria se ha acentuado en los últimos años por la creciente resistencia tanto de cepas *Plasmodium* a los fármacos convencionalmente utilizados en su tratamiento, como de los mosquitos vectores a los insecticidas (Greenwood *et al.* 2008). Las políticas de control antipalúdicas están dirigidas a reducir de la morbilidad y mortalidad, minimizar la transmisión de la enfermedad mediante la reducción del reservorio de parásitos en el humano y prevenir la dispersión de resistencia a los fármacos antimaláricos. Es por ello que, entre las estrategias que actualmente se manejan para controlar y/o erradicar el paludismo, se encuentran el desarrollo de nuevos fármacos antimaláricos, el control vectorial y, quizá la más destacable, la búsqueda de una vacuna eficaz que permita una inmunización a largo plazo.

2.4.1. Control vectorial

El control vectorial es la forma principal de controlar la transmisión a nivel de comunidad, y constituye la primera línea de defensa contra la infección individualmente. Actualmente, los programas de salud pública optan por la distribución a gran escala de mosquiteras impregnadas de insecticidas de larga duración. Otra opción es la fumigación de interiores con insecticidas de acción residual, mayoritariamente piretroides, cuyo objetivo es reducir la supervivencia de los vectores que penetran en las casas o dormitorios. Sin embargo, desafortunadamente en los últimos años se ha observado una creciente resistencia de los mosquitos a estos insecticidas (WHO 2012) (Figura 3).

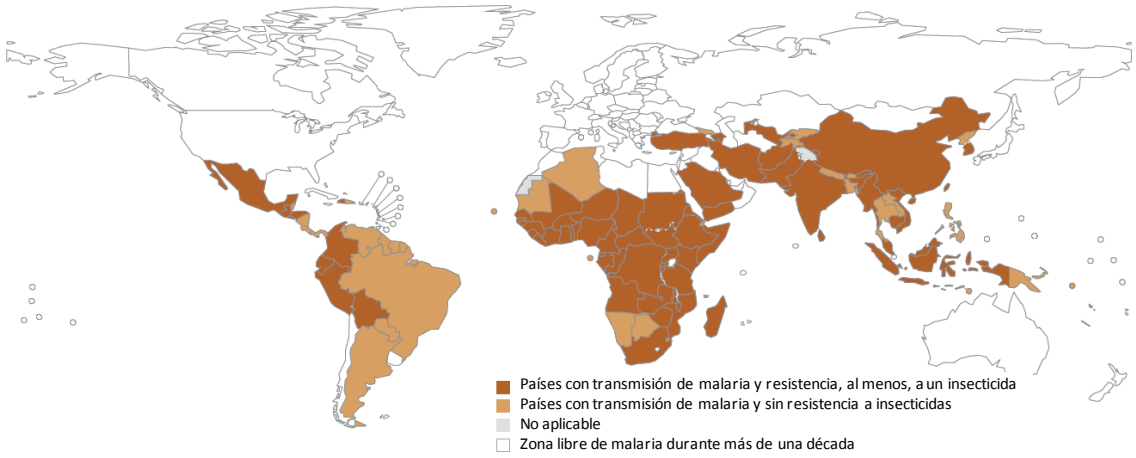

Figura 3. **Países con transmisión de malaria donde se ha identificado resistencia a insecticidas en al menos uno de sus mayores vectores** (WHO 2012).

2.4.2. Tratamientos antipalúdicos recomendados por la OMS

La OMS recomienda que antes de comenzar un tratamiento contra malaria, la presencia del parásito haya sido diagnosticada de forma diferencial a través de técnicas microscópicas, test de diagnóstico rápido o reacción en cadena de la polimerasa (PCR) si fuera posible, para distinguirla de otras enfermedades febriles. De esta manera se podrían reducir los efectos adversos del tratamiento, las interacciones con otros fármacos y la aparición de nuevas resistencias; además mejoraría la calidad de los datos sobre la eficacia del tratamiento y el seguimiento, detección e informe de casos de malaria (WHO 2010).

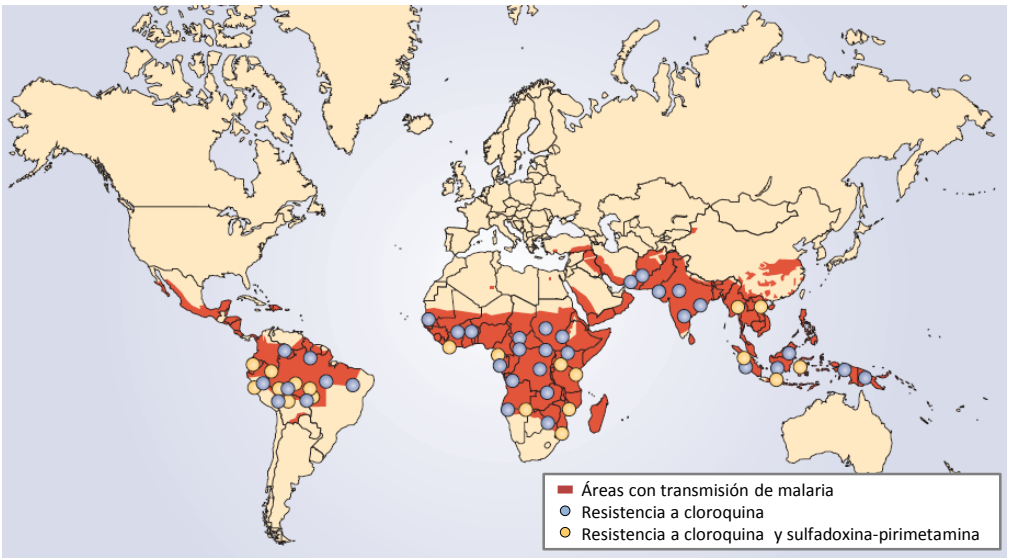

Figura 4. Distribución de malaria y áreas con resistencia a los fármacos cloroquina y sulfadoxina-pirimetamina (WHO 2001).

Desde los años 60, los fármacos antimaláricos en uso han ido sucumbiendo a la aparición de resistencia por las especies *P. falciparum*, *P. malariae* y *P. vivax* (Figura 4). Desafortunadamente, *P. falciparum* ya ha mostrado resistencia a todas las drogas antimaláricas utilizadas en su tratamiento: amodiaquina, cloroquina, mefloquina, quinina, sulfadoxina-pirimetamina y, más recientemente, a derivados de artemisina (WHO 2010) (Figura 5).

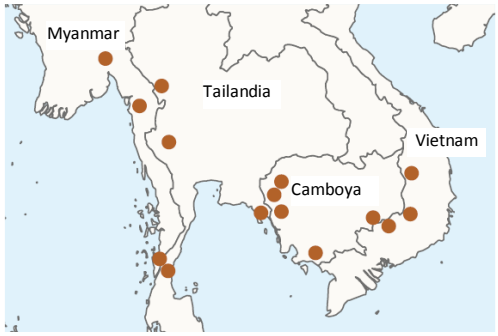

Figura 5. Lugares con resistencia confirmada o sospechada a la artemisinina, detectados en estudios sobre eficacia terapéutica. Años 2007-2012 (WHO 2012). Desde 1970 la frontera entre Camboya y Tailandia ha sido el epicentro de la emergencia de resistencias a los fármacos antimaláricos (WHO 2007).

En la actualidad la OMS recomienda que la infección no complicada por *P. falciparum,* tanto de adultos como de niños, sea tratada con TCAs. Como primera elección de tratamiento actualmente, y dependiendo de las resistencias existentes en cada territorio, está la administración de artemeter-lumefantrina, artesunato-amodiaquina, artesunato-mefloquina y artesunato-sulfadoxina-pirimetamina (Tabla 2). Como segunda línea de tratamiento, se puede utilizar la combinación de artesunato o quinina con tetraciclina, doxiciclina o clindamicina (Tabla 2).

Las terapias de grupos de alto riesgo, como son las mujeres embarazadas, han de seguir directrices diferentes. En el primer trimestre de embarazo la malaria ha de tratarse con quinina-clindamicina, y como última opción TCAs, que son las prioritarias en el segundo y tercer trimestre. En el tiempo de lactancia se deben evitar la dapsona, primaquina y tetraciclinas. Una intervención adicional recomendada para mujeres embarazadas en áreas de elevada tasa de transmisión de *P. falciparum* es el TIP, que implica la administración de al menos dos dosis de sulfadoxina-pirimetamina durante el segundo y tercer trimestre de embarazo (Tabla 2). Recientemente, dicha estrategia se ha ampliado en las recomendaciones de la OMS a bebés en áreas de riesgo (WHO 2011).

Por otra parte, los viajeros que regresan con infecciones de *P. falciparum* no complicadas a zonas no endémicas deben ser tratados con atovacuona-proguanil; artemeter-lumefantrina o quinina combinada con doxiciclina o clindamicina (WHO 2010)(Tabla 2).

Las inyecciones de artesunato, o secundariamente artemeter o quinina, se reservan para los casos de malaria severa en adultos y niños, seguidas por una TCA oral tan pronto como sea posible o quinina-clindamicina o doxiciclina (WHO 2010).

La malaria no complicada causada por *P. vivax* debe ser asistida prioritariamente con cloroquina en áreas en las que no se haya descrito resistencia a ella, o en este último caso con la apropiada TCA. Además, en aquellos pacientes que regresan a zonas no endémicas es adecuado el tratamiento con primaquina para eliminar los posibles hipnozoítos del hígado (Tabla 2), siempre que el paciente no sea deficiente en glucosa-6-fosfato deshidrogenasa (G6PD) (WHO 2010).

Los tratamientos profilácticos, aunque no confieren una protección completa, sí reducen el riesgo de una infección letal (WHO 2010). Todos los fármacos antimaláricos tienen contraindicaciones específicas y existen pocos estudios sobre los efectos secundarios asociados a la profilaxis de más de 6 meses, por lo que de momento sólo se recomienda a viajeros de estancia de corta duración. Los viajeros que enferman de malaria por *P. falciparum* no suponen un caso anecdótico y los afectados son mayoritariamente inmigrantes que viajan para visitar su lugar de origen, seguidos de turistas. El fármaco que conviene utilizar depende del área, pero, a grande rasgos, en las áreas con parásitos aún sensibles a cloroquina éste será el fármaco de elección, y en las áreas de resistencia la OMS recomienda la atovacuona-proguanil, la doxiciclina y/o la mefloquina (Tabla 2).

2.5. Identificación de moléculas con potencial actividad antimalárica

La aparición de resistencia a la artemisinina, compuesto clave para el actual tratamiento antipalúdico en áreas endémicas, hace imprescindible la búsqueda de terapias farmacológicas alternativas frente a la malaria. Sin embargo, el desarrollo de nuevas moléculas con actividad antimalárica para su uso en humanos es complejo y puede prolongarse durante 13-15 años (Figura 6).

El grupo de Gamo *et al*. de la farmacéutica GlaxoSmithKline's (Gamo *et al.* 2010) y el grupo de Guiguemde *et al*. del hospital *St. Jude Children's Research Hospital* (Guiguemde *et al.* 2010) han dado un primer paso al investigar, respectivamente, la actividad de alrededor de 13.000 y 1.100 compuestos que inhiben en un 80% el crecimiento de *P. falciparum* a una concentración de 2-7 µM y que han incluido en la base de datos del Instituto de Bioinformática Europeo ChEMBL (ChEMBL 2013) para el uso de toda la comunidad científica. Dichos estudios han conducido al descubrimiento de nuevas estructuras químicas que actúan en el parásito y, por tanto, pueden ser la base de nuevos fármacos antimaláricos.

Tabla 2. **Fármacos antimaláricos principales y su uso** (Fidock *et al.* 2004; Fidock 2010; Delves *et al.* 2012).

Nombre	Clase química	Uso clínico en malaria	Estadio	Localización de su diana	Mecanismo
Artemisininas (artemeter, artesunato, dihidroartemisinina)	Lactona sesquiterpeno con puente endoperóxido	Terapias combinadas basadas en artemisinas (TCAs)	E	Citosol/ Vacuola digestiva	Desconocido
Lumefantrina	Arilamino-alcohol	Combinación con artemer en África	E, M	Desconocido	¿Interacción con el grupo hemo, síntesis de ácidos nucleídos y proteinas? (Ehrhardt and Meyer 2009)
Amodiaquina	4-Aminoquinolina	Combinación con artesunato en África	E, G	Vacuola digestiva	Digestión de la hemoglobina (Jullien *et al.* 2010)
Mefloquina	4-Metanolquinolina	Combinación con artesunato en África; uso como profiláctico	E	Desconocido (Jeffress and Fields 2005)	Desconocido (Jeffress and Fields 2005)
Quinina/quinidina	4-Metanolquinolina	Tratamiento de malaria severa, a menudo con antibióticos	E	Membrana del parásito	Síntesis fosfolipídica, transporte de membrana
Atovacuona	Naftoquinona	Combinación con proguanil como profiláctico	E, M	Mitocondria	Transporte de electrones
Cloroquina	4-Aminoquinolina	Tratamiento de *P. vivax;* tratamiento de malaria no	E	Vacuola digestiva	Digestión de la hemoglobina
Pirimetamina	Diaminopirimidina	Tratamiento preventivo intermitente, combinado con sulfadoxina	E, M	Citosol	Metabolismo del folato
Sulfadoxina	Sulfonamida	Tratamiento preventivo intermitente, combinado con pirimetamina	E	Citosol	Metabolismo del folato
Primaquina	8-Aminoquinolina	Eliminación de estadios hepáticos incluidos los hipnozoítos de *P. vivax y P. ovale*	H	¿Mitocondria? (Wells *et al.* 2010)	¿Transporte de electrones? (Wells *et al.* 2010)
Doxiciclina	Tetraciclina (Tan *et al.* 2011)	Combinación con artesunato en África; uso como profiláctico	E (Tan *et al.* 2011)	Apicoplasto	¿Síntesis proteica? (Tan *et al.* 2011)
Proguanil	Biguanida	Combinación con atovacuona como profiláctico	E	Citosol	Metabolismo del folato

E, fases en el eritrocito; H, hipnozoíto ; G, gametocitos; M, fases en el mosquito; ¿?, no bien definido

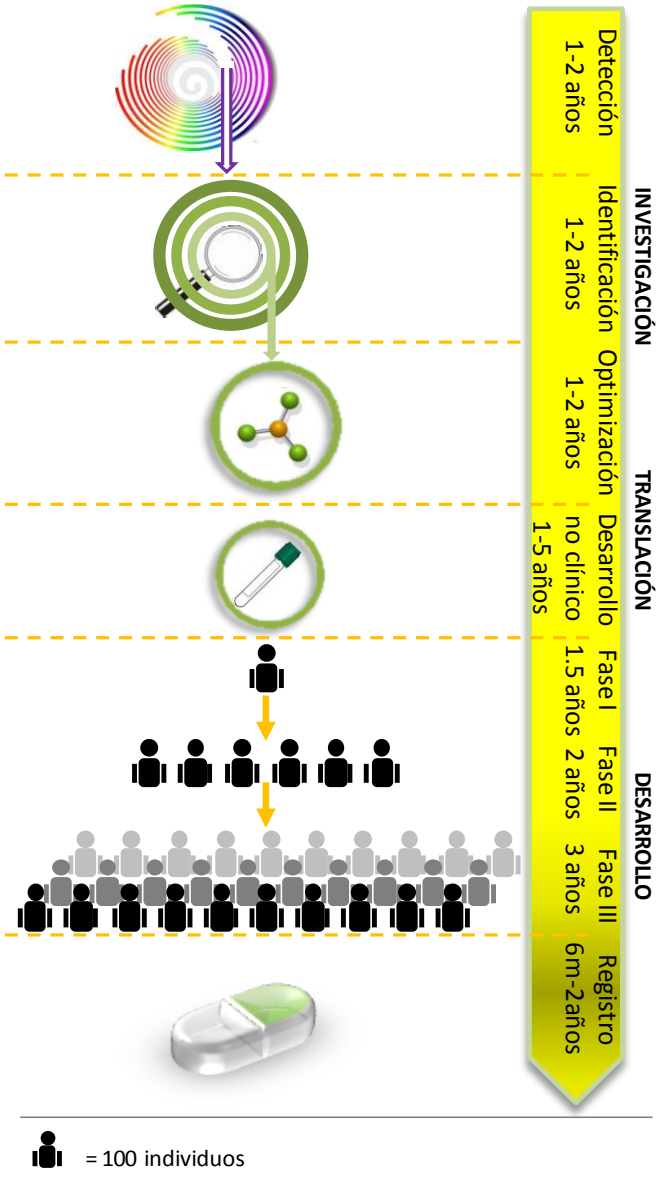

Figura 6. Proceso de desarrollo de medicamentos antimaláricos. En primer lugar se detectan los compuestos capaces de matar al parásito, se analiza su actividad *in vitro* e *in vivo* en el laboratorio y entonces se optimizan las propiedades del compuesto. Seguidamente, se analiza la seguridad del compuesto en el laboratorio, y se puede iniciar entonces el estudio clínico que comprende tres fases: en la fase I, en un pequeño grupo de voluntarios sanos, se revisa la seguridad, la tolerabilidad y la farmacocinética; en la fase II el fármaco es administrado a un número relativamente reducido de pacientes con la enfermedad y se evalúa la eficacia clínica; la fase III determina la eficacia clínica en un número mayor de pacientes. Si los resultados son satisfactorios se solicita el registro del nuevo fármaco a una autoridad reguladora (MMV 2013).

Con el fin de acelerar y facilitar el largo proceso de desarrollo de nuevos fármacos, se han desarrollado muchas iniciativas, entre las que destaca especialmente la formada por *Medicines for Malaria Venture* (MMV) en la que colaboran más de 290 entidades públicas y privadas de alrededor de 50 países. Su objetivo concreto es liberar nuevas medicinas que sean eficaces, accesibles, asequibles y apropiadas para su uso en zonas endémicas. Los nuevos compuestos se valoran considerando nuevas perspectivas: eficacia contra cepas de *P. falciparum* que tengan resistencia a los fármacos actuales, actividad potencial para tratamientos intermitentes (en niños y embarazadas), seguridad en su

administración a niños menores de 6 meses de edad y mujeres embarazadas, eficacia contra *P. vivax*, contra malaria severa o contra la transmisión del *Plasmodium* (MMV 2013).

Como se observa en la figura 7, la mayoría de los medicamentos aprobados en fase IV en la actualidad son terapias combinadas con derivados de artemisinina (Eurartesim, Pyramax, ASAQ, Coartem), aunque también existen monoterapias con artesunato que valoran aspectos como la vía de administración de fármacos ya existentes, y el SP+AQ que contiene sulfadoxina-pirimetamina y amodiaquina.

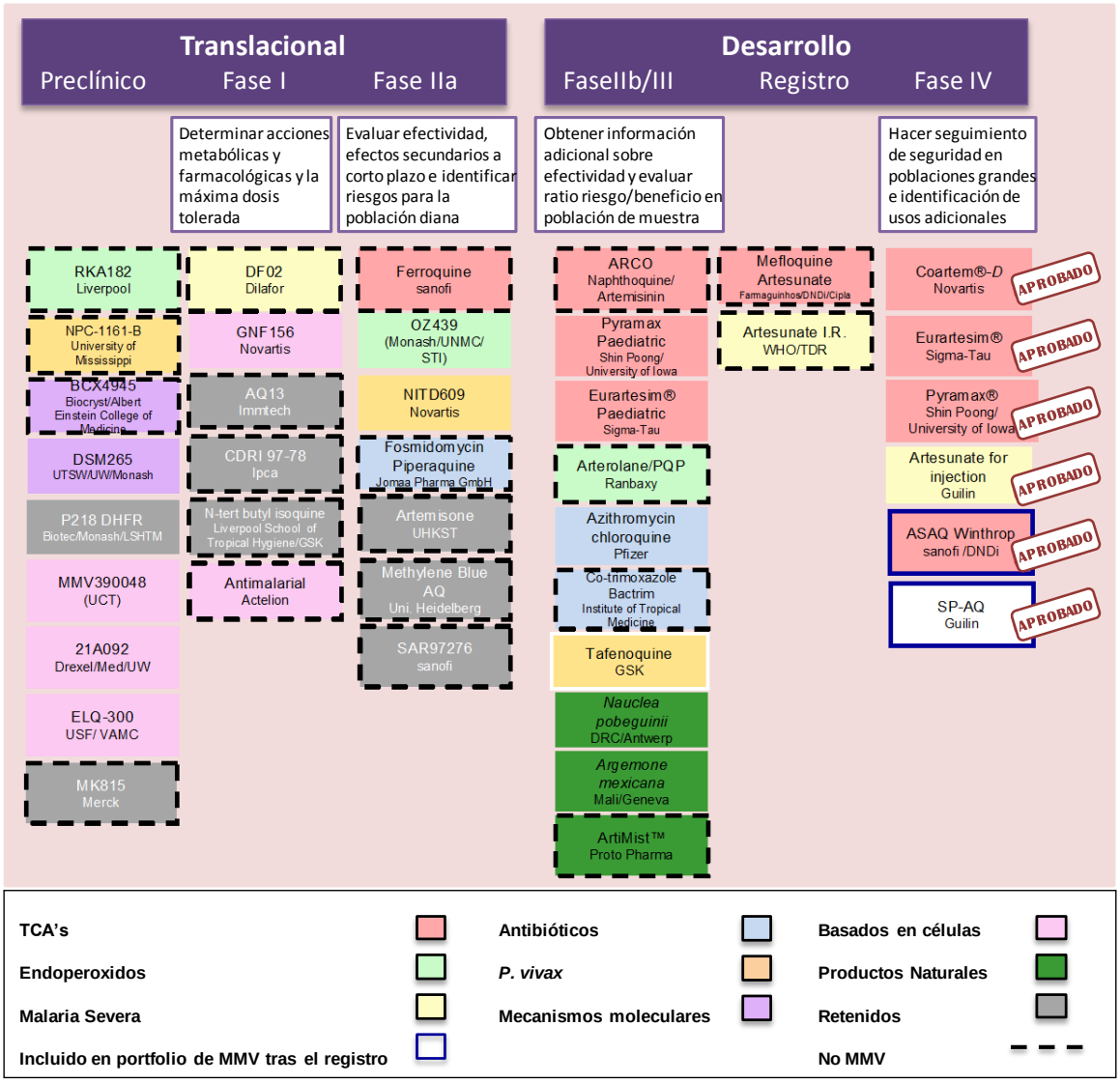

Figure 7. Esquema global de medicinas antimaláricas bajo desarrollo organizado por fase de desarrollo (a marzo del 2013). Incluye todos los proyectos en seguridad preclínica y estudios fármacocinéticos regulados formalmente. En la segunda fila se describen los objetivos de cada fase de desarrollo. Los estudios realizados con la colaboración de MMV se muestran en bloques sin bordes, los que no han implicado MMV figuran con líneas discontinuas y en color morado ("retenidos") los compuestos que no han publicado progresos en su desarrollo durante los últimos 12 meses (MMV 2013).

Además de la búsqueda de fármacos que actúan sobre dianas conocidas, son muchas las posibilidades de encontrar nuevas dianas moleculares para el desarrollo de agentes antipalúdicos. La supervivencia del parásito en el entorno del hospedador humano requiere diversas adaptaciones del mismo que, a su vez lo hacen susceptible a la acción farmacológica (Ridley 2002). Los agentes antimaláricos disponibles en la actualidad bloquean el desarrollo del *Plasmodium* al afectar al correcto funcionamiento de diferentes compartimentos subcelulares (Figura 8; Tabla 2). Las nuevas dianas farmacológicas en estudio también están íntimamente relacionadas con las funciones biológicas de las distintas organelas del parásito. De particular interés son: la vacuola digestiva lisosomal, donde tiene lugar la proteólisis de la hemoglobina y la detoxificación del grupo hemo; el apicoplasto, cuya funcion exacta es aún desconocida, pero se relaciona con rutas metabólicas (Lim and McFadden 2010) y la mitocondria, que presenta un sistema de transporte de electrones (Ridley 2002). A nivel citosólico también existen numerosas enzimas cuya actividad es susceptible de ser reguladas farmacológicamente (Greenwood *et al.* 2008). Más de la mitad de los compuestos incluidos en el Instituto de Bioinformática Europeo ChEMBL se dirigen contra enzimas del parásito (ChEMBL 2013). De igual manera, los procesos relacionados con la invasión del parásito y la liberación de esporozoítos se consideran actualmente como buenas opciones para la búsqueda de nuevas dianas farmacológicas (Blackman 2000; Ridley 2002).

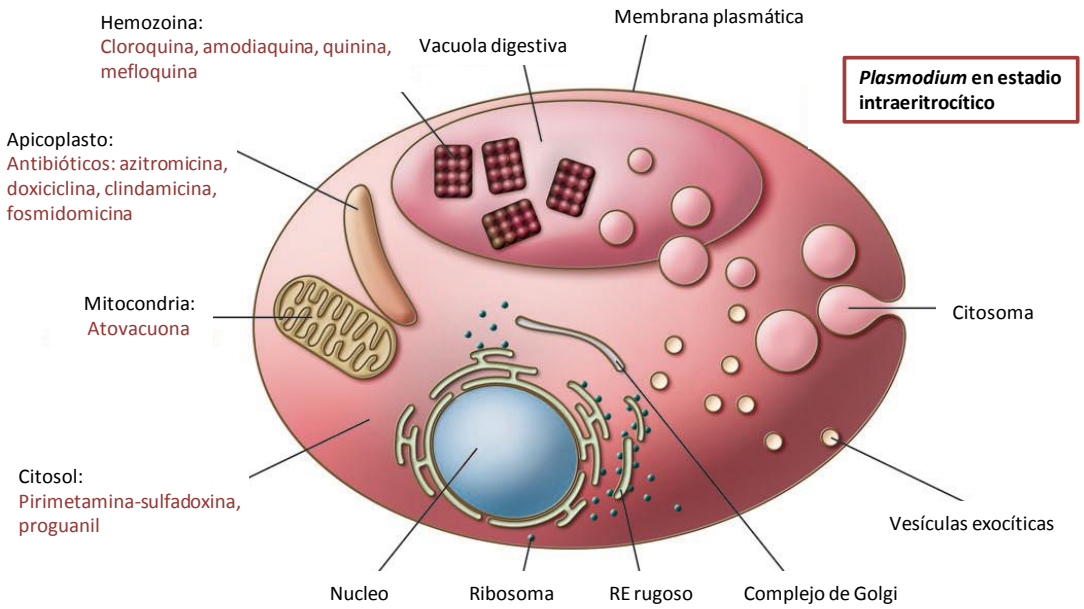

Figura 8. Representación de un trofozoíto de P. falciparum destacando los compartimentos intracelulares clave para la acción de algunos de los fármacos antimaláricos mas utilizados. Los fármacos antimaláricos logran sus efectos al interrumpir las funciones de distintos orgánulos subcelulares. La cloroquina, amodiaquina, quinina, mefloquina se concentran en la vacuola digestiva donde se piensa que se unen a la -hematin e interfieren en la detoxificación del grupo hemo. Los antibióticos como azitromicina, doxiciclina, clindamicina, fosmidomicina actúan dentro del apicoplasto donde inhiben la translación de proteínas que implica la muerte lenta de los parásitos tratados. La atovacuona inhibe el transporte de electrones en la mitocondria, mientras que la pirimetamina, sufadoxina y proguanil actúan sobre enzimas del citosol. Hay diversos fármacos, entre los que se incluyen la artemisinina y sus derivados, cuyas dianas y lugares de acción permanecen bajo investigación (Fidock *et al.* 2004; Greenwood *et al.* 2008).

La mayoría de fármacos antimaláricos actuales no han sido desarrollados conociendo su diana molecular con antelación, sino a través de la identificación de productos naturales con actividad antimalárica (por ejemplo, quinina y artemisinina), derivados químicos de éstos (cloroquina y artesunato) o compuestos activos contra otro tipo de infecciones (como antifolatos y tetraciclinas) (Fidock *et al.* 2004). Las dianas que tienen estructuras diferentes en el parásito y el humano tienen la ventaja de ser relativamente selectivas para el parásito, aunque si son compartidas por ambos, es más probable que dicha diana sea más conocida y la investigación pueda beneficiarse de otros proyectos dirigidos a su estudio. En otros casos, el parásito puede presentar dianas similares a otros microorganismos para los que ya se hayan descubierto inhibidores (Fidock *et al.* 2004).

En éste último caso podemos englobar a las ARNt (ARN de transferencia) sintetasas del parásito como dianas de tratamientos. Algunos estudios han demostrado que las ARNt sintetasas, localizadas en el citosol, mitocondrias y apicoplasto, son enzimas esenciales para la viabilidad celular y potenciales dianas aceptadas para el desarrollo de fármacos antimaláricos (Schimmel *et al.* 1998; Hurdle *et al.* 2005; Istvan *et al.* 2011). Se encargan de conjugar a cada ARNt con su aminoácido afín para que éste pueda incorporarse a una cadena proteica en crecimiento. En la última década, los inhibidores de estas enzimas se han considerado agentes antimicrobianos (Hurdle *et al.* 2005), pero también antimaláricos, debido a que estas enzimas se expresan en los tres estadios intraeritrocitícos, anillo, trofozoíto y esquizonte, aunque su máxima expresión ocurre en el último (Jackson *et al.* 2012).

La borrelidina es un antibiótico procedente de la bacteria *Streptomyces* spp. con capacidad inhibidora de treonil-ARNt sintetasas (Hutter *et al.* 1966), mientras que la mupirocina inhibe isoleucil ARNt sintetasas (Hughes and Mellows 1978) y es un producto natural de *Pseudomonas fluorescens* (Figura 9). Ambas han mostrado su actividad antimalárica contra *P. falciparum in vitro* a concentraciones bajísimas, en el rango nanomolar (Otoguro *et al.* 2003; Ishiyama *et al.* 2011; Istvan *et al.* 2011). La borrelidina presenta además actividad antimalárica *in vivo* contra *P. berghei* y *P. yoelii* al ser administrada subcutáneamente y oralmente a ratones (Otoguro *et al.* 2003).

La borrelidina inhibe la maquinaria de biosíntesis proteica, tanto a nivel citoplasmático como del apicoplasto, del *Plasmodium* capaz de detener el crecimiento del parásito de forma inmediata, principalmente en fase de trofozoíto (Ishiyama *et al.* 2011). Sin embargo, la mupirocina actúa inhibiendo la maquinaria de traducción del apicoplasto produciendo un efecto de "muerte lenta" en la que los parásitos que se detecta en subsecuentes ciclos de replicación (Istvan *et al.* 2011; Jackson *et al.* 2012).

Además de su actividad antimalárica, la borrelidina presenta actividades farmacológicas diversas: antibiótica (Berger *et al.* 1949), inhibidora de angiogénesis (Wakabayashi *et al.* 1997), anti-metastásica (Funahashi *et al.* 1999), antimitótica (Tsuchiya *et al.* 2001), antiviral (Dickinson *et al.* 1965), herbicida e insecticida (Dorgerloh *et al.* 1988) y antitumoral (Habibi *et al.* 2011; Habibi *et al.* 2012).

Asimismo, se ha descrito que actúa como inhibidor de quinasas dependientes de ciclina en levaduras Cdc28/Cln2 (Tsuchiya *et al.* 2001) y activador de caspasas 3 y 8 eucariotas (Kawamura *et al.* 2003).

Sin embargo, la toxicidad que presenta la borrelidina en células hepáticas imposibilita su potencial traslación para uso human (Vino and Lokesh 2008).

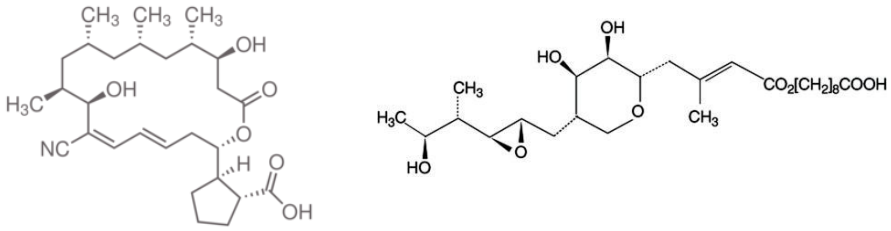

Figura 9. Estructura química de la borrelidina y mupirocina respectivamente. La borrelidina es un macrólido que se caracteriza por tener un anillo macrocíclico de lactona, y la mupirocina es el ácido pseudomónico A.

2.5.1. Vacuna

El desarrollo de una vacuna eficaz frente a la malaria supone aún un importante reto científico. El complejo ciclo biológico del parásito, así como su alta variabilidad antigénica, le proporcionan unos mecanismos de evasión inmunitaria que dificultan la producción de una vacuna (Engwerda *et al.* 2005).

Las distintas fases del ciclo del *Plasmodium* ofrecen diversas aproximaciones para la producción de vacunas con diferentes efectos y distintos tipos de protección. Las vacunas dirija contra estadios exoeritrocíticos serían capaces de inducir inmunidad estéril. Éstas pueden bien ser específicas de esporozoítos o de la fase hepática del parásito, aunque hay algunos antígenos (Ags) que se solapan entre ambos. Las vacunas dirigidas contra la fase intraeritrocítica del parásito reducirían los síntomas de la infección y la morbilidad y mortalidad en población de áreas endémicas. Por otro lado, las vacunas que bloquean la transmisión de la malaria matarían a los gametocitos que se forman en la sangre del humano o prevendrían la fertilización o el desarrollo del parásito en el mosquito. De esta forma no se protegería al individuo vacunado sino a la comunidad que vive en torno a él, por lo que las vacunas de bloqueo de la transmisión serían sólo para la población en zona endémica de malaria (Komisar 2007) (Figura 10).

La *Malaria Vaccine Techonology Roadmap,* emprendida en 2006 gracias a la financiación de la Fundación Bill y Melinda Gates, es una estrategia global que tiene dos propósitos: conseguir el desarrollo de una vacuna que produzca al menos un 50% de protección contra los casos de enfermedad grave para el 2015, y otra que prevenga un 80% de los casos clínicos para el 2025 (MVI-PATH 2013).

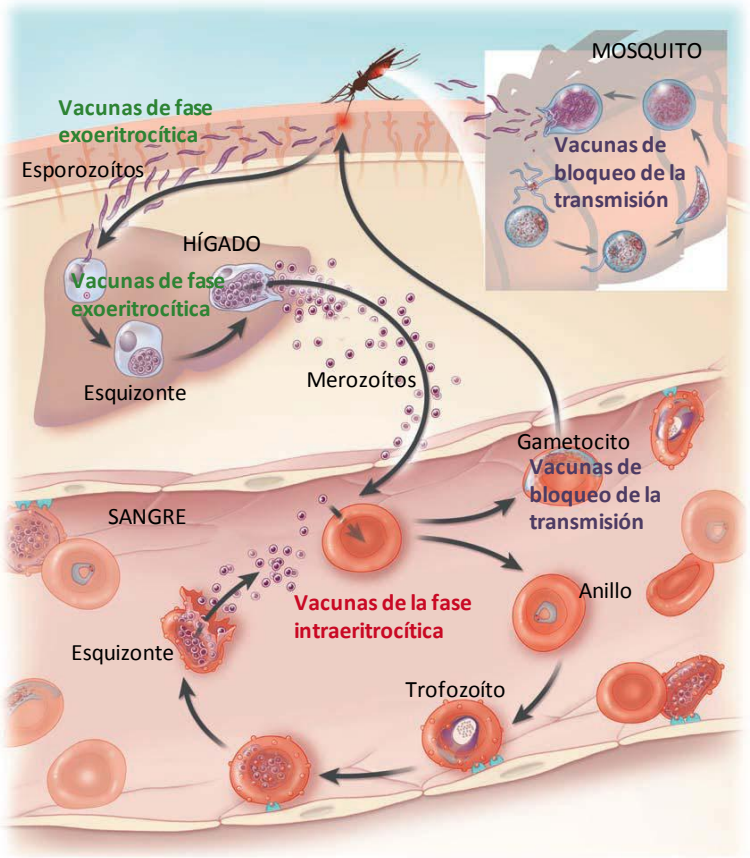

Figura 10. Interrupción del ciclo del *Plasmodium* por vacunas, adaptado de (Rosenthal 2008).

Actualmente más de 60 vacunas candidatas en desarrollo (Figura 11) (MVI-PATH 2013), siendo la vacuna GSK RTS,S AS01/AS02, desarrollada por GlaxoSmithKline, la única que ha llegado a la fase clínica III. Esta vacuna exoeritrocítica, está formulada con una porción de la proteína de superficie del circunsporozoíto (CSP) de *P. falciparum* unida a un fragmento del Ag de superficie del virus de la hepatitis B que utiliza el sistema adyuvante AS02A o AS01 y cuya co-inoculación con ADN del parásito favorece la respuesta inmune celular y humoral (Garcon *et al.* 2003; Bojang *et al.* 2005; Ansong *et al.* 2011). En 2011, los resultados del estudio clínico de fase III de la RTS,S en niños de entre 5 y 17 meses de edad mostraron que tres dosis reducen un 56% el riesgo de contraer malaria y un 47% en el caso de la malaria grave, aunque dicha protección duraba solo unos poco meses (Agnandji *et al.* 2011). Resultados posteriores revelaron además que, la vacuna aplicada a bebés de 6 a 12 semanas de edad tiene una eficacia moderada, al reducir el riesgo de sufrir malaria en un 31% (Agnandji *et al.* 2012). Idealmente, una vacuna exoeritrocítica podría prevenir la enfermedad y también la transmisión, pero necesitaría inducir grandes cantidades de Ac de gran afinidad para atrapar con efectividad al esporozoíto en la piel (Riley and Stewart 2013).

Tras el fracaso de RTS,S y otras vacunas en las fases I y II, muchos investigadores abogan por el retorno a una vacuna del organismo vivo, completo y atenuado (Hoffman *et al.* 2010; Vaughan *et al.* 2010; Matuschewski *et al.* 2011; Lindner *et al.* 2012), pero la posibilidad de que pueda revertir a un

fenotipo virulento o recombinar con formas salvajes para formar nuevos genotipos, así como la dificultad para ser almacenados complica su desarrollo (Hoffman *et al.* 2010).

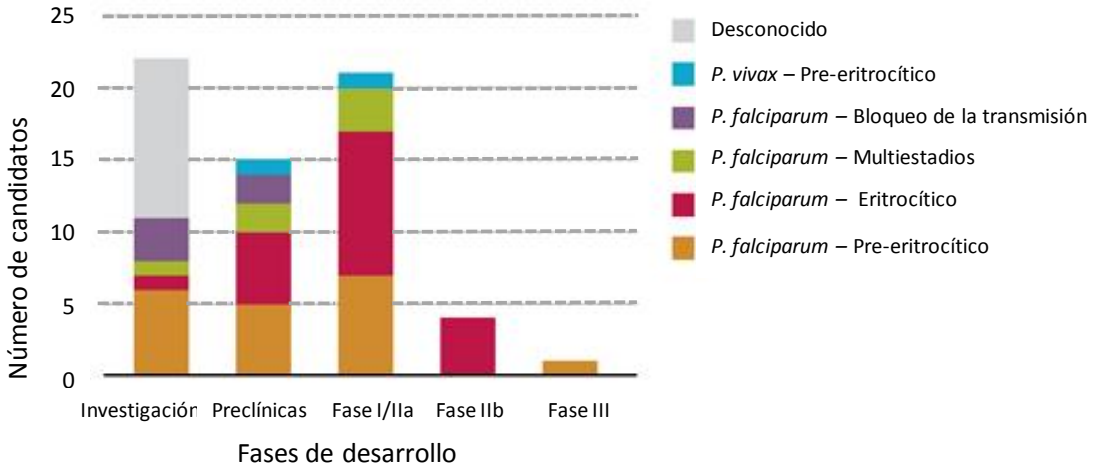

Figura 11. Vacunas en desarrollo en 2010 (MVI-PATH 2013).

Hasta ahora ninguna vacuna ha demostrado protección frente a reinfecciones por parásitos heterólogos. El fracaso de los distintos candidatos se deriva probablemente del uso de Ags demasiado polimórficos y expresados solamente en periodos breves del ciclo del parásito (Richie and Saul 2002). Además, las vacunas candidatas representan menos del 0.5% del genoma completo (Doolan *et al.* 2008), y más del 50% de las vacunas diseñadas en la actualidad se basan solamente 3 Ags: CSP, proteína de superficie del merozoíto (MSP) y Ag de la membrana apical 1 (AMA-1), utilizados de forma independiente. Cabe destacar que se ha descubierto un receptor expresado universalmente en las células rojas que sirve como conector de los merozoítos, el PfRh5, y puede comprender una magnífica diana para el desarrollo de vacunas eritrocíticas (Crosnier *et al.* 2011). En la comunidad científica existe el consenso de que la protección debería conseguirse por la combinación de distintas subunidades de varias fases del desarrollo del parásito (fase pre-eritrocítica, asexual y sexual) (Patarroyo *et al.* 2008).

Un dato a tener en cuenta en las formulaciones venideras es que los Ags expresados exclusivamente en los estadios sexuales (dentro del mosquito) son mínimamente polimórficos, ya que el sistema inmune de los mosquitos no los someten a presión selectiva al actuar de forma igualitaria sobre todos los parásitos mediante mecanismos inespecíficos. Esto hace de los Ags de estadios sexuales (gametocios, gametos u ooquinetos) dianas atractivas para reducir la transmisión, aunque no para proteger contra la enfermedad (Vaccines 2011).

Aunque se sabe que los anticuerpos (Acs) pueden eliminar eritrocitos infectados de la circulación (Cohen *et al.* 1961; Sabchareon *et al.* 1991) y que están asociados con la protección (Crompton *et al.* 2010), los Ags diana de los Acs protectores aún no se conocen bien (Targett 2005). La *inmunómica* constituye una nueva estrategia de estudio capaz de proporcionar un mejor conocimiento de los Ags que interactúan con el sistema inmunológico de un hospedador (el *inmunoma*) con el fin de identificar los epítopos más inmunogénicos responsables de inducir una inmunidad protectora (De Groot and Berzofsky 2004; Sette *et al.* 2005).

Para examinar las proteínas parasitarias reconocidas por Acs protectores, se utilizan técnicas de *inmunoblot* en 1 o 2 dimensiones, cromatografía líquida o secuenciación por espectrometría de masas con resultados interesantes (Singh *et al.* 2009; Fontaine *et al.* 2012; Kamali *et al.* 2012; Costa *et al.* 2013). Sin embargo, estos estudios se limitan a proteínas localizadas en determinadas fracciones subcelulares o a proteínas muy abundantes y detectan difícilmente epítopos en una determinada conformación (Vigil *et al.* 2010; Fontaine *et al.* 2012).

Gracias a la publicación de los genomas de seis especies de *Plasmodium* y algunos de los correspondiente proteomas (Carlton *et al.* 2002; Florens *et al.* 2002; Gardner *et al.* 2002; Lasonder *et al.* 2002; Hall *et al.* 2005; Carlton *et al.* 2008; Lasonder *et al.* 2008; Pain *et al.* 2008; Tarun *et al.* 2008), se han implementado nuevas estrategias para identificar, desde las secuencias genómicas, nuevos Ags que confieren inmunidad protectora (Davies *et al.* 2005; Vigil *et al.* 2010), y que pueden ser esenciales para el desarrollo de vacunas o de técnicas diagnósticas. En primer lugar, el clonaje de alto rendimiento y un sistema de expresión adecuado permiten actualmente la construcción de un *microarray* que representa alrededor de un 23% del *proteoma* (proteínas totales del parásito) de *P. falciparum* (1204 proteínas conocidas o hipotéticas). Los Ags fijados en el *microarray* deben ser entonces reconocidos por Acs protectores que indiquen qué proteínas son más inmunogénicas. Para obtener los Acs protectores se utilizan sueros de distintos modelos de inmunidad protectora basados en la vacunación con organismos completos atenuados, como esporozoítos de *Plasmodium* atenuados por irradiación (Hoffman *et al.* 2002) o ingeniería genética (Mueller *et al.* 2005), o con infecciones con parásitos sin atenuar en fase hepática o intraeritrocítica , sometidas a tratamiento con distintos fármacos (Pombo *et al.* 2002; Roestenberg *et al.* 2009; Roestenberg *et al.* 2011). También se utilizan sueros de individuos sometidos a vacunas de subunidades proteicas, y de aquellos con protección naturalmente adquirida (Sowa *et al.* 2004; Gray *et al.* 2007; Doolan *et al.* 2008; Crompton *et al.* 2010; Trieu *et al.* 2011). Destaca así la identificación de proteínas serodominantes de individuos expuestos naturalmente a la malaria (Doolan *et al.* 2008; Crompton *et al.* 2010) e individuos protegidos tras la inoculación de esporozoítos atenuados con radiación (Targett 2005; Doolan *et al.* 2008) tanto conocidas como hipotéticas, lo que amplía considerablemente el repertorio de productos de genes que podrían ser vacunas candidatas potenciales.

2.6. Inmunidad a la malaria en humanos

El sistema inmunitario es capaz de proteger eficazmente contra las enfermedades que causan muchos agentes patógenos tras una única infección, pero en el caso de la malaria, éste no es capaz de desarrollar una inmunidad completamente eficaz. Está descrito que la inmunidad que se adquiere de forma natural contra la infección de *P. falciparum* en humanos es de corta duración y no esterilizante (Okell *et al.* 2009); y que solo pueden adquirirla individuos adultos tras años de repetidas infecciones (Doolan *et al.* 2009), excepto mujeres embarazadas, que sufren una inmunosupresión natural durante la gestación (Menendez 1995). Estudios adicionales han mostrado que tampoco la inmunidad frente a *P. vivax* persiste un largo tiempo en ausencia de reexposiciones al parásito (Mueller *et al.* 2009).

Se han descrito diferentes categorías de inmunidad contra *Plasmodium* en humanos: anti-enfermedad, que confiere protección ante los síntomas clínicos; anti-parásito, que defiende de la parasitemia; y la premunición, que es la protección inmunológica contra nuevas infecciones al mantener un bajo nivel de parasitemia generalmente asintomática (Sergent and Parrott 1935; Doolan *et al.* 2009).

El riesgo de padecer malaria, así como sus manifestaciones clínicas, está altamente influenciado por numerosos factores relacionados con el hospedador entre los que se encuentran aspectos genéticos, el género, la edad, las medidas de defensa ante las picaduras de los mosquitos, así como la prevalencia de polimorfismos en los eritrocitos y las coinfecciones por helmintos (Schofield and Grau 2005; Akpogheneta *et al.* 2008; Doolan *et al.* 2009) (Figura 12). Por otro lado, hay numerosos mecanismos que contribuyen a que el parásito evada la respuesta inmunológica del hospedador, entre los que se encuentra su complejo ciclo biológico que incluye etapas de crecimiento intracelulares. El contacto entre el parásito y el sistema inmune sucede teóricamente en el bazo, sangre periférica y los nódulos linfáticos durante la fase intraeritrocítica, y en la sangre periférica e hígado durante la fase exoeritrocítica. A pesar de ello, el parásito es capaz de dificultar su eliminación (Smith *et al.* 1995; Sherman *et al.* 2003; Hisaeda *et al.* 2005; Schofield and Grau 2005) a través de su adherencia en la microvasculatura de distintos tejidos del hospedador como la placenta (Hviid 2010), el bazo (Martin-Jaular *et al.* 2011) y el cerebro (Pongponratn *et al.* 2003; Idro *et al.* 2005). Por otro lado, el parásito ha evolucionado para presentar una gran diversidad de Ags. El sistema inmune humano ha seleccionado mediante presión evolutiva a los genes que codifican los Ags inmunodominantes más polimórficos durante el ciclo biológico (Epstein *et al.* 2007; Mackinnon and Marsh 2010). De esta manera, los genes conservados probablemente no son dianas de una inmunidad protectora. Además, debido a que las fases infectivas del parásito en el hospedador humano son haploides, las mutaciones son expresadas inmediatamente y los clones más resistentes son seleccionados y se expanden rápidamente protegidos en el interior de los eritrocitos lo que permite su transmisión a los mosquitos. Esta selección se potencia aún más en el interior del mosquito, donde sucede una extensiva recombinación genética durante la fase sexual del parásito (Mackinnon and Marsh 2010).

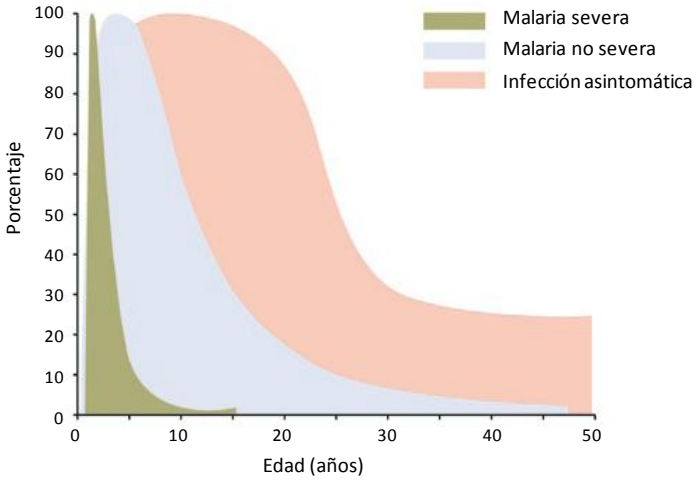

Figura 12. Índices de inmunidad a la malaria por *P. falciparum* en poblaciones de áreas de transmisión endémica. La prevalencia de infecciones asintomáticas, de casos clínicos no severos y de casos severos dependen de la exposición a la infección y la susceptibilidad del individuo; todos muestran la evidencia de una adquisición de resistencia a la infección al incrementarse la edad de la persona, pero es notable q cada indicador tiene distinta relación con cada edad. Los datos han sido normalizados y se presentan como el porcentaje máximo de casos para el índice de cada población. Datos tomados de estudios en Kilifi (Kenia) (Marsh and Kinyanjui 2006; Langhorne *et al.* 2008).

Una respuesta inmune protectora puede estar dirigida contra diversas dianas del *Plasmodium*, como son los Ags de superficie de los esporozoítos y las células hepáticas infectadas en la etapa exoeritrocítica, y de los merozoítos y eritrocitos infectados en la fase intraeritrocítica. Cada estadio se caracteriza por la expresión de proteínas específicas, requiriéndose mecanismos efectores inmunológicos con especificidades diferentes para eliminar las distintas formas parasitarias (Li *et al.* 2001). Por otro lado, aunque la infección palúdica es capaz de estimular cada componente del sistema inmune desencadenando una respuesta que puede contribuir a eliminar el parásito, también puede contribuir a la patogénesis y al desgaste del hospedador dependiendo de la naturaleza y extensión de esta respuesta. Desafortunadamente, el estudio de perfiles serológicos en la población humana tras la infección por malaria ha revelado que la especificidad de la respuesta inmunológica ante el parásito es muy variada entre individuos con un mismo estado clínico (Gray *et al.* 2007; Doolan *et al.* 2008) por lo que la identificación de respuestas efectoras asociadas a cada estado clínico es, hasta el momento, muy complicada.

2.6.1. Inmunidad durante la fase exoeritrocítica

La primera fase de la infección en el humano tiene lugar en el hígado, es asintomática y dura pocos días (Langhorne *et al.* 2008). Se ha descrito que la respuesta inmunológica frente a las formas parasitarias exoeritrocíticas no resulta imprescindible para el desarrollo de inmunidad a la malaria, ya

que la infección con formas únicamente intraeritrocíticas de una persona inmune es capaz de evitar el desarrollo de la parasitemia y de los síntomas consecuentes (Bruce-Chwatt 1963).

A diferencia de la fase intraeritrocítica, los esporozoítos y estadios hepáticos apenas inducen la respuesta innata de una forma natural, debido probablemente a que carecen de Ags capaces de inducir la respuesta innata, o a que estos están secuestrados o simplemente a que son muy poco numerosos (Riley and Stewart 2013).

El 95% de los linfocitos T circulantes presentan receptores de Ag (TCR, del inglés *T cell receptor*) con cadenas αβ e incluyen dos subpoblaciones mayoritarias dependiendo de la molécula de superficie que requieren para su activación, CD4 en el caso de los llamados linfocitos T cooperadores y CD8 en el caso de los linfocitos T citotóxicos. La escasa respuesta inmune inducida por los esporozoítos es mediada principalmente por los linfocitos T citotóxicos que pueden reconocer los Ags parasitarios asociados a moléculas del complejo principal de histocompatibilidad de clase I (MHC I). Estos linfocitos T citotóxicos pueden ser activados por células dendríticas (CDs) que hayan capturado esporozoítos o Ags de éstos en los ganglios linfáticos que drenan el lugar donde el mosquito los inoculó. Así, una vez que los linfocitos T CD8 son activados por las CDs en los ganglios, migran al hígado donde destruyen directamente a las células infectadas y pueden quedar como células de memoria durante meses, preparadas para ser re-activadas en pocas horas ante una nueva invasión de esporozoítos (Chakravarty *et al.* 2007). Además, los linfocitos T CD8 también pueden activarse en el propio hígado donde aún no se sabe con claridad si dicha activación está mediada porlos propios hepatocitos infectados, que actuarían como células presentadoras de Ag, o si son CDs reclutadas al hígado que hayan fagocitado un hepatocito infectado que haya muerto por apoptosis mediante presentación cruzada (fenómeno por el que las células dendríticas presentan Ags exógenos por moléculas MHC I) (Leiriao *et al.* 2005; Good and Doolan 2007).

Aunque no sean necesarias al inicio de la activación de las células CD8, los linfocitos T cooperadores contribuyen en la proliferación y diferenciación de los linfocitos T citotóxicos mediante la producción de interleuquina 4 (IL-4) (Carvalho *et al.* 2002; Morrot and Zavala 2004). Los Ags esporozoarios inoculados con adyuvantes potentes inducen además altos niveles de inmunoglobulinas (Ig) (Bojang *et al.* 2001; Polhemus *et al.* 2009), lo que indica que estos estadios poseen proteínas antigénicas siempre que se presenten en un contexto inflamatorio (Liehl and Mota 2012). También se ha descrito que los Acs son capaces de impedir la invasión de hepatocitos por *P. falciparum in vitro* (Nussenzweig and Nussenzweig 1985; Chappel *et al.* 2004; Silvie *et al.* 2004).

2.6.2. Inmunidad a *P. falciparum* en fase intraeritrocítica

En la respuesta inmunológica frente a la fase intraeritrocítica del *Plasmodium*, los mecanismos innatos constituyen la primera línea de defensa ante la primera infección y éstos incluyen la activación del sistema del complemento, monocitos, macrófagos, CDs, las células citotóxicas naturales (en inglés *natural killer,* NKs) y células T citotóxicas naturales (NKT, *natural killer T cells*) (Stevenson and Riley 2004). También las células T con TCR de tipo γδ (γδT), que conforman el 5% del total de linfocitos circulantes, tienen un papel fundamental de puente entre la respuesta innata y adaptativa. Estos actores limitan el crecimiento del parásito (Stevenson and Riley 2004), pero el control final del mismo, así como su eliminación, dependen de los Acs que adquieren una eficacia creciente con el tiempo, presumiblemente gracias a la adquisición y maduración de su afinidad al reconocer a los clones más prevalentes del parásito (Riley and Stewart 2013) (Figura 13).

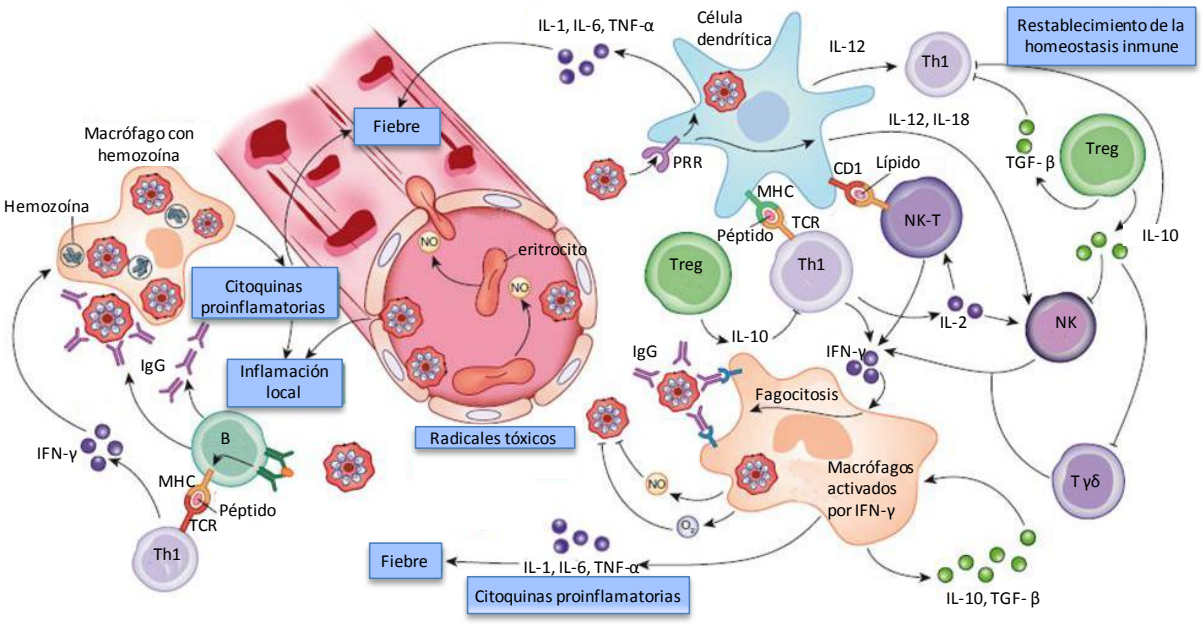

Figura 13. Inducción de respuestas inmunológicas humoral y mediada por células T contra la malaria. Las CDs detectan a los parásitos y a las células rojas infectadas a través de receptores de reconocimiento de patrones PRRs y los fagocitan para presentar sus Ags a las células T. Además, segregan citoquinas que inician una inflamación que dirige la diferenciación de células Th1 y puede llevar a la patogénesis. Las células Th1 promueven la diferenciación de células B para que produzcan Ac y también segregan IFN-γ que activa a los macrófagos. Los macrófagos fagocitan a los parásitos y a las células rojas infectadas opsonizados y los matan a través de mecanismos dependientes de NO y O2. La inflamación induce la expresión de moléculas de adhesión endotelial a las que se unen los eritrocitos infectados. La secreción de citoquinas antiinflamatorias de macrófagos y células Tregs reduce la inflamación. Tregs, células T reguladoras, TCR, receptor de células T.

Sin duda, tanto la inmunidad como la patofisiología de la malaria en humanos está ampliamente influenciada por las citoquinas producidas por las células que actúan en la respuesta innata (Clark *et al.* 2006). Estudios *in vitro* han demostrado que los eritrocitos infectados inducen la

producción de citoquinas por los leucocitos circulantes en menos de 10 horas (Scragg *et al.* 1999). Por otro lado, Hermsen *et al*. han encontrado en el suero de individuos infectados con *P. falciparum* que se produce una acumulación de citoquinas proinflamatorias cuando se liberan los parásitos desde el hígado al torrente sanguíneo (Hermsen *et al.* 2003). Entre las citoquinas producidas en respuesta a la infección palúdica destacan el factor de necrosis tumoral (TNF-α) y el interferón gamma (IFN-γ) que, aunque se han asociado a una etapa grave de la enfermedad, también se consideran cruciales para el control inicial de la parasitemia, tanto en la malaria murina como humana (Perkins *et al.* 2011). La secreción de IFN-γ por células NK, NKT y células γδT tiene efectos citotóxicos en el crecimiento del parásito mediante mecanismos aún desconocidos, e incrementa directamente la fagocitosis por los macrófagos (Urban *et al.* 2005). El TNF-α, producido principalmente por macrófagos, se correlaciona tanto con la patología de la malaria como con la inducción de protección al activar distintos leucocitos, como neutrófilos o a los mismos monocitos/macrófagos (Perlmann and Troye-Blomberg 2002; Perkins *et al.* 2011). Así, aunque la respuesta proinflamatoria aguda puede limitar la replicación inicial del parásito en los eritrocitos, ésta debe ser controlada por mecanismos antiinflamatorios capaces de prevenir procesos inmunopatológicos, entre los que cabe destacar la secreción de IL-10 y el factor de crecimiento transformante (TGF-β) por las células T CD4 (Langhorne??? 2005; Finney *et al.* 2010). Sin embargo, y a pesar de los indicios, aún se desconoce si la inmunidad natural a la malaria necesita realmente de respuestas regulatorias para controlar la inflamación minimizando así los síntomas clínicos (Riley and Stewart 2013).

Los macrófagos y monocitos tienen un papel importante, tanto a nivel de inmunidad innata como adaptativa, actuando a través de distintos mecanismos (Stevenson and Riley 2004). Como efectores de la respuesta innata, los macrófagos y monocitos pueden fagocitar eritrocitos infectados por vía no opsónica, probablemente interaccionando con Ags de *P. falciparum* a través de sus receptores de reconocimiento de patrones moleculares CD36 o de tipo Toll (TLR) y liberando citoquinas inflamatorias, destacando así la función que ejercen en ausencia de Ac citofílicos u opsonizantes (Serghides *et al.* 2003; Akira and Takeda 2004; Ayi *et al.* 2005; Krishnegowda *et al.* 2005). Además, los macrófagos y monocitos activados generan localmente altas cantidades de oxido nítrico (NO) que actúa como agente citostático, inhibiendo, aunque de forma reversible, el crecimiento de *P. falciparum* y otras especies de *Plasmodium,* como como *P. chabaudi y P. berghei* que afectan a roedores (Stevenson and Riley 2004). Respecto a su relación con la respuesta adaptativa, los macrófagos y monocitos son capaces de mediar una respuesta de citotoxicidad celular dependiente de Acs (CCDA) (Lunel and Druilhe 1989; Bouharoun-Tayoun *et al.* 1990; Chimma *et al.* 2009). Esta respuesta genera el reconocimiento de los Ags de superficie de los merozoítos o eritrocitos infectados como principal diana de los Acs y la activación de éstas células fagocíticas a través del receptor de la región Fc de los Acs (FcγR) con la consecuente liberación de mediadores solubles, como TNF-α que bloquea el desarrollo de los trofozoítos presentes en su entorno (Bouharoun-Tayoun *et al.* 1995). Además de haberse observado la cooperación de macrófagos y monocitos con Ac en la inhibición de los parásitos *in vitro*, se ha relacionado la presencia

de isotipos citofílicos de Ac con un riesgo menor a la malaria (Bouharoun-Tayoun and Druilhe 1992; Oeuvray *et al.* 1994; Taylor *et al.* 1995; Aribot *et al.* 1996; Ferreira *et al.* 1996; Sarthou *et al.* 1997). Por último, los macrófagos pueden activar células T CD4 presentando el Ag por medio del complejo principal de histocompatibildad de clase II (MHC II) expresado en su membrana (Serghides *et al.* 2003). A pesar de todas las funciones mencionadas, en la malaria humana la función de los monocitos y macrófagos puede estar alterada por los eritrocitos infectados o la hemozoína (Schwarzer *et al.* 1992; Schwarzer and Arese 1996). Concretamente, la expresión superficial de MHC II en los monocitos humanos estimulados con IFN-γ se ve disminuida en presencia de eritrocitos infectados por *P. falciparum* (Schwarzer *et al.* 1998).

La interacción entre los parásitos en fase intraeritrocítica y las células que conforman la respuesta inmune innata influye en la elaboración de la respuesta adaptativa (Wykes *et al.* 2007). En este sentido, las CDs, en comparación con otras células presentadoras de Ag profesionales como células B y macrófagos, son las principales responsables en presentar Ags a las células T CD4 colaboradoras y estimular la producción de citoquinas inflamatorias (Wykes *et al.* 2007). Las CDs residen en casi todos los tejidos donde juegan un papel esencial en la vigilancia de Ags extraños, gracias a que poseen receptores de reconocimiento de patrones moleculares como receptores TLR, de tipo Nod, de lectina tipo C o CD36 (Urban *et al.* 2001; Iwasaki and Medzhitov 2010). Tras la interacción con un Ag, las CDs maduran incrementando la expresión de MHC II, de moléculas coestimuladoras (CD40, CD80, CD86) y de moléculas de adhesión (Banchereau *et al.* 2000; Urban *et al.* 2005; Ing *et al.* 2006; Iwasaki and Medzhitov 2010) y son capaces entonces de polarizar la diferenciación de células T CD4 vírgenes hacia las subpoblaciones Th1, Th2, Th17, T foliculares o T reguladoras (Treg) (Zhu and Paul 2008; Coquerelle and Moser 2010). Además, las CDs son importantes en la activación de células NK, y su producción de citoquinas puede regular la función de otros tipos celulares con el fin de regular y amplificar las respuestas innatas y adaptativas. En la malaria humana aún se desconoce si la infección por *Plasmodium* induce un efecto inhibidor o activador sobre las CDs, aunque se ha sugerido que el efecto final depende de la especie y cepa del parásito, la severidad de la infección, del hospedador, del tiempo desde la infección, y del tamaño del inóculo (revisado por (Wykes and Good 2008; Stevenson *et al.* 2011)).

Las células NK se encuentran fundamentalmente en sangre periférica, bazo y médula ósea (Moretta *et al.* 2002). A menudo son las primeras células en responder a los eritrocitos infectados por *P. falciparum* al producir IFN-γ tras el contacto directo con el parásito, como han demostrado estudios *in vitro* (Artavanis-Tsakonas and Riley 2002; Artavanis-Tsakonas *et al.* 2003), y al lisar los eritrocitos infectados por *P. falciparum* mediante la secreción de granzima A y B (Hermsen *et al.* 2003; Bottger *et al.* 2012). Recientemente, se ha sugerido que la producción de IFN-γ por las células NK en respuesta a la infección por *P. falciparum in vitro* depende de las de células T de memoria, lo que cuestiona el papel de aquellas en la respuesta innata (McCall *et al.* 2010).

También las células NKT participan en la defensa contra *Plasmodium,* aunque se desconoce si forman parte esencial de la respuesta a la malaria y los Ags parasitarios que son capaces de reconocer

(Stevenson and Riley 2004; Vasan and Tsuji 2010). Se caracterizan por presentar marcadores característicos de células NK, como el receptor NK1.1, y de células T, como el receptor TCR tipo αβ, a través del cual detectan Ags lipídicos asociados con CD1 (Perlmann and Troye-Blomberg 2002). Se activan tanto durante fase hepática como intraeritrocítica de la malaria y producen grandes cantidades de IFN-γ e IL-4 (Artavanis-Tsakonas and Riley 2002; Stevenson and Riley 2004).

Las células γδT, a pesar de ser linfocitos T, parecen ostentar un papel en la respuesta innata. A diferencia de las T αβ, las células γδT proliferan rápidamente en etapas tempranas de la infección y, aunque provengan de un individuo nunca expuesto a malaria, son capaces de activarse al entrar en contacto con Ags del parásito e inhibir su replicación mediante exocitosis de gránulos (Troye-Blomberg *et al.* 1999; Farouk *et al.* 2004). Son activadas por citoquinas como IL-2, IL-4 y producen fundamentalmente citoquinas proinflamatorias, como IFN-γ de una forma temprana (Artavanis-Tsakonas and Riley 2002; Perlmann and Troye-Blomberg 2002).

La habilidad de las células presentadoras de Ag para capturar y procesar el Ag parasitario determina la magnitud y la calidad de la respuesta de células T. Aunque el conocimiento de la respuesta inmune celular adaptativa que se desarrolla durante la malaria es todavía escaso, se ha demostrado mediante modelos de experimentación animal que las células T CD4 son esenciales en el desarrollo de una inmunidad protectora (von der Weid and Langhorne 1993; van der Heyde *et al.* 1996; Langhorne *et al.* 1998). Las células T CD4, tanto Th1 como Th2, son células colaboradoras que se diferencian según las citoquinas que secretan y la función que desempeñan. Las Th1 producen IFN-γ, IL-2 y linfotoxina y son responsables de activar a los macrófagos; por el contrario, las Th2 producen IL-4, IL-5, IL-6, IL-10 y IL-13 y contribuyen a la maduración de las células B hacia células plasmáticas y al cambio de isotipo y maduración de la afinidad de las Igs (Beeson *et al.* 2008). El tercer tipo de células efectoras T CD4 son las Th17, inducidas por IL-6, IL-21, IL-23, and TGF-β (Ivanov *et al.* 2007), cuyo papel en malaria es desconocido (Radosevic *et al.* 2010; Metenou *et al.* 2011). Los pacientes con VIH, que presentan menor número de células T CD4, presentan mayores parasitemias y síntomas clínicos, lo que evidencia el importante papel de las células T CD4 (Ndungu *et al.* 2005).

Se debe considerar la contribución de subpoblaciones de células T CD4 minoritarias, como las células Tregs, que juegan un papel esencial al mantener la homeostasis inmunológica y controlar una excesiva respuesta inmune (Sakaguchi *et al.* 1995). Las células Tregs humanas están definidas por la expresión de CD25 (cadena α del receptor de IL-2 de alta afinidad) y Foxp3 (un factor de transcripción). Recientemente, se ha determinado mediante ensayos *in vitro* que eritrocitos infectados por *P. falciparum* inducen dos tipos de poblaciones de células Tregs: unas expresan niveles intermedios de Foxp3 y producen citoquinas como IFN-γ, IL-4 y IL-17, mientras otras tienen altos niveles de Foxp3, no producen citoquinas proinflamatorias y necesitan TGF-β1 e IL-10 para su generación. Además, ninguna de ellas es capaz de activarse a través del TCR *in vitro*, lo que sugiere que no es un mecanismo específico

de Ag (Scholzen *et al.* 2009). *In vivo*, las células Tregs se asocian con altas parasitemias de *P. falciparum*, considerándose por ello un factor de virulencia (Walther *et al.* 2005; Couper *et al.* 2008).

La otra gran familia de células T αβ, las células T CD8, reconoce Ags presentados por MHC I por lo que su papel no está muy definido en las fases intraeritrocíticas del parásito. Se ha demostrado que se encuentran activados durante la malaria cerebral, aunque aún se desconoce si participan en su patogénesis (Miyakoda *et al.* 2008), sin embargo no parecen estar involucrados en la protección frente a estadios intraeritrocíticos del *Plasmodium* (Vinetz *et al.* 1990).

Las principales dianas de la respuesta inmune durante la fase intraeritrocítica de la malaria son los merozoítos libres y los parásitos intraeritrocíticos que, al no presentar moléculas MHC I ni MHC II en su membrana (Jayawardena *et al.* 1983), no pueden ser dianas directas de linfocitos T CD4 o CD8 efectores (Langhorne??? 2005). Los Acs desempeñan un papel protector ante la malaria por *P. falciparum,* al haberse demostrado que los ensayos de transferencia de suero de individuos adultos inmunes a niños infectados no inmunes son una estrategia eficaz de tratamiento (Cohen *et al.* 1961). Si bien esta estrategia no se puede desarrollar a gran escala, demuestra la facilidad de inducir inmunidad frente a la malaria a través de la transferencia de la inmunidad humoral (Smith and Taylor-Robinson 2003). Además, modelos de ratón carentes de células B revelan la importancia de estas células en el paludismo, ya que éstos son incapaces de eliminar definitivamente infecciones de *P. yoelii* (Weinbaum *et al.* 1976) y *P. chabaudi* (von der Weid *et al.* 1996). Las células B, además de ser productoras de anticuerpos, tienen otros papeles importantes en la inmunorregulación, incluyendo la secreción de citoquinas y la presentación de antígenos. Las células B residen en diferentes tejidos y presentan distintos fenotipos según el nivel de maduración y activación. Los linfocitos B inmaduros se originan en la médula ósea y se transforman en células B transitorias 1 (T1) al abandonar dicho órgano, llegando por el torrente sanguíneo hasta el bazo donde dan lugar a las células B transitorias 2 (T2). En estos estadios, sólo son capaces de expresar IgM en su membrana. Las células T2 continúan su desarrollo para formar, tanto células maduras que recirculan en búsqueda de un Ag o permanecen en los ganglios linfáticos (Loder *et al.* 1999)o en la zona folicular del bazo y se denominan células B foliculares (FO), como células B de la zona marginal, conocidas como células B marginales (MZ) (Loder *et al.* 1999; Chung *et al.* 2003). Las células B maduras pueden expresar IgM y IgD en su membrana, pero el reconocimiento de un Ag inicia su activación con una serie de cambios genéticos, llamados “mutaciones somáticas”, que implican el cambio de isotipo de los Acs de membrana, que suele ser el IgG en el caso de una infección por malaria. Las respuestas dependientes de células T llevan a la formación de centros germinales (CGs) en los folículos, en donde se generan células plasmáticas y células B de memoria (Sagaert *et al.* 2007). Las células B marginales generan por su parte células plasmáticas de vida corta, responsables de Ac de baja afinidad que constituyen una primera línea de defensa (Martin and Kearney 2000).

Existe además otra familia de células B, llamadas B-1, que se localiza principalmente en el peritoneo y forman variablemente alrededor de 5% de las células B del bazo, pero están ausentes de los

ganglios linfoides. Las células B-1 se diferencian de las B-2 (que incluyen las células B FO, MZ, CGs, T1 y T2) en que su función principal es la producción de IgM como una fuente primaria de Acs frente a diferentes infecciones y no sufren mutaciones somáticas (Berland and Wortis 2002; Baumgarth 2010).

Los Acs ayudan en la eliminación del parásito mediante distintos mecanismos que se describen a continuación:

- Bloqueo de la invasión de los glóbulos rojos por los merozoítos (Miller *et al.* 1975; Epstein *et al.* 1981; Blackman *et al.* 1990).
- Inhibición de la citoadherencia de eritrocitos que contienen esquizontes (Perlmann and Troye-Blomberg 2002).
- Opsonización de los merozoítos o eritrocitos infectados para ser fagocitados por células efectoras que son activadas a través del receptores FcR (Bouharoun-Tayoun *et al.* 1990; Braga *et al.* 2005).
- CCDA: respuesta inmune en la que los Acs opsonizan al patógeno haciéndolo diana de la liberación de productos de lisis por monocitos, macrófagos, neutrófilos, eosinófilos y células NK (Brown *et al.* 1986). Conecta la inmunidad celular con la humoral promovida por respuesta Th1, esta última estimulada por patógenos acumulados dentro de vesículas de macrófagos y células dendríticas.
- Inhibición celular mediada por Acs: respuesta de tipo CCDA que inhibe el crecimiento de las formas sanguíneas en el interior de los eritrocitos a través de la liberación de factores solubles, como TNF-α, por monocitos y macrófagos (Lunel and Druilhe 1989; Bouharoun-Tayoun *et al.* 1990).

La colaboración de las Igs con la respuesta celular adquiere un papel importante en la respuesta a la malaria en humanos ya que se ha desmostrado que la protección frente a *P. falciparu*m depende de la proporción entre los niveles de Acs citofílicos (IgG1 e IgG3) y no citofílicos (IgG2 y IgG4) (Aribot *et al.* 1996). Los citofílicos son aquellos Acs que tienen una afinidad adicional hacia distintos tipos de células que no está relacionada con la afinidad específica hacia los Ags que los han inducido. La inmunidad adquirida a la malaria implica la adquisición de Acs específicos de un amplio espectro de Ags del parásito (Bull *et al.* 2000), pero a su vez, *Plasmodium* da lugar a una temprana activación policlonal de células B que genera elevadas cantidades de Acs no específicos de *Plasmodium* en sangre cuya implicación en la inmunidad innata aún se desconoce (Perlmann and Troye-Blomberg 2002).

Durante la infección palúdica también se ha detectado un aumento en la producción de IgE, aunque ésta se ha asociado a la patogénesis ya que la concentración de IgE es mayor en pacientes con malaria grave o cerebral que con malaria no complicada, quizá debido a que provoca, a través de sus inmunocomplejos, la producción de TNF-α y NO por monocitos y células endoteliales en la microvasculatura (Perlmann and Troye-Blomberg 2002). Además, el *Plasmodium* es capaz de promover la activación policlonal de los linfocitos e inducción preferencial de Acs que no tienen función protectora

(Donati *et al.* 2004). Un elevado número de estudios defienden que la respuesta de los Acs contra Ags específicos de *Plasmodium* es de corta duración y puede depender de la continua presencia de parásitos en el organismo (Conway *et al.* 2000; Bull and Marsh 2002), lo que sugiere que la generación de la memoria inmunológica y la longevidad de la respuesta inmune mediada por células B y/o T estaría alterada.

2.6.3. Memoria inmunológica

La memoria inmunológica, definida como la habilidad del sistema inmune de responder más rápida y robustamente ante la reexposición a un Ag que en su primer encuentro, es consecuencia de la inmunidad adaptativa. Sin embargo, en la malaria se adquiere lentamente y es de corta vida (Doolan *et al.* 2009).

La posibilidad de adquirir memoria inmunológica frente a estadios exoeritrocíticos del parásito se ha demostrado ampliamente a través de infecciones experimentales en roedores, primates no humanos y en humanos, mayoritariamente con parásitos atenuados. Una irradiación correcta de esporozoítos provoca mutaciones en el ADN que, aunque no afectan a la capacidad de invadir células hepáticas, no les permite terminar su desarrollo y de esta manera no causan malaria intraeritrocítica (Vanderberg *et al.* 1968). Los humanos inmunizados con esporozoítos de *P. falciparum* irradiados, quedan protegidos frente a nuevas inoculaciones de esporozoítos normales (Clyde *et al.* 1973; Rieckmann *et al.* 1974; Hoffman *et al.* 2002). El mecanismo de esta protección se debe probablemente a células CD8 e INF-γ, como se ha demostrado en modelos animales (Vaughan *et al.* 2010).

La inmunización mediante picaduras de mosquitos infectados de *P. falciparum* bajo tratamiento profiláctico de cloroquina (que sólo actúa contra fase intraeritrocíticas del parásito), ha destacado como potenciales efectores de protección, durante al menos 2 años, a las células T CD4 productoras de IFN-γ, TNF-α e IL-2 (Roestenberg *et al.* 2009; Roestenberg *et al.* 2011). De forma similar, tras la administración de la vacuna RTS,S se ha observado una correlación interesante entre el número de células T CD4 que producen TNF-α o IL-2 y el nivel de protección (Olotu *et al.* 2011), pero la baja duración de ésta puede ser debida a que, hasta ahora, no se ha identificado una producción de Acs diferencial entre las respuestas de individuos protegidos y no protegidos (Kester *et al.* 2009; Agnandji *et al.* 2011; Ansong *et al.* 2011; Lumsden *et al.* 2011). Tras la inmunización con la RTS,S a niños entre 1 y 4 años, los Acs específicos de CSP de *P. falciparum* disminuyen rápidamente transcurridos 6 meses desde la vacunación, desapareciendo la protección frente a la malaria clínica tras 6 meses (Alonso *et al.* 2004; Alonso *et al.* 2005; Bejon *et al.* 2008), aunque la protección a la malaria clínica se prolonga hasta los 45 meses (Sacarlal *et al.* 2009). Por último, la inmunización mediante el tratamiento de dos inoculaciones de esporozoítos con azitromicina ha dado lugar a una protección robusta basada en células T CD8 e INF-γ (Friesen *et al.* 2010).

Ahora bien, la exposición repetida a esporozoítos normales por infecciones naturales en áreas endémicas no induce una protección de éstas características, probablemente porque la dosis de esporozoítos en infecciones naturales sea demasiado pequeña (Vaughan *et al.* 2010). Bajo determinadas situaciones, la exposición frecuente a *Plasmodium* en áreas endémicas induce la acumulación de células B de memoria específicas del parásito que en adultos pueden permanecer presentes hasta 8 años (Migot *et al.* 1993; Langhorne *et al.* 2008). Sin embargo, una proporción de estas células puede mostrar características de células agotadas que responden en menor medida a los estímulos que las células B de memoria clásicas (Traore *et al.* 2009) (Weiss *et al.* 2009). En niños, las células B de memoria son muy escasas, por lo que su protección en infecciones asintomáticas puede estar sustentada por respuesta humorales de vida corta (Dorfman *et al.* 2005).

En infecciones experimentales, los estadios intraeritrocíticos atenuados también inducen inmunidad en distintos mamíferos, incluidos humanos (Doolan *et al.* 2009). La inoculación de dosis ultra-bajas de eritrocitos infectados de *P. falciparum* bajo tratamiento quimioterapéutico, induce una inmunidad estéril ante infecciones homólogas, basada en una respuesta Th1 con producción de IFN-γ y NO pero no de Acs (Pombo *et al.* 2002).

Por otro lado, también las células T tienen subpoblaciones de memoria que pueden jugar un papel importante en la inmunidad protectora (Stockinger *et al.* 2006). No obstante, el hecho de encontrar células T de memoria no significa que exista necesariamente memoria inmunológica atribuida a células T, ya que diversos factores como la persistencia del antígeno, mecanismos regulatorios o la exposición inicial al Ag, pueden condicionar la magnitud de su respuesta (Cockburn and Zavala 2007).

2.7. Modelos animales para el estudio de la fase intraeritrocítica de la malaria

2.7.1. Líneas de ratón

Los estudios sobre la biología y la patología de la malaria humana deberían idealmente realizarse en humanos, pero, por consideraciones éticas, esto no es siempre posible. Consecuentamente, los modelos animales se han vuelto imprescindibles para elucidar en la medida de lo posible los mecanismos patogénicos de la malaria, así como la respuesta inmunológica del hospedador frente a la infección.

Teniendo en cuenta que *P. falciparum* no infecta mamíferos no-simios, se han desarrollado modelos animales de malaria con diferentes especies de parásito y hospedador que se distinguen por las características de la infección de malaria que desarrollan. El modelo más extendido es el de roedor

por las ventajas que ofrece respecto a los modelos en simios: son de fácil manejo y baratos de mantener, el sistema inmune de ratón se conoce muy bien, admiten determinadas intervenciones que no son posibles en humanos y hay gran similitud genética entre las especies de *Plasmodium* que afectan a roedores y *P. falciparum,* lo que permite una gran reproducibilidad (Carlton *et al.* 2005; Taylor-Robinson 2010).

Ahora bien, siempre se deben tener en cuenta que existen diferencias entre el sistema inmune del ratón y el humano, tanto a nivel celular en los receptores o enzimas expresados, componentes de las vías de señalización, respuesta celular a quimioquinas y citoquinas, desarrollo celular, diferenciación Th1/Th2, etc. (revisado por (Mestas and Hughes 2004)); como a nivel de humoral. Los ratones producen IgA, IgD, IgE, IgM, y cuatro isotipos de IgG: IgG1, IgG2a, IgG2b e IgG3 (excepto las cepas de ratón C57BL/6, C57BL/10, SJL, y NOD que no presentan IgG2a, sino IgG2c (Martin and Lew 1998)), mientras que los humanos presentan IgA1, IgA2, IgD, IgE, IgM y los isotipos de IgG son IgG1, IgG2, IgG3 e IgG4.

Actualmente, la composición genética de todas las cepas de ratón en uso en los laboratorios proviene de un mosaico de subespecies en proporciones desiguales, forzada por una cría dirigida y en confinamiento. A pesar de la heterogeneidad genética de las poblaciones humanas, las cepas de ratones más utilizadas son genéticamente homogéneas (consanguíneas) ya que resultan del apareamiento ininterrumpido entre hermanos y hermanas por más de 20 generaciones. Así, las líneas de ratones más clásicas, como son los BALB/c, alcanzan un 100% de genes homocigotos; de esta manera, los laboratorios que utilizan animales consanguíneos investigan en una genética definida que les permite correlacionar la variabilidad en los parámetros experimentales con factores exclusivamente no genéticos.

Al contrario, los roedores de laboratorio no consanguíneos, como son los ICR, presentan un alto grado de heterocigosis que se corresponde con una elevada variabilidad genética (Benavides and L. 2003) (Festing, 1992; 1993; Berry y Cutler, 2007). La cepa ICR:CD-1 es original de Suecia (Lynch 1969) y se ha utilizado desde 1947 en el estudio del cáncer (Hauschka and Mirand 1973). Esta cepa de ratón se emplea escasamente en el análisis de infecciones por malaria comparativamente con otras como BALB/c, sin embargo anteriores estudios en nuestro laboratorio han demostrado cierta heterogeneidad en la respuesta de ICR a las infecciones de malaria (Moneriz *et al.* 2011; Kamali *et al.* 2012).

2.7.2. Parásitos *Plasmodium* de roedores

Los modelos de malaria en ratón han sido desarrollados utilizando parásitos originales de roedores de África (Figura 14) lo que significa que, a diferencia de los humanos, ninguno es un patógeno natural de animales de laboratorio, y en consecuencia no ha habido adaptación conjunta de los parásitos y el hospedador (Sanni *et al.* 2002).

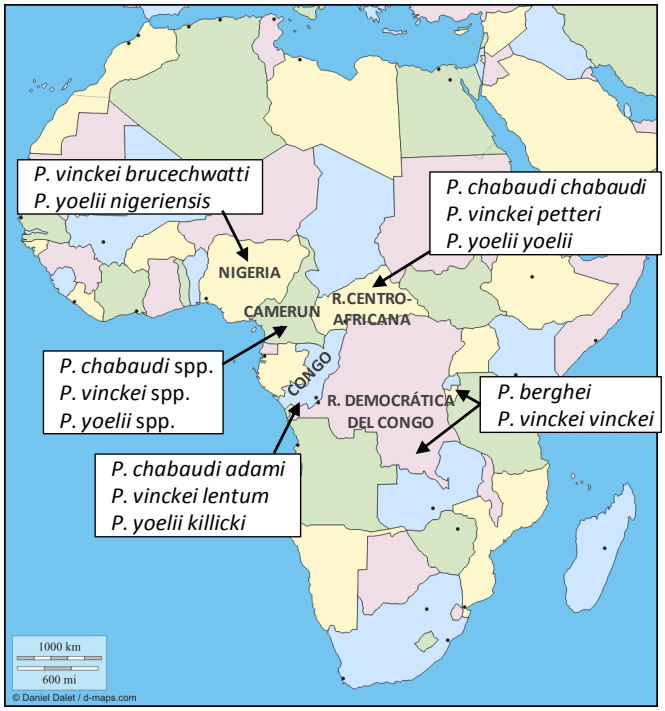

Figura 14. Origen de los parásitos de roedor en diferentes zonas de África (Landau and Chabaud 1994; Carlton *et al.* 2001). Hay tres especies de *Plasmodium* que infectan roedores y que se utilizan ampliamente como modelos experimentales de malaria en fase intraeritrocítica: *P. chabaudi., P. yoelii,* y *P. vinckei*.

Hay cuatro especies de *Plasmodium* que infectan roedores y que se utilizan ampliamente como modelos experimentales de malaria: *P. chabaudi*, *P. yoelii, P. vinckei* y *P. berghei* (Tabla 3). Las diferencias entre estas cuatro especies radican en la morfología, tiempo y tipo de desarrollo (sincrónico/asincrónico), tamaño de los diferentes estadios e isoenzimas que contienen, preferencia por infectar un grado de madurez diferente de glóbulos rojos (reticulocitos o glóbulos rojos maduros), temperatura de esporogonia en el mosquito, etc. (Janse 2006). Estas características influyen en la interacción parásito-hospedador y son las responsables de los diferentes cursos que tomarán la infección, su virulencia y la patogenia asociada. A pesar de que ningún modelo por si sólo refleja exactamente las características de la infección humana, su uso en conjunto proporciona información valiosa sobre Ags parasitarios, mecanismos inmunológicos protectores, biología y patología general de la malaria, además de contribuir al descubrimiento y validación de fármacos (Taylor-Robinson 2010).

- ***P. yoelii*** presenta 3 subespecies, *P. yoelii yoelii*, *P. yoelii killicki* y *P. yoelii nigeriensis,* muy utilizadas en modelos para el desarrollo y caracterización de vacunas candidatas (Doolan *et al.* 1998), en el estudio de mecanismos inmunes y patogénesis, en la investigación de la invasión de glóbulos rojos y, más recientemente, en estudios de ligamiento genético (Stevenson and Riley 2004; Hernandez-Valladares *et al.* 2005). Esta especie invade preferentemente reticulocitos, aunque también puede

invadir eritrocitos maduros. El ciclo intraeritrocítico dura 18 horas (Thorat *et al.*) (Gautret *et al.* 1994) y es muy asincrónico, a diferencia de *P. falciparum* cuyo ciclo es de 48h y sincrónico (Sherman 1998). La subespecie más utilizada es *P. yoelii yoelii,* entre la que se distinguen: 17XL y YM, dentro de las convencionalmente consideradas letales, y 17XNL y 265 entre las no letales (Li *et al.* 2001; Singh *et al.* 2002). Las diferencias en la letalidad se localizan tanto a nivel genético (Otsuki *et al.* 2009) como a nivel transcripcional (Preiser and Jarra 1998).

- ***P. chabaudi*** es similar a *P. falciparum* por: 1) su capacidad para infectar eritrocitos; 2) desarrollarse de modo sincrónico *in vivo*; 3) provocar el abandono de la circulación de los eritrocitos más viejos; 4) dar lugar a una o más recrudescencias en los ratones que sobreviven a una primera infección aguda; 5) presentar Ags sanguíneos análogos; 6) haber secuestro de parásitos en el hígado y el bazo durante la infección; 7) invadir tanto los glóbulos rojos maduros como los reticulocitos. *P. chabaudi* presenta un ciclo intraeritrocítico de 24 h. Esta cepa es particularmente valiosa en estudios de mecanismos inmunes y de inmunorregulación por citoquinas, para identificar loci y para estudios en los que se necesitan determinadas fases de crecimiento (Hernandez-Valladares *et al.* 2005) (Stevenson and Riley 2004). Cuando los roedores están expuestos al ritmo solar, la esquizogonia tiene lugar a medianoche. Existen 2 subespecies ampliamente estudiadas: *P. c. chabaudi* y *P. c. adami.*

- ***P. vinckei*** es la más distribuida entre la especies murinas, pero sin embargo es la menos estudiada. *P. vinckei* presenta un ciclo intraeritrocítico muy sincrónico de 24 h de duración y se desarrolla principalmente en los normocitos. Existen 4 subespecies: *P. v. vinckei, P. v. petteri, P. v. lentum* y *P. v. brucechwatti.* Las dos primeras se han utilizado en estudios quimioterapeúticos y en la identificación de nuevas dianas terapeúticas potenciales para el desarrollo de fármacos antimaláricos (Hernandez-Valladares *et al.* 2005).

- ***P. berghei*** invade preferentemente reticulocitos y posee un ciclo asincrónico. *P. berghei* puede ser manipulado genéticamente en el laboratorio usando tecnologías de ingeniería genética. En consecuencia, este parásito es de uso frecuente para el análisis de la función de los genes de la malaria aplicando la tecnología de modificación genética (Janse 2006). *P. berghei* es un también un modelo útil para el estudio experimental de la malaria cerebral. Ratones susceptibles infectados con *P. berghei* ANKA mueren durante la segunda semana con síntomas neurológicos severos y alteraciones microvasculares en el cerebro, que son síntomas comunes a las infecciones en humanos. Asimismo, *P. berghei* ANKA se ha utilizado en ensayos de ligamiento genético para mapear loci susceptibles a la malaria para aclarar así los mediadores de la patología cerebral letal (Hernandez-Valladares *et al.* 2005). Finalmente, las infecciones por *P. berghei* se han usado también en programas de investigación para el desarrollo y selección de fármacos contra la malaria (Fidock *et al.* 2004).

Tabla 3. Infección de malaria experimental en diferentes cepas de ratón (Li *et al.* 2001; Sanni *et al.* 2002).

Plasmodium	Cepa/ clon	Cepa ratón	Eritrocitos infectados	Letal
yoelii yoelii	17XL	Swiss BALB/c A/J CBA C57BL/6	Eritrocitos y reticulocitos	Sí
		DBA/2J		No
	17XNL	CBA C57BL/6 BALB/c DBA	Eritrocitos y reticulocitos	No
	YM	CBA C57BL/6 BALB/c DBA A/J	Eritrocitos y reticulocitos	Sí
	265	BALB/c C57BL/6	Eritrocitos y reticulocitos	No, raramente
chabaudi chabaudi	AS	CBA C57BL/6 BALB/c NIH	Eritrocitos	No
		A/J DBA/2		Sí
	CB	CBA C57BL/6	Eritrocitos	Sí (20-50%)
chabaudi adami	556 KA	BALB/c C57BL/6	Eritrocitos	No
vinckei vinckei		BALB/c	Eritrocitos	Sí
vinckei petteri	CR		Eritrocitos	No
berghei	ANKA	CBA/T6 C57BL/6 BALB/c DBA/2J	Eritrocitos y reticulocitos	Sí
	K173	CBA/T6 C57BL/6 BALB/c DBA/2J	Eritrocitos y reticulocitos	Sí

2.7.3. Respuesta inmunológica a la fase intraeritrocitaria de *P. yoelii yoelii*

Las distintas cepas de ratón responde a menudo de manera diversa a una misma cepa de *Plasmodium* (Wang *et al.* 2009). Así, la cepa *P. yoelii* 17XL es letal en ratones CBA, C57BL/6 y BALB/c (Wang *et al.* 2009), pero no en ratones DBA/2 (Sanni *et al.* 2002; Wang *et al.* 2009), lo que parece indicar que la letalidad depende de la forma de interacción con el hospedador. A pesar de que la rapidez de la infección por *P. yoelii* se ha relacionado con el patrón de invasión del eritrocito por el parásito (Preiser *et al.* 1999), está aceptado que la resolución de la infección de *PyL* también está influenciada por la respuesta inmune del hospedador (Couper *et al.* 2007).

Varios estudios sobre la infección de ratones NIH han descrito que en la infección por *PyNL* parecen activarse tanto la respuesta Th2 como la Th1 con una producción de Acs caracterizada por la producción temprana de IgG2a (citofílico) y tardía de IgG1 (no citofílico), mientras que *PyL* provoca la activación sólo de la Th2 y da lugar a niveles reducidos de IgG2a, pero no de IgG1 (Smith and Taylor-Robinson 2003). Otros estudios han corroborado que durante el contacto con *PyL* no se produce una correcta activación de células Th1 y Th2, pero ésta puede ser inducida por una vacunación que estimule la producción de citoquinas por ambas poblaciones (De Souza and Playfair 1995; De Souza *et al.* 1996). En la infección por ambas cepas parece ser preferible la producción de citoquinas proinflamatorias al comienzo de la infección, ya que la neutralización de citoquinas antiinflamatorias, como IL-10 y TGF-β, durante la infección por *PyL* se correlaciona con un mejor control de la parasitemia (Omer and Riley 1998; Omer *et al.* 2003) y, en una línea similar, la exposición a *PyNL* se caracteriza por una respuesta temprana con IFN-γ y TNF-α (De Souza *et al.* 1997; Omer and Riley 1998; Choudhury *et al.* 2000; Omer *et al.* 2003). Tanto las células NK como las γδT producen IFN-γ (De Souza *et al.* 1997; Choudhury *et al.* 2000). La función de las células NK parece ser crucial ya que su ausencia en infecciones no letales por *P. yoelii* aumenta su virulencia, mientras que la participación de las células γδT, aunque actúen antes que de los linfocitos T αβ específicos sean activados, podría ser prescindible en el control de la parasitemia (Choudhury *et al.* 2000).

A diferencia de lo que se observa tras la infección de *P. falciparum* en humanos, *P. yoelii* no hace disminuir la expresión de MHC II, CD80 ni CD86 en las células presentadoras de Ags profesionales, que son las CDs, los macrófagos y las células B, por lo que aparentemente habría una correcta maduración y activación de éstas (Luyendyk *et al.* 2002; Wykes *et al.* 2007). Es muy relevante el estudio de Couper *et al*., en el cual se describe que los macrófagos juegan un papel esencial en el control de la primera oleada de parasitemia de *PyNL* o *PyL* en ratones C57BL/6, mientras que la ausencia de linfocitos T, B, células NK o de la producción de IFN-γ no se estiman influyentes individualmente (Couper *et al.* 2007). Los macrófagos son activados por IFN-γ y células CD4 para fagocitar parásitos intraeritrocíticos y merozoítos libres (Stevenson and Riley 2004). Además, se ha observado que la repuesta de macrófagos inducida por *PyNL* dura más tiempo que la inducida por *PyL* (Fu *et al.* 2012).

También se ha descrito un comportamiento diferente en las CDs dependiendo de la letalidad de la infección. Tanto el flujo de éstas células hacia el bazo como la capacidad de estimular a los linfocitos T es menor en infecciones letales de *P. yoelii* que en las no letales y, además, ratones infectados con la cepa letal *P. yoelii* YM logran superar la infección al recibir CDs de un ratón infectado de *PyNL*, pero no de *P. yoelii* YM (Wykes *et al.* 2007).

Los linfocitos T CD4 ayudan a la activación de las células B frente a Ags proteínicos específicos y posteriormente a la producción de Igs específicas frente a dichos Ags por las células plásmaticas. Aunque el papel de las células T CD4 puede estar influenciado por la cepa de ratón (Langhorne *et al.* 2008), los experimentos de depleción selectiva de dichos linfocitos y su transferencia a ratones

inmunodeprimidos han puesto de manifiesto su importancia en cuanto al control de la infección se refiere (Amante and Good 1997; Xu *et al.* 2002). En la misma línea, los ratones SCID (carentes de células T y B) o NUDE (carentes de células T) no son capaces de controlar la parasitemia de *PyNL* a largo plazo y la infección acaba siendo letal, lo que demuestra el papel fundamental de células T y B en la inmunidad a *P. yoelii* (Choudhury *et al.* 2000). Sin embargo, se cree que su efectividad pudiera ser todavía mayor si no fuera porque las células T CD4 específicas de Ags del parásito sufren un aumento de apoptosis tras la infección por distintas especies de *Plasmodium*, debido no solo a la estimulación por el Ag, sino también a la contribución del IFN-γ (Xu *et al.* 2002). Por último, se ha constatado que las células T CD4 de memoria por sí solas son capaces de proteger contra la malaria murina, aunque la duración de su respuesta todavía se desconoce (Cockburn and Zavala 2007).

Existen experimentos de transferencia que han evidenciado el papel protector que tienen las células T CD8 en la infección por *P. yoelii* (Imai *et al.* 2010) y su activación y proliferación en respuesta al *PyNL* (Chandele *et al.* 2010) y otros que por el contrario, han concluido que estas células no funcionan como mediadores de protección (Vinetz *et al.* 1990).

La inmunización con eritrocitos infectados de *P. yoelii* 265 bajo tratamiento de cloroquina promueve una protección estéril, que actúa tanto en los estadios intraeritrocíticos como en los exoeritrocíticos. No se conocen los mecanismos exactos, pero la producción de NO y el papel de las CD4 y CD8 y, en menor medida de INF-γ, parecen imprescindibles (Belnoue *et al.* 2008). En cuanto al papel de las células Tregs, los estudios realizados para conocer su papel durante una infección por *Plasmodium* han mostrado resultados muy heterogéneos, probablemente debido a que el marcador más utilizado para medir esta población celular, el CD25, también se expresa en células T activadas y efectoras (Finney *et al.* 2010).

Por su parte, los linfocitos B y los Acs juegan un papel decisivo en la eliminación de los parásitos tras el primer pico de parasitemia (Langhorne *et al.* 1998), si bien necesitan la participación de mediadores incluyendo células del sistema innato y células T. Es posible que los mecanismos de eliminación del parásito por medio de Acs sean diferentes en humanos y roedores, ya que el uso de ratones deficientes en Fcy ha permitido concluir que los Acs median directamente en la protección frente a *PyL*, sin ser esencial la fagocitosis mediada por Fc o CCDA (Rotman *et al.* 1998). Es interesante destacar que las células B de memoria y las células plasmáticas de larga vida, generadas en ratones vacunados con la proteína MSP-1, sufren apoptosis tras la infección por *P. yoelii,* lo que podría mostrar otro mecanismo de evasión inmunológica del parásito (Wykes *et al.* 2005).

3. JUSTIFICACIÓN Y OBJETIVOS/ JUSTIFICATION AND OBJECTIVES

La malaria es todavía uno de los problemas sanitarios más importantes a nivel mundial, y particularmente grave, en las poblaciones de las zonas tropicales. A pesar de los programas globales de control y erradicación, que se centran en los tratamientos quimioprofilácticos, desarrollo de vacunas y control vectorial, todavía se producen alrededor de 216 millones de casos de malaria anuales, de los cuales 655000 tienen un desenlace fatal, en su mayoría niños menores de 5 años y mujeres embarazadas. Las estrategias de lucha contra la malaria están dificultadas por diferentes factores como son la aparición de cepas de *Plasmodium* resistentes a los fármacos convencionalmente utilizados, de los mosquitos vectores a los insecticidas, así como por otra serie de factores demográficos, socioeconómicos y políticos. Además, el objetivo de desarrollar una vacuna efectiva sigue siendo una promesa para el futuro, en parte debido al complejo desarrollo biológico del parásito y a la variedad de antígenos expuestos, que le permite evadir los mecanismos de defensa inmunitarios. Por ello, conocer detalladamente cómo se activa y desarrolla la inmunidad frente a la infección palúdica contribuiría en gran medida a alcanzar este objetivo.

El estudio de la respuesta inmune ante una primera infección de malaria en humanos se ha llevado a cabo, fundamentalmente, analizando parámetros sanguíneos en poblaciones de áreas endémicas de malaria o durante infecciones experimentales en voluntarios no inmunes bajo tratamiento farmacológico. Sin embargo, aún no se conocen con precisión cuales son los factores inmunológicos asociados a los diferentes perfiles de infección observados en humanos, que ayudarían a predecir el pronóstico y gravedad de la enfermedad y por tanto a mejorar el tratamiento de la misma.

Teniendo en cuenta estos antecedentes, los **objetivos** del presente trabajo fueron los siguientes:

1. Estudiar la respuesta inmune celular y humoral frente a una primera infección de *P. yoelii yoelii* 17XL en la cepa de ratón ICR
2. Analizar la memoria inmunológica que se desarrolla frente a dicha infección.
3. Examinar la actividad *in vitro* de la borrelidina frente a *P. falciparum*.
4. Evaluar el efecto del tratamiento con los antibióticos borrelidina y mupirocina contra la infección por *P. yoelii yoelii* 17XL en un modelo estándar de malaria en ratón.
5. Estudiar la respuesta humoral tras la infección de malaria tratada con borrelidina en ratones.

Malaria is one of the most important parasitic infectious disease in the world, and predominantly in tropical areas. Global programs adopted to control and ultimately eradicate malaria have mainly focused on prophylaxis, chemotherapy, vaccine development and vector control programs. However, in countries where malaria is endemic, about 216 million cases were estimated in 2010 causing nearly 655,000 deaths, mostly in children under 5 years old and pregnant women(WHO 2012). Strategies and action plans to fight malaria are being hampered by emerging parasite resistance to classical drugs, insecticide-resistant mosquitoes, as well as by demographic, socioeconomic and politic factors. Besides, the development of a malaria vaccine remains a promise for the future, in part due to the immune evasion strategies employed by the parasite which include a complex life cycle and high diversity of exposed antigens. Consequently, research programs to explore the development of immunity against malaria would contribute to achieving this goal.

Information on malaria immunity against a primary infection in humans has been mainly provided from the analysis of peripheral blood parameters in people from the malaria-endemic areas or in non-immune volunteers subjected to experimental infections under drug treatment. However, few it is known about the immune factors associated with the different infection profiles observed in humans, which would help to predict the prognosis and severity of the disease and therefore, to improve treatment.

Thus, the specific **aims** of this project were the following:

1. To study the cellular and humoral immune response against a primary *P. yoelii yoelii* 17XL infection in the ICR mice strain.
2. To analyse the immune memory developed against this infection.
3. To examine the activity of the antibiotic borrelidin against *P. falciparum in vitro.*
4. To evaluate *in vivo* the effects of the borrelidin and mupirocin treatments against the *P. yoelii yoelii* 17XL infection in a standard malaria model in mice.
5. To study the humoral response developed upon borrelidin treatment of malaria-infected mice

4. TRABAJOS EXPERIMENTALES

Insights into the preclinical treatment of blood-stage malaria by the antibiotic borrelidin

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Running head: Preclinical antimalarial treatment with borrelidin

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SUMMARY

Background and purpose: Blood-stage *Plasmodium* parasites cause morbidity and mortality from malaria. Parasite resistance to drugs makes development of new chemotherapies an urgency. Aminoacyl-tRNA synthetases have been validated as antimalarial drug targets. We explored long-term effects of borrelidin and mupirocin in lethal *P. yoelii* murine malaria.

Experimental approach: Borrelidin and mupirocin treatments were evaluated for long-term immunological response upon an initial 4-days suppressive test. Prophylactic and curative properties were examined and the type of inhibitory effect on the parasites was further analyzed.

Key results: Borrelidin protected against lethal malaria at 0.25 mg kg^{-1} day^{-1}. Antimalarial activity of borrelidin concurred with accumulation of trophozoite in peripheral blood. All infected mice treated with borrelidin recovered from the disease and subsequently developed immunity that protected them from re-infection upon further parasite challenges 75 and 340 days after the initial infection. This long-term immunity in borrelidin-treated mice showed the features of near imperceptible parasitemia after re-infections and large increase in total serum levels of antiparasite IgGs with augmented avidity. Long-term memory IgGs mainly reacted against high and low molecular weight parasite antigens. Immunofluorescence microscopy revealed that circulating IgGs bound predominantly to late intracellular stage parasites, mainly schizonts.

Conclusions and implications: Low borrelidin doses protect mice from lethal malaria infections and lead to protective immune responses after treatment. The development of borrelidin regimes in combination therapies and selective modifications of the borrelidin molecule to specifically inhibit plasmodial threonil tRNA synthetase would eventually improve therapeutic strategies for malaria.

ABBREVIATIONS

ARS, aminoacyl-tRNA synthetases; Cq, chloroquine; $IC_{50,}$ 50% inhibitory concentration; IleRS, isoleucyl t-RNA synthetase; ip, intraperitoneal injection; iRBCs, infected red blood cells; NAI, naturally acquired immunity; pi, post infection; *Py17XL, P. yoelii 17XL;* ThrRS, threonil t-RNA synthetase.

INTRODUCTION

Among all parasite diseases, malaria causes the highest morbidity and mortality in the world. In countries where malaria is endemic, about 216 million cases were estimated in 2010 causing nearly 655,000 deaths, mostly in children under 5 years old and pregnant women. Global plans adopted to control, eliminate and ultimately eradicate malaria have mainly focused on prophylaxis, chemotherapy, vaccine development and vector control programs. Unfortunately, these strategies are being hampered by emerging parasite resistance to old and newly introduced drugs (Bloland 2001) so new long-acting antimalarial drugs are urgently needed for combination therapies. Parasite load, the innate host resistance to the infection (Stevenson and Riley 2004), the naturally acquired immunity and the capacity of the parasite to evade the host immune response (Doolan *et al.* 2009) are known to play an important role in the course of infection and in the outcome of the treatment. Although the acquisition of immunity against *Plasmodium falciparum* after a single infection can be generated, it is incomplete, non-sterilizing and transient, requiring repeated infections to be retained, and it is compromised in pregnant women and almost inexistent in children (Doolan *et al.* 2009). This condition provides clinical protection against new infections by maintaining a low-grade (Druilhe and Perignon 1997) and generally asymptomatic parasitemia in adults (Collins and Jeffery 1999). Since the naturally acquired immunity (NAI) is an efficient resource against severe disease or lethality in continuously malaria-exposed adults (Doolan *et al.* 2009), efforts directed to prophylactic interventions based on facilitating an efficient immunological response would eventually help to control malaria disease (Achtman *et al.* 2005). Following this goal, experimental inoculations of very small doses of intact sporozoites in volunteers during chloroquine treatment have shown to confer higher and longer levels of protection than vaccination with radiation-attenuated sporozoites (Hoffman *et al.* 2002; Roestenberg *et al.* 2009; Roestenberg *et al.* 2011). More than 30 years ago, the same combination of sporozoites and chloroquine was reported to be successful for the immunological protection of mice (Beaudoin *et al.* 1977). These studies highlight the potential of the inoculation of parasites combined with antimalarial treatment to favor the native exposure of antigens for the development of protective immunity (Sauerwein *et al.* 2010; Borrmann and Matuschewski 2011). In any case, it seems that this kind of immunoprotection cannot be induced in untreated malarial infections, since the high parasitemia level reached could impair the development of protective immunity (Ocana-Morgner *et al.* 2003; Wilson *et al.* 2006). Taken together, these data support the development of new antimalarial strategies based on sustained NAI by drug treatment.

To this respect, we focused on inhibitors of aminoacyl-tRNA synthetases (ARS), essential enzymes for cell viability that have been identified and validated as antimalarial drug targets (Schimmel *et al.* 1998; Hurdle *et al.* 2005; Istvan *et al.* 2011; Hoepfner *et al.* 2012). Mupirocin is an inhibitor of isoleucyl tRNA synthetase (IleRS) (Hughes and Mellows 1978) while borrelidin is an inhibitor of

prokaryotic threonil tRNA synthetase (ThrRS) (Hutter *et al.* 1966) and yeast cyclin-dependent kinase Cdc28/Cln2 (Tsuchiya *et al.* 2001) as well as an activator of eukaryotic caspase-3 and caspase-8 (Kawamura *et al.* 2003). Several pharmacological activities have been reported for borrelidin: antibiotic (Berger *et al.* 1949), angiogenesis inhibitor (Wakabayashi *et al.* 1997), anti-metastatic (Funahashi *et al.* 1999), antimitotic (Tsuchiya *et al.* 2001), antiviral (Dickinson *et al.* 1965), herbicidal and insecticidal (Dorgerloh *et al.* 1988) and antitumoral (Habibi *et al.* 2012). Both antibiotics, borrelidin and mupirocin, have been shown to display substantial *in vitro* inhibitory activity against *P. falciparum* with a 50% inhibitory concentration (IC_{50}) value in the nanomolar range (Otoguro *et al.* 2003; Istvan *et al.* 2011). In addition, recent data suggest that while mupirocin inhibits apicoplast-specific translation producing a delayed-death, the borrelidin effect is not restricted to an organelle-specific phenotype and promotes an immediate parasite growth arrest (Jackson *et al.* 2012). On the other hand, borrelidin has also antimalarial *in vivo* activity against *P. berghei* and *P. yoelii* ssp. when administered subcutaneous and orally, although its effect has been only studied during the first 4 days post-infection during a primary contact (Otoguro *et al.* 2003).

Here, we analyzed the effect of both borrelidin and mupirocin antibiotics administration during exposure of mice to lethal blood-stage malaria, focusing in the immune response acquired in comparison with the standard antimalarial drug chloroquine, a rapid parasiticide widely used in the past for human malaria treatment.

METHODS

Rodent parasites and animals

All animal care and experimental procedures carried out at the Universidad Complutense de Madrid complied with Spanish (R.D. 32/2007) and European Union legislation (2010/63/CE) and were approved by the Animal Experimentation Committee of this institution. The experiments here described involving animals are reported following the ARRIVE guidelines for pharmacological studies (Kilkenny *et al*., 2010). The rodent malaria parasite *P. yoelii* 17XL (*Py17XL*) was kindly provided by Dr Virgilio Do Rosario (Instituto de Higiene e Medicina Tropical, Universidade Nova

de Lisboa) and stored in liquid nitrogen after serial blood passages in mice. Inbred BALB/cAnNHsd pathogen-free female mice, aged 6–8 weeks and 16–18 g of weight were purchased from Harlan Laboratories (Italy) and housed at random in airy racks containing Lignocel® three-fourths bedding (Rettenmaier & Sohne, Rosenberg, Germany) and kept under constant standard conditions of light (12:12 h light : dark cycles), temperature (22–24°C) and humidity (around 50%) at the Animal

House of the Universidad Complutense de Madrid. All mice were fed a commercial diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories) *ad libitum*.

In vivo antimalarial activity

Firstly, the *in vivo* antimalarial activity of borrelidin (Fluorochem, Hadfield, UK), mupirocin (GlaxoSmithKline, Brentford, Middlesex, UK) and chloroquine (Sigma-Aldrich, St. Lois, MO, USA) was assessed using a 4-day suppressive test as previously described (Peters and Robinson 1999). Briefly, mice were inoculated with $2x10^7$ *Py17XL*-infected red blood cells (iRBCs) from infected mice by intraperitoneal injection (ip). The dose used for *in vivo* drug treatment was calculated based on the *in vitro* IC_{50} of each drug considering the toxicity and solubility data of each compound. Then, mice were daily treated for 4 days by ip injection – using a 30 G one-half needle under an approximately 10–15° angle – in the lower quadrant of the abdomen off midline. Borrelidin (0.25 $mg \cdot kg^{-1} \cdot day^{-1}$; n = 10), mupirocin (2.5 $mg \cdot kg^{-1} \cdot day^{-1}$; n = 10) or chloroquine in two doses (1 $mg \cdot kg^{-1} \cdot day^{-1}$; n = 5 or 30 $mg \cdot kg^{-1} \cdot day^{-1}$; n = 10), were used, starting 2 h after the infection. The tested drugs were prepared at appropriate doses in aqueous vehicle containing 7% Tween-80 and 3% ethanol. Control animals received aqueous vehicle by the same route (n = 5). Mice for each group were selected at random and treatments were carried out at the animal room. Parasitemia was monitored daily (between 9:00 and 10:00 h) by microscopy examination of Wright's-stained thin-blood smears using the Plasmoscore 1.3 software (Burnet Institute, Melbourne, Australia) (Proudfoot *et al.* 2008). To assess immunity against re-infection, cured mice were challenged with the same parasite dose 75 and 340 days after the primary infection and parasitemia was monitored for a further 30 days. Three independent experiments were conducted.

To study the curative properties of assayed drugs after parasitemia establishment, borrelidin (0.25 mg kg^{-1} day^{-1}) and chloroquine (30 mg kg^{-1} day^{-1}) were administered daily for 4 days beginning when blood parasitemia achieved 10% values (from day 3 pi to day 6 pi). Data from two independent experiments with n = 5 mice per group.

In vitro determination of antimalarial type of action

Drug activity assays were performed using *P. falciparum* strain Dd2 (clone MRA-150; Malaria Research and Reference Reagent Resource Center: http://www.mr4.org) which was maintained in continuous culture following the protocol previously described by Radfar *et al*. (Radfar *et al.* 2009). To determine the type of antimalarial activity, we used the previously described procedures (Bahamontes-Rosa *et al.* 2011) for drug exposure length and parasite culture intervals. Briefly, parasites in the ring stage were seeded at 1% parasitemia and 2% hematocrit and exposed for 48 h to borrelidin or chloroquine at 20 times the IC_{50} value (25 nM and 3 µM respectively) previously described (Moneriz *et*

al. 2009; Jackson *et al.* 2012). Parasites were then harvested and washed three times with 10 ml washing medium RPMI 1640 (Sigma-Aldrich, St. Lois, MO, USA) supplemented with 100 µM hypoxanthine (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich) and 12.5 µg/mL gentamicine (Sigma-Aldrich) to completely remove the drug from the cultured medium. Then they were cultured in culture medium (Radfar *et al.* 2009) without drug for a further 8 days. After drug withdrawal, culture medium was changed daily. Parasitemia and life cycle stages were monitored by microscopy examination of Wright's-stained thin-blood smears using Plasmoscore software. Four independent experiments were conducted.

Characterization of specific *P. yoelii 17XL* antibodies in mice serum

i. Extracting parasite proteins from infected whole blood

Py17XL protein lysates were extracted from the erythrocytes of infected mice showing > 50% parasitemia. Mice were anesthetized and whole blood was collected from the aorta into tubes containing ethylenediaminetetraacetic acid (EDTA) 0.1 M as anticoagulant and kept at -80ºC until protein extraction. Protein isolation began with erythrocyte lysis using 10 vol of saponin 0.1% (w/v) in phosphate buffered saline (PBS). After centrifugation (320 x g, 5 min, 4ºC) and washing twice in cold PBS, the pellet was treated with 2-3 vol of extraction buffer (50 mM Tris-HCl, pH 8.0; 50 mM NaCl; 0.5 % Mega 10) containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and subjected to four freeze-thaw cycles. Finally lysates were centrifuged (780 x g, 10 min, 4ºC) and total *Py17XL* protein samples stored at -20ºC until use. Protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

ii. IgG concentrations

Specific anti-*Py17XL* antibodies in sera from mice were quantified using mouse-immunoglobulin G (IgG) ELISA detection kits following the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX, USA). Briefly, microtiter plates were coated overnight with 100 µl/well of *Py17XL* protein extracts from iRBCs (described in subsection i) at 5 µg/ml in carbonate-bicarbonate buffered solution (Sigma). For specific IgG antibody quantification, diluted mouse serum was incubated for 1 h at room temperature (dilutions 1:150-1:800). IgG binding was detected with goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) at a 1:50,000 dilution. The enzymatic reaction was developed using 3,3´,5,5´tetramethyl benzidine (TBM) as enzyme substrate. Absorbance readings of reaction products were obtained at 652 nm in a Varian Cary 50 Bio spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Sera from naïve mice, uninfected drug-treated mice and untreated infected mice were used as negative controls. Purified myeloma-derived mouse IgG (Bethyl Laboratories) was used to generate a sigmoid logistic four-parameter standard curve.

iii. IgG avidity

To test antibody avidity, five different concentrations (0, 1, 2, 3 and 4 M) of the chaotropic agent NaSCN (Sigma-Aldrich, St. Lois, MO, USA) were used to independently disrupt antigen-antibody binding during the ELISA protocol described immediately above, as previously described (Pullen *et al.* 1986). *Py17XL* protein extracts from iRBCs (described in section i) were used as antigen. After the serum incubation step, wells were washed three times using PBS plus 0.05% Tween-20. Next, each NaSCN concentration was added to a different well. Plates were allowed to stand at room temperature for 15 min and extensively washed (x6) with PBS containing 0.05% Tween-20. Subsequent steps were performed as described above in the ELISA protocol. After incubation with various NaSCN concentrations, spectrophotometric readings at 652 nm were translated into percentage immunoglobulin binding with respect to values obtained at 0 M NaSCN. The avidity index is given as the NaSCN concentration value that produced a 50% reduction in immunoglobulin binding.

iv. Western blot analysis

10 µg of parasite proteins were fractionated on 10% SDS-PAGE (Bio-Rad) and transferred onto nitrocellulose membranes following standard procedures. Blots were blocked for 2 h in 5% non-fat skimmed milk in PBS and then incubated with mice serum antibodies (1:10,000) overnight. Anti-mouse IgG HRP linked (Amersham Bioscience, Buckinghamshire, UK) secondary antibody was incubated for 1 h at room temperature at a 1:5,000 dilution and the antigen-antibody reaction visualized using the SuperSignal chemiluminescent substrate (Pierce) and exposure to X-ray film. Sera from naïve mice, uninfected drug-treated mice and untreated infected mice were used as negative controls.

v. Immunofluorescence assay

To identify IgG antibodies specificity to intraerythrocyte stage parasites, thin-blood smears were prepared using iRBCs from mice at 40% parasitemia, and subsequently fixed in freshly prepared 90% acetone-10% methanol for 2 min. The parasites were blocked with 3% bovine serum albumin and 10% goat serum in PBS for 1 h at room temperature, and subsequently incubated with a 1:2,500 dilution of mouse serum in the same conditions. Finally, smears were incubated with goat anti-mouse IgG labeled with Alexa Fluor 488 (1:400) and DAPI (0.3 µM) for 1 h at room temperature, and then mounted according to standard procedures. Two different controls were established following the same procedures but substituting the primary antibody with the same volume of IgG serum from uninfected mice or with PBS. Also, since the acetone-methanol fixation method used in these immunofluorescence assays permeabilizes RBC membranes, additional staining controls were prepared to determine whether IgG also recognized surface antigens on the iRBCs. Thus, thin-blood smears were fixed in 4% paraformaldehyde (PFA) and permeabilized or not with 0.1% Triton X-100 (Sigma-Aldrich, St. Lois, MO, USA).

Labeling was detected by confocal immunofluorescence microscopy using a Leica CTR 6500 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Alexa Fluor (Invitrogen, Carlsbad, CA) was monitored by excitation with the 488-nm wavelength laser and DAPI (Invitrogen, Carlsbad, CA) were excited at wavelengths of 405 nm.

Statistical analysis

Data are presented as means ± standard errors of the means (SEM). Groups were compared using non-parametric Mann Whitney test. The statistical significance was set at $P \leq 0.05$.

RESULTS

***In vivo* antimalarial activity of borrelidin and mupirocin against lethal *P. yoelii 17XL* infection.**

Drugs were administered in mice the first four days following a primary contact with *Py17XL* infection. As shown in figure 1, borrelidin was the only antibiotic successful at curing lethal malaria infection in mice comparable to the positive control of chloroquine 30 mg/kg (chloroquine-30). In contrast, individuals treated with mupirocin or low chloroquine dosage (1 mg/ kg) were unable to halt parasite growth which was detectable from day 2 pi and subsequently caused their death by days 4.2 ± 0.1 and 5 ± 0.3 pi respectively, in similar trend to untreated mice (death on day 4.6 ± 0.3 pi). All mice treated with borrelidin and standard chloroquine dose (30 mg/kg) regimes inhibited parasite growth during the 4 days of its administration (<1% parasitemia in peripheral blood) ($P < 0.0001$ between parasitemia of untreated and both, chloroquine-30 and borrelidin treated mice at day 3 pi). We found that the ED_{90} value for borrelidin was around 0.25mg/kg against the lethal *P. yoelii* strain 17XL, slightly lower than for other *P. yoelii* strains (Otoguro *et al.* 2003). Different effects on the control of parasitemia were observed after borrelidin and chloroquine-30 treatments withdrawal at day 4 pi. Whereas in borrelidin-treated mice parasitemia values started to rise during the subsequent days up to a maximum of 35% attained on day 11 pi, the parasite growth was notably reduced in chloroquine-30 treated mice, which reached a peak below 10% at day 10 pi ($P < 0.0001$ between parasitemia of chloroquine-30 and borrelidin treated mice at day 10 pi). Thereafter, a progressive decrease was observed until no parasites could be microscopically detected at day 12 pi in chloroquine-30 treated mice and significantly later in borrelidin treated mice around day 20 pi ($P = 0.0003$).

Parasite stages distribution in treated *P. falciparum* cultures

The two antimalarial drugs that were proficient *in vivo* to cure lethal malaria in mice were also assayed *in vitro* on the drug-resistant *P. falciparum* strain Dd2. Following the approaches used in previous drug activity studies, the cultures were exposed to borrelidin and chloroquine for 48 h (one complete parasite life cycle) and then cultured along 4 subsequent life cycles (8 additional days) (Figure 2) (Goodman *et al.* 2007; Bahamontes-Rosa *et al.* 2011). The concentration used was 20-fold the corresponding IC_{50} values of borrelidin and chloroquine for *P. falciparum* Dd2.

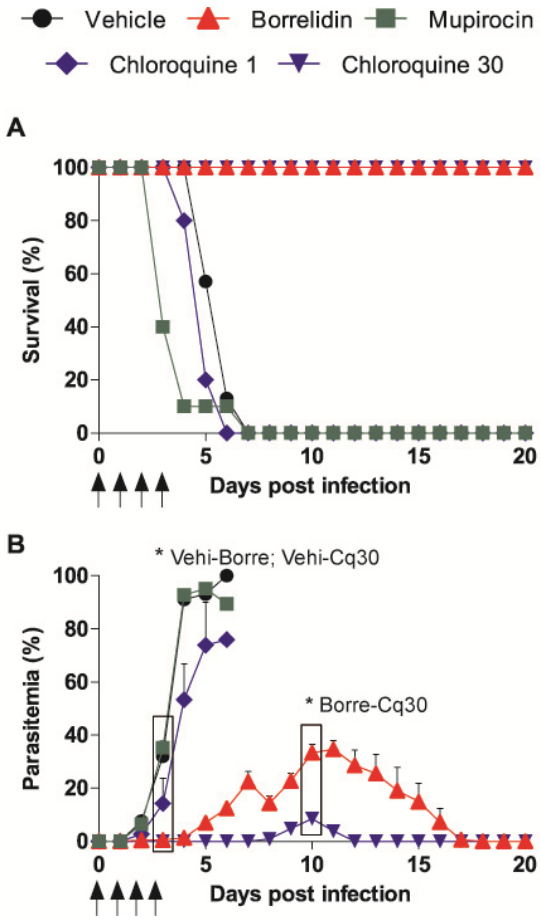

Figure 1. Survival and parasitemia course in infected mice subjected to 4-days suppressive antimalarial treatment. BALB/c mice infected with 2 x 10^7 *P. yoelii 17XL* iRBCs were treated for 4 days with vehicle (black ●) (n = 5),1 mg kg^{-1} day^{-1} chloroquine (blue ♦) (n = 5), 30 mg kg^{-1} day^{-1} chloroquine (blue ▼) (n = 10), 25 mg kg^{-1} day^{-1} borrelidin (red ▲) (n = 10) or 2.5 mg kg^{-1} day^{-1} mupirocin (green ■) (n = 10). (**A**) Survival and (**B**) parasitemia percentages are shown for each group as mean ± SEM. Arrows indicate the four ip injections of drug/vehicle. Data from three independent experiments. * $P < 0.05$, significant differences between indicated groups.

Microscopic observation of the treated cultures (Figure 2A) revealed a reduced parasite growth after 48 h treatment with borrelidin (19-fold) and chloroquine (> 100-fold) compared to control culture (P = 0.034; P = 0.016 respectively) with abundant presence of shrunk forms of the parasite cell. These remaining parasites treated with borrelidin struggled throughout the 4 following days to recover their viability, which was only partially regained after 240 h (4 parasite life cycles) (P = 0.05). Analysis of intraerythrocytic stages distribution after antibiotic withdrawal in survival parasites showed a high percentage of ring forms (> 75%) (P = 0.028 after 72 and 96 h) suggesting a specific effect of borrelidin on mature stages (Figure 2B). Remarkably, the antibiotic treatment also induced an altered cellular development in a fraction of surviving early-stage parasites which seems to lead to death in the following schizogonic cycles (72-144 h) (Figure 2C). Control cultures followed a normal growth during the first 3 life cycles. Afterwards, the high parasite growth did not allow new invasion cycles without the addition of new erythrocytes and consequently the conditions in cycle 4 and 5 were not comparable.

Parasite stages distribution in borrelidin treated mice

To follow potential changes in the typical asynchronic *P. yoelii* infection, analysis of parasite stages distribution was performed in the iRBCs from the different groups of treated mice from days 0 to 10 pi by microscopic examination of thin-blood smears. Control mice and mupirocin-treated mice displayed identical *P. yoelii* asynchronicity (Figure 3A, B). Both groups showed a high proportion of ring-stage parasites during the first 2 days of increasing parasitemia while mature forms (trophozoite/schizonts) were mainstream in the last 2 days before death. This observation is in agreement with the 22-25 hours required to complete the P. *yoelii* infection cycle in the erythrocyte. In the first two days, when parasitemia was not too high, healthy schizonts containing merozoites were able to continue invading any intact RBCs that were still available. However, when erythrocytes were in short supply because of the high parasitemia level (more than 80%), merozoites could not easily find red cells to generate new rings and therefore the remaining mature forms, including schizonts, were the predominant observed forms (P = 0.01 schizont vs rings at day 5 pi). Similar results were found in mice treated with sub-therapeutic chloroquine doses (data not shown). The borrelidin-treated mice showed a different picture (Figure 3C). Thus, at day 3 pi, mice treated with borrelidin showed a significant dominance of trophozoite-stage parasites (86.6 ± 8.2%) (P = 0.002) that doubled the corresponding forms in the untreated (33.6 ± 4.2%) (P = 0.004) or in the mupirocin-treated (34.8 ± 3.3%) (P = 0.004) groups. Conversely, at this time, ring-stage parasites in borrelidin treated animals (22.2 ± 11.1%) decreased by around 2.5-fold compared to untreated (52.9 ± 1.4%) and mupirocin-treated (57.8 ± 3.7%) mice (P = 0.06). In agreement with the above figures, borrelidin-treated vs. untreated or mupirocin-treated mice also showed differences in schizont-stage parasite percentages. In the borrelidin treated mice trophozoites persisted as the most abundant form until day 10 pi (P < 0.05 trophozoites vs

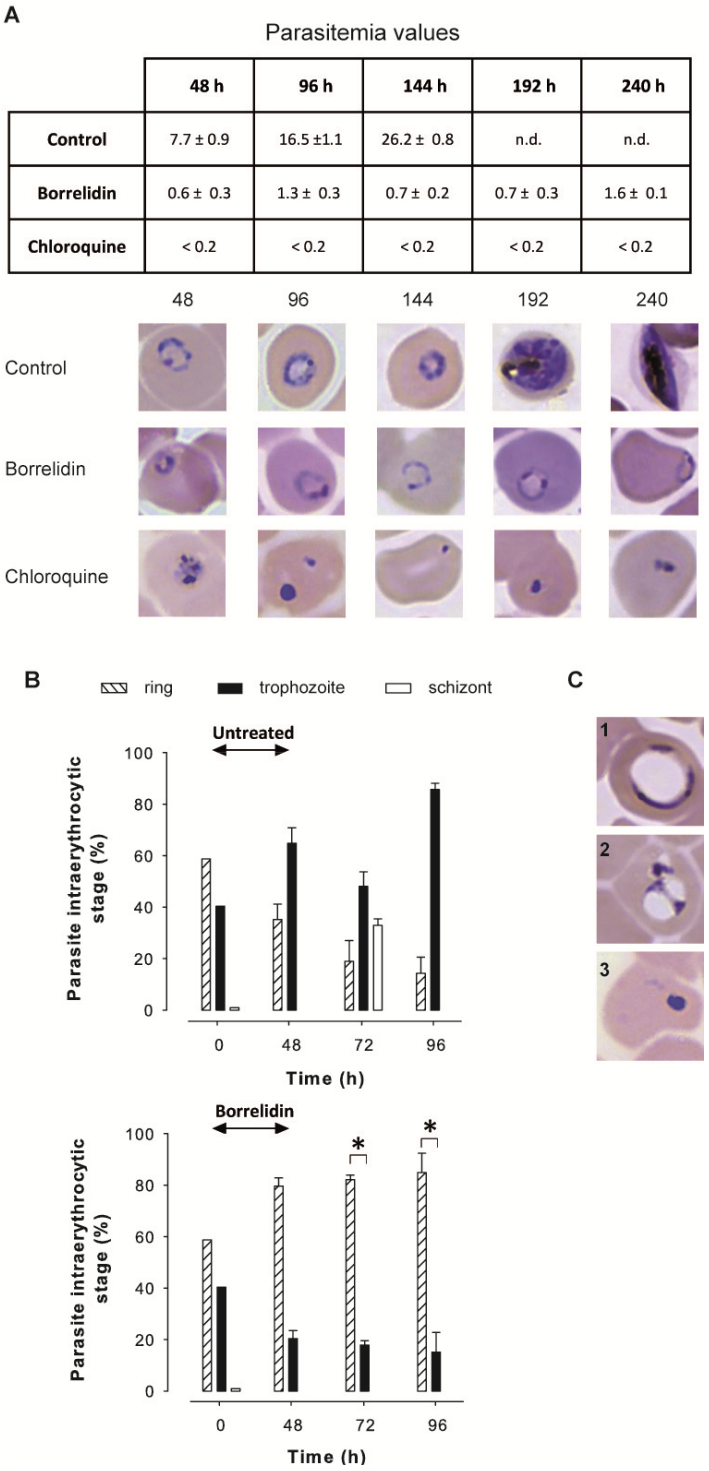

Figure 2. *In vitro* antimalarial activity in a 240 h growth assay. *P. falciparum* Dd2 parasites were treated with borrelidin (25nM) or chloroquine (3 µM) for 48 h and then were further cultured in medium in the absence of the drugs. (**A**) Variation of parasitemia and parasites images and (**B**) percentages of parasite intraerythrocitic stages show the morphology and evolution of the parasites at the different time points over 96 h with respect to the control. Bars show the percentages of ring- (hatched), trophozoite- (black) and schizont-stage (white) parasites in iRBCs. (**C**) Representative images of altered borrelidin-treated parasites. Results are expressed as the mean ± SEM in two independent experiments. n.d. = not determined parasitemia in control cultures due to the saturation of parasites after two cycles that made growth not comparable to the drug-treated cultures. Four independent experiments were conducted. * $P < 0.05$.

schizonts at days 7, 8, 9 and 10 pi; and trophozoites vs rings at day 7 and 10 pi), when parasitemia levels peaked.

Long-term immunity in cured mice

To ascertain whether the borrelidin treatment influences the development of protective malaria immunity to re-infection, cured mice were re-infected on day 75 pi and parasitemia was monitored for the following 30 days. The mice cured with the chloroquine-30 regime were also re-infected and used for comparison. As shown in figure 4A, all the borrelidin-treated animals showed long-term full protection against the second lethal infection that was lethal to vehicle controls. In fact, blood parasitemia levels (lower than 0.01% in most mice) were barely perceptible (Figure 4B). A lower protection was obtained in chloroquine-30 treated animals.

After the secondary challenge, parasite growth was also transient and maintained at extremely low rates (< 0.01%) with the exception of one mouse corresponding to the only one that during the primary infection did not show microscopic parasitaemia. This mouse was unable to control re-infection dying on day 84 pi. Interestingly, parasitemia in this mouse began to be detected on day 79 pi (0.2%) whereas control mice of the same age exhibited a mean value of 75% parasitaemia by this day. Both borrelidin and chloroquine-30 cured mice from this re-infection were challenged again on day 340 pi, and all of them showed full protection (Figure 4A, B).

Protective humoral immune response acquired in cured mice

To explore the humoral immune response developed after borrelidin or chloroquine-30 treatments, we determined the concentrations of specific IgGs in sera obtained once the blood parasitemia had been cleared after each infection on days 21, 85 and 350 pi. The low specific IgG levels detected in borrelidin after the first infection underwent a significant increase (P = 0.004) after the second challenge, and these levels persisted after the third antigenic challenge (P = 0.024) (Figure 5A). Similar results were obtained in chloroquine-30 group (Figure 5A). Parasite-specific IgG antibodies in sera from naïve mice taken before first infection (day 0) and from untreated deceased mice taken on day 4 pi, were undetectable.

Qualitative traits of humoral response during consecutive infections were studied by measuring the antibody avidities, defined as the strength with which an antibody binds to an antigen (Goldblatt *et al.* 1998). Avidity was determined by ELISA after treatment with several concentrations of NaSCN, a chaotropic agent that disrupts antigen-antibody interactions (Figure 5B).

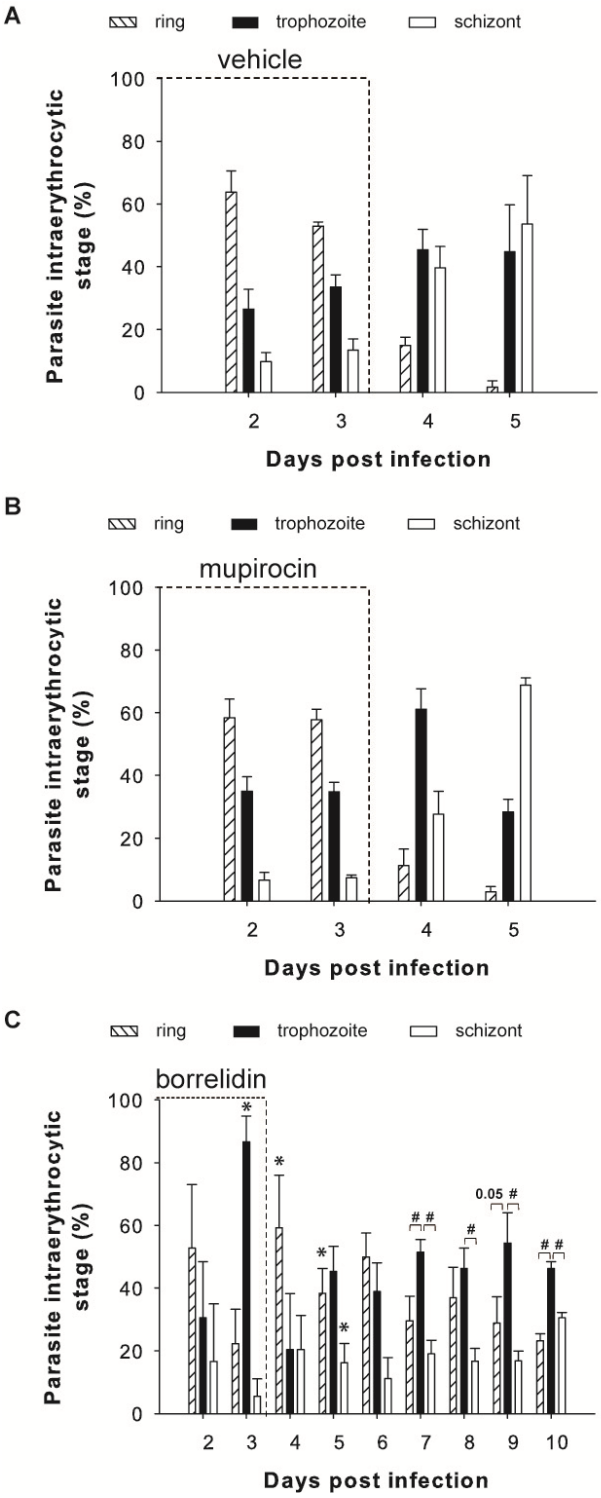

Figure 3. Distribution of intraerythrocytic parasite stages in blood samples from treated and untreated mice. Bars show the percentages of ring- (hatched), trophozoite- (black) and schizont-stage (white) parasites in iRBCs from day 2 to 5 pi in (**A**) vehicle-injected (n = 5) and (**B**) mupirocin-treated mice (n = 10) or (**C**) to day 10 pi in borrelidin-treated mice (n = 10). Data were obtained by microscopy inspection of Wright's-stained thin blood smears. Results, expressed as the mean ± SEM, represent the percentages of cells of each parasite stage among a total of 500 erythrocytes per slide. Three independent experiments were performed. * $P < 0.05$, significant differences versus same parasite form in vehicle-injected mice; # $P < 0.05$, significant differences between parasite forms in borrelidin-mouse group.

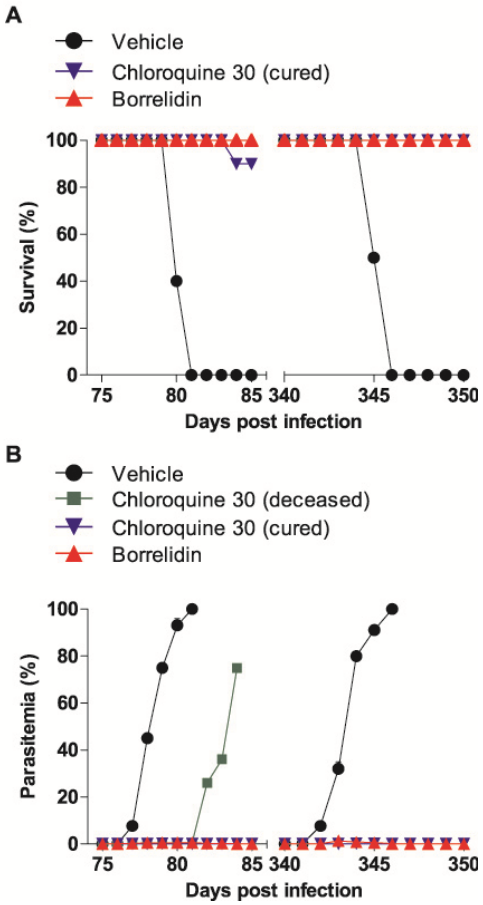

Figure 4. Survival and parasitemia course of the borrelidin and chloroquine-treated mice in response to a second and third re-infection. Borrelidin and chloroquine-treated mice (n = 10) surviving primary infection were re-infected twice on days 75 and 340 pi with the same doses of *P. yoelii* 17XL and (**A**) survival and (**B**) parasitemia were then daily monitored for 30 days pi. Naïve infected mice (black ●) (n = 5), chloroquine 30-cured (blue ▼), chloroquine 30-deceased mice (green ■) and borrelidin-treated group (red ▲) are shown. Results are expressed as mean ± SEM.

Thus, low avidity antibody binding is disrupted at lower NaSCN concentrations than high avidity binding. Antibody avidity (AI) was displayed as an avidity index corresponding to the molar concentration of NaSCN at which 50% of the bound antibodies is eluted off. As shown in figure 5B, in borrelidin-treated animals the avidity index increased significantly from the primary infection (AI = 0.88 M) up to 2-fold in the second (AI = 1.83 M; *P* = 0.036) and 1.6-fold in the third challenges (AI = 1.44 M). From the second to third challenge, a small but significant decrease in the IgG avidity index was detected (*P* = 0.036).A similar trend was observed in chloroquine-30 group (Figure 5B). Clear avidity maturation occurred during the second infection (AI = 1.69 M; 2-fold increase), but some avidity dropped also in the third infection (AI = 1.02 M on day 350 pi). Thus, although the avidity of the specific IgG response substantially increased after the second infection, without parasite boosting for a long period of time this response can slightly decay no matter the treatment applied.

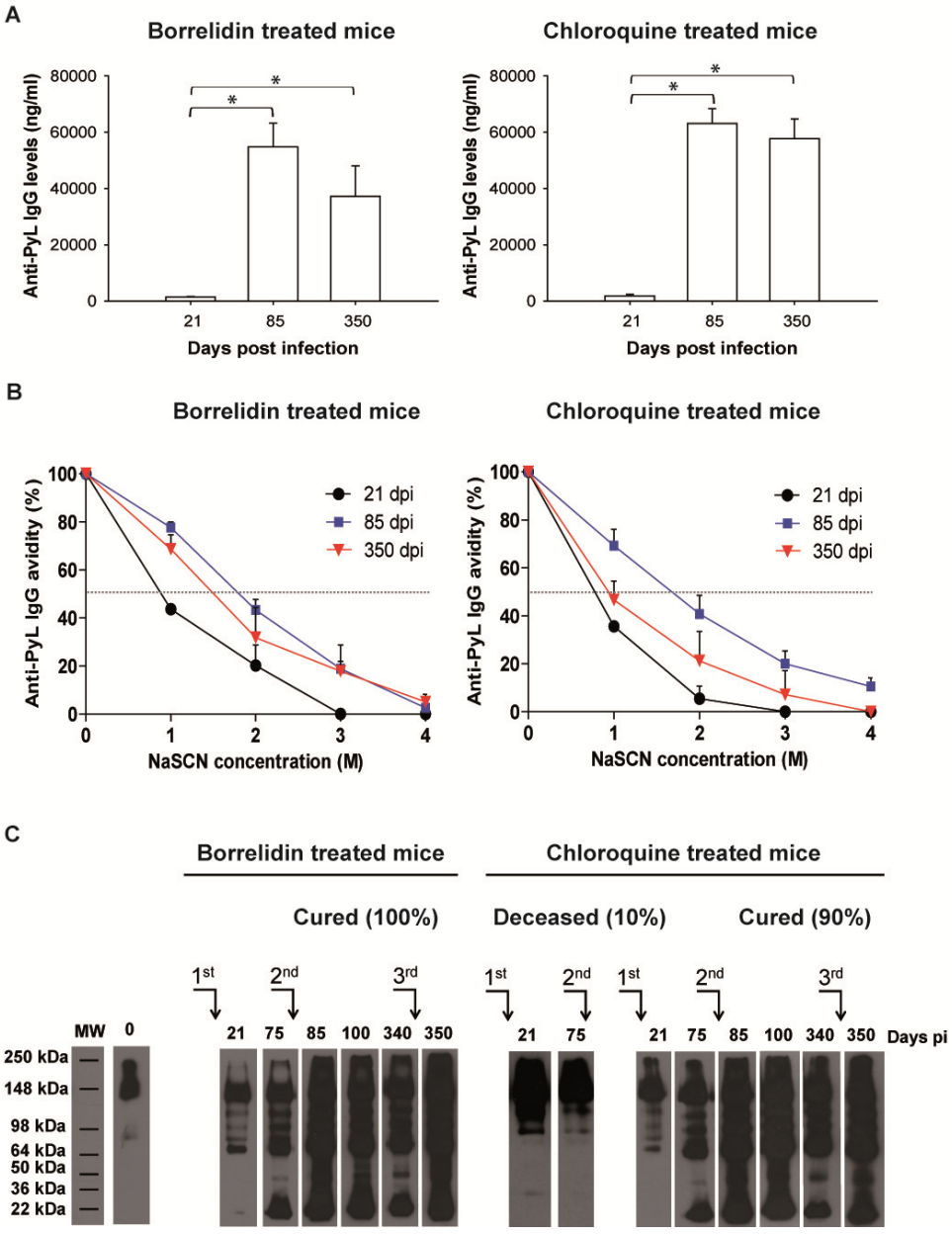

Figure 5. Parasite-specific IgG responses in borrelidin and chloroquine 30-treated mice. (**A**) Anti-*P. yoelii* IgG concentration in sera obtained from the differently treated mice after total clearance of the parasite from the first (21 dpi), second (85 pi) and third (350 pi) infections. n = 6 samples of each experiment were measured in duplicate by ELISA. (**B**) Anti-*P. yoelii* IgG avidity values of sera (n = 3-6) after total clearance of the parasite from the first (21 pi) (black ●), second (85 pi) (blue ■) and third (350 pi) (red ▼) infections. Data are shown as mean ± SEM. Avidity of n = 3 samples of each experiment were determined in duplicate using NaSCN in ELISA. (**C**) Time course immunoblot analysis of anti-*P. yoelii* IgG in sera from borrelidin- treated mice**.** Total protein extracts (10 µg) from *P. yoelii* were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and developed with sera collected during the three infections. Arrows indicate the time of infection/re-infection. MW: molecular weight markers.

Immunoblot analysis of total *P. yoelii* proteins was performed using the sera from different infection time-points. The profile of immunodetected parasite proteins in cured mice after both borrelidin and chloroquine-30 treatments revealed a progressive increase in specific IgG levels, whose

signal was boosted after each infection (days 21, 85 and 350 in Figure 5C). Moreover, IgG antibodies recognized an ever-wider range of parasite antigens as the number of re-infections increased. Strongest signals were detected in the immunogenic profiles in the molecular weight ranges 22-36, 64-98 and 140-150 kDa. Antibodies against parasite proteins in the 36-64 kDa range only appeared after re-infections and were less stable without parasite exposure as it is shown by their notable decrease after the second challenge on day 340 pi. In contrast, a reduced variety of antigenic proteins were recognized with the serum from the chloroquine-30 treated mouse that died after secondary infection (Figure 5C), indicating the development of an immune response enough to delay the parasite growth but not to guarantee the mouse survival (Figure 4). Serum from naïve mice, taken before first infection, showed an unspecific reaction with some high molecular weight proteins of iRBCs. Similar results were obtained with sera from uninfected borrelidin-treated mice and untreated deceased mice (data not shown).

Specificity of protective IgG response against intraerythrocytic parasite stages

To identify the intraerythrocytic parasite stages bound by the specific IgGs presented in the sera of protected mice, immunofluorescence microscopy analysis was performed on blood smears of iRBCs (Figure 6). Mature stages were identified by DAPI fluorescence of the nucleus (in blue) since during parasite growth, the nucleus divides and the resulting nuclei are transferred to merozoites before their release (Matteelli *et al.* 1997). The images obtained showed that specific IgGs (in green) from borrelidin and chloroquine-30 cured mice from day 85 pi preferentially bind to late parasite stages and 100% of the schizonts and merozoites are recognized by these antibodies (Figure 6B and 6C, images 2, 3 and 2´, 3') Early parasite stages exhibited a weak immunostaining (Figure 6B and 6C, images 1 and 1´). The binding of IgG antibodies to iRBCs was observed only when the iRBC membranes were permeabilized (data not shown). Preimmune sera under the same experimental conditions did not show any signal (Figure 6B).

Curative properties of borrelidin against lethal blood-stage malaria

As the drug-treatment which follows a classic 4-day suppression test begins just two hours after infection, it reflects the prophylactic properties of drugs. Thus, to study the curative drug effects a 4 days drug-treatment of mice starting when mice showed around 10% of parasitemia (day 3 pi) was carried out. Borrelidin was slower at decreasing parasitemia rates than chloroquine (Figure 7B) and the infection caused the death of 25% of treated mice (Figure 7A), although with parasitemia levels less severe than in untreated mice. Parasitemia peaks in both groups were similar to those achieved after the 4-days prophylactic test (Figure 1B). All survivor mice were re-infected on day 135 pi and successfully overcame the infection (data not shown).

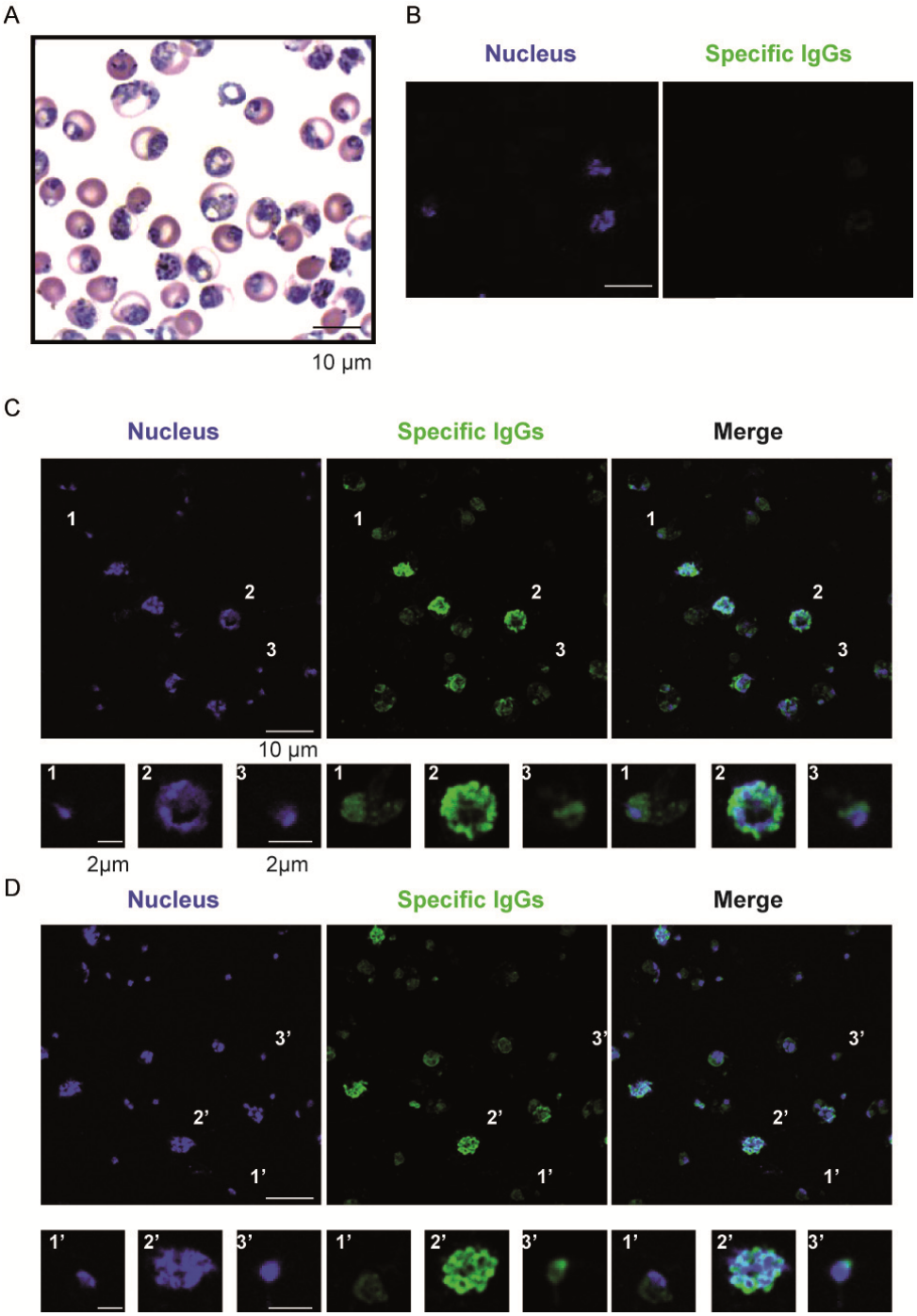

Figure 6. Immunofluorescence microscopy analysis of IgG specificity against blood-stage *P. yoelii* infection. (**A**) Thin-blood smears showing high parasitemia from untreated *P. yoelii* infected mice. (**B-D**) Fixed thin-blood smears showing high parasitemia from untreated *P. yoelii* infected mice were stained with DAPI (blue) to identify parasite DNA and incubated with different sera. (**B**) No signal was obtained when control preimmune serum was used. (**C**) Borrelidin-treated mice serum (1:2500) or (**D**) chloroquine 30-treated mice serum (1:2500) from day 85 pi was detected by Alexa Fluor 488-labeled anti-mouse IgG (green) (1:400). Double staining of DAPI and Alexa Fluor 488-labeled anti-mouse IgG shows the co-localization of late stage intraerythrocyte parasites with antibody binding. Images are representative of n = 3 duplicate samples from experiments. *Scale bars*, panels C-D: 10 µm. Images 1 and 1' are representative of early parasite stages; Images 2 and 2' show the presence of stained schizonts; Images 3 and 3' correspond to *P.* yoelii stained invasive forms (merozoites). *Scale bars*, panels 1-3 and 1-3´: 2 µm.

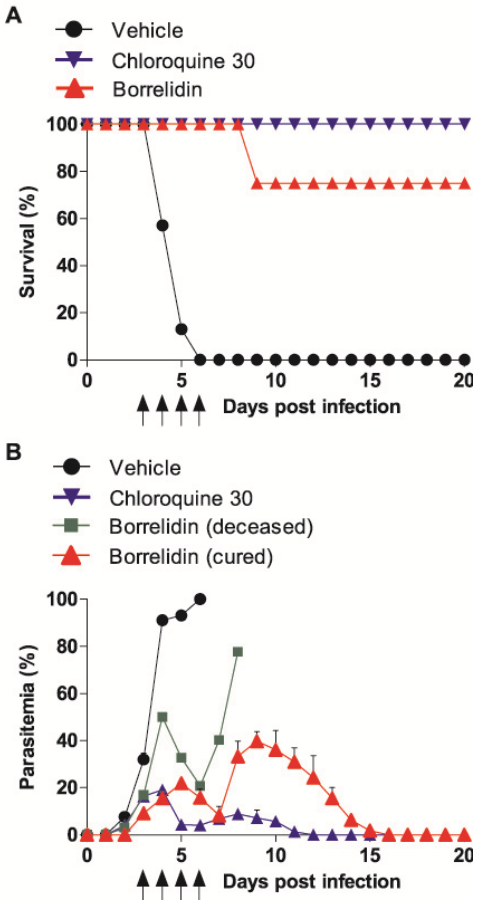

Figure 7. **Survival and parasitemia course in infected mice with 10% parasitemia subjected to antimalarial treatment**. BALB/c mice infected with 2 x 10^7 *P. yoelii 17XL* iRBCs acquired 10% parasitemia (monitored by microscopic examination) prior to be treated during 4 days with vehicle (black ●), 30 mg kg^{-1} day^{-1} chloroquine (blue ▼) or 0.25 mg kg^{-1} day^{-1} borrelidin (red ▲). Deceased borrelidin-treated mice are also labeled (green ■). Mean (**A**) survival and (**B**) parasitemia percentages are shown for each group. One representative experiment is shown (n = 5). Arrows indicate the four ip injections of drug/vehicle.

DISCUSSION AND CONCLUSIONS

Our results demonstrate that prophylactic administration of borrelidin during the exposure to live blood-stages of lethal *P. yoelii* 17XL is enough to halt the infection and protect mice from death. In contrast, mupirocin was unable to control primary malaria infection *in vivo* which could be attributed to its rapid hydrolysis in blood plasma, its binding to serum and the decrease of biological activity at pH near 8 (Thomas *et al.* 2010) and thus, by now it is clinically restricted to topical use (Sutherland *et al.* 1985). Remarkably, a borrelidin concentration 120-fold lower compared to chloroquine showed the same efficiency according to surviving rate after primary infection. Both borrelidin and chloroquine induced a fast inhibition of *P. falciparum* growth *in vitro* within the first 48h confirming a not delayed-

death effect (Jackson *et al.* 2012) usually attributed to antibiotics that inhibit prokaryote translation (Barthel *et al.* 2008). These results are also consistent with previous data describing the stage specificity of borrelidin to trophozoite and schizont stages (Ishiyama *et al.* 2011; Jackson *et al.* 2012) probably because the ARS maximal expression happens in mature life cycle stages (Jackson *et al.* 2012). Remarkably, although we used borrelidin at a 5-fold concentration and for the double of time than in previous studies (Ishiyama *et al.* 2011; Jackson *et al.* 2012), a fraction of *P. falciparum* parasites at ring and young trophozoite stages were capable of survive and resume the multiplication in the 5th cycle. Therefore, the effect of borrelidin on ring blood-stage parasites would better fit with a static activity as they suffered growth arrest that was recovered after the drug withdrawal in contrast to mature stages that would be susceptible of a cidal activity which leads to a swift death (Bahamontes-Rosa *et al.* 2011). However, since aberrant parasite forms were also found in the two following life cycles to antibiotic withdrawal, the normal development of a fraction of surviving early-stage parasites can also be affected. This altered development is also observed after other antibiotic treatments (Barthel *et al.* 2008). Results derived from *in vivo* assays also may support a static activity for borrelidin, which is a typical effect of ARS inhibitors (Critchley *et al.* 2005). The examination of intraerythrocytic parasite stages along borrelidin treatment in mice during *Py17XL* infection reflected an accumulation of trophozoites in peripheral blood at the end of the treatment (day 3 pi). This accumulation could be attributed not only to the *in vitro* effect of borrelidin on mature stages which prevent the appearance of new rings (Ishiyama *et al.* 2011), but also on the development of rings and young trophozoites which instead of completing the cycle from day 2 to 3 pi were delayed in our *in vivo* assays and only grew until the trophozoite stage.

Besides direct antimalarial activity, our results showed that borrelidin treatment sustained protective humoral response during a primary infection. All borrelidin treated mice were capable of controlling parasitaemia and subsequently they developed an efficient immune response with the production of specific antibodies that completely eliminated parasites after re-infection. Static compounds seem to allow the host immune system to participate in the battle against infection by increasing the period of antigen presentation (Scholar and Pratt 1939), which is particularly important to develop malaria immunity (Urban *et al.* 2005; Amante *et al.* 2011). Currently, some of the antimalarial drugs in use such as atovaquone or pyrimethamine have static activity (Bahamontes-Rosa *et al.* 2011) and their use, combined with other compounds, is recommended by the World Health Organization (World-Health-Organization 2010). The treatment with the antibiotic borrelidin allowed a robust humoral response that 100% prevented from subsequent lethal infections in all animals. Chloroquine cidal activity led to a protective humoral response in 90% of mice after first challenge similar to the borrelidin-treated mice group, but 10% of mice died during re-infection. Thus, antimalarial treatments or doses that may provide rapid elimination of parasites in blood would eventually reduce the residence time of native parasite antigen for the efficient presentation to the immune cells. This could be the case of the single chloroquine 30-treated mouse that died in one of the re-infection experiments, which in

turn diminished malaria parasite recognition by the raised antibodies as it was shown in its corresponding immunoblot.

Experimental inoculations of malaria in humans and mice have demonstrated that a drug controlled exposure to blood- or liver-stage parasites can result in protection (Pombo *et al.* 2002; Roestenberg *et al.* 2009; Friesen *et al.* 2010; Sauerwein *et al.* 2010). In mice, treatments that maintain the chronicity of the infection show a high surviving rates after re-infection, whereas mice receiving radical treatments that completely abolish parasite multiplication, die in a second challenge (Long *et al.* 2002). Moreover, parasite levels seems to be very important since ultra-low presence of blood-stage *P. falciparum* in volunteers is not capable of eliciting antibodies, but induce efficient cell-mediated immunity (Pombo *et al.* 2002). Any efficient malaria immune response is complex and involves several cell and humoral factors. Cell-mediated immune mechanisms are fundamental to the control of the first wave of infective *Plasmodium* parasites (Achtman *et al.* 2005). Conversely, the particular importance of antibodies in malaria immunity has been shown by serum transfer experiments in humans (Cohen *et al.* 1961) and mice (Jayawardena *et al.* 1978).

Thus, the generation of immunological memory in the borrelidin and chloroquine-30 treated mice is supported by the robust antibody response in the re-infections, the presence of switched specific antibodies during 9 months of barely detectable parasitemia after a 2^{nd} and 3^{rd} re-infection (Kinyanjui *et al.* 2004; Achtman *et al.* 2007; Weiss *et al.* 2009) and by the increase in specific IgGs avidity after re-infections (Berek 1993). The decrease of antigen-IgG binding strength observed after the 3^{rd} infection was more evident in chloroquine than borrelidin-treated mice and could reflect a progressive loss of immune response after the absence of parasite contact during 9 months as it has been described in humans (Linares *et al.* 2011). These data are also in agreement with non-lethal *Plasmodium* re-infections in mice that do not increase antibody avidity at long-term (Bull *et al.* 2002). In addition, in both treated mouse groups the repertoire of *Py17XL* antigens recognized by the specific IgGs raised was also amplified after each re-infection, similarly to the acquired malaria immunity in humans which is likely to depend on the accumulation of a wide repertoire of antigenic specificities over a long time (Kinyanjui *et al.* 2004) and which parallels with a gradual gaining of clinical immunity (reviewed in (Bull *et al.* 2002)). After both treatments, mice showed IgG binding to merozoite antigens and internal antigens of *Py17XL* iRBCs, as recently some of them have been identified in a similar experimental set (Kamali *et al.* 2012). Since internal antigens are only exposed in disrupted target cells they are detected as secreted antigens which seems to induce antibody responses more efficiently than membrane and cytoplasmic antigens due to an enhanced ability to reach the lymph node (Boyle *et al.* 1997). However, antibodies to intracellular proteins are usually considered markers of past infection and could only indicate an increased parasite killing. There are some exceptions of human and rodent antibodies with reactivity to intracellular antigens of *Plasmodium* or other parasites which can induce humoral protection (Vedi *et al.* 2008; Crompton *et al.* 2010). Thus, given that the immunogenic antigens encoded by the parasite are

largely unknown (Langhorne *et al.* 2008), we do not discard that some intracellular *Plasmodium* antigens released into the iRBCs might cooperate in immune protection.

Interestingly, IgGs binding to antigens in the medium 36-64 KDa range vanished after infection in all mice. (Mota *et al.* 2001; Pombo *et al.* 2002; Elliott *et al.* 2005)Similarly, human antibody patterns in seasonal malaria transmission show strong preference towards high MW antigens (Thelu *et al.* 1991) and IgGs from mice suffering non-lethal malaria infections recognize more trophozoite or schizont- than ring-infected erythrocytes (Mota *et al.* 1998).

Finally, although borrelidin prophylactic administration turned out to be more effective in protecting mice from lethal malaria than a therapeutic dosage applied when the acute infection was already established, it can be expected that further research for the improvement of the borrelidin regime in combination therapies and towards chemical modifications of the borrelidin polyketide molecule, would eventually generate better therapeutic strategies and more selective analogs for inhibiting ThrRS from *Plasmodium* species, respectively.

In conclusion, our results provide new insights into the potential use of borrelidin as antimalarial drug and contribute to validate ThrRS as a target for prophylaxis or therapy against malaria. We show that a low borrelidin dose treatment has parasite-stage specific actions and leads to develop a robust long-term protective response in 100% of treated animals.

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Differential immune response associated to malaria outcome is detectable in peripheral blood following *Plasmodium yoelii* infection in mice

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SUMMARY

Malaria infection in humans elicits a wide range of immune responses that can be detected in peripheral blood, but we lack detailed long-term follow-up data on the primary and subsequent infections that lead to naturally acquired immunity. Studies on antimalarial immune responses in mice have been based on models yielding homogenous infection profiles. Here, we present a mouse model in which a heterogeneous course of *Plasmodium yoelii* lethal malaria infection is produced in a non-congenic ICR strain to allow comparison among different immunological and clinical outcomes. Three different disease courses were observed ranging from a fatal outcome, either early or late, to a self-resolved infection that conferred long-term immunity against re-infection. Phenotypic changes produced in different leukocyte populations, cytokine profiles and specific humoral responses detected in peripheral blood revealed that monocytes, dendritic cells and immature B cells were the main cell subsets present in highly-parasitized mice and $CD4^+CD25^+$ T cells expanded at an earlier time point than in surviving mice. In contrast, survivors showed a more controlled production of a panel of cytokines and mostly stable circulating innate cells followed by the expansion of activated circulating T cells and switched-class B cells with a long-term protective humoral response. Our findings prompt the examination of circulating markers of protection during malaria infection that have implications for preclinical studies on antimalarial vaccines and treatments.

ABBREVIATIONS

Ab, antibody; Ag, antigen; ASC, Ab-secreting cells; DCs, dendritic cells; ED, early deceased mice; FO, follicular; FSC-SSC, forward scatter-side scatter gate; iRBCs, infected red blood cells; i.p., intraperitoneal; LD, late deceased mice; Max, maximum; MZ, marginal zone; PB, peripheral blood; pi, post-first infection; *PyL, P. yoelii yoelii* 17XL; *PyNL, P. yoelii yoelii* 17XNL*;* RT, room temperature; S, surviving mice; SEM, standard error; T1, transitional 1; T2, transitional 2; Tregs, T regulatory cells; WBC, white blood cells.

INTRODUCTION

The pathophysiological mechanisms that lead to a given outcome in malaria patients are thought to be influenced by epidemiological and immunological factors (Doolan *et al.* 2009) along with the mechanisms of immune evasion evolved of the parasite (Hisaeda *et al.* 2005). Natural acquired immunity against *Plasmodium falciparum* is incomplete, non sterilizing and can be progressively acquired only after years of repeated infection in adults, but generally not in pregnant women or young children, and does not persist over long periods of time (Doolan *et al.* 2009). In the immune response to malaria, innate mechanisms are able to limit parasite density (Stevenson and Riley 2004), but Abs and T cells are required to completely eliminate blood-stage parasites. APCs are particularly important to activate T CD4 cells which fight against the parasite by producing inflammatory cytokines which activate other cells such as macrophages and helping B cell activation to produce Abs (Good and Engwerda 2011). These Abs have a protective role in malaria (Cohen *et al.* 1961) and act by blocking merozoite invasion, by cell-Ab cooperation or by inhibiting cytoadherence of schizont- infected RBCs (iRBCs). Peripheral blood (PB) sampling has so far been the main provider of information on human immune responses against malaria since it is the only readily accessible source of leukocytes. However, WBC may not reflect the global response to malaria since the activated cells during the infections may appear in secondary lymphoid organs. Hence, a better understanding of measurable immune system cells and proteins in PB could help identify malaria clinical states in humans. Although studies in animal models have provided useful information on the about protective immunity to malaria, most rodent malaria studies have examined lymphoid organs rather than circulating cells because of the large quantity of cells available in these organs. This determines that the extrapolation of experimental data to the human response to infection is not straightforward. A wide variety of host-parasite models have addressed malaria immunity since any single rodent model replicates all the features of human malaria (Craig *et al.* 2012). Despite high genetic variability in human populations, most bioassays in mice have used combinations of *Plasmodium* species and inbred mouse strains, which explains the homogeneous outcomes obtained.

By convention, *P. yoelii yoelii* 17XL (*PyL*) is considered a uniformly lethal parasite strain when used to infect the inbred mouse strains most commonly used, including Balb/c, C57BL/6, Swiss and CBA (Li *et al.* 2001). Consequently, to date little evidence has been compiled on natural resistance to *PyL* parasites, only DBA/2 strain survives *PyL* infection after developing only moderate parasitemia (Wang *et al.* 2009). Previous results from our laboratory show spontaneous recovery from lethal *PyL* infection of around 20% of the mice from the non-consanguineous ICR strain (Moneriz *et al.* 2011). In the present study, we aim to formally characterize this new malaria model and identify potential immune response profiles associated to the different infection courses and final outcome. After a first *PyL* challenge, 20% of outbred ICR mice naturally developed a protective humoral response that confers long-term

immunity against homologue re-infections. Besides, repeated individualized cytometric analysis of WBC revealed that cell mobilization and phenotypes vary in mice showing different infection severities and outcomes. Thus, monocytes, dendritic cells (DCs) and immature B cells where mainly present in blood samples from highly parasitized mice and $CD4^+CD25^+$ T cells expansion occurred earlier than in surviving mice. In contrast, the most remarkable changes in blood cell composition observed in survivors was the increase in circulating activated T cells and class-switched B cells occurring after 2 wk of the infection. Collectively our data reveal dramatic WBC changes that take place during malaria infection and describe, for the first time, the heterogeneous infection and blood immune response to the disease in ICR mice.

METHODS

Ethics statement

All procedures involving animals were carried out according to Spanish (Ley 32/2007) and European Union legislation (2010/63/CE). The protocols for our *in vivo* experiments received institutional review board approval (Universidad Complutense de Madrid).

Animals and parasites

Seven wk old female Hsd:ICR (CD-1) and seven wk old BALB/cAnNHsd female mice were purchased from Harlan Ibérica. All animals were pathogen-free and were kept in the animal house of the Universidad Complutense de Madrid, with free access to food and water. The rodent malaria parasite *P. yoelii yoelii* 17XL (*PyL*) was kindly provided by Dr Virgilio Do Rosario (Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa) and stored in liquid nitrogen after serial blood passages in mice.

Experimental infection

Hsd:ICR(CD-1) mice were infected i.p. with $2x10^7$ *PyL*-iRBCs obtained from donor *PyL*-infected mice. Parasitemia was monitored sequentially in each mouse by performing Wright's eosin methylene blue solution-stained thin tail blood smears. Total parasite clearance in cured mice was also confirmed by PCR analysis and i.p. sub-inoculation of 50 µl of blood from the mice into naïve Balb/c recipients. Mice that recovered from 1^{st} infection were reinfected on days 60 and 420 post-first infection (pi) following the same infection protocol. RBCs were counted sequentially in each mouse using a hemocytometer. Age-matched uninfected mice were used as controls. Three independent experiments are shown (each n = 20).

Cell preparations

Single-cell suspensions were prepared sequentially from PB of each mouse for flow cytometry analysis. Around 40 µl of blood were collected from each mouse in PBS containing 0.1 M EDTA. After RBC lysis with ACK Lysing Buffer (Gibco), WBC were divided into aliquots of minimum 50,000 cells for staining with different mixes of fluorescent Abs. Viable cell counts were always made by Trypan Blue exclusion using a hemocytometer. Total bleeding was always ≤100 µl/mouse/wk to minimize the biological effects of blood loss (Weaver *et al.* 2002).

Flow cytometry labeling

Cells from individual mouse were separately incubated with anti-CD16/32 (clone 93; eBioscience) to block non-specific binding, and then stained with different combinations of FITC, PE, PE-Cy5, PerCP-Cy5.5 or APC conjugated anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-CD45 (30-F11), anti-CD43 (S7), anti-IgM (II/41), anti-CD45R/B220 (RA3-6B2) (from BD Pharmingen); anti-CD44 (IM7), anti-CD5 (53-7.3), anti-CD11b (M1/70), anti-CD23 (B3B4), anti-CD25 (PC61.5), anti-IgD (11-26c), anti-Mac-3 (M3/84), anti-MHC II (M5/114.15.2) (from eBioscience); and anti-CD11c (N418) (from AbD Serotec). Events were acquired on FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FCS Express software. Adequate isotype controls were used for all Abs (eBioscience). In all tests, cells were firstly gated on a forward scatter-side scatter gate (FSC-SSC) to exclude debris and secondly on a CD45+ gate to select the leukocytes.

***P. yoelii* protein extraction from infected whole blood**

PyL protein lysates were extracted from the whole blood of infected Hsd:ICR(CD-1) mice showing >50% parasitemia. Whole blood was collected in tubes containing 0.1 M EDTA and kept at -80ºC until protein extraction. The extraction protocol began with erythrocyte lysis using 10 vol of saponin 0.1% (w/v) in PBS. After twice washing in cold PBS, the pellet was treated with 2-3 vol of extraction buffer (50 mM Tris-HCl, pH 8.0; 50mM NaCl; 0.5 % Mega 10; 3% MEGA 10) containing a protease inhibitor cocktail (Roche) and subjected to four freeze-thaw cycles. Finally, lysates were centrifuged and *PyL* total protein samples stored at -20ºC until use.

PCR-quantification of parasite DNA in blood

P. yoelii DNA was extracted from peripheral iRBCs using the NuncPrep™ Chemistry Isolation of DNA from Whole Blood protocol of the ABI PRISM® 6100 Nucleic Acid Prepstation (Applied Biosystems) according to the manufacturer's instructions. Oligonucleotide primers and probes for the *P. yoelii yoelii 18S* ribosomal gene subunit (GenBank Accession No. U44379) were taken from (Witney *et al.* 2001).

Parasite DNA quantification was assessed employing the 5' fluorogenic nuclease assay (TaqMan) using a FAMTM dye-labeled specific probe. The primers/probe used were (5´-3´ sequences): Forward, CTTGGCTCCGCCTCGATA; Reverse, TCAAAGTAACGAGAGCCCAATG; Probe, CTGGCCCTTTGAGAGCCCACTGATT. PCR reactions were done in triplicate. Amplification, data acquisition and data analysis were carried out using the ABI 7700 Prism Sequence Detector system (Applied Biosystems).

Western blotting

10 µg of *PyL* total protein extract were fractionated on 10% SDS-PAGE (Bio-Rad), transferred to nitrocellulose membranes and blocked with 5% non-fat skimmed milk in PBS. Membranes were incubated overnight at 4ºC with the different collected sera at 1:5000 dilutions and then with the secondary HRP-labeled anti-mouse IgG (Amersham Bioscience) at a 1:5000 dilution.

ELISAs

Total IgM, IgG and *PyL*-specific IgG Abs were quantified by Ab isotype-specific ELISA. Total IgM and IgG were quantified using anti-mouse IgM or IgG as the capture Ab (Bethyl Laboratories) while *PyL* specific IgG isotypes were quantified using 0.5 µg/well of *PyL* total protein lysates as coating Ag prepared in carbonate-bicarbonate buffered solution (Sigma). Coating Ags were incubated for 2 h at RT and subsequently overnight at 4°C. From this step onwards, the manufacturer's protocol was followed (Bethyl Lab.). Briefly, plates were blocked with 1% BSA in Tris-buffered saline solution and duplicate diluted serum samples were added for 1 h at RT (1:5000 for IgM; 1:50000 for IgG; 1:40-1:2000 for *PyL*-specific IgGs). Total IgM and IgG Abs were detected with HRP-labeled goat anti-mouse IgM or IgG at a 1:75000 dilution and IgG subclasses were determined with 1:45000 diluted HRP- labeled goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 Abs. The enzyme reaction was developed using 3,3´,5,5´tetramethyl benzidine (TBM) as the enzyme substrate (Thermo Scientific). Samples were read at 450 nm in a Varian Cary 50 Bio spectrophotometer (Agilent Technologies). Sera from uninfected mice were used as negative controls. Purified myeloma-derived mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM (Bethyl Lab.) were used to generate a logistic four-parameter sigmoidal standard.

Adoptive transfer experiments

In groups of 4-5 animals, 6-7 wk-old female Balb/c naïve mice were injected i.v. with 150-200 µg of total IgG from pooled sera obtained from late deceased mice on days 8-11 pi; or from surviving or uninfected mice on day 70 pi (10 days after the 2nd infection). Mice injected with PBS were used as infection controls. After 2 h of passive transfer of serum, mice were challenged with 2x10^{7} *PyL* iRBCs.

One mouse in each group was left uninfected as a healthy control. Two independent experiments were performed.

Cytokine antibody arrays

To determine cytokines and chemokines in the mouse sera, the Mouse Cytokine Ab Array II kit (AAM-CYT-2-2, RayBio) was used according to the manufacturer's protocol. Pooled serum samples from each group of mice (of days 3 or 7 pi) were applied to the membranes and, after incubation with the detection Ab, membranes were developed with streptavidin-HRP followed by a chemiluminescence reagent (Thermo Scientific). Membranes were then exposed to X-ray film. Pixel densities were calculated for each spot of the array using Quantity One software (Bio-Rad Laboratories) and mean values for duplicate spots were compared.

Statistical analysis

To assist sorting of the infected animals into the 3 experimental groups (ED, LD and S), linear regression analysis was used to determine the slope of the parasitemia increase during the first 15 days of the infection. Data were compared using the Student's t-test or Mann-Whitney non-parametric test in Prism 5 software (GraphPad Software Inc.). Significance was set at $p < 0.05$. Data are shown as means ± SEM.

RESULTS

Primary *P. yoelii 17XL* infection leads to three malaria infection profiles in ICR mice

The i.p. infection with $2x10^7$ *PyL* in ICR outbred mice resulted into three different infection profiles according to their parasitemia and survival kinetics (Table I). A diagram showing the experimental design is provided in Fig. 1.

A 20% of mice spontaneously resolved the infection and were designated as surviving mice (S). S mice showed a slow increase in parasitemia with a peak of 59% and resolved the infection by day 22 pi (Fig. 2A). The infection was lethal before day 15 pi in the remaining 80% of the animals. Among deceased mice two different infection profiles were observed: Early deceased mice (ED) showed rapid-onset fulminating parasitemia with a peak of 83% and died before day 8 pi (Fig. 2A), being significantly associated the day of their death and the slope of the parasitemia increase ($p < 0.05$; R = 0.82). Different from this behavior, the rest of deceased mice designated as late deceased mice (LD) underwent a slow increase in parasitemia, similar to that of S mice, which peaked at 54%, but followed fatal outcome

around day 11 pi. The slope of parasitemia growth was significantly different between ED mice and LD or S mice (both $p < 0.0001$), but no differences were found between LD and S mice (Fig. 2B). In LD mice the time of death was not associated with the slope of the parasitemia ($p > 0.05$; $R^2 = -0,05$). Total clearance of parasites in S mice after infection was confirmed by microscopy examination of blood smears, PCR and sub-inoculation of blood in naïve Balb/c mice. To ascertain whether the intrinsic properties of the parasites might contribute to the development of the heterogeneous infection profile observed in ICR, $2x10^7$ *PyL* iRBCs were collected from ICR mice with high-level parasitemia (22% on day 3 pi) and low-level parasitemia (< 0.5% on day 3 pi and 35% on day 6 pi) to inoculate into 5 Balb/c inbred mice. The course of infection in Balb/c groups was identical (data not shown).

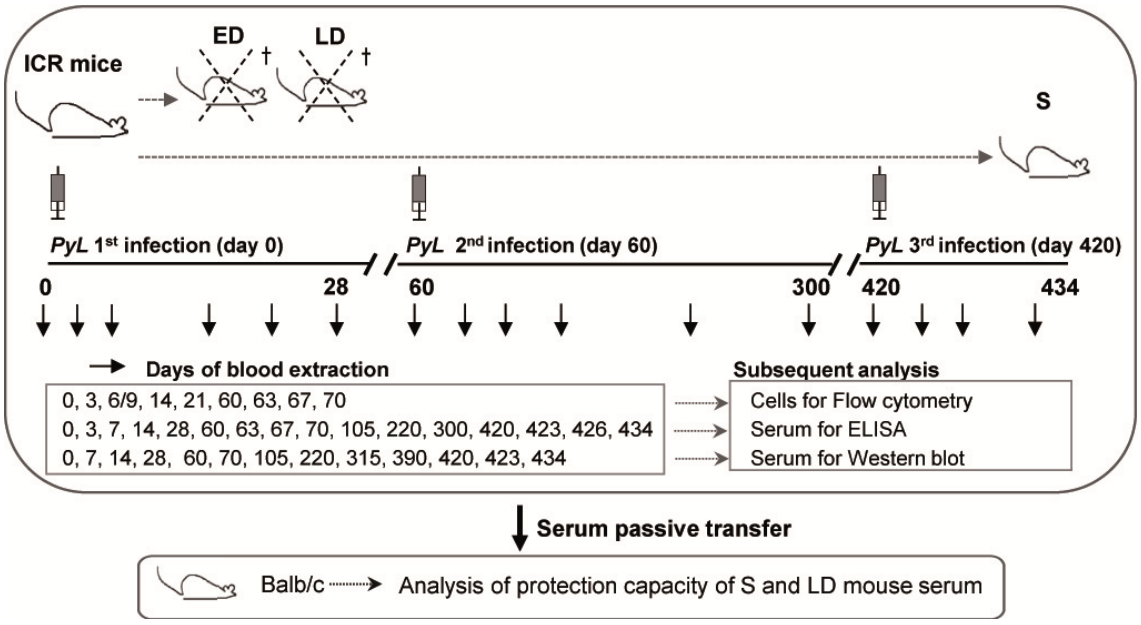

FIGURE 1. Experimental design used to examine *P. yoelii* 17XL infection in ICR mice. ICR mice were characterized as early deceased (ED), late deceased (LD) or surviving (S) depending on parasitemia rates and outcomes after primary infection with $2x10^7$ *PyL* iRBCs. S mice were reinfected on days 60 and 420 pi. Blood was extracted at the indicated time-points during infection for the different tests using age-matched uninfected mice as controls. The sera of S and LD mice were passively transferred to naïve BALB/c mice.

After their recovery, S mice were reinfected twice on days 60 and 420 pi using the same challenge *PyL* doses. 100% of these animals survived both reinfections and none of them exhibited parasites in PB (Fig. 2A). Anemia was detected in all groups of animals (Fig. 2C). In ED mice, RBC loss was significantly evident from day 3 pi until death ($p = 0.01$) whereas in LD mice, the drop in RBCs started on day 9 pi ($p = 0.02$). In S mice, RBC counts fell from days 6 ($p = 0.03$) to 14 pi ($p = 0.04$), but thereafter recovered and counts were comparable to initial levels. In both LD and S mice, infection induced an increase in WBC (Fig. 2C). In S mice, WBC increased 3.6-fold by day 21 pi ($p = 0.03$), but by the start of the 2nd infection counts returned to baseline.

TABLE 1. *P. yoelii* 17XL infection in ICR mice leads to three different infection profiles: early death (ED), late death (LD) or survival (S).

	ED mice	LD mice	S mice
% all mice (n = 55)	61.8 (n=34)	20 (n=11)	18.2 (n=10)
Day of death	5.2 ± 0.2 *	11.1 ± 0.6	----
Peak leukocyte number (x10^6/ml)	21.8 ± 2 *	34.4 ± 7 *	64.1 ± 5.1
Day of leukocyte peak	1.56 ± 0.4 *	6.28 ± 1.6 *	21
Max. iRBCs (%)	83.3 ± 2.1 *	53.7 ± 5	58.8 ± 6.3
Day of max. iRBCs	4.81 ± 0.2 *	8.91 ± 0.6	12 ± 0.7
Max. RBC loss (%)	37.6 ± 6.4	60.2 ± 11	71.8 ± 7.2
Max. RBC loss (10^9/ml)	3.8 ± 0.5 *^{S}	4.8 ± 1.3	5.1 ± 1
Day of max. RBC loss	3.2 ± 0.2 *	7.5 ± 1.2	13.4 ± 2.7

* Significant differences between groups ($p < 0.05$), except group indicated.

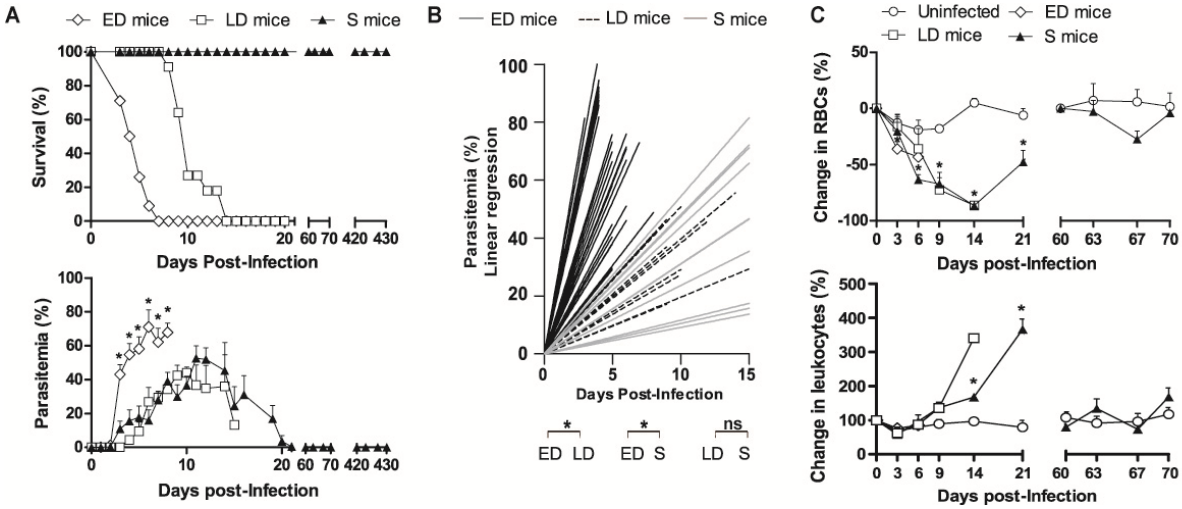

FIGURE 2. Mice survival and kinetics of parasitemia, anemia and leukocytes in blood of ED, LD and S mice infected with *P. yoelii* 17XL. ICR mice infected with 2x10^7 *PyL* iRBCs were classified as early deceased (ED), late deceased (LD) or surviving (S) depending on their (*A*) survival and parasitemia. * $p <$ 0.05 between ED mice and the remaining groups. (*B*) Linear regression of the parasitemia of each mouse. * Significance between the mean 1/slope of each group. (*C*) Changes from baseline (day 0) produced in circulating numbers of RBCs and WBC. * $p < 0.05$ comparing to uninfected mice. Only one LD mouse was still alive on day 14 pi. Data express mean ± SEM of three independent experiments with n = 20.

Circulating monocytes and DCs show a marked increase in ED mice

Both DCs (Stevenson *et al.* 2011) and macrophages (Couper *et al.* 2007) have been shown to have a protective effect in malaria infection. However, the acute phase of lethal *PyL* may impair DC function (Wykes *et al.* 2007). Changes produced in PB activated monocytes (Mac-3$^+$ MHC II$^+$) and DCs (CD11c$^+$ MHC II$^+$) during *PyL* infection and possible links to different outcomes were assessed (Fig. 3A). ED mice showed most changes in PB innate immune cells. Thus, 10-fold and 5-fold increases were observed in the frequencies of monocytes and DCs respectively on day 6 pi (Fig. 3B, C; $p < 0.01$), when parasitemia was at its maximum level (Fig. 2A). Total cell numbers showed similar kinetics, with an 18-fold increase in monocytes and 10-fold increase in DCs detected on day 6 pi in ED mice (both $p < 0.01$). Remarkably, S mice showed the earliest monocyte expansion on day 3 pi ($p = 0.01$) although this increase was reduced relative to the expansion in ED mice on day 6 pi.

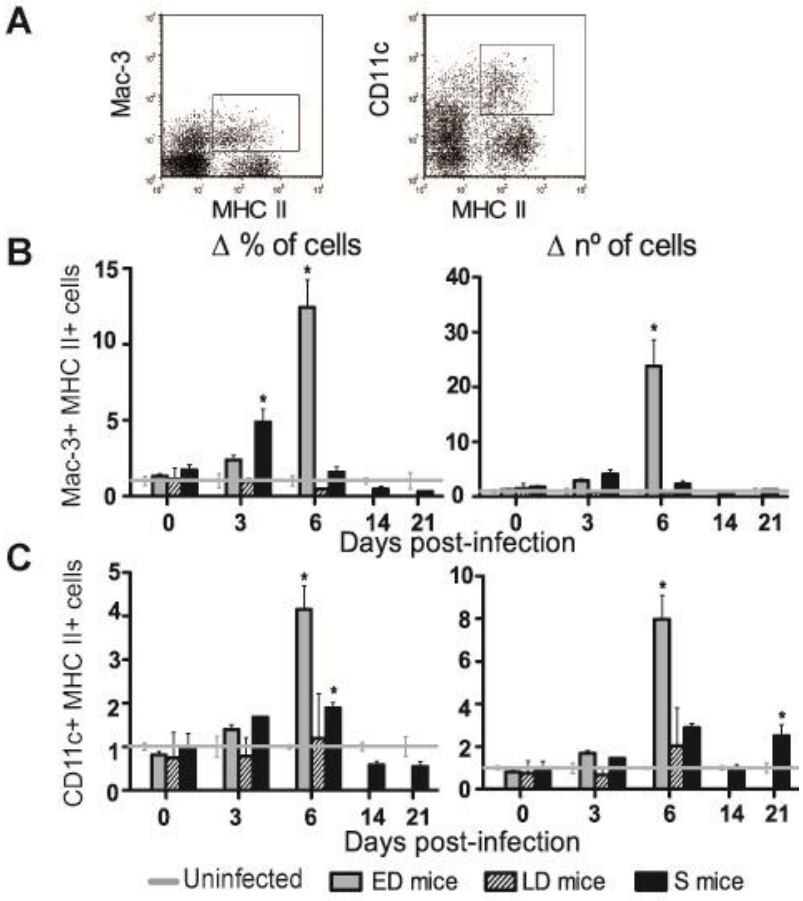

FIGURE 3. Monocytes and DCs increase in blood of ED mice during acute infection. WBCs were isolated from the PB during the 1st *PyL* infection in ICR mice and (*A*) monocytes (Mac3$^+$ MHC II$^+$) and DCs (CD11c$^+$ MHC II$^+$) were detected by flow cytometry. Animals were classified depending on the infection profiles as early deceased (ED), late deceased (LD) or surviving (S) and (*B, C*) their cell frequencies with respect to total leukocytes and numbers were recorded. Data express mean ± SEM of 2 independent experiments, each with n > 3 mice per time point. The data for each infected mouse was normalized to the data recorded in 5 uninfected mice per experiment. * $p < 0.05$ with respect to uninfected mice.

S mice show the enhanced mobilization of CD8 and CD4 T cells

It is widely accepted that CD4 T cells are essential to control blood-stage malaria infection and that CD8 T cells play a role during the liver-stage of the parasite cycle (Doolan and Hoffman 2000). However, the contribution of the latter T cells to blood-stage infection remains unclear. In S mice, $CD4^+$ and $CD8^+$ T cells showed similar kinetics in blood (Fig. 4A, B). Percentages of circulating CD4 and CD8 T cells decreased from day 9 pi onwards during the 1st infection ($p < 0.05$, except for CD8 T cells on day 14 pi). In contrast, total numbers of CD4 T cells were elevated on day 21 pi ($p = 0.01$) and numbers of CD8 T cells increased on days 14 and 21 pi ($p = 0.03$). During the 2nd infection in S mice, initial CD4 T cells levels were recovered, but CD8 T cells remained in lower number and proportion than in uninfected mice. ED mice showed a reduction in the number ($p < 0.01$) and proportion ($p < 0.01$) of CD8 T cells and unchanged CD4 T cell levels, whereas LD mice showed a similar trend to that observed in S mice.

During primary infection elevated levels of activated lymphocytes are detected in the peripheral blood of surviving mice

CD44 is a ligand for hyaluronic acid, which is up-regulated in activated/memory cells mediating rolling and adhesion during the traffic of activated lymphocytes to target sites of immunity (Siegelman *et al.* 1999). Percentages and numbers of activated leukocytes ($CD44^+$) markedly augmented in S mice, from day 9 to day 21 pi (all $p = 0.03$) (Fig. 4C). To differentiate peripheral T cells, which can be naïve or previously activated Ag-experienced memory cells, we examined their expression of the CD44 receptor. In S mice, the T $CD4^+$ population showed a higher frequency of $CD44^+$ cells on day 21 pi ($p = 0.01$) and higher $CD44^+$ cell numbers on days 14 ($p = 0.03$) and 21 pi ($p = 0.04$). T $CD8^+$ $CD44^+$ cells showed similar increases on day 21 pi ($p = 0.03$ both frequency and number) (Fig. 4D).

The time point of circulating $CD4^+CD25^+$ T cell expansion varies with outcome

Expression of the IL-2Rα chain, CD25, is a widely used, but not exclusive marker for T regulatory cells (Treg) (Shevach 2002) and the suppressor activity of $CD4^+$ $CD25^+$ cells is well documented (Thornton and Shevach 1998)(Fig. 5A). The frequencies and numbers of $CD4^+$ $CD25^+$ cells were early increased in mice with fatal malaria (Fig. 5B). In S mice, only an increase in cell numbers was detected at the end of the 1st infection ($p = 0.03$) once the parasitemia had been controlled, and these returned to the normal range after the 2nd challenge.

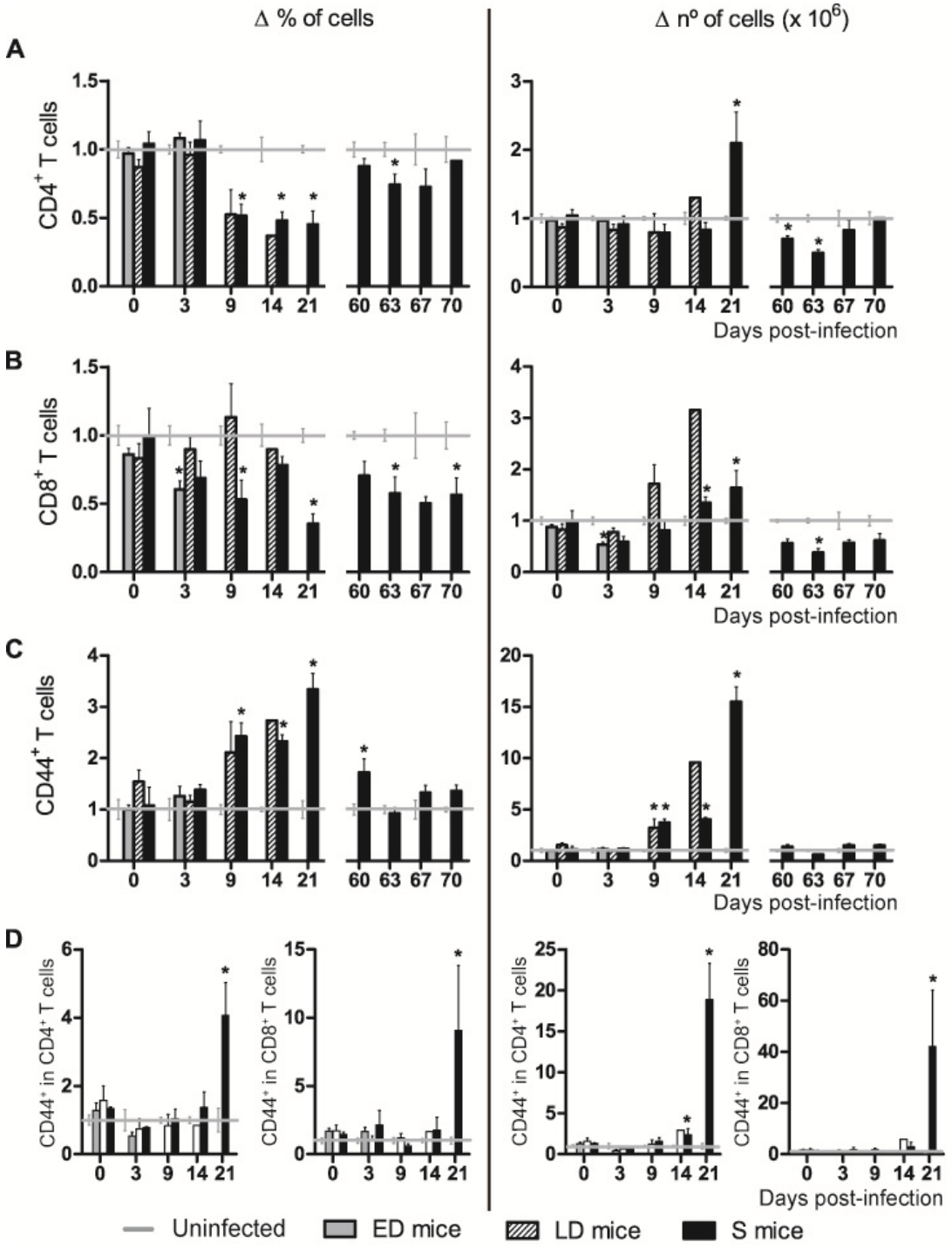

FIGURE 4. Kinetics of CD4 and CD8 T cells and CD44 expression in blood of *PyL* infected ICR mice. WBCs were isolated from the PB of mice infected with $2x10^7$ *PyL* iRBCs showing different infection profiles: early deceased (ED), late deceased (LD) or surviving (S). Survivors were reinfected on day 60 pi. Frequencies (left panel) and absolute numbers (right panel) of (*A*) CD4 T, (*B*) CD8 T cells, (*C*) total $CD44^+$ cells and (*D*) $CD44^+$ expressing cells in CD4 and CD8 T populations. Values indicate mean (± SEM) of 2 independent experiments, each with n > 3 mice per time point (except day 14 pi with only one LD mouse). The data for each infected mouse was normalized to the data recorded in 5 uninfected mice per experiment. * $p < 0.05$ with respect to uninfected mice.

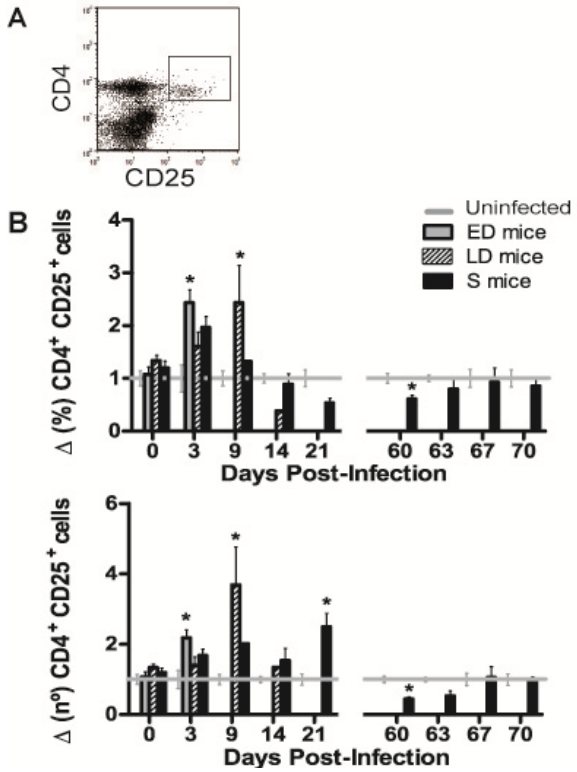

FIGURE 5. CD4$^+$ CD25$^+$cells in blood of *PyL* infected mice. ICR mice infected with 2x10^7 *PyL* iRBCs were classified as early deceased (ED), late deceased (LD) or surviving (S) depending on the infection profiles. Survivors were reinfected on day 60 pi. Blood leukocytes from each mouse were isolated and (*A*) CD4$^+$ CD25$^+$ cells were detected by flow cytometry. (*B*) Proportions with respect to total leukocytes and absolute numbers recorded in ED mice, LD mice and S mice were normalized to the data recorded in uninfected mice (n = 5 per experiment). Data express mean (± SEM) of 2 independent experiments, each with n > 3 mice per time point (except day 14 pi with only one LD mouse). * $p < 0.05$ with respect to uninfected mice.

Transitional, mature and switched-class B cell kinetics differ according to infection severity

Mice lacking B cells reveal the importance of B cells in malaria as they are unable to clear *P. yoelii* (Weinbaum *et al.* 1976) and *P. chabaudi* infections (von der Weid *et al.* 1996). To explore the maturation of B cells during blood-stage malaria infection according to severity, the expression of surface IgM and IgD was determined (Fig. 6A). When immature B cells leave the bone marrow, they develop into transitional 1 (T1) (IgM^{hi} IgD^{low}) B stage cells, which via the bloodstream reach the spleen. They then become transitional 2 (T2) (IgM^{hi} IgD^{hi}) cells and progress to mature cells (IgM^{low} IgD^{int}), which re-circulate to find an Ag or remain in the follicular (FO) zone of the spleen or progress to marginal zone (MZ) B cells (Loder *et al.* 1999; Chung *et al.* 2003). After Ag-induced differentiation, B cells commonly switch from expressing IgM to IgG Abs, generating IgM$^-$ IgD$^-$ cells that include short or long-lived plasma cells and memory B cells (Sagaert *et al.* 2007). In blood, cells gated as IgM^{hi} IgD^{low} also include B-1 cells (Wardemann *et al.* 2002). In our study, CD11b$^+$ CD5$^-$ B-1b cells (B-1a cells, which are CD5$^+$, were absent from blood) were detected at very low percentages (always < 4.5% of IgM^{hi} IgD^{low} cell levels) while

CD11b$^-$ CD5$^-$ T1 B cells comprised the majority of the IgM^{hi} IgD^{low} cell gate (always > 70% of IgM^{hi} IgD^{low} cell levels) (Fig. 6B). All subtypes except T2 cells were detected in the PB of all mice. Although elevated T1 B cell numbers were detected in blood from day 3 in both ED ($p < 0.01$) and S mice ($p = 0.03$), the increase was highest in mice with the worse prognosis (Fig. 6C). Total mature cells presented minor changes; first a decline in all infected mice on day 3 pi and then an increase in LD and S mice on day 6 pi ($p = 0.03$). Isotype-switched B cell numbers showed a small increase in ED ($p = 0.03$) and LD mice ($p < 0.01$) on day 6 pi, but in S mice at the end of infection, the increase produced was 8-fold ($p < 0.01$).

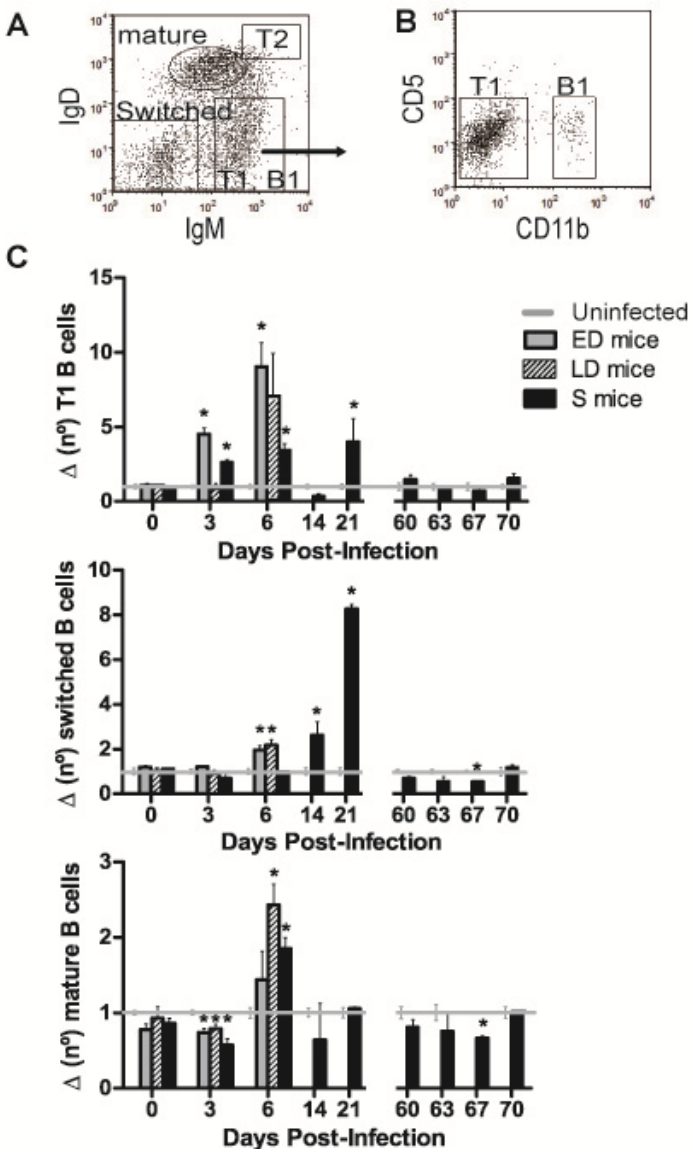

FIGURE 6. Changes in B cell subpopulations in blood of *PyL* infected ICR mice. ICR mice infected with 2x10^7 *PyL* iRBCs were classified as early deceased (ED), late deceased (LD) or surviving (S). Survivors were reinfected on day 60 pi. (*A*) Representative flow cytometry dot plot of transitional 1 (T1) and B-1 (IgM^{hi} IgD^{low}), transitional 2 (T2) (IgM^{hi} IgD^{hi}), mature naïve (IgM^{low} IgD^{int}) and class-switched B cells (IgM$^-$ IgD$^-$) identified among B220$^+$ B cells from blood. (*B*) T1 and B-1 cells distinguished through CD5 and CD11b expression. (*C*) Numbers of B cell subpopulations in mice normalized to the data recorded in uninfected mice (n = 5 per experiment). Data express mean (± SEM) of 2 independent experiments, each with n > 3 mice per time point. * $p < 0.05$ with respect to uninfected mice.

The B220^{+} MHC II^{+} subset and proportion of B220^{low} cells

MHC class II is constitutively expressed by mature B cells at modest levels, but is dramatically overexpressed on activated B cells and lost after differentiation to plasma cells. Thus, the ability of B cells to up-regulate MHC II expression following activation is likely to be critical for their ability to function as APCs. We examined activated B cells (B220^{+} MHC II^{+}) in blood and a decrease was observed in their frequency in all infected mice, but only significantly so in S mice on days 14 (p = 0.01) and 21 pi (p < 0.01) (Fig. 7A). Absolute B220^{+} MHC II^{+} cell numbers in S mice, even when a gross increase in WBC was observed, represented half the initial cell numbers on day 14 (p = 0.02) and day 21 pi (p = 0.04). Normal levels of active B cells in PB were recovered at the beginning of the 2nd infection (data not shown).

We could distinguish two populations of B220^{+} MHC II^{+} cells according to the B220 levels: B220^{high} MHC II^{+} (B^{high}) and B220^{low} MHC II^{+} (B^{low}) as shown in the dot plot in Fig. 7B. Descriptions in the literature already exist of the distribution of B220$^{high/low}$ populations in mice suffering non-lethal *P. yoelii* 17XNL (*PyNL*) infection (Kanda *et al.* 2010), amyloidosis (Kawabe *et al.* 2004) or mammary tumor virus infection (Ardavin *et al.* 1999) and in healthy neonatal or old mice (Tachikawa *et al.* 2008) and ovariectomized mice (Masuzawa *et al.* 1994). Our healthy mice constantly showed about 25% B^{high} cells in PB whereas the B^{low} subset only represented around 1.5% of the total WBC count (data not shown); the normal proportion of B^{high}/ B^{low} cells being around 9:1 among total activated B cells (Fig. 7B). However, malaria infection promoted a rise in B^{low} cells and drop in B^{high} cells in all mice from day 3 or 6 pi.

Next, we determined the expression of the different receptors on the B220^{low} cells in all infected mice: all B220^{low} cells were CD23^{-} and CD5^{-}, 12.8 ± 2.5% were IgD^{+}, 48.2 ± 5.4% were IgM^{+} and 17.8 ± 3% were CD43^{+} (Fig. 7C). Interestingly, the expression of IgM and IgD in B220^{low} and B220^{high} cells in PB revealed that B220^{high} were mainly mature cells (IgM^{low} IgD^{+} CD5^{-}) whereas B220^{low} were ~50% B-1 and T1 B cells (IgD^{-} IgM^{+} CD5^{-}) and ~50% IgD^{-} IgM^{-} CD5^{-} cells (Fig. 7D). Although some studies have defined the B220^{low} population as CD43^{-} B-1b cells (Tachikawa *et al.* 2008), classic B-1 cells are IgM^{+} CD23^{-} CD43^{+} IgD^{-} (reviewed in (Berland and Wortis 2002; Baumgarth 2010)). To examine the presence of B-1 cells in the B220^{low} population, we distinguished CD23^{-} IgM^{+} CD43^{-} T1 cells from classic CD23^{-} IgM^{+} CD43^{+} B-1 cells and only 3.11 ± 0.7% of B-1b cells were detected (Fig. 7E).

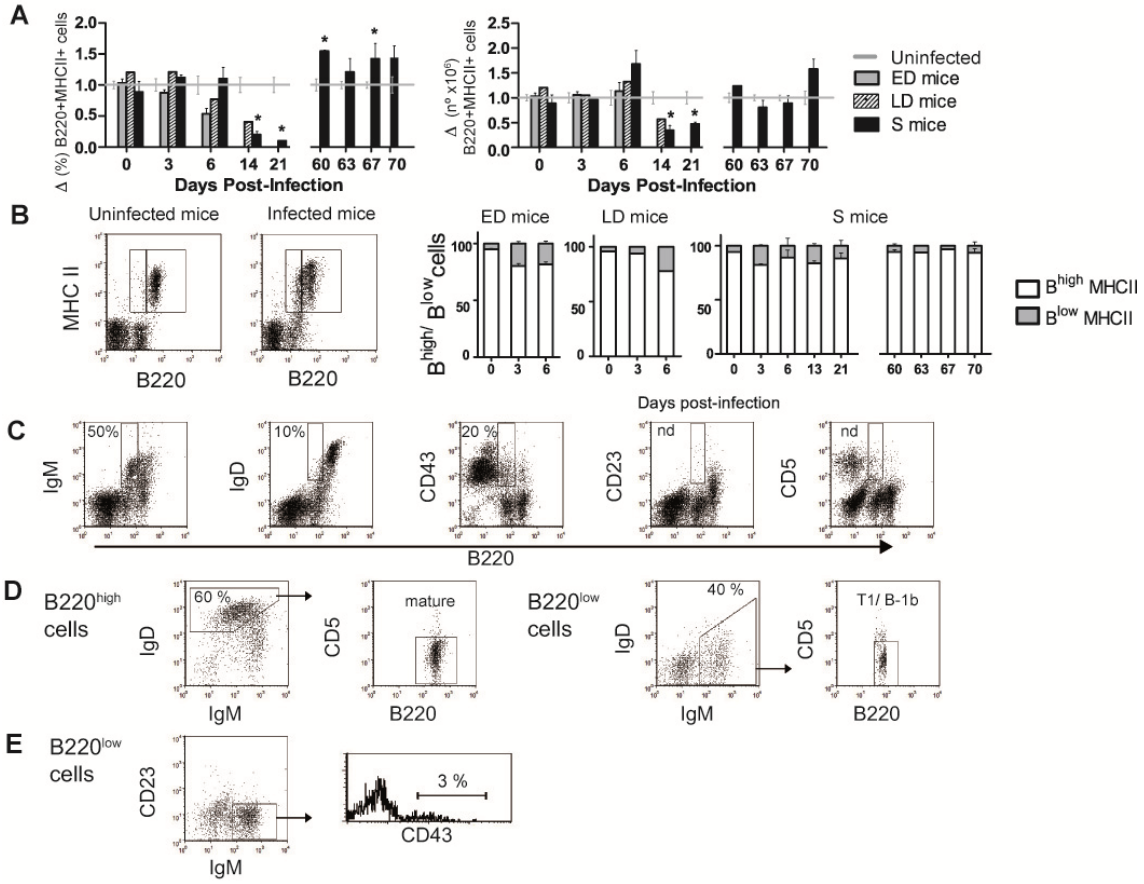

FIGURE 7. Blood kinetics of MHC II$^+$ B cells and their subsets during *P. yoelii 17XL* infection. ICR mice were infected with 2x10^7 *PyL* iRBCs and classified as early deceased (ED), late deceased (LD) or surviving (S). Survivors were reinfected on day 60 pi. WBCs were isolated and (*A*) B220$^+$ MHC II$^+$ cells percentages and absolute numbers were normalized to data obtained in uninfected mice (n = 5 per experiment). * $p < 0.05$ with respect to uninfected mice. (*B*) Representative flow cytometry dot plots identifying B220^{low} MHC II$^+$ and B220^{high} MHC II$^+$ populations in infected mice. Graph shows the contribution of each subpopulation to the total number of B220$^+$ MHC II$^+$ cells. As uninfected mice (n = 5) maintained in all time-points same proportions as day 0, for simplicity the graphic has not been shown. (*A, B*) Data express mean (± SEM) of 2 independent experiments,each with n > 3 mice per time point. (*C*) Dot plot showing IgM, IgD, CD43, CD23 and CD5 expression in B220^{low} cells. (*D*) Identification of mature (IgM^{low} IgD$^+$ CD5$^-$) and T1 and B-1b cells (IgD$^-$ IgM$^+$ CD5$^-$) in B220^{high} and B220^{low} cell gates. (*E*) In the B220^{low} cell gate, B-1 cells (CD23$^-$ IgM$^+$ CD43$^+$) were distinguished. (*C, D, E*) Representative percentages of measures in 2 independent experiments (n = 10).

Antibodies in serum during three consecutive *PyL* infections

Abs are crucial components of the protective immune response against malaria in human and animal models (Cohen *et al.* 1961; Jayawardena *et al.* 1978). *PyL* infection modifies Ab production in LD and S animals (Fig. 8A). While serum IgM levels peaked in the first infection, IgG Ab production started to increase after the 2nd wk of infection and peaked after the 2nd challenge in S animals. Among the serum *PyL*-specific IgG isotypes, IgG2b were most abundant in S and LD mice (day 14 pi), though IgG2a and IgG1 also reached high levels in S mice (Fig. 8B). In the 1st infection, LD and S mice differed mainly

according to the presence of IgG2a in the latter on day 14 pi. The 2nd challenge promoted a rapid 3-4.5-fold expansion in IgG2b, IgG2a and IgG1 and the appearance of IgG3 Abs, which were always the least abundant subclass during all infections. Third *PyL* challenge, induced again the Ab production. To assess specific IgG reactivity, we conducted a time-course IgG immunoblot analysis using total *P. yoelii* proteins (Fig. 8C). IgG Abs recognized a wide range of parasite Ag, the strongest signals appearing in the high molecular weight range. Serum from mice showing a fatal outcome unspecifically reacted with some high molecular weight iRBC proteins. Similar results were obtained using serum from uninfected mice and untreated deceased mice (data not shown).

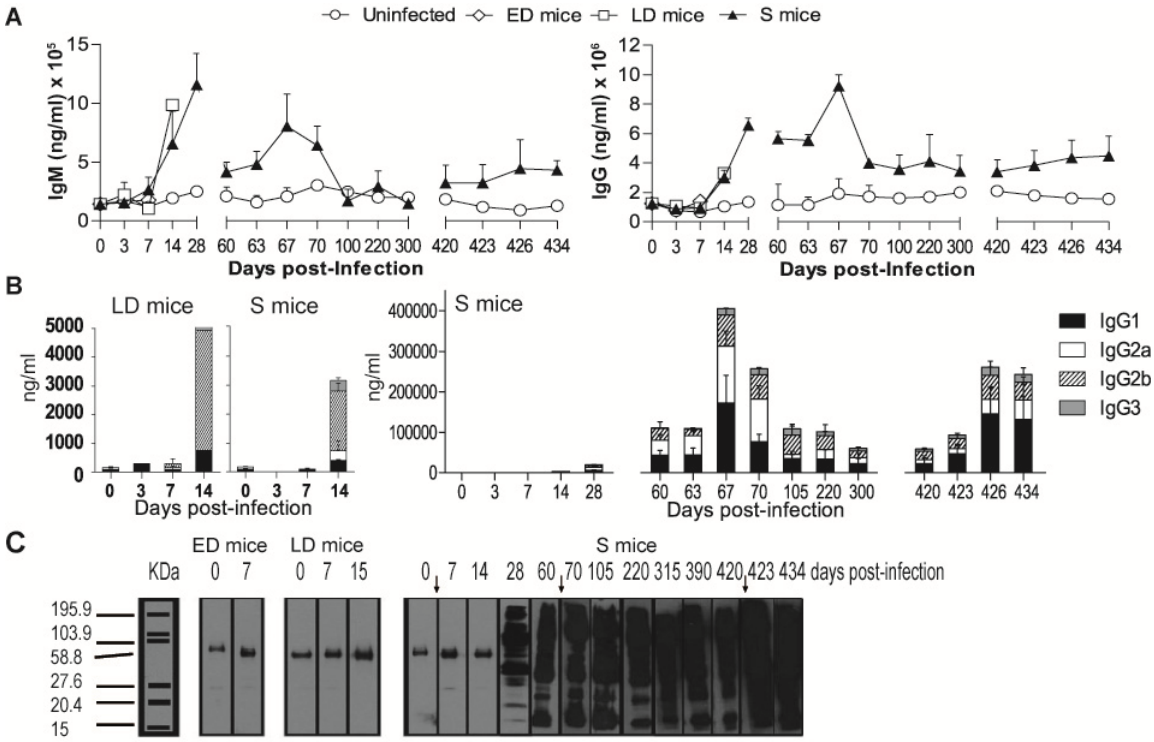

FIGURE 8. Humoral response following *PyL* infection in ICR mice. ICR mice were infected with 2x10^7 *PyL* iRBCs and classified as early deceased (ED), late deceased (LD) or surviving (S). Survivors were reinfected on days 60 and 420 pi. (*A*) Total serum IgG and IgM concentrations and (*B*) *PyL*-specific IgG isotypes were analyzed by ELISA during infections. Data express mean (± SEM) of n > 5 mouse sera per time point. (*C*) Representative Western blots show the specificity of serum IgGs against *PyL* infected RBCs. Western blots were prepared using protein extracts of *PyL* iRBCs and anti-mouse IgG-HRP as secondary Ab.

Serum from surviving mice partially protects BALB/c mice from *PyL* infection

To confirm the protective role of anti-*PyL* Abs in S mice, we performed passive transfer assays in BALB/c mice, which is a sensitive strain to *PyL*. Animals were inoculated with 200 µg of serum IgGs taken on day 74 pi from S mice or age-matched uninfected mice or on days 8-11 pi from LD mice and then infected 2 h later with 2x10^7 *PyL* iRBCs. Transfers using PBS were performed as controls. No

protection from infection was conferred by serum from LD mice, uninfected mice or PBS, but pooled sera from S mice was able to cure 40% of transferred naïve mice confirming the protective capacity of anti-*PyL* Abs elicited in spontaneously cured ICR mice (Table 2).

TABLE 2. Passive transfer of serum to BALB/c mice.

Inoculate	Outcome	Mice %	Day of death	Day of max. iRBCs	Max. iRBCs
PBS	Dead	100%	9.3 ± 0.3	8.3 ± 0.3	76 ± 5.5
US	Dead	100%	6.3 ± 1.6	5.7 ± 2	89.7 ± 3
LDS	Dead	100%	6.7 ± 2	6 ± 2.4	91.9 ± 7
SS	Dead	60%	14.7 ± 5.4	13.3 ± 5	81.9 ± 5.3
	Cured	40%	---	15.5 ± 2.1	73.4 ± 0.7

LDS, serum from late deceased mice
SS, serum from surviving mice
US, serum from uninfected mice
Max, maximum

Circulating cytokines in early deceased and surviving mice

The balance between pro- and anti-inflammatory responses is essential to limit an immune-mediated disease (Artavanis-Tsakonas *et al.* 2003). In human malaria, evidence exists of a link among cytokine profiles in sera, disease severity and parasitemia (Day *et al.* 1999; Cox-Singh *et al.* 2011). We compared serum cytokine profiles during the 1st wk of infection in S and ED mice by protein microarrays. S and ED mice produced all the cytokines examined on days 3 and 7 pi, but their levels were different (Fig. 9). At 3 days pi, S mice secreted higher levels of cytokines such as hematopoietic IL-3, the Th2 cytokine IL-4, and the Th1 cytokines IFN-γ and IL-2 than ED mice. Conversely at 7 days pi, ED mice showed higher levels of most of the markers, possibly as the result of their state of terminal decline and consequent physiological dysregulation. At this time point, S mice showed similar cytokine levels in serum to those observed on day 3 pi, though most were overall reduced. Greatest reductions from days 3 to 7 pi were produced in IL-4, TNF-α, IL-13, IL-2, IL-3, IFN-γ and IL-17 while VEGF (vascular endothelial growth factor), eotaxin, IL-6 and sTNFR1 (soluble tumor necrosis factor receptor 1) levels were slightly higher.

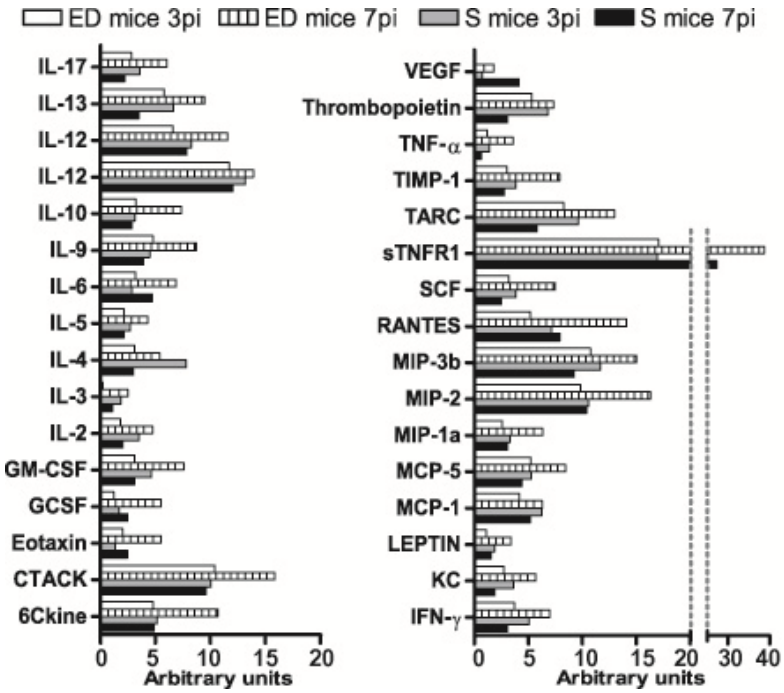

FIGURE 9. Cytokine antibody array analysis. ICR mice infected with $2x10^7$ *PyL* iRBCs were classified as early deceased (ED) mice or surviving (S) mice. Pooled sera from 4-5 mice in each group collected on days 3 and 7 pi were subjected to the RayBio mouse cytokine Ab array II and signals normalized to those obtained using positive Ab array controls.

DISCUSSION AND CONCLUSIONS

Here, we show that the serial analysis of blood immune factors in an animal model yielding different outcomes of infection can provide useful information on the course of the immune response and pathological processes. Our experiments reveal for the first time that the outbred ICR mouse strain mounts different immunobiological responses against primary *PyL* infection associated to the course of infection. Three different infection profiles were observed according to parasitemia and survival: a fulminating parasite growth that led to a fatal outcome before day 8 pi in 60% of animals (designated early deceased, or ED mice) or a more sustained parasitemia that was lethal before day 15 pi in 20% of animals (designated late deceased, or LD mice) yet self-resolved in a further 20% (designated surviving, or S mice). In S mice, parasitemia followed the typical non-lethal *Plasmodium* kinetics for rodents, peaking after 2 wk and clearing by the 3^{rd}-4^{th} wk. In the LD mouse group, the parasitemia course as well as changes in WBC populations were similar to those observed in S mice, suggesting that the death of LD animals may be in part due to ineffective erythropoiesis, as described in both human and rodent malaria infection (Lamikanra *et al.* 2007). Although mice surviving a malaria infection usually become immune to following infections, 5% of Balb/c naïve mice that recover from *P. yoelii nigeriensis* primary infection remain susceptible to reinfection a month later (Singh *et al.* 2000). After parasite clearance, homologue

reinfections in S mice revealed that these animals develop a protective long-standing immune response for 14 months.

In our model, *PyL* parasite contact promoted a late leukocytosis, consistent with the results of prior murine studies (Helmby *et al.* 2000; Nduati *et al.* 2010), but contrary to reports of acute malaria in humans in which leukocytopenia and lymphopenia are characteristic features (Lisse *et al.* 1994; Worku *et al.* 1997). This discrepancy and the drastic changes in mouse blood cell composition observed during blood-stage *P. chabaudi* (Helmby *et al.* 2000; Nduati *et al.* 2010) and *PyL* infections (present study) point to the importance of timing of blood sampling during a malaria infection. Some authors have even recently suggested that immunological studies based on WBC in mice may provide data comparable to the infection process in humans (Craig *et al.* 2012).

Our serial cytometric phenotyping of WBC served to identify different cell kinetics in ICR mice according to disease outcome. The first defense barrier against a 1st wave of *Plasmodium* is the innate cell response that promotes the subsequent T cell-mediated response (Stevenson and Riley 2004). Macrophages play a critical role in the immune response to malaria due to their ability to phagocytose iRBCs in the absence of cytophilic or opsonizing Abs, to activate T cells through Ag presentation on MHC II and to release inflammatory cytokines. Particularly in *PyL* infections, the key role of macrophages has been clearly demonstrated (Couper *et al.* 2007). The contributions of DCs include Ag uptake and stimulating T-helper cells (Banchereau and Steinman 1998). In the present study, although ED mice experienced the highest increase in blood levels of both innate cell populations during the 1st wk of infection, the innate response elicited was unable to control the high parasitemia (over 60%). In human malaria, monocyte and macrophage functions are altered by iRBCs or hemozoin. In particular, the surface expression of MHC II on human monocytes culture-stimulated with IFN-γ is impaired by iRBCs (Schwarzer *et al.* 1998). However, our data indicate that the inhibition of MCH II expression during murine *Plasmodium* contact *in vivo* is unaltered, as described in other consanguineous mouse strains (Luyendyk *et al.* 2002). Hence, the low efficiency of monocytes in our ED mice could be the result of an insufficient level of IFN-γ compared to levels in surviving mice, as detected in PB by microarrays at the start of infection. In agreement, higher IFN-γ levels in splenocyte cultures have been reported in resistant DBA/2 than susceptible Balb/c mice during *PyL* infection (Chen *et al.* 2009). Controversial results have been obtained regarding whether DCs are activated or inhibited by *Plasmodium* species in mouse and human studies, suggesting the effect is dependent on the parasite species and strain, the severity of infection, the host, the time after infection and the size of the inoculum (reviewed in (Wykes and Good 2008; Wang *et al.* 2009)). The prompt increase observed here in circulating innate system cells in ED mice indicates that high numbers of circulating monocytes or DCs are not needed to control the infection, rather they could be markers of an inadequate innate response at early time points in acute malaria infection. Hence, the general “stress condition” caused by severe infection in ED mice probably

contributed to the dysregulation of immune responses, also reflected by the over production of the majority of cytokines measured.

Neither T nor B lymphocytes in general seem to be required to control the 1st wave of *P. yoelii* infection (Couper *et al.* 2007). However, the suppressive role of CD4+CD25+ cells (Thornton and Shevach 1998; Belkaid and Rouse 2005) make of this population an important player during bacterial (Kursar *et al.* 2007), viral (Kinter *et al.* 2004), helminthic (Taylor *et al.* 2005; Finney *et al.* 2007) or protozoan infections including malarial (Belkaid *et al.* 2002; Hisaeda *et al.* 2004; Kinter *et al.* 2004; Taylor *et al.* 2005; Finney *et al.* 2007). In the $CD4^+CD25^+$ population are included effector CD4 T cells and Treg cells which play an important role through suppression of the Th1 response (Scholzen *et al.* 2009). In our study, the expansion of PB $CD4^+CD25^+$ cells observed in mice with a fatal outcome occurred before the parasitemia peak, whereas in surviving mice it was only observed when parasitemia had almost cleared. According to our results, excessive $CD4^+CD25^+$ cells at the onset of infection could impair the development of effective protective immunity, yet would be beneficial in later stages by preventing a prolonged inflammatory response (Scholzen *et al.* 2009). Notably, a similar conclusion has been reached in human studies (Torcia *et al.* 2008). Further cell studies focusing in Tregs ($CD4^+CD25^+FoxP3^+$) in this model could yield interesting results since in human malaria infection, Tregs expansion seems to be linked to parasite load (reviewed in (Scholzen *et al.* 2009)) rather than to infection outcome. In our mice model $CD4^+CD25^+$ cells correlate with disease outcome, in line with a previous study using *PyL* susceptible or resistant mouse strains (Wu *et al.* 2007).

In S mice, minor changes in circulating innate cells, but a late increase in activated T and class-switched memory B cells together with the high production of specific Abs conferred these survivors a very different immunological profile to that shown by ED mice. Consistent with data derived from non-lethal *P. chabaudi* infection (Helmby *et al.* 2000), the expansion of activated T cells ($CD44^+$) in blood at the very end of infection could be the outcome of cell migration from the spleen, given prior reports of the continued proliferation of CD4 and CD8 $CD44^+$ T cells in spleen from day 8 after non-lethal *P. yoelii* infection (Chandele *et al.* 2010). In transfer experiments, the role of CD8 T cells in *P. yoelii* malaria models has been found to be protective (Imai *et al.* 2010) and their activation and proliferation in response to *PyNL* has been described (Chandele *et al.* 2010). On the contrary, other passive transfer studies have concluded that these cells do not mediate protection (Vinetz *et al.* 1990). Though the functionality or specificity of CD8 T cells remains to be investigated, the increase in the total number of $CD8^+$ cells with an activated phenotype detected here in S mice could point to a protective role or at least suggest they do not impair a proper immune response.

In S mice, the decline in circulating $B220^+$ $MHCII^+$ cells paralleled to class-switched B cells increase in the PB could suggest the exit of B cells from the circulating population to lymphoid organs upon stimulation by parasite Ag, causing them to switch class to Ag-experienced and memory B cells. A subset of B cells expressing low levels of B220 protein was observed in infected mice. Although it has

been speculated that $B220^{low}$ cells might be beneficial in malaria *PyNL* infections (Kanda *et al.* 2010), similarly increased PB levels of these cells were observed in all our mouse groups and we characterized them mainly as immature B cells and not as B-1 B cells as previously described (Kanda *et al.* 2010).

The inability of erythrocytes to process Ag prevents iRBC destruction by a specific MHC-restricted T-cell response. Immunity to blood stage malaria parasites is thus primarily conferred by humoral immune responses. In our study, IL-4, a cytokine that directs the differentiation of Th0 to Th2 subsets (Taylor-Robinson and Phillips 1998), was augmented sooner in serum from S mice than ED mice and this would eventually promote the production of specific Abs (Stevenson and Tam 1993). In S mice, an efficient humoral immune response was mounted during 1^{st} infection and maintained for more than a year after the second infection. The concept that immunity to *PyNL* (Jayawardena *et al.* 1978; Freeman and Parish 1981) and to lethal *P. yoelii nigeriensis* (Singh *et al.* 2000) is largely humoral, as shown by passive transfer of hyperimmune sera, is now extended to include the *PyL* strain by our passive transfer results.

The development of immunological memory in the S animal group was patent since both reinfections produced a rapid protective specific Ab response. All the IgG isotypes examined, IgG1, IgG2a, IgG2b and IgG3, were detected in the sera of S mice, consistent with findings in *PyNL*-infected ICR mice (White *et al.* 1991). Studies in humans suggest that the presence of malaria-specific Abs may be dependent on the presence of chronic parasitemia (Akpogheneta *et al.* 2008), but the clearance of blood parasitemia in *PyL*-ICR mice after each infection was confirmed by microscopy, PCR and re-inoculating blood in naïve mice. The persistence of parasites in the spleen or other organs was, however, not investigated. Our immunoblots revealed that the repertoire of *PyL* Ag was recognized by the specific IgGs raised after each reinfection. This has also been observed in acquired immunity to human malaria and is likely to depend on the build-up of a wide range of antigenic specificities over a long period (Kinyanjui *et al.* 2004). In our malaria model, parasite proteins exhibiting antigenicity spanned a wide MW range. Remarkably, circulating Abs against high MW *PyL* Ags were preferably maintained after several months without parasite re-exposure. In a recent proteomic study, we identified some of these *PyL* Ags with Abs from protected S mice, as a new strategy to develop multi-antigen-based vaccine therapies (Kamali *et al.* 2012).

The results obtained in our rodent malaria model indicate rapid cell changes in the PB of ICR mice during blood-stage *PyL* malaria related to the severity of the infection and outcome. During the first days of the infection, the immune response observed in peripheral blood of ICR mice with the worst prognosis to *PyL* parasites consisted of a rapid increase in circulating $CD4^{+}CD25^{+}$ cells and immature B cells followed by a dramatic rise in activated innate cells and a small increase in class-switched B cells. In contrast, during same days the immune response observed in survival mice is characterized by the controlled production of cytokines and mostly unchanged circulating innate cell kinetics. Moreover, after first week an increase in circulating activated T cells and class-switched B cells together the

generation of a long-term protective humoral response. These findings are encouraging for human studies since they identify WBC as markers of severity and outcome in lethal malaria infection and could have implications for assessing new vaccine and treatment candidates since the desired immune response could be early detected in PB. Here, we demonstrate that outbred mice strains that show different clinical outcomes are valuable animal models to distinguish between potentially effective and ineffective immune responses to malaria. Thus, once described this model, future experiments are necessary to study in detail the immunological mechanisms of malaria susceptibility and protection in ICR mice.

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Increased follicular, B-1 and memory B cells are maintained at long term after a single self-resolved *P. yoelii* lethal infection in ICR mice

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Short Title: B cells in *P. yoelii* lethal infection

SUMMARY

ICR mice have heterogeneous susceptibility to lethal *Plasmodium yoelii 17XL* (*PyL*) infection from the first days of infection. To determine changes in immune cellularity in spleen fostered by the different prognostic parasitemia we examined low and high parasitized mice during the first days of the primo-infection and, in the case of surviving mice, 500 days after the primary *PyL* challenge. Changes in total cellularity of spleen during the first 6 days of infection were observed to correlate with parasitemia. In addition, B cell subsets were also modified across the different infection fates**,** maintaining a constant proportion along the first week of *PyL* infection but increasing switched B cells in mice with high parasitaemia as early as day 3 post-infection. Moreover, *PyL* infection influenced follicular (FO) B cells response more largely than marginal zone B cells in the spleen, revealing an increase in their absolute numbers from day 3 pi, being larger in mice with high parasitemia. FO B cells maintained enlarged levels than naïve mice even after 500 days of the primo-infection, similarly to B-1 cells. The changes in B-2 and B-1 B cells number in the peritoneal cavity during the first week of infection was particularly observed in mice with high parasitemia despite that the infection did not interfered in the B-1:B-2 cells proportion. More significantly, *PyL*-specific memory B cells, investigated by determining *PyL* specific IgG in the supernatant from cultures of splenocytes, remained in the spleen 500 days after the primo-infection, with B cell population in the germinal centres being absent. These results are consistent with the persistence of a reservoir of memory B cells in surviving mice upon a single malaria infection.

ABBREVIATIONS

Ab, antibody; BM, Bone marrow; FO, follicular; FSC-SSC, forward scatter-side scatter gate; GC, germinal center; iRBCs, infected red blood cells; ip, intraperitoneal; LDA, limiting dilution assay; MZ, marginal zone; OD, optical density; pi, post-infection; PCs, plasma cells; *PyL, P. yoelii yoelii* 17XL; RT, room temperature; SD, standard deviation; SEM, standard error; T1, transitional 1; T2, transitional 2.

INTRODUCTION

In endemic *P. falciparum* malaria, human immunity against severe clinical symptoms can be developed after one or two infections (Gupta *et al.* 1999). However, the control of parasite multiplication by immune mechanisms is acquired only after repeated infections over a number of years as a non-sterile condition. Besides, factors as age, genetics, gender, prevalence of common RBC polymorphisms, helminth co-infection and the transmission dynamics of the parasite can influence the overcoming of the infection (Schofield and Grau 2005; Akpogheneta *et al.* 2008; Doolan *et al.* 2009).

Most of the mouse models used to study malaria immunity shows a homogeneous lethal outcome after *PyL* infection (Li *et al.* 2001). Little information is available about self-cured *PyL* infections. Two main studies have shown respectively, a B and T cell response in a 5% of BALB/c naïve mice that resolved a *P. yoelii nigeriensis* infection without developing immune memory (Singh *et al.* 2000), and a potential nitric oxide role in the protection of *PyL*- infected DBA/2 mice (Wang *et al.* 2009).

In previous work, we demonstrate that *PyL* infection in ICR mice promotes different profiles of parasitemia and outcome with characteristic white blood cells changes (Azcárate *et al.*). The infection is lethal in 80% of the animals before day 15 pi, whereas the remaining 20% of mice resolve the infection spontaneously (Azcárate *et al.*). Spleen has a critical role fighting malaria infection both in humans and mice (Buffet *et al.* 2011; Del Portillo *et al.* 2012). Spleen red pulp cells remove senescent, damage and parasite-infected RBCs, meanwhile white pulp cells assist immune response (Carsetti *et al.* 2004). In fact, splenomegaly is a major clinical marker of the endemicity in *P. falciparum* transmission regions (Snow *et al.* 1997) which is caused by an expansion and congestion of red pulp (Oo *et al.* 1987), a large influx of macrophages into the white pulp (Urban *et al.* 1999) and an expansion of the splenic B cell population (Hansen *et al.* 2003). Among the immune cells, B lymphocytes are key effectors in rodent malaria immunity as mice lacking B cells are unable to clear *P. yoelii* (Weinbaum *et al.* 1976) and *P. chabaudi* infections (von der Weid *et al.* 1996). Since 80% of the fatal cases by *PyL* infection in outbreed ICR mice take place during the first week at high parasitemia level (Azcárate *et al.*), in the present study, we investigated the effect on the B cell population of the malaria infection during the first days to search for markers linked to parasitemia (Azcárate *et al.*). The most significant changes in B cell subsets of spleen

and peritoneal cavity (PerC) cells were observed at short term in highly parasitized mice. Furthermore, we also followed the long-term effect of the primo-infection in the B lymphocytes of surviving ICR mice. Flow cytometry analysis after 16 months of malaria infection revealed that follicular and B-1 cells were the B subsets that remained at high values. Most importantly, surviving mice showed the presence of memory B cells (MBCs) in spleen 500 days after clearance of a single infection. Our study provides evidences on gross B cell changes that are promoted by malaria infection in lymph organs which could be involved in severity or tolerance to primo-infection in murine malaria.

METHODS

Ethics statement

All procedures involving animals were carried out according to Spanish (Ley 32/2007) and European Union legislation (2010/63/CE). The protocols for *in vivo* experiments received approval by the Animal Experimentation Committee of Universidad Complutense de Madrid. The number of animals was calculated using Statgraphics software to keep around 80% of statistic power with 95% confidence level, and always following the 3Rs principles.

Parasites, mice and experimental infection

The rodent malaria parasite *P. yoelii yoelii* 17XL (*PyL*) was kindly provided by Dr Virgilio Do Rosario (Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa) and stored in liquid nitrogen after serial blood passages in mice. Hsd:ICR (CD-1) pathogen-free female mice, aged 7 weeks were purchased from Harlan Ibérica (Barcelona, Spain) and housed at random in airy racks containing Lignocel® ¾ beeding (Rettenmaier & Sohne, Rosenberg, Germany) and kept under constant standard conditions of light (12:12 h light:dark cycles),temperature (22-24ºC) and humidity (around 50%) at the Animal House of the Universidad Complutense de Madrid. All mice were fed a commercial diet (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories) *ad libitum*.

Mice were infected by intraperitoneal (ip) inoculation of $2x10^7$ *PyL*-infected red blood cells (iRBCs) obtained from donor *PyL*-infected ICR mice. Infection progression was daily monitored from the second day onwards by staining blood smears with Wright's eosin methylene blue solution followed by counting parasitemia under microscope. Mice were sacrificed at day 3 and 6 post-infection (pi) attending levels of parasitemia. Mice with low parasitemia at day 6 pi were allowed to survive to the infection and survivors were sacrificed and examined at day 500 pi. Age-matched uninfected mice were

included as controls (n=5). The clearance of parasitemia in surviving mice was confirmed at day 60 pi by ip sub-inoculation of blood into naïve BALB/c pathogen-free recipients (Harlan Ibérica) and by PCR analysis. Three independent experiments with twenty ICR mice were performed.

Cell preparations

Spleen and peritoneal cavity (Perc) cells were obtained after sacrifice at 0, 3 and 6 days pi. Spleen cells were isolated by pressing the tissue through a 70-µm nylon cell strainer (BD Biosciences, San Diego, CA, USA) with complete Iscove's medium containing 10% fetal calf serum (FCS) (Gibco, N.Y., USA), 100 units/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 6 mM HEPES (Sigma, St Louis, MO, USA) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma) followed by lysis of RBCs with ACK Lysis Buffer (Gibco). PerC exudate cells were harvested by flushing the peritoneum with 3 ml of RPMI medium 1640 (Sigma) containing 10% FCS and 3ml of air. Bone marrow (BM) cell suspensions were obtained by flushing femurs with RPMI medium 1640 containing 10% FCS and the obtained solution was filtered through a 70-µm nylon cell strainer. Lymphocytes from all cell suspensions were counted and aliquoted into units of 3 x 10^{6} cells for flow cytometry staining. Viable cell counts were always determined by Trypan Blue exclusion test using a hemocytometer.

Immunophenotyping of leukocytes by flow cytometry analysis

Cells were first incubated with anti-CD16/32 (clone 93; eBioscience, San Diego, CA, USA) for 10 min on ice to prevent non-specific binding and then stained with FITC, PE, PE-Cy5, PerCP-Cy5.5 or APC anti-IgM (II/41), anti-CD138 (281-2), anti-CD21/CD35 (7G6), anti-CD43 (S7), anti-GL7 or anti-CD45R/B220 (RA3-6B2) (from BD Pharmingen); anti-CD23 (B3B4), anti-CD38 (90), anti-IgD (11-26c) or anti-MHCII (M5/114.15.2) (from eBioscience) for 30 min on ice. Washing steps were made with FACS buffer (PBS with 2% FCS). Stained cells were fixed in 2% paraformaldehyde and flow cytometric acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences). Data was analyzed using FCS Express software (De Novo Software). Sample cells were gated on a forward scatter-side scatter gate (FSC-SSC) to exclude debris. Absolute cell numbers were calculated based on population frequencies and total cell numbers. Adequate isotype controls were used for all antibodies (eBioscience).

Protein extraction from *P. yoelii*

RBCs were isolated from the whole blood of infected Hsd:ICR(CD-1) mice showing >50% parasitemia to obtain protein lysates from the parasite. Mice were anesthetized and whole blood was collected from brachial vessels in tubes containing 0.1 M EDTA and kept at -80ºC until protein extraction. The extraction protocol began with erythrocyte lysis using 10 vol. of saponin 0.1% (w/v) in

PBS. After twice washing in cold PBS, the pellet was treated with 2-3 vol. of extraction buffer (50 mM Tris-HCl, pH 8.0; 50mM NaCl; 0.5 % Mega 10; 3% MEGA 10) containing a protease inhibitor cocktail (Roche, Indianapolis, USA) and subjected to four freeze-thaw cycles. Finally, lysates were centrifuged and *PyL* total protein samples stored at -20ºC until use. Protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Mitomycin C-treated feeder cells

In order to have feeder cells for the memory B cell culture, spleen cells from naïve mice were isolated with RPMI 10% FCS followed by RBCs lysis and adjust to a 2 x 10^7/ml concentration. Mitomycin C was added to the cell solution to a final concentration of 50 µg/ml and cells were further incubated at 37°C in 6% CO_2 during 20 min. After washing four times, cell viability was estimated by a trypan blue exclusion assay and cells concentration was adjusted to a final 10^7 cells/ml.

Determination of *PyL*-specific memory B-cell (MBC) frequency

PyL-specific MBC frequencies were determined by ELISA-quantifying the supernatant reactivity to *PyL* proteins applying limiting dilution assay (LDA). The LDA assay and cells culture were adapted from previous protocols (Ndungu *et al.* 2009; Blanchard-Rohner *et al.* 2010; Nduati *et al.* 2010). Two-fold serial dilutions of splenic cell suspensions, from 2 x 10^6 to 2.5 x 10^5 cells/well, were prepared. Replicates of 15 wells for each dilution were cultured in 96-well round-bottom plates (Corning, New York, USA) in 200µl complete Iscove's medium (as described above) containing 0.4 µg lipopolysaccharide (Sigma), 1 x 10^6 feeder splenocytes treated with mytomicin and 20 µl of a culture supernatant from concanavalin A-stimulated ICR splenocytes prepared as previously described (Glasebrook and Fitch 1980). Cells were incubated at 37°C in 6% CO_2 for 6 days. At the end of the culture period, plates were centrifuged and the individual supernatants were cleared from debris, harvested and frozen.

For the detection of *PyL*-IgGs in supernatants, ELISA plates were coated with 100 µl/well of *PyL* total protein lysates diluted at 5 µg/ml in carbonate-bicarbonate buffered solution (Sigma). After overnight incubation in humid chamber at room temperature (RT), plates were blocked with Tris-buffered saline (pH 8.0) containing 1% BSA (Sigma) for 1 h at RT. 35 µl of culture supernatant were transferred to each ELISA wells for 1h at RT. Supernatants from cultures of healthy mice spleen cells were used as negative control and immune serum diluted 1/800 in complete medium were used as positive controls. Plates were washed and bound IgGs were detected by goat anti-mouse IgGs conjugated with horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA) at 1:1000 dilution, followed by addition of 3,3´,5,5´Tetramethyl Benzidine substrate buffer (Thermo Scientific, Rockford, IL, USA). The optical density (OD) was read at 652 nm on a Varian Cary 50 Bio spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Based on the OD obtained, the frequency of *PyL*-specific MBC in

each PBMC population was estimated by determining the fraction of negative wells for each cell density based on the Poisson analytical equation as previously described (Blanchard-Rohner *et al.* 2010). The cut-off value of OD to score a well as positive was defined as the OD mean + 3 x standard deviation (SD) obtained in the negative control wells (wells containing feeder cells and medium) (Blanchard-Rohner *et al.* 2010).

Quantification of parasite DNA in blood

To confirm parasite elimination from blood in cured mice, *P. yoelii* total DNA was extracted from peripheral iRBCs using the NuncPrep™ Chemistry Isolation of DNA from Whole Blood protocol in the ABI PRISM® 6100 Nucleic Acid Prepstation, following the manufacturer's instructions (Applied Biosystems, Life Technologies Ltd, Paisley, UK). Parasite DNA quantification was assessed with the 5' fluorogenic nuclease assay (TaqMan) using a FAMTM dye-labeled specific probe. The sequence of the oligonucleotide primers and probe used for the *P. yoelii yoelii 18S* ribosomal gene subunit (GenBank Accession No. U44379) were obtained from previously published procedures (Witney *et al.* 2001). The specific set of primers and probe used to identify the *P. yoelii yoelii 18S* ribosomal gene were (5´-3´ sequences): Forward-primer, CTTGGCTCCGCCTCGATA; Reverse-primer, TCAAAGTAACGAGAGCCCAATG; Internal-probe, CTGGCCCTTTGAGAGCCCACTGATT. The 20 µl-PCR mixture contained 1X TaqMan Master Mix (Applied Biosystems), 300 nM of each primer, 200 nM of probe and 1 µL of DNA template. The amplification conditions were 10min at 95ºC for enzyme activation and 40 cycles of 20s at 95°C and 30s at 58°C and 30s at 62ºC. Samples were always run in triplicate. Amplification, data acquisition and data analysis were carried out using the ABI 7700 Prism Sequence Detector system (Applied Biosystems).

Statistical analysis

Mann-Whitney non-parametric test was used for assessing statistical significance on Prism 5 software (GraphPad Software Inc.). A probability of $P<0.05$ was considered significant when comparing the results. Results are indicated as average ± SEM. Unless otherwise specified, all tests compare the group indicated against control uninfected mice. Measurements in infected-survivor mice at day 500 pi were compared with age-matched controls.

RESULTS

Changes in splenic B cell populations are associated to prognostic parasitaemia.

ICR mice have heterogeneous susceptibility to lethal *PyL* infection that is evident from the first days of infection (Azcárate *et al.*). To evaluate and compare changes in immune cell composition in the

spleen promoted by different parasitemia levels, ICR mice were inoculated with 2 x 10^7 *PyL*-iRBCs and examined during the first days of the primo-infection and, in the case of surviving mice, 500 days after the primary *PyL* challenge. Animals were classified as low or high parasitemic depending on their detectable blood parasitemia on day 3 and 6 pi. Low parasitemic mice exhibited 7.5 ± 4.8% significantly different from high parasitemic group, which showed 62.12 ± 4.8% parasitemia (both day 3 and 6 pi P = 0.01) (Figure 1A). Mice with low and high parasitemia on day 3 and those high parasitemic on day 6 pi were examined. Mice with low parasitemia at day 6 pi were allowed to recover from infection and survivors were examined at day 500 pi.

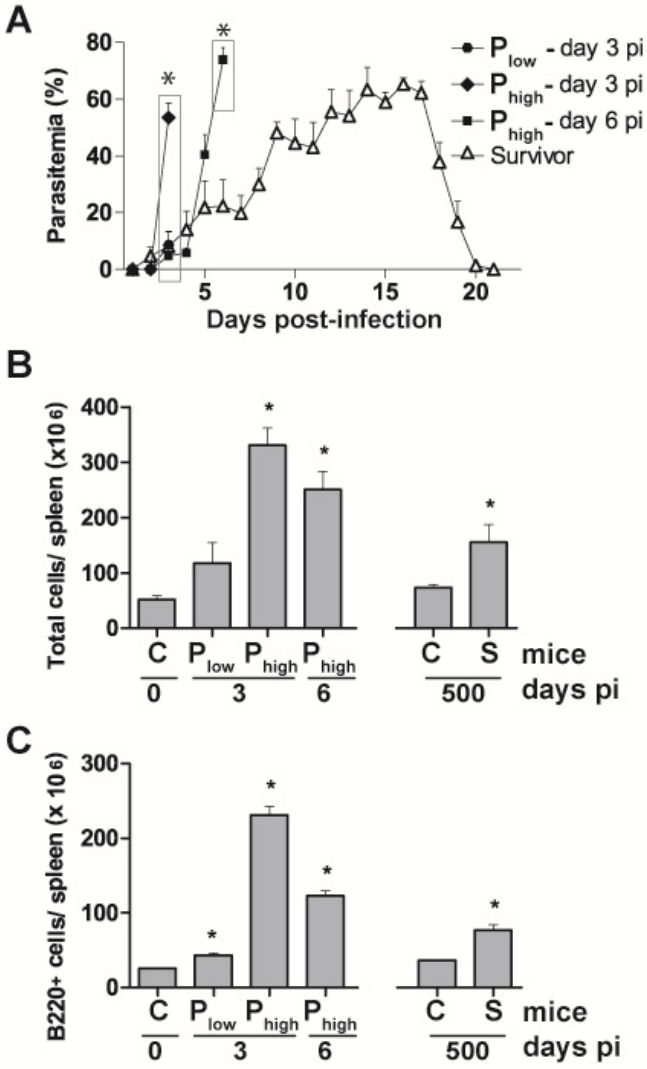

FIGURE 1. Parasitemia and spleen cell number in mice infected with *P. yoelii* 17XL. ICR mice were infected with 2x10^7 *PyL* iRBCs and grouped depending on the high "P_{high}" or low "P_{low}" parasitemia exhibited on days 3 and 6 pi. Mice with low parasitemia at day 6 pi were allowed to recover from infection and survivors "S" were examined and compared to age-matched control healthy mice "C" at day 500 pi. (**A**) Parasitemia,*p < 0.05 between high and low parasitemic mice; (**B**) total spleen cell number; and (**C**) B cell number ($B220^+$) detected by flow cytometry in spleen. * p < 0.05 comparing to uninfected mice. Data express the mean value ± SEM of three independent experiments, each with three to seven mice per time point.

Changes in total cellularity of spleen during the first 6 days of infection were observed to correlate with parasitemia (Figure 1B). At day 3 pi, splenocytes in high parasitemic mice increased significantly 3-fold compared to low parasitized animals (P = 0.002). This difference was significantly maintained in mice at day 6 pi (P = 0.006 respect day 0 pi). Interestingly, survival animals also showed a significant 2-fold increase in their spleen cellularity compared with healthy age-matched mice (P = 0.02), which was discernible 500 days after the primo-infection with no any other further contact with *P. yoelii*. There were not differences between the number of spleen cells in control healthy mice at days 0 and 500 of the experiment.

We then analysed the B cell population, as it is determinant for the clearance of parasitemia (Weinbaum *et al.* 1976; von der Weid *et al.* 1996) and for immune memory development (Zubler 2001). Although the infection did not alter the $B220^{+}$ B cells frequency in spleen (data not shown), the absolute B cell number drastically increased in all high parasitemic mice (P < 0.001) and even in those with low parasitemia at day 3 pi (P = 0.01) (Figure 1C). Again, the single *PyL* infection led to a 2-fold significant increase in the number of B cells at day 500 pi (P = 0.02).

B cell subpopulations changed in the spleen across different infection fates

Developmental stages of B cells were identified according to their IgM and IgD expression pattern as shown in Figure 2A. Immature transitional 1 (T1) B cells are defined as IgM^{hi} IgD^{low} cells, but in spleen this gate also includes B-1 and marginal zone (MZ) B cells (Loder *et al.* 1999; Korner *et al.* 2001). Transitional 2 (T2) are designated as IgM^{hi} IgD^{hi} expressing cells and mature naïve B cells are IgM^{low} IgD^{int}. Mature B cells re-circulate to find an antigen and after antigen-induced differentiation they generate IgM^{-} IgD^{-} isotype-switched cells. B cell subsets maintained a constant proportion in a *PyL* independent manner along the first week of *PyL* infection (~15% T1/B-1/MZ, ~40% mature and ~10% T2) with the exception of switched B cells which increased around 3-fold on mice with high parasitaemia at day 3 and 6 pi (both P < 0.001). Regarding absolute number of B cell subsets, they were all significantly expanded at day 3 and 6 pi (P < 0.001) in these animals, while low parasitized mice showed a lower, but significant increase in mature and switched cell numbers as early as day 3 pi (both P = 0.01). Finally, mice which survived the infection showed an augmented number of mature B cells in the spleen at day 500 pi (P = 0.02), although the relative proportions of B cell subsets were similar to those in uninfected mice.

Plasma cells (PCs), identified as $CD138^{+}$ cells, were barely detected in the first week and on day 500 pi in spleen (data not shown). This is consistent with other studies where PCs are absent in the spleen until day 10 pi and after 45 days from first malaria infection (Nduati *et al.* 2010).

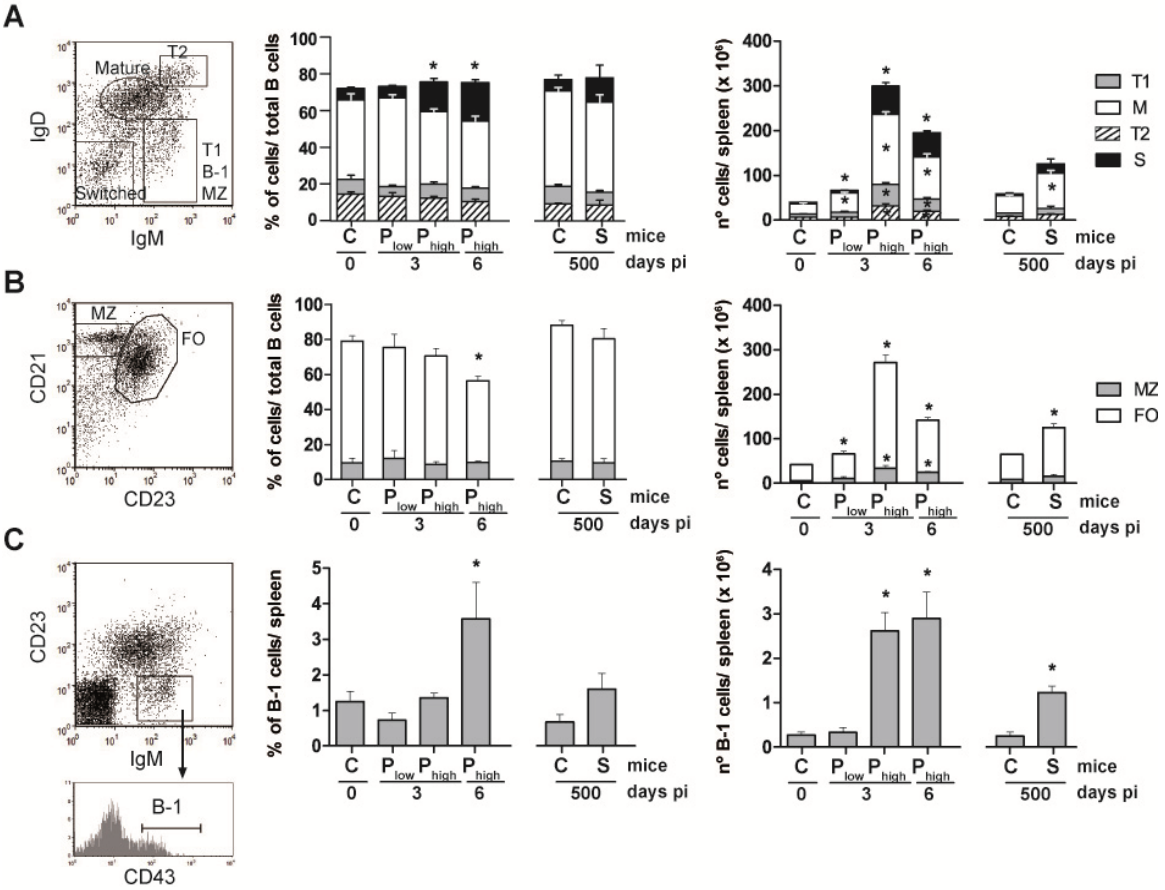

FIGURE 2. B cell subsets in the spleen during infection. Data from ICR mice infected and grouped as indicated in Figure 1 are labeled to indicate high (P_{high}) or low (P_{low}) parasitemia and surviving (S) or control (C) mice. Percentages and absolute numbers of (**A**) transitional 1 (T1) (IgM^{hi} IgD^{low}), transitional 2 (T2) (IgM^{hi} IgD^{hi}), mature (M) (IgM^{low} IgD^{int}) and class-switched (S) (IgM^{-} IgD^{-}) B cells identified among $B220^{+}$ cells; (**B**) follicular (FO) ($CD21^{low}$ $CD23^{+}$) and marginal (MZ) ($CD21^{+}$ $CD23^{-}$) B cells gated from $B220^{+}$ cells and (**C**) B-1 cells (IgM^{+} $CD23^{-}$ $CD43^{+}$) in spleen were examined by staining for flow cytometry. Data express the mean value ± SEM of three independent experiments, each with three to seven mice per time point. * $p < 0.05$ between infected and uninfected mice.

***P. yoelii* 17 XL infection altered follicular (FO) B cells more largely than marginal zone B cells in the spleen**

FO B cells are main mediators of T-dependent antigen responses, contrary to MZ B cells which mediate T-independent responses (Martin and Kearney 2000). The fate of these compartments was studied by the staining with CD21, a complement receptor, and the low-affinity IgE receptor CD23. FO B cells are recirculating mature naïve cells that are $CD21^{low}$ $CD23^{+}$ while MZ B cells are $CD21^{+}$ $CD23^{-}$ (Figure 2B). In naïve mice, MZ B cells comprised 9.6 ± 2.5 % and FO B cells 69.3 ± 3.1 % of spleen. Although MZ B cell frequency remained unchanged during the first week of infection, mice suffering from high

parasitemia increased its number on days 3 and 6 pi ($P < 0.001$). Analysis of FO cells revealed a frequency decrease at day 6 pi ($P < 0.001$), but an increase in absolute numbers in infected mice from day 3 pi, being larger in mice with high parasitemia ($P < 0.001$). FO B cells numbers, even after 500 days of the infection, maintained enlarged levels than naïve mice ($P = 0.02$).

Increased B-1 B cells in the spleen of high parasitemic animals

Two families are distinguished among B cells, B-1 B cells locate mainly in the PerC whereas B-2 B cells include FO, germinal center (GCs), MZ, T1 and T2 B cells (although the term B-2 has been currently reduced to denominate FO B cells in the literature). Frequencies and numbers of B-1 B cell in spleen, that are $IgM^+ CD23^- CD43^+$ (Berland and Wortis 2002; Baumgarth 2010), remained unchanged in mice suffering from low parasitemia (Figure 2C). Contrary, high parasitemic animals showed an increase in the frequency on day 6 pi ($P = 0.02$) and in cell numbers in both days 3 and 6 pi ($P < 0.001$). The increased numbers of B-1 cells were maintained by surviving mice at day 500 pi ($P = 0.02$).

Changes in B-2 and B-1 cell number in the peritoneal cavity during the first week of infection

Murine PerC cells are able to act against parasitized erythrocytes *in vitro* (Tosta and Wedderburn 1980; Brown and Kreier 1982). PerC cellularity increased in mice with high parasitemia, exceptionally at day 3 pi ($P = 0.03$) (Figure 3A). We discriminated B-2 cells, gated as $B220^+ IgM^- IgD^+ CD5^-$ cells, from the $IgM^+ IgD^-$ B-1 cells in the PerC (Wardemann *et al.* 2002). The infection did not interfered in the B-1:B-2 cells proportion which, in both healthy and infected mice, was around 2:1 (Figure 3B) and B-1 cells counted for 11.0 ± 1.7 % of total leuckocytes (data not shown). However, mice with high parasitemia showed a significant increase in the absolute numbers of B-2 (4-fold increase) and B-1 cells (3.5-fold increase respect uninfected mice) at day 3 pi ($P < 0.001$) (Figure 3B). At day 500 pi infection-surviving mice showed the same kinetics in B-1 and B-2 cells than control mice.

***PyL*-specific memory B cells remained in the spleen 500 days after a single infection**

The activation of mature B cells by T cell-dependent antigens is characterized by the formation of GCs, where MBCs are mainly differentiated (Aiba *et al.* 2010). GC B cells can be identified by CD38 and GL7 staining. In mouse, CD38 is downregulated on antigen-specific GC B cells and in mature PCs, but it is re-expressed on the resting memory B cell population (Lund *et al.* 1995; Oliver *et al.* 1997; Ridderstad and Tarlinton 1998). On the other hand, GL7 peanut agglutinin, distinguishes antigen-experienced B cells (Cervenak *et al.* 2001).

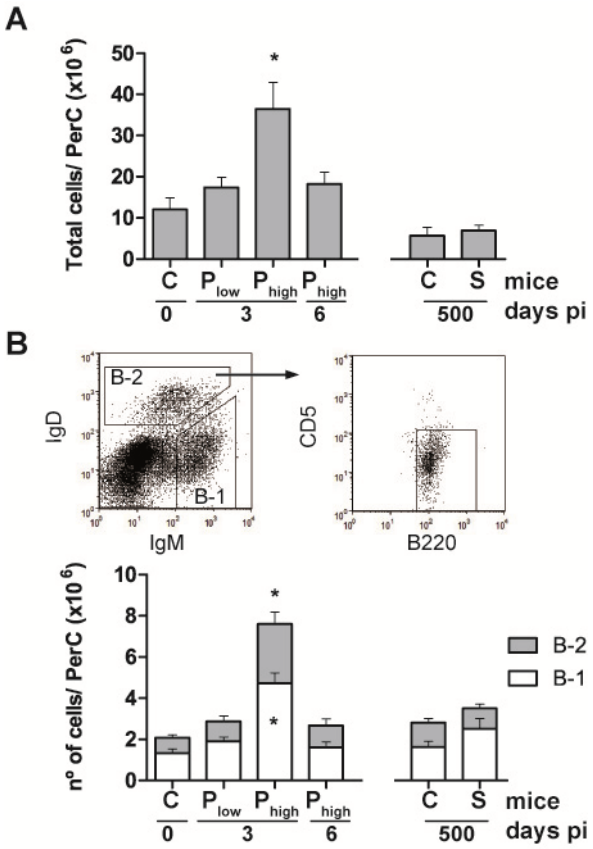

FIGURE 3. Peritoneal cavity B cells. Data from ICR mice infected and grouped as indicated in Figure 1 are labeled to indicate high (P_{high}) or low (P_{low}) parasitemia and surviving (S) or control (C) mice. (**A**) Absolute number of total cells and (**B**) of B-1 (IgM^+ IgD^-) and B-2 ($B220^+$ IgM^- IgD^+ $CD5^-$) B cells from peritoneal cavity were examined by staining for flow cytometry. Data express the mean value ± SEM of three independent experiments, each with three to seven mice per time point. * $p < 0.05$ between infected and uninfected mice.

We observed that GC B cell population, designated as $GL7^+$ $CD38^-$ MHC II^+ cells (Aiba *et al.* 2010), was absent from the spleen of surviving mice at day 500 pi (Figure 4A). In previous work we have shown that ICR mice surviving a single *PyL* infection are able to develop immunological memory that protects them against a second infection applied 2 months later (Azcárate *et al.*). Although it is of great interest to determine whether MBCs could be maintained at long-term, unfortunately, there is not a cytometric antibody (Ab) combination that definitively define murine MBCs since this phenotype is highly heterogeneous (Anderson *et al.* 2007; Tomayko *et al.* 2010). Number of $B220^+$ $CD38^+$ cells in spleen of survivor mice at day 500 pi was double than in control mice ($P = 0.02$) (Figure 4B), but as they could be either naive or memory, *PyL*-specific MBCs were investigated by determining *PyL* specific IgG in the supernatant from cultures of stimulated MBCs (Blanchard-Rohner *et al.* 2010). Supernatant from cultured splenocytes from naïve mice did not score positively in this assay, while splenocytes from mice which had cleared a primary infection on day 20 pi showed the presence of MBCs at day 500 pi (Figure

4C). This result is consistent with the persistence of a reservoir of MBCs in surviving mice upon a single infection with *PyL*.

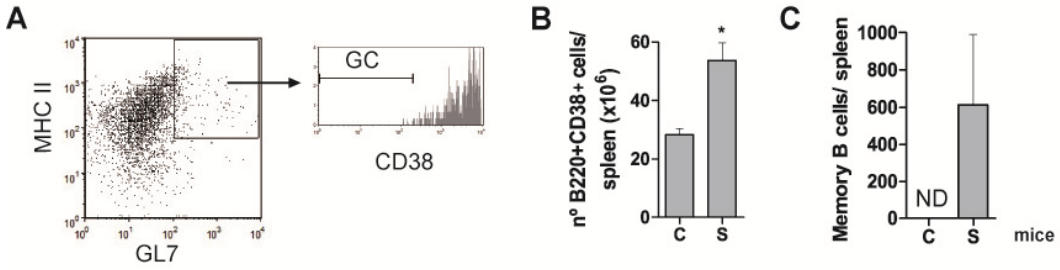

FIGURE 4. Memory B cells in spleen. Surviving mice "S" from *PyL* infected ICR mice were examined and compared to age-matched control healthy mice "C" 500 days after infection. (**A**) Gating strategy for germinal centre (GC) cells detection by flow cytometry. (**B**) Absolute numbers of $B220^{+}$ $CD38^{+}$ cells in spleen. * $p < 0.05$ between infected and uninfected mice. (**C**) Total numbers of *PyL*-specific IgG memory B cells after polyclonal stimulation in limiting dilution cultures of splenocytes. Data express the mean value ± SEM of two independent experiments, each n = 4 mice.

Plasma cells were absent in bone marrow 500 days after a single infection

To ascertain whether a single infection with *PyL* is capable of eliciting long live plasma cells, this cellular type was analysed in BM after 500 pi. No differences were detected in BM total cell numbers between malaria infected or control animals (Figure 5). Since Ab secreting cells may migrate to BM to differentiate into long-lived cells (Manz *et al.* 1997), we analyzed the number of PCs ($B220^{+/lo}$ $CD138^{+}$) in BM and, although this population increased in surviving mice in comparison to healthy controls, the differences were not significant (Figure 5).

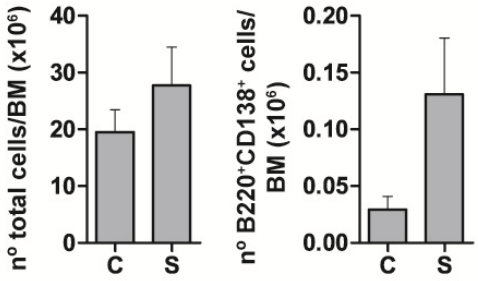

FIGURE 5. Bone marrow cells. Surviving ICR mice "S" from *PyL* infection were examined and compared to age-matched control healthy mice "C" 500 days after infection. Number of total cells and $B220^{+}$ $CD138^{+}$ cells in bone marrow were examined by flow cytometry. Data express the mean value ± SEM of two independent experiments, each n = 4 per group.

DISCUSSION AND CONCLUSIONS

To investigate malaria immunity in rodent models, susceptible mice are usually compared to drug-treated infected mice, non-lethal *Plasmodium* infected mice, immunized mice or homogeneously malaria resistant strain mice. In previous work we characterized a model of *PyL*-infected ICR mouse which associate differential immune responses detected in blood with a diversity of malaria prognosis, from survival to early decease. In this model it is shown that most severe fatal cases have significantly higher parasitemia levels than surviving mice from day 3 pi (Azcárate *et al.*). Here, we studied the evolution of splenic B cell population comparing infection groups at day 3 pi and at long term upon survival. In our model, we observed that spleen and PerC cells can be differentially affected depending on parasitemia level and length of the infection.

In this malaria infection model with *PyL*, the increase of cell numbers in spleen was directly proportional to the parasitemia rates during the first week as it has been previously shown in *P. chabaudi* (Helmby *et al.* 2000; Castillo-Mendez *et al.* 2007) and non-lethal *P. yoelii* (Chandele *et al.* 2010) infections. As early as day 3 pi, the spleen of *PyL*-infected ICR mice following high parasitemia had 3-fold increase in cell number than healthy mice, despite that total leukocytes do not increase in blood (Azcárate *et al.*). On the contrary, surviving mice expand white blood cells numbers from day 14 pi in blood (Azcárate *et al.*) suggesting a migration of cells from the spleen to the circulating blood compartment after the first week of the infection, which is consistent with other malaria models (Helmby *et al.* 2000). Although naturally surviving mice maintained the splenomegaly for more than 16 months after a single infection, the number of white cells in blood remain constant even applying a second infection (Azcárate *et al.*). Thus, the analysis of blood cells in malaria infections cannot always reflect internal white blood cells kinetics since we observed that parasitemia, time of infection and individual response may modify spleen/blood compartments.

All infected ICR mice, even those with low parasitemia levels, increased the number of B lymphocytes in spleen during the first days of infection, in line with the high rate of B cell proliferation observed in human splenomegaly caused by malaria and resulting in an expanded population of activated B cells (Bates and Bedu-Addo 1997). The changes in the phenotype of B cells in our mice were associated to the parasitemia level and the length of infection. Immunity to blood-stage malaria is largely humoral (Good and Doolan 1999), therefore it is consistent that major changes of cell numbers and frequencies were observed in FO B cells that play an important role in the generation of long humoral responses against T-dependent antigens. MZ subset, which generate short-living PCs responsible of low-affinity antibodies as a first-line defence against the parasite (Martin and Kearney 2000), suffered minor changes. It is interesting that B-1 cells, a natural high IgM producer that do not contain somatic mutations (Berland and Wortis 2002; Baumgarth 2010), was maintained at high numbers in surviving mice after a single infection.

How the immunological memory is developed in humans is still uncertain (Doolan *et al.* 2009). Malaria immunity after the inoculation of intact parasites in humans has been deeply studied through artificial *P. falciparum* sporozoites inoculations (Roestenberg *et al.* 2009; Roestenberg *et al.* 2011; Teirlinck *et al.* 2011) or infections with ultra-low doses of blood-stage parasites under concomitant regime of drugs (Pombo *et al.* 2002). In both studies the submicroscopic presence of blood-stage parasites explains the lack of humoral response in volunteers, and T cell predominant response. Actually, in endemic areas the immune response is mainly targeted against blood-stage Plasmodium parasites rather than liver-stage parasites, probably due to the low dose of parasites transmitted per mosquito bite (reviewed in (Vaughan *et al.* 2010)).

Since the surface phenotype of murine MBCs display considerably heterogeneity (Anderson *et al.* 2007), the functional cell assessment is an accurate method for their definition. The development of immunological memory in ICR surviving mice is assumed from previous experiments according to the lack of parasites after a second infection, the maintenance of high levels of specific-IgG antibodies in serum during one year after two infections and their protective role by passive transference to naïve BALB/c mice (Azcárate *et al.*). Since vaccine experiments in humans against smallpox, influenza or malaria have shown that there is a decline of specific MBCs per month after vaccinations (Crotty *et al.* 2003; Wrammert *et al.* 2008; Crompton *et al.* 2010), we were interested about the lenght of immunology memory in our mouse model. To elucidate if a single infection is enough to generate a long-lived response in ICR mice, *PyL*-specific IgG memory B cell frequencies were determined 500 days pi in spleen by LDA (Blanchard-Rohner *et al.* 2010). Our data showed, for the first time, that MBCs are efficiently induced by primary lethal malaria infection in self-cured mice and. Although in other models MBCs can be also favored by chronic infection due to the their continuous exposure to the antigen (Stephens *et al.* 2009), in our ICR-*PyL* model the MBCs were maintained in the spleen longer than 16 months after *PyL* complete clearance from blood. Other malaria-infection models with *P. chabaudi* demonstrate B memory cells present in the spleen after 8 months of the single infection (Ndungu *et al.* 2009).

It is in the GCs of the spleen follicles where recirculating B cells differentiate into long-living PCs and where MBCs continuously proliferate to PCs, which sustain high affinity Ab responses (Sagaert *et al.* 2007). The fact that B cells were not observed in the GCs irrespective the changes in FO or MZs B cells in our study is in agreement with the timing of GC reaction (Sagaert *et al.* 2007) and with the observation in *P. chabaudi* infections that GCs are visible after 8 days pi (Achtman *et al.* 2003). Besides, 16 months after the *PyL* infection probably exceed the period of time in which GCs can persist, that is of 60 days for *P. chabaudi* infection (Achtman *et al.* 2003) and 4 months for mammary virus infection (Luther *et al.* 1997).

Together, this study contributes to the characterization of B cell kinetics during the different parasitemia courses shown by lethal *P. yoelii* infection in outbreed mice ICR. Moreover, from the cell

changes detected in organs during the very first days of infection, to the changes observed in B cell subsets after almost 2 years of the malaria primo-infection in self cured mice, including the presence of MBCs, demonstrate a variety of effects and responses under identical infection. Our observations highlight the importance of considering potential heterogeneous responses in blood and spleen to gain new insights into the immunity against lethal strains of Plasmodium, including *P. falciparum* in humans.

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5. DISCUSIÓN

5.1. Actividad antimalárica de la borrelidina, mupirocina y cloroquina

Debido a que la resistencia a los fármacos antimaláricos se ha convertido en uno de los mayores desafíos para el control de la malaria, es necesaria la búsqueda de nuevas alternativas terapéuticas. En el pasado, el uso de antibióticos ha controlado a las cepas resistentes de otros compuestos antimálaricos. Las tetraciclinas son uno de los antibióticos con actividad antimalárica (Rieckmann *et al.* 1971). Entre ellas destaca la doxiciclina cuya eficacia contra el *P. falciparum* se ha demostrado en experimentos *in vitro* (Basco and Le Bras 1993; Pradines *et al.* 2000) y en estudios clínicos (Gras *et al.* 1993; Baudon *et al.* 1999). Su mecanismo de acción consiste en el bloqueo específico de la expresión del genoma del apicoplasto, lo que resulta en la distribución de apicoplastos no funcionales en la progenie de los parásitos tratados, y por ello en un efecto de "muerte lenta" (Dahl *et al.* 2006). Los antibióticos macrólidos (cuya estructura química se caracteriza por contener un anillo de lactona de gran tamaño (Cobos-Trigueros *et al.* 2009)), como la eritromicina y su derivado semisintético, la azitromicina, también poseen actividad antimalárica *in vitro* (Geary and Jensen 1983; Gingras and Jensen 1992) y en el humano (Kuschner *et al.* 1994; Taylor *et al.* 1999). La diana de la azitromicina en el parásito es la maquinaria de traducción del apicoplasto y, como en el caso anterior, su efecto es considerablemente mayor en la progenie que en los parásitos tratados (Sidhu *et al.* 2007). Por otro lado, las fluoroquinolonas también tienen actividad contra *P. falciparum in vitro* (Krishna *et al.* 1988; Tripathi *et al.* 1993; Yeo and Rieckmann 1994) e *in vivo* (Watt *et al.* 1991), en este caso mediante la inhibición de la ADN girasa (topoisomerasa II) (Maxwell 1992). En resumen, los antibióticos desempeñan su efecto antimalárico mayoritariamente en la progenie de los parásitos tratados con un consecuente retraso de su acción (Dahl and Rosenthal 2007).

En el presente trabajo nos hemos centrado en dos antibióticos, borrelidina y mupirocina cuya actividad antimalárica contra especies de Plasmodium parásitas de humano y roedor (Otoguro *et al.* 2003; Ishiyama *et al.* 2011; Istvan *et al.* 2011) han sido previamente demostradas, y radican en su poder inhibitorio de aminoacil-ARNt sintetasas (ARSs). La ARS es una enzima que participa en la síntesis proteica al catalizar la reacción siguiente, dependiente de adenosin trifosfato (ATP) y magnesio ($Mg2^{+}$):

$$\text{Aminoácido} + \text{ARNt} + \text{ATP} \longrightarrow \text{Aminoacil-ARNt} + \text{AMP} + \text{PPi}$$

La activación de los aminoácidos y la formación de aminoacil-ARNt tiene lugar en dos pasos, en el primero el aminoácido reacciona con el ATP para formar un aminoacil-AMP y a continuación se transfiere el aminoácido activado al extremo 3` del ARNt correspondiente. Por lo general, existe una sola ARS para cada aminoácido. La reacción es específica tanto a nivel de aminoácido como de ARNt, lo que permite establecer una relación unívoca entre un aminoácido determinado y una secuencia anticodón, característica del ARNt al que se une. Es en base a esta relación como cada uno de los

codones del ARNm dirige la inserción de un aminoácido específico a través de la secuencia anticodón del ARNt a la cual se acoplan. La ARS reconoce los diferentes ARNt por medio de unos determinados nucleótidos, y también, en algunos casos, por la propia secuencia anticodón. Por otro lado, el aminoácido es reconocido por su tamaño y sus propiedades (Teijón *et al.* 2006).

La borrelidina es un antibiótico macrólido que inhibe las treonil-ARSs de Plasmodium (Hutter *et al.* 1966), que se localizan en citosol y en apicoplasto. No hay evidencias de treonil-ARS en la mitrocondria, por lo que se deduce que los ARNt serían cargados en citosol o apicoplasto para ser entonces importados a la mitocondria. Por otro lado, la mupirocina es un ácido pseudomónico e inhibe únicamente la isoleucil-ARS del apicoplasto (Hughes and Mellows 1978; Jackson *et al.* 2012). La diferencia en la localización de las ARSs diana de ambos antibióticos provoca que la borrelidina tenga un efecto inmediato en el crecimiento de *P. falciparum*, mientras que la mupirocina se asemeje a la mayoría de antibióticos antimaláricos en la lentitud de su efecto.

Para analizar el efecto de los antibióticos borrelidina y mupirocina sobre el desarrollo de la enfermedad y su influencia en la adquisición de respuesta inmune, procedimos a estudiar el resultado del tratamiento en ratones BALB/c infectados con la cepa letal *P. yoelii yoelii* 17XL (*PyL*) en fase intraeritrocítica, en comparación con el tratamiento con cloroquina a distintas dosis. El tratamiento de los ratones infectados se llevó a cabo según las pautas de administración descritas en el test supresor de 4 días (Peters and Robinson 1999). La evolución de la enfermedad se examinó mediante análisis diarios de parasitemia y supervivencia. Los resultados obtenidos indican, en primer lugar, que todos los animales infectados y tratados con borrelidina o con cloroquina a la mayor dosis, 30 mg/Kg (cloroquina-30), sobrevivieron a la primera infección; mientras que los tratados con mupirocina o con la dosis menor de cloroquina, 1mg/Kg (cloroquina-1), fallecieron en la primera semana de infección similarmente a los ratones no tratados. El hecho de que la mupirocina no sea capaz de controlar una primera infección de malaria *in vivo*, puede ser debido a su rápida hidrólisis en plasma, su unión al suero o a la disminución de su actividad biológica a pH cercano a 8 (Thomas *et al.* 2010) por lo que su empleo en humanos, por el momento, se restringe al uso tópico (Sutherland *et al.* 1985).

A diferencia del resto de grupos, el tratamiento con borrelidina inhibió la multiplicación del parásito durante los 4 días de tratamiento en los ratones infectados, lo cual es coincidente con los efectos de la administración del antibiótico en infecciones de *P. yoelii* y *P. berghei* en ratones (Otoguro *et al.* 2003), con el grupo tratado con cloroquina-30. A pesar de la actividad que posee la borrelidina a tan baja concentración, se desconocía su efecto en el tratamiento de ratones más allá de los 4 pi, por lo que continuamos el análisis de la infección hasta el día 20 pi. Tras retirar el tratamiento de borrelidina, la parasitemia aumentó antes y alcanzó un máximo 3 veces mayor que en los ratones tratados con cloroquina-30. Finalmente, los parásitos disminuyeron hasta ser indetectables microscópicamente primero en el grupo tratado con cloroquina y alrededor de una semana después en los que recibieron borrelidina. Estos resultados demuestran que la administración profiláctica de la borrelidina durante 4

días, comenzando el mismo día de la infección con el parásito *PyL*, es suficiente para frenar la infección y para que los ratones desarrollen una respuesta inmunológica capaz de controlar la multiplicación del parásito y evitar la muerte. Es destacable que la borrelidina, a una concentración 120 menor que la cloroquina, mostró la misma eficacia que ésta en cuanto a supervivencia tras una primera infección (las dosis de ambas fueron la misma respecto a sus respectivas dosis inhibitoria 50, IC_{50}).

La diferencia entre las curvas de parasitemia de los dos grupos de ratones supervivientes nos indujo a estudiar el efecto de los tratamientos in vitro. Se trataron glóbulos rojos infectados en el estado de anillo, procedentes de cultivos sincrónicos de la cepa Dd2 (resistente a cloroquina) de *P. falciparum*, con concentraciones de borrelidina o de cloroquina 20 veces mayor que sus IC_{50} durante durante un ciclo infectivo (48 h), transcurrido el cual se procedió a determinar las formas parasitarias presentes mediante análisis microscópicos. En ambos casos se induce una inhibición rápida del crecimiento del parásito. Este resultado confirma los resultados obtenidos por Jackson *et al*. en su estudio de la borrelidina (Jackson *et al.* 2012). Tras el tratamiento durante 2 días, se mantuvieron los cultivo libres de los compuestos durante 4 ciclos infectivos adicionales (8 días) para el seguimiento del desarrollo parasitario. Se observa que la recuperación del crecimiento de los parásitos tras el tratamiento con la borrelidina es más temprana que con la cloroquina, lo que coincide con los resultados obtenidos en el presente trabajo *in vivo*. Se analizó además la distribución de las distintas formas del parásito. Es destacable que, aunque en nuestro estudio se cultivó el parásito con la borrelidina a una concentración 5 veces mayor y por el doble de tiempo que en los ensayos realizados en estudios previos (Ishiyama *et al.* 2011; Jackson *et al.* 2012), una fracción de parásitos en fase de anillo sufre un arresto del crecimiento durante 4 ciclos, pero es capaz de sobrevivir y retomar su crecimiento pasados 8 días tras la retirada del antibiótico. Otra fracción degenera en formas aberrantes de parásitos jóvenes que estarían afectadas mortalmente. Así, el efecto de la borrelidina en el estadio de anillo del parásito intraeritrocítico podría considerarse parcialmente parasitostático (inhibe el crecimiento y multiplicación del parásito sin matarlo), mientras que los estadios maduros del parásito serían susceptibles de una actividad parasiticida de la borrelidina (mata al parásito reduciendo carga microbiana) (Bahamontes-Rosa *et al.* 2011).

Con el fin de evaluar los efectos de la borrelidina en las distintas formas parasitarias de *PyL* in vivo, procedimos a examinar las fases intraeritrocíticas del parásito en los ratones del ensayo mediante el análisis microscópico de las extensiones sanguíneas durante los diez primeros días de la infección. Los ratones inoculados con el medio vehículo o tratados con la mupirocina, muestran una acumulación de parásitos en forma de anillo al final del tratamiento (día 3 pi), seguida por la acumulación de formas maduras antes de morir, provocada seguramente por la poca disponibilidad de eritrocitos a causa de la infección que imposibilita la formación de nuevos anillos, pero no afecta a las formas maduras restantes que ya estaban en crecimiento. Por el contrario, los ratones tratados con la borrelidina muestran la acumulación de trofozoítos en sangre periférica al final del tratamiento (día 3 pi) que persiste, en

general, entre los días 7 y 10 pi. Esta acumulación puede ser atribuida al efecto de la borrelidina en los estadios maduros observado *in vitro*, que previene la apariencia de nuevos anillos (Ishiyama *et al.* 2011), así como al retraso del desarrollo de anillos y trofozoítos jóvenes que no llegarían a completar un ciclo entero desde el día 2 al 3 pi, creciendo únicamente hasta el estadio de trofozoíto en nuestro ensayo *in vivo*. Los resultados de este estudio ponen por primera vez de manifiesto que el efecto específico de estadio de la borrelidina hacia los trofozoítos y esquizontes (Ishiyama *et al.* 2011; Jackson *et al.* 2012), probablemente debido a su mayor expresión de la ARS (Jackson *et al.* 2012), se cumple también durante el tratamiento *in vivo*. Por último, con el fin de investigar si la administración curativa de la borrelidina es tan eficaz como la profiláctica, procedimos a tratar durante cuatro días (3-6 pi) una infección homóloga en el mismo modelo animal comenzando cuando los ratones mostraban un 10% de parasitemia. En este caso, la borrelidina es más lenta que la cloroquina en ejercer su efecto antimalárico lo que reduce su efectividad a una protección del 75% de los ratones.

Los ratones alcanzan mayor parasitemia tras la administración de la borrelidina, tanto profiláctica como curativa, que tras el tratamiento de la cloroquina. Esto puede ser debido a que el antibiótico ejerce un efecto letal mayoritariamente en los parásitos maduros, lo que es más rápidamente reversible por el conjunto de los parásitos que el cursado por la cloroquina, que es letal en todos los estadios intraeritrocíticos excepto los merozoítos (Langreth *et al.* 1978; Cambie *et al.* 1991). Además, parámetros farmacocinéticos como la vida media en suero y su concentración de máxima inhibición podrían influir en los resultados obtenidos(Van Bambeke and Tulkens 2001).

5.2. Respuesta humoral tras tratamiento de malaria con borrelidina y cloroquina

La influencia de los tratamientos antimaláricos en la adquisición de inmunidad por el hospedador es un aspecto fundamental en la infección por malaria puesto que la primera defensa ante la infección, en los países endémicos, es la respuesta inmune. En el campo de los estudios clínicos se ha demostrado que el tratamiento quimioterapéutico de la malaria es una estrategia eficaz de inmunización. La repetición de infecciones subclínicas de malaria, seguidas de un tratamiento farmacológico, facilita el desarrollo de la inmunidad a la malaria en humanos (Schellenberg *et al.* 2005). Además, la inoculación experimental de pequeñas dosis de esporozoítos de *P. falciparum* bajo el tratamiento preventivo con cloroquina en humanos (Roestenberg *et al.* 2009) confiere mayor nivel de protección comparado con la vacunación mediante esporozoítos irradiados (Hoffman *et al.* 2002). Similarmente, la inmunización de ratones con cepas no letales de *P. yoelii* bajo el tratamiento con cloroquina induce la protección contra parásitos intra y exoeritrocíticos (Belnoue *et al.* 2008). Incluso, los fármacos antimaláricos clásicos pueden afectar directamente al sistema inmunitario en mecanismos como la presentación antigénica, la fagocitosis, la producción de citoquinas, nitrógeno o intermediarios

de oxígeno y la adherencia de los eritrocitos parasitados, entre otros (revisado por (Muniz-Junqueira 2007)).

Con el objetivo de conocer la influencia de la borrelidina sobre la adquisición de inmunidad a la malaria, los ratones que sobrevivieron a la primera infección por *PyL* tras el tratamiento con borrelidina o cloroquina-30, fueron reinfectados de forma homóloga el día 75 pi. La adquisición de parasitemia fue examinada durante los 30 días posteriores a esta segunda inyección de parásitos. Todos los animales tratados con borrelidina sobrevivieron asintomáticamente a la infección con una parasitemia transitoria y casi imperceptible microscópicamente, lo cual indica la inmunidad a la malaria de todos los animales. En el caso de los ratones tratados con cloroquina-30, se observó también una parasitemia extremadamente baja tras la segunda infección, con la excepción de un ratón que desarrolló una parasitemia la cual fue letal en el día 10 pi. Todos los ratones supervivientes de la segunda infección del ensayo, sobrevivieron a una tercera infección aplicada al cabo de un año. El retraso de la muerte del ratón tratado con cloroquina-30 respecto a los controles infectados por primera vez, sugiere cierta activación inmunológica, pero la ausencia de parasitemia visible microscópicamente durante su primera infección fue probablemente la responsable de la falta de protección eficaz. Los resultados coinciden con infecciones de *P. berghei* en ratón en las que el tratamiento que mantienen la cronicidad de la primera infección promueven altas tasas de supervivencia ante la reinfección, mientras que los tratamientos que eliminan completamente al parásito en la primo-infección no protegen ante la reinfección (Long *et al.* 2002).

Los datos de concentración de los Acs IgG específicos en los sueros de los ratones tratados con borrelidina y cloroquina-30 obtenidos tras las tres infecciones revelaron que los niveles de IgG fueron mucho mayores tras las reinfecciones que en el primer contacto con el parásito y fueron equivalentes entre ambos grupos, lo que indicaría que, además de la actividad antimalárica directa, la protección está mediada por una respuesta humoral y que ésta es similar en ambos grupos de tratamiento. La presencia de grandes cantidades de Acs específicos durante 9 meses tras la segunda infección que son capaces de eliminar rápidamente al parásito (Kinyanjui *et al.* 2004; Achtman *et al.* 2007; Weiss *et al.* 2009) y el incremento de la avidez de las IgGs tras las reinfecciones (Berek 1993) nos indican la generación de memoria inmunológica frente a la infección por *PyL* en el 100% de ratones tratados con borrelidina y en el 90% de los tratados con cloroquina. Ambos tratamientos mostraron la mayor avidez en la segunda infección, lo que puede ser debido a una cierta pérdida de la respuesta inmune a la infección un prolongado periodo de tiempo (9 meses) sin contacto con el parásito entre la segunda y tercera infección, como se ha demostrado también en humanos (Linares *et al.* 2011) y cepas no letales de *Plasmodium* en ratón (Bull *et al.* 2002).

La variedad de antígenos detectados por las IgGs del suero de los ratones supervivientes fue evaluado mediante técnicas de *inmunoblot* que revelaron el aumento de éstos con cada infección, contrariamente al ratón tratado con cloroquina-30 que murió tras la segunda infección, que mostró un

reducido reconocimiento de antígenos. Este resultado coincide con la adquisición de inmunidad clínica en los humanos, que es paralela a la acumulación de distintas especificidades antigénicas a lo largo del tiempo (Bull *et al.* 2002; Kinyanjui *et al.* 2004).

Los resultados demuestran que los tratamientos antimaláricos a dosis que promueven la eliminación rápida de los parásitos en la sangre pueden reducir el tiempo de residencia de los parásitos nativos en la sangre dificultando la presentación antigénica a las células inmunes. Así, los niveles de parasitemia durante la infección es un factor muy importantes para el desarrollo de la respuesta inmune ya que la inmunización de humanos a la malaria a través de infecciones experimentales de *P. falciparum* revelan que la presencia de Acs -particularmente transcendentales en la inmunidad a la malaria en humanos (Cohen *et al.* 1961) y ratones (Jayawardena *et al.* 1978)- es dependiente de la presencia de parásitos detectable microscópicamente en sangre (Pombo *et al.* 2002; Roestenberg *et al.* 2011; Teirlinck *et al.* 2011). Los compuestos con actividad estática pueden incrementar el tiempo de presentación antigénica necesaria para el desarrollo de la inmunidad a la malaria (Scholar and Pratt 1939; Urban *et al.* 2005; Amante *et al.* 2011).

Se localizaron por microscopía de fluorescencia los antígenos reconocidos por las IgGs de los ratones inmunes en los merozoítos e interior de los eritrocitos infectados. Las proteínas de los merozoítos, especialmente AMA-1 y variantes de MSP, son conocidas por su alta antigenicidad que ha pretendido ser aprovechada para la elaboración de vacunas (Riley and Stewart 2013). Por el contrario, los antígenos internos del eritrocito se consideran marcadores de infección y de un probable incremento de la eliminación de los parásitos, pero no de la existencia de una inmunidad protectora debido a que éstas proteínas son solo detectadas al ser secretadas con la rotura de las células rojas que las contienen (Boyle *et al.* 1997). Sin embargo, hay estudios previos que han detectado excepcionalmente antígenos intracelulares de *Plasmodium* u otros parásitos que confieren inmunidad humoral protectora (Vedi *et al.* 2008; Crompton *et al.* 2010), por lo que no se puede descartar la relevancia de los antígenos intracelulares detectados por nuestros ratones en la protección.

5.3. Utilidad de la borrelidina como agente antimalárico

Según los resultados obtenidos la borrelidina se presenta como una potencial alternativa experimental que mejoraría las estrategias de tratamiento con antimaláricos:

a) *Reducción de la parasitemia durante la fase aguda de la enfermedad*. Nuestros resultados contribuyen a validar la treonil-ARNt sintetasa como una diana para terapia tanto profiláctica como curativa de la malaria en modelos animales. Sin embargo, los ensayos realizados indican que el fármaco no evita que el animal infectado alcance niveles de parasitemia considerables. La terapia combinada es una consigna obligatoria en el tratamiento de malaria, en especial para evadir los mecanismos de

resistencia del parásito a las drogas, lo que sería doblemente aconsejable en este caso, ya es requisito de los nuevos fármacos que eliminen completamente al parásito, al menos a niveles submicroscópicos. Otros compuestos con actividad parasitostática como la azitromicina o atovacuona (Bahamontes-Rosa *et al.* 2011) son tratamientos frecuentemente utilizados en humanos combinados con otros compuestos de acción más rápida (WHO 2010).

b) *Inducción artificial de inmunidad contra infecciones homólogas de malaria mediante la inoculación de parásitos intactos en fase intraeritrocítica bajo tratamiento quimioterapéutico*. El efecto antimalárico del compuesto, al ser aplicado los 4 primeros días de infección, es protector frente a la letalidad de la infección y permite el desarrollo de una respuesta humoral protectora frente a las reinfecciones. En áreas donde la transmisión de malaria es de intensidad moderada o alta se puede presuponer que las infecciones por Plasmodium serán naturalmente adquiridas por lo que la administración de un compuesto cada cierto tiempo, como se plantea en los TPI, puede atenuar a los parásitos manteniéndolos a bajos niveles y promoviendo inmunidad (Schellenberg *et al.* 2001). Para ello se requiere una vida media relativamente alta del agente terapéutico. La ventaja de la TPI es que favorece la respuesta inmunológica contra las cepas existentes en cada área o contexto, pero su desventaja es que la respuesta inducida ante una cepa de Plasmodium puede no ser protectora ante otras. Tanto si la infección es experimental o natural, el aumento del tiempo de la exposición de una amplia variedad de antígenos parasitarios al sistema inmune, al mismo tiempo que se restringe en cierta medida el desarrollo de los síntomas más graves y los niveles de parasitemia mortales, parecen ser la clave de este tipo de aproximaciones (Sutherland *et al.* 2007).

c) *Uso de la borrelidina para la identificación de antígenos protectores en la respuesta inmune adquirida*. La borrelidina favorece la presencia de parásitos a niveles no letales en sangre, con lo que se prolonga la exposición de múltiples antígenos al sistema inmune y puede mejorar el desarrollo de una respuesta protectora en el huésped efectiva a largo plazo. La identificación de estos antígenos podría ser útil para el diseño de nuevas vacunas dirigidas contra una gran variedad de epítopos.

d) *Uso de la borrelidina como tratamiento o estrategia vacunal a la malaria en humanos*. Existe el acuerdo generalizado de que los nuevos fármacos antimaláricos deben cumplir idealmente los requisitos de: rápida eficacia, toxicidad mínima, actividad frente a parásitos actualmente resistentes, y propiedades farmacocinéticas compatibles con la administración de una dosis diaria (bajo coste) (Fidock *et al.* 2008). La dosis letal al 50% (LD_{50}) es una medida estándar de la toxicidad de un compuesto y representa la dosis individual requerida para matar al 50% de la población de muestra. En roedores, la LD_{50} de la borrelidina tras su administración subcutánea es de 75 mg/Kg en ratón (Glasby 1993) y 39 mg/Kg por vía intravenosa en ratón (Glasby 1993), más tóxica comparada con la cloroquina, que administrada por vía subcutánea en ratón es de 200 mg/kg. Afortunadamente, la potente actividad antiangiogénica de la borrelidina ha incrementado la investigación sobre la síntesis de análogos que presenten un incremento de actividad unido a una mayor permeabilidad celular, solubilidad en agua y

menores efectos secundarios para su administración (Moss *et al.* 2007).

5.4. Modelo animal de malaria: ratones ICR infectados por *PyL*

La malaria experimental se estudia mayoritariamente con cepas de ratón consanguíneas (Langhorne *et al.* 2002; Stevenson and Riley 2004) porque desarrollan homogéneamente determinadas características deseables tras la infección. Los ratones ICR son animales no consanguíneos cuya infección con distintas cepas de *Plasmodium* resulta en pronósticos variables. La infección i.p. con especies como *P. berghei* NYU-2 (Murphy and Lefford 1979), *P. berghei* NK65 (Ishih *et al.* 2003; Ishih *et al.* 2006) provoca el desarrollo de una infección que es siempre fulminante, mientras que con *P. chabaudi AS* mueren el 60%, 80% o 100% de los animales con dosis crecientes de 10^5, 10^6 y 10^7 de eritrocitos infectados, respectivamente (Ishih *et al.* 2003) y con *PyNL* siempre sobreviven (Murphy 1980).

Resultados previos en nuestro laboratorio habían mostrado una respuesta heterogénea de los ratones ICR ante la infección por *PyL* (Moneriz *et al.* 2011). Con el objetivo de caracterizar el perfil de la infección de este nuevo modelo de malaria experimental, los ratones ICR fueron inoculados i.p. con $2x10^7$ eritrocitos infectados y se analizó diariamente el nivel de parasitemia en todos animales del ensayo microscópicamente. La infección originó tres perfiles diferentes atendiendo a las cinéticas de parasitemia y supervivencia de los ratones. La malaria fue letal en un 80% de los animales antes del día 15 p.i. y dentro de este grupo se diferenciaron, a su vez, 2 grupos de animales. Por un lado, se observaron animales con mortalidad prematura (ED), los cuales sufrieron una muerte rápida antes del día 8 pi, al alcanzar un nivel de parasitemia del 83%; y por otro lado, ratones con mortalidad tardía (LD) que alcanzaron un máximo de parasitemia del 54%, pero que no consiguieron superar la infección y murieron alrededor del día 11 p.i. Un 20% de los ratones se curó espontáneamente de la infección y estos se definieron como ratones supervivientes (S). Estos animales mostraron un lento incremento de la parasitemia similar a los LD, que disminuyó hasta eliminarse totalmente en el día 22 p.i. La pendiente de la curva de parasitemia fue significativamente diferente entre los ratones ED y el resto de grupo de ratones. Los niveles de parasitemia y el resultado de la infección se asemeja a la malaria de áreas no endémicas en que existe una correlación entre la severidad de la enfermedad y la densidad de parasitemia (Doolan *et al.* 2009). La parasitemia en los ratones S siguió una curva típica de infecciones maláricas con cepas de *Plasmodium* no letales, al alcanzar su máximo tras dos semanas de infección y desaparecer en la tercera o cuarta semana. Tras confirmar el aclaramiento de parásitos en la sangre, se reinfectó dos veces a los ratones S, en los días 60 y 420 pi, y no desarrollaron de nuevo parasitemia. A pesar de que, generalmente, los ratones que sobreviven a una primera infección de malaria suelen quedar inmunizados frente a infecciones homólogas, hay excepciones descritas de ratones BALB/c que controlan de forma natural una primera infección de una cepa letal de *P. yoelii,* pero que son susceptibles a una nueva infección meses después (Singh *et al.* 2000).

Todos los grupos de animales desarrollaron anemia como consecuencia de la infección. La destrucción de eritrocitos por la replicación de parásito (Lamb and Langhorne 2008) parece ser la causa más importante de la anemia humana en malaria (Mackintosh *et al.* 2004), pero no la única ya que se ha descrito también una interrupción en reemplazamiento de glóbulos rojos, así como la existencia de una apariencia anormal en ellos (diseritropoyesis) y una destrucción prematura de los eritrocitos no infectados (eritrofagocitosis) (Abdalla *et al.* 1984; Jakeman *et al.* 1999; Price *et al.* 2001) responsable de la alteración del número total de estas células en circulación (Lamikanra *et al.* 2007). Tanto la diseritropoyesis como la eritrofagocitosis ha sido también observada en ratones (Clark and Chaudhri 1988) (Yap and Stevenson 1992; Evans *et al.* 2006). Además la respuesta proinflamatoria en el hospedador, tanto humano como murino, parece tener un papel en el establecimiento de la anemia (Lamb and Langhorne 2008).

Debido a que un elevado número de leucocitos en la sangre es a menudo indicativo de una infección (Alberts 2005), procedimos su recuento en el transcurso de la primera) y segunda infección. Además, contamos el número de células recuperadas de bazo y cavidad peritoneal en ratones con distintos niveles de parasitemia a lo largo de la primera semana de infección. El bazo tiene varias funciones en el control de la infección por malaria humana y murina (Buffet *et al.* 2011; Del Portillo *et al.* 2012). La pulpa roja del bazo elimina células viejas o dañadas y eritrocitos infectados por el parásito, mientras que la pulpa blanca participa en la respuesta inmunológica (Carsetti *et al.* 2004), especialmente facilitando la generación de respuestas de memoria (Cozine *et al.* 2005). En la primera semana de infección solo los ratones altamente parasitados presentaron un aumento del número de células mononucleares en bazo y cavidad peritoneal. La esplenomegalia es un marcador fundamental de la infección clínica de *P. falciparum* en las áreas de transmisión (Snow *et al.* 1997) que está causado por la expansión y congestión de la pulpa roja (Oo *et al.* 1987), y una migración incrementada de macrófagos a la pulpa blanca (Urban *et al.* 1999) y a una expansión de la población de células B del bazo (Hansen *et al.* 2003). Además, en ratón el bazo tiene también actividad eritropoiética que contribuye al incremento del tamaño (Lamikanra *et al.* 2007). Contrariamente, el número de leucocitos en sangre no mostró ninguna variación durante la primera semana en los ratones infectados, independientemente de la parasitemia. Esto demuestra la variación que puede existir entre los parámetros inmunológicos medidos en sangre, como suele ser el caso de estudios en humanos, con los observados en bazo, fuente principal de información sobre estudios en modelos animales de malaria.

A partir de la segunda semana, los ratones infectados mostraron un aumento de leucocitos en sangre periférica tal y como ha sido observada en estudios de malaria por *P. chabaudi* en ratones BALB/c (Helmby *et al.* 2000; Nduati *et al.* 2010) y que puede estar provocado por la hemozoína (Jaramillo *et al.* 2004). Los valores de leucocitos en sangre de los ratones control sanos coincidieron con los de referencia de la casa comercial (Harlan), rondando valores de 4.2×10^9/ml. La leucocitosis observada en nuestros ratones contradice a estudios sobre malaria aguda en humanos que muestran

una leucopenia y la linfopenia predominantes (Lisse *et al.* 1994; Worku *et al.* 1997). Esta discrepancia, unida a los cambios en la composición de la población leucocitaria sanguínea durante infecciones por *P. chabaudi* (Helmby *et al.* 2000; Nduati *et al.* 2010) y *PyL* (nuestro estudio), destacan la importancia del momento en que se recogen las muestras de sangre durante una infección por malaria. Incluso, algunos autores han sugerido que son recomendables estudios inmunológicos basados en los leucocitos circulantes de ratón para proveer datos comparables con los obtenidos durante el proceso infeccioso en humanos (Craig *et al.* 2012).

5.5. Respuesta inmunológica de ratones ICR tras la infección con *PyL*

Con el objetivo de evaluar cambios cuantitativos y fenotípicos en los leucocitos circulantes asociados a los distintos pronósticos de infección de los ratones ICR, procedimos a la obtención de suspensiones de glóbulos blancos a partir de 40 µl de sangre de cada uno los animales del ensayo en el transcurso de la primera (días 0, 3, 6, 9, 14, 21 pi) y segunda infección (días 60 63, 67, 70 pi) para su análisis por citometría de flujo. Se tiñeron alícuotas de al menos 50.000 células cada una, con diferentes combinaciones de Acs fluorescentes. Para identificar sin error a la población leucocitaria sanguínea, debido a su bajo número en las alícuotas y a la alta concentración de hemozoína y eritrocitos lisados en sangre en los ratones con alta parasitemia, fue necesaria la detección de la población leucocitaria con su marcador CD45. Los ratones no infectados se comportaron como individuos sanos en los que, al haber un recambio celular (MacLennan 1998), el tamaño de todas las poblaciones celulares detectadas por citometría durante nuestros ensayos permaneció constante.

La primera barrera de defensa a una primera infección por *Plasmodium* es la respuesta de las células innatas que promueve seguidamente la respuesta mediada por células T (Stevenson and Riley 2004). Ante un primer contacto con el parásito, el papel de la respuesta innata es sin duda la clave de la supervivencia, ya que la mayoría de los ratones ICR fallecieron entre los día 4-7 p.i. y la respuesta adaptativa no es funcional a tan corto plazo (Janeway *et al.* 2003). Por ello, en primer lugar evaluamos los cambios en la presencia en sangre periférica de macrófagos, detectados por la expresión de la proteína de superficie Mac-3 que se ve incrementada tras la diferenciación de monocitos a macrófagos, y de CDs, a través de la expresión de la subunidad CD11c de una integrina que está presente en células dendríticas de órganos linfoides y sangre. Ambas poblaciones fueron identificadas también por la expresión de la molécula que les distingue como células presentadoras de antígeno, el MHC II. A pesar de que los ratones ED presentaron una mayor circulación de células maduras de la inmunidad innata, macrófagos (Mac-3^+ MHC II^+) y CDs ($CD11c^+$ MHC II^+), en el día 6 p.i. que coincidieron con los máximos niveles en su parasitemia (60%), éstos no fueron suficientemente efectivos para controlar las altas parasitemias desarrolladas.

Durante la malaria humana, la presencia de eritrocitos infectados por *P. falciparum* dificulta la estimulación de la expresión de MHC II en la superficie de monocitos (Schwarzer *et al.* 1998) y la hemozoína inhibe la diferenciación de monocitos hacia células dendríticas (Urban and Todryk 2006), mecanismos que disminuirían las respuestas de linfocitos T (Schwarzer *et al.* 1998). Sin embargo, en nuestros ratones la expresión de MHC II no se vió alterada durante la infección con *Plasmodium* y las altas parasitemias no evitaron la diferenciación a CDs, lo que coincide con otros estudios en otras cepas consanguíneas de roedor (Luyendyk *et al.* 2002). El rápido incremento de macrófagos y CDs en la circulación periférica de ratones con alta parasitemia por malaria sería un marcador temprano de una respuesta inadecuada al inicio de la infección aguda en nuestro modelo. En los ratones S y LD, el perfil inmunológico fue muy diferente al mostrado por animales ED, ya que mostraron una circulación más estable de células innatas.

En malaria humana existe una relación entre los perfiles de citoquinas en suero y la severidad y parasitemia de la infección (Day *et al.* 1999; Cox-Singh *et al.* 2011). Para comparar los niveles de citoquinas y quimioquinas durante los primeros días de infección entre los ratones con peor y mejor pronóstico tras la primera infección, identificamos 32 citoquinas secretadas en el suero a través de un kit de *microarray* comercial. El menor nivel de IFN-γ en los sueros de los ratones ED que los S, a pesar de la mayor parasitemia registrada, podría indicar una baja estimulación de la respuesta inflamatoria en el día 3 pi que puede afectar negativamente a la eficiencia de los macrófagos en los ratones ED. En este sentido, se han observado niveles mayores de IFN-γ en cultivos de esplenocitos de cepas de ratón resistentes a la malaria por *PyL* que en cepas susceptibles (Chen *et al.* 2009). Por otro lado, la sobreproducción de la mayoría de citoquinas medidas en los ratones ED en el día 7 pi respecto a los ratones S, que mostraron una producción más controlada, demuestra que la infección severa por *PyL* en los ratones ICR promueve un "estado de estrés" general que probablemente contribuye a una desregulación de la respuesta inmune (Seixas and Ostler 2005).

En general, ni los linfocitos T ni los B se consideran necesarios para controlar la primera ola de parasitemia (Couper *et al.* 2007). Sin embargo, el papel supresor de respuestas Th1 de las células T caracterizadas como $CD4^+CD25^+$ (Thornton and Shevach 1998; Belkaid and Rouse 2005) es muy importante en infecciones bacterianas (Kursar *et al.* 2007), virales (Kinter *et al.* 2004), helmínticas (Taylor *et al.* 2005; Finney *et al.* 2007) o protozoarias incluida la malaria (Belkaid *et al.* 2002; Hisaeda *et al.* 2004; Kinter *et al.* 2004; Taylor *et al.* 2005; Finney *et al.* 2007). La proteína CD25 es el receptor de IL-2, una citoquina que estimula la proliferación de linfocitos B y T y de células NK y que está producida por células T activadas (Amu *et al.* 2006). El CD25 se expresa aproximadamente en el 10% de células T CD4 periféricas de ratones sanos adultos (Amu *et al.* 2006). Dentro de la población $CD4^+CD25^+$ se incluyen células T CD4 efectoras y Tregs; éstas últimas son especialmente relevantes por su capacidad de inhibir la respuesta Th1 (Scholzen *et al.* 2009). En nuestro estudio, observamos un incremento del número de células $CD4^+CD25^+$ en sangre periférica de ratones con un mal pronóstico antes de que se alcanzase el

máximo de parasitemia, mientras que en los ratones S este incremento surgió cuando los parásitos estaban prácticamente eliminados. Según nuestros resultados, un número de células $CD4^+CD25^+$ excesivo al comienzo de la infección podría impedir el desarrollo de una inmunidad protectora efectiva, pero sería beneficioso en etapas tardías de la infección al evitar una respuesta inflamatoria prolongada. Es interesante señalar que se han obtenido conclusiones similares en estudios humanos (Torcia *et al.* 2008) y modelos animales (Wu *et al.* 2007).

Respecto al resto de la población de linfocitos T, los T CD8 fueron los únicos en mostrar una disminución significativa en la primera semana de infección en los ratones ED, si bien la tendencia en los ratones S era también descendente. Existen experimentos de transferencia que han evidenciado el papel protector que tienen las células T CD8 en la infección por *P. yoelii* (Imai *et al.* 2010) y su activación y proliferación en respuesta al *PyNL* (Chandele *et al.* 2010) y otros que por el contrario, han concluido que estas células no funcionan como mediadores de protección (Vinetz *et al.* 1990). Aunque no hemos evaluado la función y la especificidad que tienen las células T CD8 en nuestros ratones, el aumento del número total de células $CD8^+$ con un fenotipo activado en los ratones S apunta a un papel protector o que, al menos, no afectan negativamente al desarrollo de una respuesta inmunológica efectiva.

Todos los ratones infectados desarrollaron una subpoblación de células B que expresaban bajos niveles de proteína B220 en la membrana, pero su contribución a la protección de la infección es dudosa porque las caracterizamos principalmente como células B inmaduras; y no como células B-1 no clásicas como se había descrito previamente (Kanda *et al.* 2010).

La incapacidad que tienen los eritrocitos para procesar antígenos evita la destrucción del GRi por células T específicas dependientes de MHC, por lo que es la respuesta inmune humoral la que confiere inmunidad a estadios intraeritrocíticos del parásito de la malaria. En nuestro estudio, la IL-4, citoquina que dirige la diferenciación de células Th0 a Th2 (Taylor-Robinson and Phillips 1998), aumentó antes en el suero de ratones S que de ratones ED, lo que podría promover una producción más efectiva y específica de Acs (Stevenson and Tam 1993).

Las reinfecciones, que cursaron sin parasitemia visible, unido al ensayo de reinoculación de los parásitos en ratones BALB/c, por el que determinamos que las propiedades intrínsecas del parásito no estaban contribuyendo a las diferencias observadas en los distintos perfiles de los ratones ICR, reveló la existencia de una respuesta inmune protectora desde la primera infección. Además detectamos la existencia de células B de memoria 500 días después de una primera infección en ratones ICR supervivientes a ella, lo que confirmó el desarrollo de una respuesta inmune de memoria.

Todos los isotipos de las IgG examinados, IgG1, IgG2a, IgG2b e IgG3, fueron detectados en el suero de los ratones S de acuerdo a resultados previos de infecciones de ratones ICR infectados con *PyNL* (White *et al.* 1991). Además, es poco probable que su producción en nuestros ratones dependa de la presencia de niveles crónicos de parasitemia, ya que pudimos confirmar la eliminación total de

parásitos en sangre después de cada infección. Los ratones S desarrollaron una respuesta inmunológica humoral. Estudios de transferencia pasiva de suero han consolidado el concepto de que la inmunidad a *PyNL* (Jayawardena *et al.* 1978; Freeman and Parish 1981) y al parásito letal *P. yoelii nigeriensis* (Singh *et al.* 2000) es ampliamente humoral, lo que ahora se haría extensivo a la infección por la cepa *PyL,* tal como demuestran los resultados obtenidos en nuestros ensayos de transferencia de suero.

Por otra parte los ensayos de inmunotransferencia revelaron que las IgGs específicas reconocieron un repertorio de antígenos del *PyL* creciente tras cada reinfección. Estos resultados también han sido observados en el desarrollo de la inmunidad adquirida a la malaria, en humanos, que es probable que dependa de la acumulación de una amplia variedad de especificidades antigénicas a largo plazo (Kinyanjui *et al.* 2004). En un reciente estudio de proteómica hemos identificado algunos de los Ags del *PyL* reconocidos por los Acs de los ratones S como son la proteína de choque térmico-70, una disulfuro isomerasa, una plasmepsina y el factor 3 de iniciación de la translación, lo que representa una nueva estrategia de identificación de Ags para el desarrollo de nuevas vacunas (Kamali *et al.* 2012).

En resument, nuestros resultados indican que existen cambios celulares rápidos en la sangre durante la infección de ratones ICR con *PyL* en la fase intraeritrocítica, los cuales se relacionan con la parasitemia, la severidad de la infección y su pronóstico. Además la parasitemia afecta irreversiblemente a las células del bazo. Con nuestro estudio hemos podido demostrar que ratones de cepas no consanguíneas que manifiestan diferente progresión clínica frente a la malaria, pueden ser modelos experimentales valiosos para distinguir entre respuestas inmunes potencialmente efectivas e inefectivas a la malaria. Estos resultados son alentadores para estudios humanos ya que identifican a los leucocitos como marcadores de severidad y de pronóstico en infecciones de malaria letal. Todo ello podría tener implicaciones para evaluar nuevas vacunas o tratamientos ya que se podría detectar con rapidez una respuesta inmune efectiva en sangre. No obstante, una vez descrito este modelo, es necesario diseñar futuros experimentos para estudiar en detalle los mecanismos inmunológicos de susceptibilidad y protección de los ratones ICR a la malaria.

6. CONCLUSIONES/CONCLUSIONS

1. Los ratones no consanguíneos ICR infectados por la cepa letal *P. yoelii yoelii 17XL* permiten la comparación de tres evoluciones clínicas diferentes, en cuanto a parasitemia y resultado de la infección, ante una misma infección primaria. Este modelo proporciona el marco experimental adecuado para una valoración de las respuestas inmunológicas en distintos perfiles de infección.

2. En nuestro modelo experimental, tanto el fenotipo como el número de las células blancas circulantes varía en relación con la evolución clínica de cada animal. El análisis consecutivo de factores inmunológicos en sangre demuestra que el peor pronóstico se relaciona con un aumento temprano de células innatas circulantes y células B inmaduras y a una producción no controlada de citoquinas. Por otro lado, los ratones supervivientes se caracterizan por la producción controlada de citoquinas y una circulación inalterada de células de la respuesta innata seguidas por el incremento de células activadas T y B y la generación de una respuesta humoral de larga duración.

3. En nuestro modelo, las células B del bazo se ven alteradas durante la primera semana de infección en mayor o menor grado dependiendo del nivel de parasitemia. Además, una sola infección es capaz de inducir la generación de células B de memoria que se mantienen durante más de un año en dicho órgano.

4. El tratamiento profiláctico con borrelidina cura, con un 100% de efectividad, la infección por *P. yoelii yoelii 17XL* en modelos de ratón BALB/c, sensibles a ella. Además, les protege contra consecutivas infecciones homólogas al favorecer la generación de una respuesta humoral específica. El tratamiento terapeútico con borrelidina, en este mismo modelo, es menos eficaz que el tratamiento profiláctico al conferir protección al 75% de los animales.

5. La borrelidina ejerce un efecto parasiticida en los estadios intraeritrocíticos maduros de *P. falciparum in vitro*, mientras que los parásitos jóvenes quedan en un estado de inhibición estática tras el que pueden retomar el crecimiento.

1. Non congenic ICR mice infected with the lethal strain *P. yoelii yoelii 17XL* allow us the comparison between three different clinical evolutions, regarding parasitemia and outcome of the infection, after one primary infection. This model provides the appropriate experimental setting to analyse the immune responses in different infection profiles.

2. In our animal model, both the fenotype and the number of circulating white cells changes, are associated to the clinical evolution of each animal. The consecutive analysis of blood immune factors demonstrates that the worst prognosis is associated with an early increase of innate cells and immature B cells and an uncontrolled cytokine production. On the other hand, surviving mice are characterized by the controlled cytokine production and an unaltered circulation of innate cells followed by the increase of activated T and B cells and the generation of a long lasting humoral response.

3. In our model, B cells of spleen change during the first week of infection depending on the parasitemia level. Besides, a single infection is able to induce the generation of B memory cells which are maintained during more than one year in this organ.

4. The prophylactic treatment with borrelidin is 100% effective against the *P. yoelii yoelii 17XL* infection in BALB/c mouse model, a sensitive strain to the infection. Besides, treated mice remain protected against following homologue reinfections favoring the development of a specific humoral response. The therapeutic treatment with borrelidin is less efficient that the prophylactic regime, protecting a 75% of animals.

5. Borrelidin has a parasiticidal effect in mature blood-stages of *P. falciparum in vitro*, while young stages are affected by a static inhibition after which they can resume the growth.

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