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Characterization of the host immune response to a new formulation of circumsporozoite protein that protects against malaria

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Parasite Disease Group

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To my family, to my children.

À minha família, aos meus filhos.

SUMMARY

The circumsporozoite protein (CSP) is the most abundant surface protein of sporozoites, the form of *Plasmodium* that is injected in the skin of mammals upon the bite of an infected a*nopheline* female mosquito and that causes malaria. In the sequence of the circumsporozoite protein, different B and T cell epitopes have been identified and have been used in the search for a subunit malaria vaccine. At moment there is no vaccine against malaria and RTS,S, the leading vaccine candidate, gives only partial protection in african children, that are the most affected by this disease. RTS,S is based on the circumsporozoite protein and its immunity is mainly mediated by IgG antibodies.

In this study, we evaluated the humoral and cellular immune responses to a new formulation of circumsporozoite protein. Balb/C mice were immunized two times, two weeks apart, with CSP alone or in combination with adjuvants A or B. Previous work showed that mice immunized with CSP in combination with adjuvant A showed a high degree of protection against a stringent challenge with *P. yoelii* sporozoites but sterile protection was only achieved when animals were immunized with CSP in combination with adjuvant B.

The study of the humoral response involved the determination of IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 titers specific to CSP and of total IgM and IgG titers, by ELISA. The cellular response was characterized by analyzing the frequency of splenic B, CD4+ and CD8+ T cells; naïve, effector and central memory T cells; IL-2, IL-6, IL-10, TNF-α and INF-γ CD3+ T cells, by flow cytometry.

Immunization with CSP alone or with adjuvants induced the production of specific antibodies. However, animals immunized with CSP in combination with adjuvants A or B presented higher titers of specific IgGs compared to animals that just received CSP. IgG1 was the most abundant IgG isotype in all immunized groups. For animals immunized with CSP plus adjuvants A or B, IgG2a was the second most abundant isotype, followed by IgG2b and IgG3 while IgG3 was the second most abundant isotype in the group that just received CSP.

Mice immunized with CSP in combination with the adjuvant B showed a high frequency of CD3+ splenocytes producing IL-6. These results, despite preliminary and awaiting further confirmation and characterization, are very exciting since these cells may contribute to eliminate liver stage parasites.

In conclusion, we would like to propose that high titers of specific antibodies generated in the animals immunized with CSP in combination with adjuvants A or B may equally

prevent sporozoites to reach the liver. However, the sterile protection observed in the animals immunized with CSP in combination with adjuvant B might be related with the production of IL-6 that will prevent the development in the liver of sporozoites that eventually were not blocked by the antibodies.

SUMÁRIO

A proteína de superfície “circumsporozoite” (CSP) é a mais abundante nos esporozoítos de *Plasmodium*, isto é, na forma do parasita que é injetada na pele do hospedeiro mamífero através da picada de um mosquito fêmea infetado e que causa malária. Diferentes epítopos antigénicos para células B e T foram identificados nesta proteína e têm sido estudados no âmbito de uma vacina contra a malária. De momento não existe vacina contra a malária e a formulação líder designada RTS,S, confere apenas proteção parcial em crianças africanas, sendo estas as mais afetadas pela doença. Esta formulação tem como base a proteína “circumsporozoite” sendo a imunidade conferida mediada, principalmente, por anticorpos.

Neste estudo, foi feita a avaliação das respostas imunes humoral e celular de uma nova formulação de proteína “circumsporozoite”. Ratinhos Balb/C foram imunizados, duas vezes, com o intervalo de duas semanas, com apenas CSP e com CSP em combinação com os adjuvantes A ou B. Resultados prévios mostraram que os ratinhos imunizados com CSP em combinação com o adjuvante A apresentaram um elevado grau de proteção contra a infeção com esporozoítos de *Plasmodium yoelli,* mas a proteção estéril foi apenas observada nos animais imunizados com CSP em combinação com o adjuvante B.

O estudo da resposta humoral envolveu a determinação dos títulos de IgM, IgG, IgG1, IgG2a, IgG2b e IgG3 específicos para a CSP e dos títulos totais de IgM e IgG, por ELISA. A resposta celular foi caracterizada analisando a frequência esplénica das células B e das células T CD4+ e CD8+; das células T “naïve” e de memória central e efetora; e das células T CD3+ que produzem IL-2, IL-6, IL-10 TNF-α ou IFN-γ , por citometria de fluxo.

A imunização apenas com CSP ou com CSP em combinação com os adjuvantes A ou B induziu a produção de anticorpos específicos. Contudo, os animais imunizados com CSP em combinação com os adjuvantes A ou B apresentaram títulos mais elevados quando comparados com os animais que apenas receberam CSP.

Em todos os animais, o isotipo mais abundante foi IgG1. Para os animais imunizados com CSP em combinação com os adjuvantes A ou B, o segundo isotipo mais abundante foi IgG2a, seguido de IgG2b e de IgG3. O IgG3 foi o segundo isotipo mais abundante nos animais que receberam apenas CSP.

Ratinhos imunizados com CSP em combinação com o adjuvante B apresentaram uma elevada frequência de esplenócitos CD3+, produzindo IL-6. Estes resultados, apesar

de preliminares e aguardando futura confirmação e caracterização, são bastante interessantes, uma vez que estas células podem contribuir para a eliminação dos parasitas no fígado.

Em conclusão, gostaríamos de propor que os elevados títulos de anticorpos específicos para a CSP e presentes nos animais imunizados com CSP em combinação com os adjuvantes A ou B, podem igualmente prevenir a invasão do fígado pelos esporozoítos. Contudo, a proteção estéril observada nos animais imunizados com CSP em combinação com o adjuvante B, pode estar relacionada com a produção de IL-6 que irá prevenir o desenvolvimento no fígado de esporozoítos, que eventualmente não foram bloqueados pelos anticorpos.

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ABBREVIATIONS

BSA	bovine serum albumin
CD	cluster of differentiation
CSP	circumsporozoite protein
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMC	inner membrane complex
IPTG	isopropylthio-β-galactoside
LB	Luria Broth
MPL	3´-O-desacyl-4´-monophosphoryl lipid A
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic
OPD	o-phenylenediamine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QS21	*Quillaja saponaria* 21
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF	tumor necrosis factor
TSR	thrombospondin type I repeat
WHO	World Health Organization

I. INTRODUCTION

1. MALARIA

1.1. The current situation

Malaria remains a major public health problem in many developing countries and it is the most deadly parasitic disease of humans, affecting every year more than 200 million people and killing up to 600 thousand people mostly, african children. Globally, an estimated 3.4 billion people are at risk of malaria. Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5 years of age (WHO, Malaria Report 2013).

Since 2000, a tremendous expansion in the financing and coverage of malaria control programmes has led to a progress in vector control, on chemoprevention, in diagnostic testing and malaria treatment, resulting in a wide-scale reduction in malaria incidence and mortality (*figure 1*). More than half of the countries that had ongoing malaria transmission in 2000 have recorded decreases in the incidence of confirmed malaria, or in reported admissions and deaths (or both). Estimated malaria mortality rates worldwide fell by 42% between 2000 and 2012 in all age groups, and by 48% in children under 5 years of age. If the annual rate of decrease that has occurred over the past 12 years is maintained, then malaria mortality rates are projected to decrease by 52% in all ages, and by 60% in children under 5 years of age, by 2015. In 2013, there were 97 countries and territories with ongoing malaria transmission, and 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic (WHO, Malaria Report 2013). However, emergence of resistances to drug treatments and spread of resistant parasites is a major concern since no alternative antimalarial treatment will be available within the next five years and no efficient licensed vaccine currently exists.

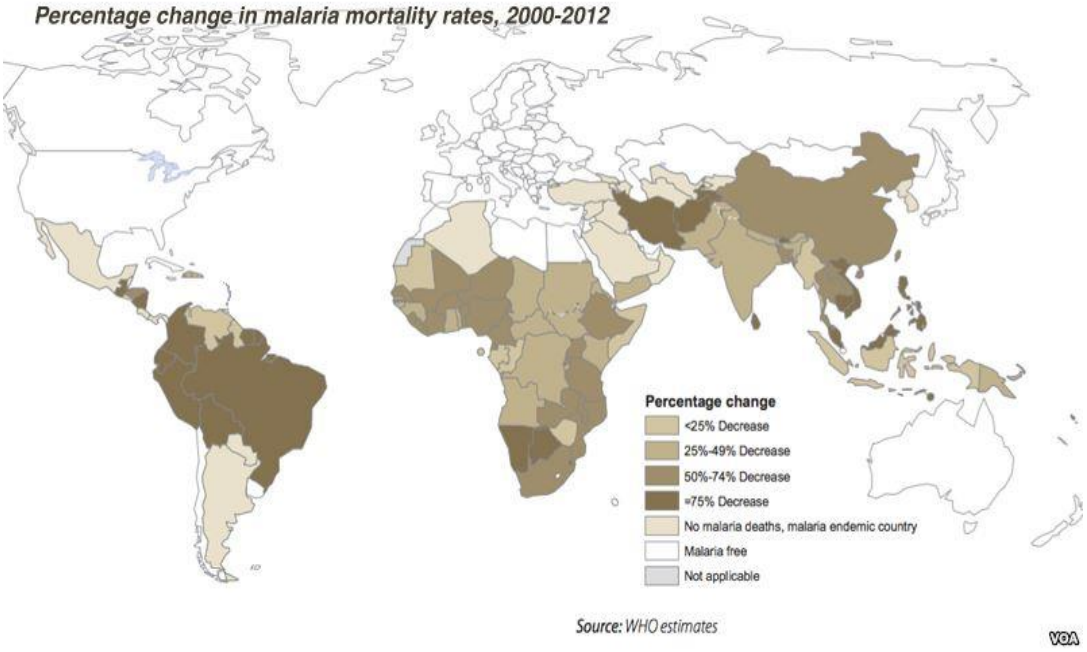

Figure 1 | Percentage change in malaria mortality rates, between 2000 and 2012 (WHO, Malaria Report 2013).

1.2. Transmission

Plasmodium belongs to the Apicomplexa phylum and is the etiological agent of malaria. The parasite is transmitted among vertebrate hosts by the blood feeder´s female mosquitoes of the genus *Anopheles* (*figure 2*). Of the approximately 430 *Anopheles* species, only approximately 30-40 transmit *Plasmodium* in nature. Anophelines that can transmit parasites are found not only in malaria-endemic areas, but also in areas where malaria has been eliminated (*figure 3*); these areas are constantly at risk of re-introduction of the disease (WHO, Malaria Report 2013).

Figure 2 | *Anopheles freeborni* mosquito pumping blood (picture from Centers for Disease Control and Prevention).

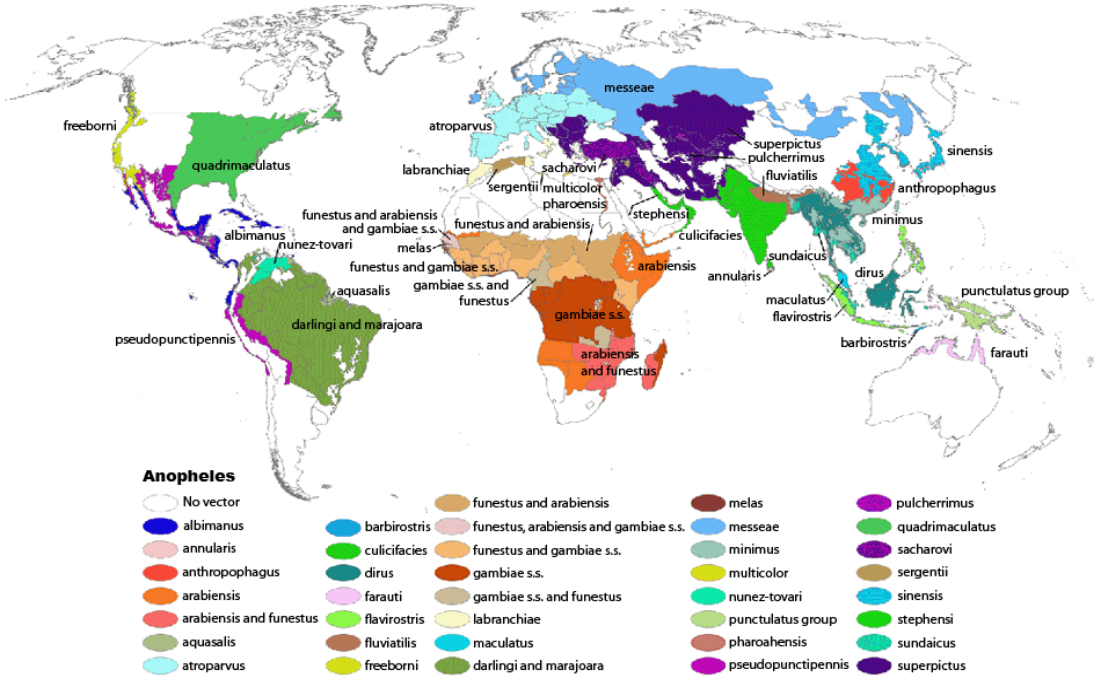

Figure 3 | Anophelines are found worldwide except Antarctica. *Plasmodium* parasites are transmitted by different *Anopheles* species, depending on the region and the environment (from Centers for Disease Control and Prevention).

1.3. Symptoms and diagnosis

Malaria symptoms are due to repetitive cycles of *Plasmodium* multiplication inside red blood cells. It is an acute febrile illness. In a non-immune individual, symptoms appear seven days or more (usually 10–15 days) after the infective mosquito bite. The first symptoms like fever, headache, chills and vomiting, may be mild and difficult to recognize as malaria (Pradhan *et al*., 2013).

Infection with *Plasmodium falciparum* causes the most severe malaria in humans. The predominant symptoms are anemia, splenomegaly and fever. Cerebral malaria, liver dysfunction, acute renal failure, acidosis, hypoglycemia, respiratory distress, and edema are also observed as complications in malaria patients, although these symptoms do not always appear. Cerebral malaria is characterized by the presence of neurological features, especially impaired consciousness. In malaria endemic areas, persons may develop partial immunity, allowing asymptomatic infections to occur (Waknine-Grinberg *et al.*, 2010; Inoue *et al.*, 2013).

Microscopic analysis of Giemsa, Wright, or Wright-Giemsa stained blood smears (*figure 4*) and direct visualization of infected red blood cells remains the "gold standard" for malaria diagnosis (Cotter *et al.*, 2013).

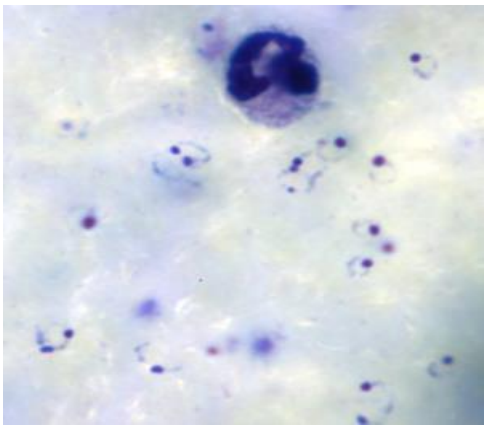

Figure 4 | Ring-form trophozoites of *Plasmodium falciparum* in a thin blood smear (from Centers for Disease Control and Prevention).

Molecular tests such as Polymerase Chain Reaction (PCR) can detect parasites in specimens where the parasitemia may be below the detectable level of blood film examination (Snounou *et al.*, 1993). Malaria parasites can also be detected on the basis of antigens or enzymatic activities associated with the parasites. These methods are often packaged as individual test kits called rapid diagnostic tests or RDTs (antigen detection method).

1.4. Treatment and prevention

Two important currently used antimalarial drugs are derived from plants whose medicinal values had been noted for centuries: artemisinin from the Qinghaosu plant (*Artemisia annua*, China, 4th century) and quinine from the cinchona tree (*Cinchona spp.*, South America, 17th century). Artemisinins in combination with other antimalarials drugs (known as artemisinin-combination therapy) are used to treat uncomplicated malaria. Artesunate, a derivate of artemisin, is used to treat severe malaria (WHO, Malaria Report 2013).

The acquisition and spread of drug resistance is a health problem and investigators are seeking out novel antimalarial inhibitors and drug targets, and defining the genetic basis of resistance to existing drugs, as a means to facilitate detection and develop novel strategies to overcome resistance (Fidock *et al.*, 2008).

Disease transmission can be reduced by preventing mosquito bites by using mosquito nets and insect repellents, or with mosquito-control measures such as spraying insecticides and draining standing water (Arama *et al*., 2014; Feachem *et al.*, 2010). In recent years, mosquito resistance to insecticides has emerged in many countries (Mathias *et al*., 2014). So, the development of new, alternative insecticides is a high priority in malaria incidence reduction.

2. *PLASMODIUM* PARASITES

2.1. The Apicomplexa phylum

Plasmodium parasites belong to the Apicomplexa phylum, which comprises a wide variety of protozoans including several pathogens to humans such as *Toxoplasma* and *Cryptosporidium*. Despite their diversity, Apicomplexa parasites share some features. Among them is the transformation into specialized stages named "zoites" which eventually invade host cells. These stages possess the apical complex, a unique structure that defines their anterior pole and is composed of secretory organelles, an apical polar ring and in some cases a conoid. Within the secretory organelles, micronemes contain several proteins whose secretion is involved in parasite motility, adhesion to and invasion of host cells, while rhoptries are packed with proteins and lipids that are released during and after invasion of host cells. These secretions participate in the process of host cell entry and establishment of the parasitophorous vacuole in which the invading parasite penetrates the cell (Blackman *et al.*, 2001; Montagna *et al.*, 2012). Apicomplexa parasites share a vestigial non-photosynthetic plastid called apicoplast. This vital organelle has been shown to participate in fatty acid and isoprenoid synthesis (Fichera *et al.*, 1997; Jomaa *et al.*, 1999; Waller *et al.*, 1998; He *et al.*, 2001).

2.2. Species that infect humans and rodents

In humans, malaria is caused by five species of *Plasmodium* such *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Most cases are caused by either *P. falciparum* or *P. vivax* (Arama *et al.*, 2014; Cox, 2010; White *et al*., 2013).

P. falciparum is the most lethal species to humans, being responsible for approximately 90% of the mortality and prevailing in Africa (Almeida *et al*., 2014; Doll *et al*., 2014). The genetic diversity of *Plasmodium falciparum* is one of key factors in the morbidity and mortality observed (Tanabe *et al.*, 2013). *P. vivax* is the most prevalent human malaria species in the Americas, Asia and Oceania (Céspedes *et al*., 2014; Versiani *et al.*, 2013). The geographical distribution, prevalence, lethality and drug

resistance risk of the human malaria parasites are summarized in *Table 1* (Doolan *et al*., 2009), which data are confirmed in malaria report of 2013, from WHO.

Species	Range	Prevalence	Lethality risk	Drug resistance risk	Relapse	Animal reservoir
P. falciparum	Pan-tropical	High	High	High	No	No
P. vivax	Pan-tropical, temperate	High	High (?)	High	Yes	No
P. malariae	Tropical	Low, focal	Low	Low	No	No (?)
P. ovale	West Africa, Southeast Asia	Rare	Low	Low	Yes	No
P. knowlesi	Southeast Asia	Rare	High (?)	Low	No	Yes

Table 1. The geographical distribution, prevalence, lethality, and drug resistance risk of the human malaria parasites (Doolan *et al*., 2009).

P. vivax parasites infect reticulocytes, resulting in long-term chronic infections and anemia but little mortality. By contrast, *P. falciparum* parasites invade cells of various ages and cause acute anemia and frequently death, as mentioned before. *P. vivax* sporozoites can either initiate a primary blood-stage infection or remain in the liver as a resting hypnozoite stage. When conditions are favorable, the activation of this stage can occur, resulting in disease (Shanks *et al.*, 2013; Vanloubbeecka *et al.*, 2013). The biological differences between *P. falciparum* and *P. vivax* can be explained, at least in part, by their independent origins as human parasites (*figure 5*; Carlton *et al.*, 2008).

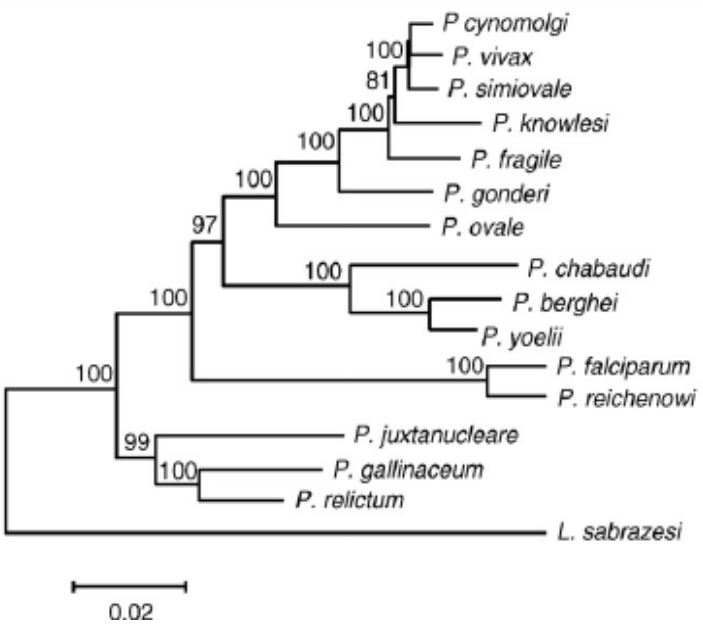

Figure 5 | A phylogenetic tree of the *Plasmodium* genus (Carlton *et al.*, 2008).

Malaria is in no way restricted to humans. Several studies have been performed with *Plasmodium* species that infect rodents, such as *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*. Since these species are essentially analogous to human parasites regarding their life cycle, physiology and structures, they represent an important model to study malaria (Keeling *et al.*, 2014; Perlmann *et al.*, 1995).

2.3. Life cycle

The life cycle of mammalian *Plasmodium* spp. (*figure 6*) can be divided into three main steps: the pre-erythrocytic phase, an asymptomatic and asexual multiplication of the parasite in the liver of the vertebrate host; the erythrocytic phase, during which parasite asexual multiplication within red blood cells leads to the symptoms of the disease; and the mosquito phase characterized by sexual and asexual multiplications transforming invertebrate host into a potential transmission vector (Arama *et al*., 2014).

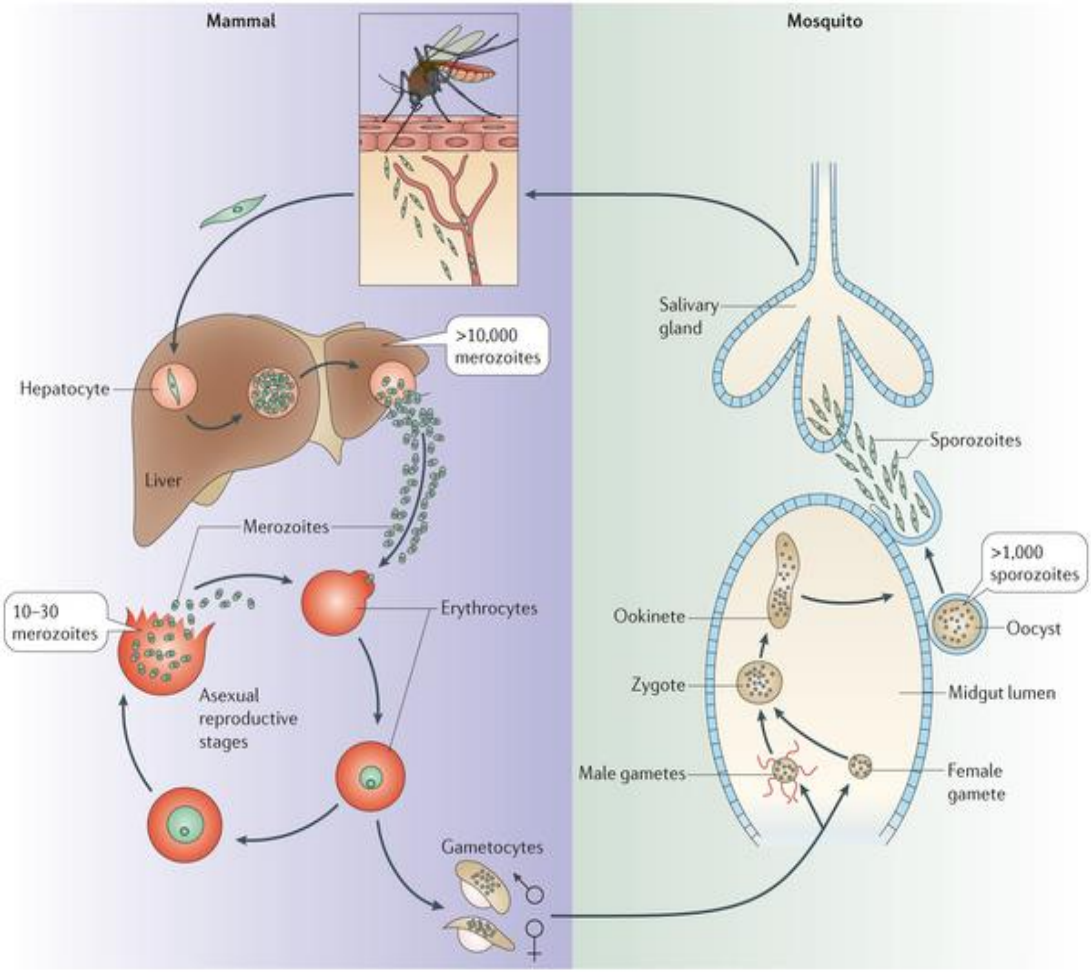

Figure 6 | The *Plasmodium* spp. life cycle (Ménard *et al*., 2013).

The pre-erythrocytic phase (*figures 6* and *7*) begins with the bite of an infected a*nopheline* female mosquito that while having a blood meal injects sporozoites in the skin. Sporozoites migrate to the liver, where they invade a hepatocyte, inside a vacuole and a single sporozoite transform in thousands of merozoites, the red blood cells infective form (Amino *et al*., 2006; Gerald *et al.*, 2011; Krych-Goldberg *et al.*, 2002; Malaguarnera *et* al., 2002; Miller *et al*., 2014).

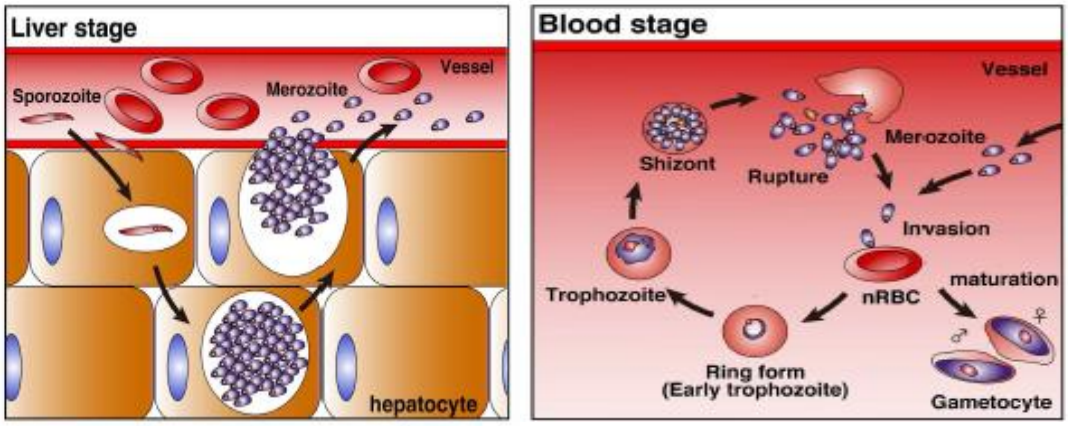

Figure 7 | The pre-erythrocytic and erythrocytic phases of the *Plasmodium* spp. life cycle (Inoue *et al.*, 2013).

After 1-2 weeks, the erythrocytic phase (*figures 6* and *7*) starts with the release thousands of hepatic merozoites. Merozoites can invade red blood cells, and develop inside them, to the so- called ring-stage, trophozoite stage and ultimately to the schizont stage. When the schizonts rupture, many merozoites are released and invade new red blood cells. This is known as the asexual blood stage of the parasites life cycle, which is associated with the symptoms and complications of malaria. The erythrocytic cycle of *P. falciparum* has a unique feature: mature trophozoites and schizonts are sequestered in the peripheral circulation, due to parasite mediated changes of the surface of the infected red blood cells, causing them to adhere to endothelial cells (sequestration) and other erythrocytes (rosetting). It is an accepted theory that this adhesion is an immune escape mechanism of the parasite, and that it also may lead to a better maturation in the microaerophilic venous atmosphere (Gerald *et al.*, 2011; Krych-Goldberg *et al.*, 2002). Some intra-erythrocytic parasites differentiate into male or female gametocytes, which are taken up by a mosquito during a blood meal. Gametocytes egress from erythrocytes, activate into gametes and fuse in the mosquito midgut lumen, forming an ookinete that crosses the gut epithilium and transforms into an oocyst, where sporozoites are formed. The oocyst bursts and liberates sporozoites, which migrate to the salivary glands to await inoculation at the next blood feed (Crompton *et al*., 2014; Mathias *et al*., 2014; Ménard *et al*., 2013).

2.4. The sporozoite

Sporozoites are extracellular forms capable of traveling in the tissues of both the mosquito and the vertebrate hosts by relying on the gliding motility and capacity to migrate through cells (Ménard *et al*., 2008). Sporozoites are elongated banana-shaped cells of about 10 μm long and 1 μm wide (*figure 8A*). Their morphology is established during development within the oocysts and correct morphogenesis is dependent on the levels of CSP (Thathy *et al.*, 2002). As mentioned previously, sporozoites are polarized cells due to their apical complex at the anterior pole. Sporozoites are also bound by a pellicle composed of parasite plasma membrane and a network of closely apposed flattened vesicles called the inner membrane complex (IMC). Microtubules, which are anchored to the apical polar ring, run longitudinally underneath the IMC (Morrissette et al., 2002).

Sporozoites glide, as illustrated in *figures 8B-D*, on solid substrates without changing their shape (Ménard, 2001). An acto-myosin motor powers this type of locomotion and movement is apparently achieved through rearward translocation of transmembrane proteins binding both the extracellular substrate and the intracellular acto-myosin motor. Sporozoites trajectory is characterized by circular movement on glass slides (*figure 8B*) or on top of cell monolayers (*figure 8C*) and by spiral movement in 3D matrix such matrigel or in the dermis (*figure 8D,E*) (Vanderberg et al., 2004; Amino et al., 2006). Sporozoites are capable of disrupting host cell plasma membranes, glide free in the cytosol and exit from the cell, a process called cell traversal (Vanderberg et al., 1990; Mota et al., 2001). Cell traversal was found to be important for sporozoites progression from the skin to the liver. Indeed, mutant sporozoites lacking cell traversal capacity could not exit the skin and escape clearance from phagocytic cells in the liver sinusoids (Amino et al., 2008; Ishino et al., 2005; Tavares et al., 2013). Sporozoites journey in the mammalian host ends up with successful invasion of hepatocytes (Aikawa M., 1977). This step is crucial for parasites transformation and multiplication in the red blood cells infective forms.

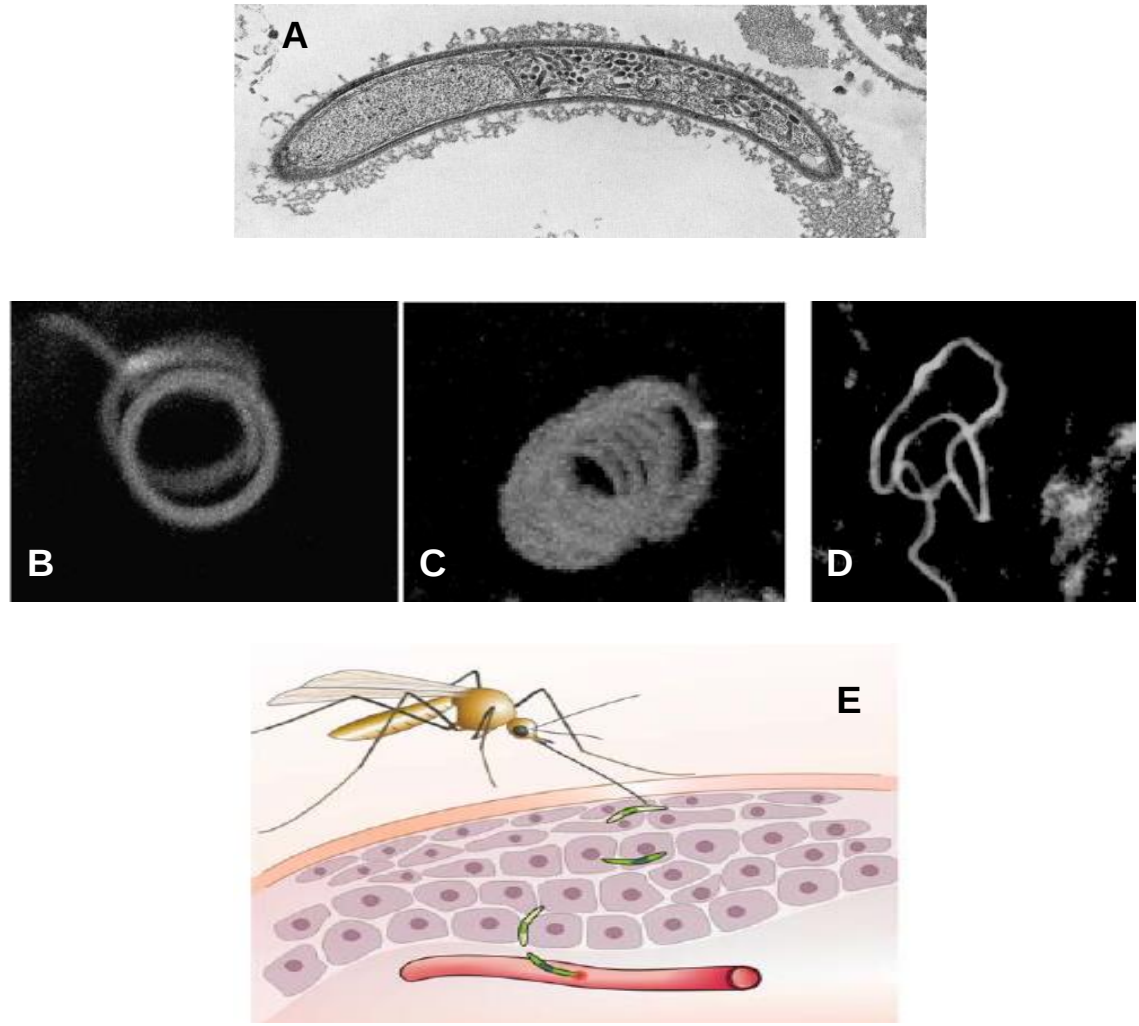

Figure 8 | *Plasmodium* sporozoites gliding motility. (A) Sporozoites morphology by electron microscopy (Aikawa, 1977). The motility of sporozoites is characterized by circular trajectories on glass slides (B) and on a fibroblast monolayer (C), and by random spiral trajectories in the skin (D). When sporozoites (green) are injected into the dermis by an infected mosquito (E), they migrate in the skin using their gliding motility and actively invade blood vessels (from Montagna *et al.*, 2012).

2.5. Circumsporozoite protein (CSP)

CSP is highly expressed in all *Plasmodium* species and its structure is highly conserved between the *Plasmodium* species that infect rodents, primates and humans (Suarez *et* al., 2001; Coppi *et al.*, 2011). CSP is the immunodominant and major surface protein of *Plasmodium* sporozoites (Moorthy *et al*., 2009) and this fact was discovered in early 1980s (Nardin *et al.*, 1978; Nussenssweig et al.,1985; Hughes, 1991). The expression of CSP starts in oocyst (Miura *et* al., 2014) and it has been proposed CSP has a role in sporozoites arrest in the liver sinusoids and in hepatocyte invasion (Ménard , 2000). Structurally, the protein can be divided in three main parts, as represented in *figure 9*. The less polymorphic N-terminal region, which contains a CD4 epitope and the site of proteolytic processing (Coppi *et al*., 2005); the highly polymorphic central repeated region, which contains a NANP repeat region, a B cell epitope; and also, the highly polymorphic C-terminal region, that has a CD8 epitope followed by a thrombospondin type I repeat (TSR) (Aragam *et* al., 2013; Doud *et al.*, 2012; Plassmeyer *et* al., 2009; Zeeshan *et al.*, 2012).

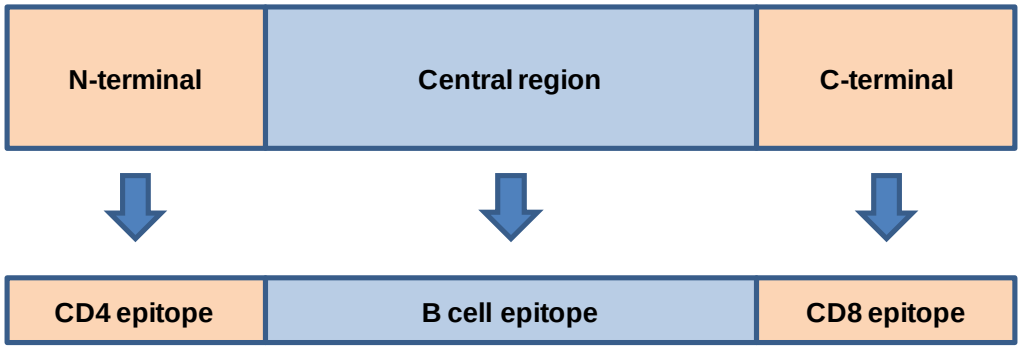

Figure 9 | CSP structure and its epitopes.

In the sporozoite, CSP has two conformational states (*figure 10*): an adhesive conformation in which the C-terminal cell-adhesive domain is exposed and a non-adhesive conformation in which the N terminus masks this domain. It was demonstrated by generating transgenic sporozoites that the cell-adhesive domain functions in sporozoite development and hepatocyte invasion. Indeed, proteolytic cleavage of CSP regulates the switch to an adhesive conformation, and the highly conserved region I plays a critical role in this process. If the CSP domain architecture is altered such that the cell adhesive domain is constitutively exposed, the majority of sporozoites do not reach their target organs, and in the mammalian host, they initiate a blood stage infection directly from the inoculation site (Coppi *et al.*, 2011).

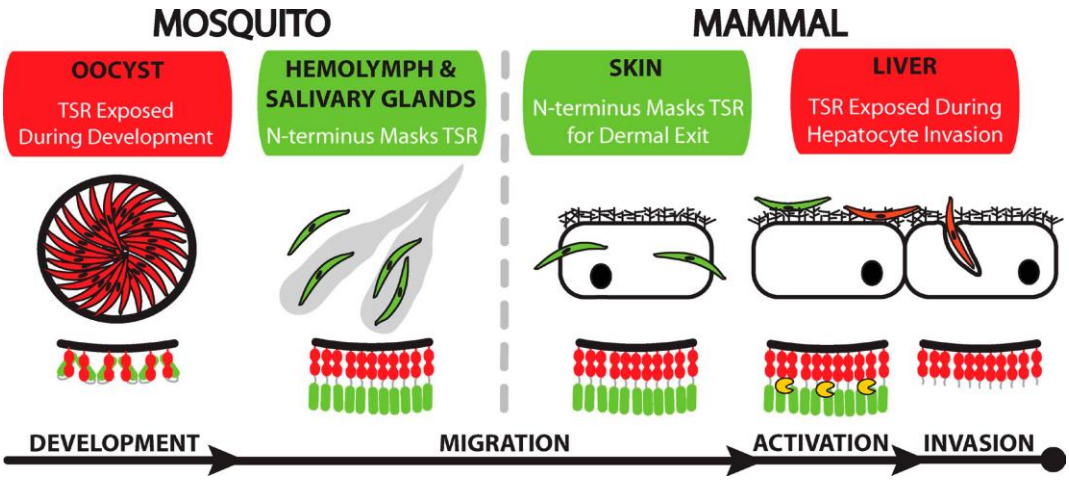

Figure 10 | The two conformational states of CSP and its functions (Coppi *et al*., 2011).

CSP is translocated by malaria sporozoites across cell membranes into the cytosol of mammalian cells, leading to inhibition of protein synthesis in the target cells. In this way, the parasite uses this strategy to limit the host's ability to present parasite antigens, attenuating immune responsiveness (Frevert *et al.*, 1998).

Young children from endemic regions and adults from non-endemic regions initially develop antibodies against the three major domains of CSP: N-terminus, central repeats and C-terminus (Bowman *et al*., 2013; Calle *et al.*, 1992; Lopez *et al.*, 1996). However repeated infections in endemic areas skew antibody response to central repeat regions and re-infections continue to occur.

As the immunodominant sporozoite antigen, CSP has been the main target for vaccine development against the pre-erythrocytic phase. The most advanced malaria vaccine, RTS,S is composed of a C-terminal CSP fragment comprising a portion of the repeats and the TSR fused with hepatitis B surface antigen (Agnandji *et al.*, 2011; Zhao *et al*., 2013).

3. IMMUNITY TO PRE-ERYTHROCYTIC STAGES

Populations that are continually exposed to malaria parasites can develop innate immunity against malaria and their mechanisms are not well understood. Unlike other acute infections, malaria does not induce a long lasting immunological memory, but it rather develops gradually and requires repeated infections to persist. The developed immunity is not sterile, malaria parasites can still survive in the host but at such low levels so that the clinical symptoms do not show. The development of this semi-immunity is influenced by age, genetic background, pregnancy, co-infections and the nutritional status of the host (Beeson *et* al., 2008; Diarra *et al.*, 2012; Good *et al.*, 2013).

Sporozoites and ensuing liver stages are seen as attractive targets for vaccination against malaria. Sporozoites when migrating from the skin to the liver can be targeted by antibodies, while pre-erythrocytic stages developing inside hepatocytes by CD8+ T cells (Torre et *al*., 2002).

First, antibodies reduce the sporozoite inoculum and parasite viability during a mosquito bite. With an experimental rodent model, it has been shown that an immune complex formed during the mosquito bite reduces the number of sporozoites deposited during feeding. The parasites injected into immune mice were immobilized and not capable of invading dermal blood vessels. When the sporozoites are inoculated intravenously, thus bypassing the skin, anti-CSP antibodies efficiently block the infection of hepatocytes. The inhibition of hepatocyte infection may be due to a reduction of the sporozoites' ability to move or attach to hepatocytes (Teixeira *et al.*, 2014).

There is however, little evidence that natural exposure to parasites induces, even partial protection against pre-erythrocytic stages (Offeddu *et al*., 2012). In contrast, strong protection in humans and rodents against a sporozoite challenge can be achieved by injection of live attenuated parasites that are not able to complete their development inside hepatocytes and consequently initiate a blood infection (Luke *et* al., 2003; Sedegah *et* al., 2007; Hoffman *et* al., 2010; Ménard *et al*., 2013). Protection induced with radiation-attenuated sporozoites has been well characterized in rodent models. CSP-specific antibodies were identified as having an important role in immobilizing sporozoites and CD8+T cells in killing infected hepatocytes (Yoshida *et al*., 1980; Stewart *et al*., 1986; Schofield *et al*., 1987; Weiss *et al*., 1988; Romero *et al*., 1989; Rodrigues *et al*., 1991; Marussig *et al*., 1997). The exact mechanism used by parasite specific CD8+T cells to kill infected hepatocytes remains unclear since cells

lacking IFN-γ, perforin or FAS ligand can still eliminate liver-stage (Chakravarty *et al*., 2008; Butler *et al*., 2010).

The development of a vaccine targeting the pre-erythrocytic stage of life cycle of *Plasmodium* would prevent the symptomatic blood stage of infection and is an attractive vaccine target for several reasons: the number of parasites transmitted by the mosquito is low and consequently, the number of infected hepatocytes is extremely low; human parasites like *Plasmodium falciparum* and *Plasmodium vivax* take nearly a week to complete development in hepatocytes, providing time sufficient time for elimination; and unlike *Plasmodium*-infected red blood cells, the infected hepatocyte is capable of presenting parasite antigens to immune effector cells; and the fact that sterile immunity against the pre-erythrocytic forms of both human and rodent parasites can be induced by immunization with radiation attenuated parasites or with low-dose of wild-type sporozoites (Van Braeckel-Budimir *et al.*, 2014; Duffy et al., 2012; Vignali *et al.*, 2009; Ménard *et al*., 2013).

At moment, there are no approved malaria vaccines and RTS,S, the most prominent vaccine candidate, just moderately (approximately 30-50%) protected children from clinical episodes of malaria in Africa (RTS,S Clinical Trials Partnership *et al.*, 2012). This vaccine is currently being evaluated in a large clinical trial in 7 countries in Africa. RTS,S vaccine is based on the *P. falciparum* CSP fused with hepatitis B surface antigen, and an adjuvant system AS01 or AS02. The adjuvant system AS01 consists in a liposomic formulation of MPL and QS21 immunostimulants. The adjuvant system AS02 consists in an oil-in-water emulsion of MPL and QS21 immunostimulants. (Alonso *et al*., 2004; Arama *et al*., 2014; Cohen *et al*., 2010; Fox *et* al., 2013; Doll *et al*., 2014; Malaria Vaccine Initiative). This vaccine is safe, well tolerated and immunogenic (Alonso *et al.*, 2004). Immunization with RTS,S vaccine induces very high IgG levels and low frequencies of CD8+T CSP specific immune responses. High levels of antibodies against CSP are thought to be the main determinant of protection conferred by RTS,S immunization (Moorthy *et al*., 2009) analogous to the transference of high amount of antibodies targeting the central repeats of CSP that is required to completely inhibit sporozoites infectivity in mice (Potocnjak *et al*., 1980). However, the RTS,S vaccine efficacy disappears over four years and is less in areas of high transmission. One possible explanation for poor vaccine performance might be the lack of cross-protection against genetic variants not included in the vaccine (Bowman et al., 2013), since CSP is highly polymorphic in natural parasite populations (Aragam *et al*., 2013). On other hand, the immune response depends of the host-parasite interactions and probably it is the reason that we have differences in protection against malaria in

heterogeneous human populations (Van Braeckel-Budimir *et al.*, 2014). Ideally a malaria vaccine should protect against multiple *Plasmodium* species (Van Braeckel-Budimir *et al.*, 2014). So it is necessary the development of new vaccine candidates that induce a CD8+ T cell response that can be used alone or in combination with RTS,S (Van Braeckel-Budimir *et al.*, 2014).

II. AIMS

The main aim of this study is the characterization of the immune response of Balb/C mice immunized with *P. yoelii* CSP in combination with two distinct adjuvants. The adjuvants will be identified as A or B throughout the work and cannot be disclosed at this stage. Indeed, this work is done in collaboration with Doctor Rogerio Amino, at Pasteur Institute in Paris and Professor Silvia Boscardin from São Paulo University. Balb/C mice will be immunized two times and two weeks apart, with recombinant *P. yoelii* CSP alone or in combination with adjuvants A or B. Previously, mice immunized with CSP in combination with adjuvant A showed a high degree of protection against a stringent challenge with *P. yoelii* sporozoites but sterile protection was only achieved when animals were immunized with CSP in combination with adjuvant B.

To characterize the host immune response induced by this new vaccination protocol we propose to study the humoral and cellular responses. To analyse the humoral response, IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 titers specific to CSP and total IgM and IgG titers will be determined by ELISA and during 20 weeks. The analysis of the cellular immune response, using multiparametric flow citometry, will be done by assessing the frequency of splenic: B, CD4+ and CD8+ T cells; naïve, effector and central memory T cells; IL-2, IL-6, IL-10, TNF-α and INF-γ CD3+ T cells.

The specific objectives of the work were:

- Expression in *E.coli* and purification of recombinant *P. yoelli* CSP;
- Balb/C mice immunization with CSP alone or formulated with adjuvants A or B;
- Determination of total IgM and IgG titers and IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 titers specific to CSP, during 20 weeks;
- Study at 6 weeks-post vaccination the frequency of splenic: B, CD4+ and CD8+ T cells; naïve, effector and central memory T cells; IL-2, IL-6, IL-10, TNF-α and INF-γ CD3+ T cells.

III. METHODOLOGY

1. Expression of recombinant CSP

The full length *Plasmodium yoelli* CSP open reading frame was codon optimized and cloned in a bacterial expression vector PET21b to produce a recombinant protein with 6x histidine residues and it was kindly provided by Prof. Silvia Boscardin from São Paulo University, Brazil. The production of recombinant protein was done in BL21 (DE3) strain of *Escherichia coli*.

In order to produce CSP in large-scale, bacteria cultures were left growing at 37ºC in LB medium supplemented with NaCl (10 g/L) and ampicillin (100 μg/mL). The induction was done with IPTG (final concentration 1 mM) and after 4 hours at 37ºC, the cultures were centrifuged at 4000 rpm for 20 minutes. The supernatants were discarded and the bacterial pellets containing CSP were stored at -20ºC.

2. Purification of recombinant CSP

The pellets previously obtained were re-suspended in buffer A [0.5 M NaCl, 20 mM Tris, 10% glycerol (v/v), pH 7.69] supplemented with 5 mM imidazole and were lysed using a sonicator. After sonication, the cell suspensions were centrifuged at 4000 rpm, 30 minutes, at 4ºC; after, the pellets were discarded and the supernatants were maintained at 4ºC.

For the purification of protein, it was used a nickel affinity column (Ni-NTA Agarose from Qiagen), according to the manufacturer instructions. First, this column was equilibrated with buffer A and then the supernatants, containing the protein, were applied to the column. The column was washed with different volumes of buffer A with increasing gradients of imidazole (25; 50; 100; 500 and 1000 mM) and the collection of protein started after washing the column with buffer A containing 25 mM of imidazole.

The fractions that contain CSP, which analysis was done by SDS-PAGE stained with Coomassie Blue, were joined and the imidazole was removed, using PD-10 desalting column (from GE Healthcare) and according to the manufacturer instructions. PBS was used as the buffer. During all these procedures, the solutions and containers were kept refrigerated.

3. Endotoxin removal and semi-quantification of CSP

Endotoxins of *E. coli* were removed by two-phase extraction with Triton X-114 according to Liu *et al.*, 1997. First, to the fractions of CSP previously purified, it was added a volume of Triton X-114 (Sigma) to make a final solution containing 1% (v/v) of this reagent. It was used a vortex to mix very well the protein and the detergent and then the solution was placed on ice for at least 15 minutes and, until becoming clear. After 1 minute vortex, the solution was placed in a 37ºC water bath for at least 30 minutes and, until becoming opaque. After centrifugation at 2000 g, for 10 minutes, the bottom phase, containing the endotoxins, was discarded and the top phase was submitted to the same protocol another three times.

After endotoxin removal, protein fractions were sterile-filtered (0.2 μm), aliquoted and stored at -20ºC. Endotoxins level was quantified, using Limulus amebocyte lysate assay (Pierce). The protein was only used, when endotoxins level remained below 0.1 EU/mL.

The semi-quantification of CSP was done by SDS-PAGE, using BSA as standard. The proteins were separated on a 10% polyacrylamide gel under denaturing and reducing conditions. The gel was stained with Coomassie Blue.

4. Mice immunization and sample collection

Female Balb/C mice were purchased from Charles River Laboratories. All experiments involving mice were approved by the Local Animal Ethics Committee of IBMC, University of Porto, Portugal and licensed by DGV (General Directory of Veterinary, Ministry of Agriculture, Rural Developmental and Fishing, Govt. of Portugal). All animals were handled in strict accordance with good animal practice as defined by national authorities (DGV, Law nu1005/92 from 23rd October) and European legislation EEC/86/609 and directive 2010/63.

The mice were immunized with 100 μl of a solution containing 5 μg of recombinant *Plasmodium yoelii* CSP, with or without Adjuvant A or Adjuvant B. Mice immunized with saline or adjuvants alone serves as a control group. The formulations were administered by intraperitoneal injection and animals were immunized twice, at weeks 0 and 2.

Blood was collected from the tail vein nick and into heparinized capillary tubes, every two weeks after first immunization, until week 12, and then the collections

became monthly. Plasma samples were obtained after centrifugation at 2000 rpm for 10 minutes, during 20 weeks. These samples were stored at -20ºC to be used in antibody determinations by ELISA.

For cellular immune response studies, the vaccination protocol described above was also done in Balb/C mice. At 6 weeks after first immunization, the mice were sacrificed and their spleens were collected in sterile conditions.

5. Antibodies determination by ELISA

The determination of total IgG and IgM titers (CSP non-specific) and the determination of CSP specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 titers were done by ELISA. All antibodies were purchased from SouthernBiotech.

The ELISA assay consisted in coating high binding 96-well plates (from Greiner) with 50 μl/well of a 1 μg/mL solution (prepared in 0.1 M carbonate/bicarbonate buffer, pH 9) of goat anti-mouse IgG or IgM antibody (for the determination of total IgG and IgM titers); or of recombinant CSP (for the determination of CSP specific IgM or IgG titers). After incubation overnight at 4ºC, plates were blocked with 200 μl/well of blocking buffer [PBS with 1% porcine skin gelatin (m/v, Sigma)] during 30 minutes at 37ºC. Then the plates were washed with PBS containing 0.1% Tween-20 (v/v, Sigma) in an automatic ELISA plate washer (BioTek ELx405). Eight twofold serial dilutions of each animal's plasma were added (diluted in blocking buffer) for a final volume of 50 μl/well. Eight controls of 50 μl/well of only blocking buffer were added and the plates were left incubating for 1 hour at 37ºC. Again, the plates were washed and then were incubated with 50 μl/well of HRP-conjugated goat anti-mouse antibody IgG/IgM/IgG1/IgG2a/IgG2b/IgG3, diluted 1:5000 (IgM, IgG1, IgG3 and IgG2a) or 1:7500 (IgG and IgG2a) in blocking buffer, for 30 minutes, at 37ºC. Once more, the plates were washed and then the plates were incubated with 100 μl/well of the substrate solution [5 mg of OPD (Sigma) and 10 μl of H_2O_2 (Sigma) per each 10 ml of citrate solution (0.15 M Na_2HPO_4 (Sigma) - 0.15 M citric acid (Sigma), pH 5)], for 10 minutes, in the dark, at room temperature. This reaction was stopped with 50 μl/well of HCl (Sigma) 3 M.

The absorbance was read at 492 nm in a plate reader (BioTek Synergy 2) and the dilution factor for which the optical density is equal or higher than 0.1 is the titer of the antibody, as has been previously described (Boscardin *et al*., 2006).

6. Spleen cells analysis

At 6 weeks after the first immunization, mice were sacrificed and cells from spleens were isolated by mechanical disruption.

6.1. Cell populations study

For cell populations studies, $1x10^6$ spleen cells were washed with 150 μl of PBS-2% fetal bovine serum (FBS) buffer and incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 (T cell populations study); and with FITC-conjugated anti-mouse IgM (B cell populations study). For memory T cells determination, cells were labeled with peridinin chlorophyll (PERCP)-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD44 and allophycocyanin (APC)-conjugated anti-mouse CD62L. Incubations were conducted in the dark, on ice, for 30 minutes (the total antibody volume used was 50 μl). Then cells were washed two times with 150 μl of PBS-2% FBS, suspended in 200 μl of PBS-2% FBS and analysed by flow cytometry (Fluorescence Activated Cell Sorting - FACS Canto II, from BD Biosciences).

6.2. Intracellular cytokines staining

Splenocytes single-cell suspensions ($1x10^6$ cells) from individual mice were incubated for 2 h, in 96-well round-bottom cell culture plates (37ºC, 5% CO_2), with 10 ng of phorbol 12-myristate 13-acetate (PMA) and 100 ng of ionomycin (final volume of 200 μl). After this incubation, each well was incubated with 2 μg of brefeldin A, for 2 h (37ºC, 5% CO_2). Cells were collected and stained with PE-cy7-conjugated anti-mouse CD3 and Brilliant Violet™ 510 (BV510)-conjugated anti-mouse CD4 as described above. The cells were then fixed with 150 μl of PBS-paraformaldehyde 2% (20 minutes, at room temperature). PBS (150 μl) was used to wash cells before incubating them with 150 μl of PBS-2% FBS-10% saponin (10 minutes, at random temperature), followed by staining with, FITC-conjugated anti-mouse IFN-γ, Pacific Blue-conjugated anti-mouse IL-2, PE-conjugated anti-mouse IL-6, APC-conjugated anti-mouse IL-10, and PERCP-conjugated anti-mouse TNF-α, using the BD Biosciences

Cytofix/Cytoperm kit, according to the manufacturer's instructions (incubation with 50 μl of antibodies was done in the dark, at random temperature, for 20 minutes). Cells were washed with 150 μl of PBS-2% FBS-10% saponin two times and washed with 150 μl of PBS-2% FBS two times also. Cells were suspended in 200 μl of PBS-2% FBS and analysed by flow cytometry (Fluorescence Activated Cell Sorting - FACS Canto II, from BD Biosciences).

7. Data processing and statistical analysis

SDS-PAGE gels were analysed with *ImageJ 1.48* image processing software and results from flow cytometry were analysed using *FlowJo 7.6.5* software. All graphs and statistical analysis (Mann-Whitney and ANOVA tests) were done using *GraphPad Prism 6.0* software. Statistical significance was established whenever $P < 0.05$.

IV. RESULTS

1. Purification and quantification of recombinant CSP

The *P. yoelli* CSP encoding sequence was codon optimized and cloned into the bacterial expression vector pET21b. The fusion protein containing a tag of six histidine residues was purified by affinity chromatography in a nickel-agarose column under non-denaturing conditions. The presence and purity of the recombinant protein on the several fractions were evaluated by SDS-PAGE Coomassie Blue stained (*figure 11 A*). Several of the more concentrated fractions were joined. The protein was then dialyzed against PBS using PD10- desalting columns and endotoxins were removed by treatment with Triton X-114 (Liu *et al.*, 1997). The protein was quantified by semi-quantitative SDS-PAGE, using BSA as standard for the construction of a calibration curve (*figure 11 B*).

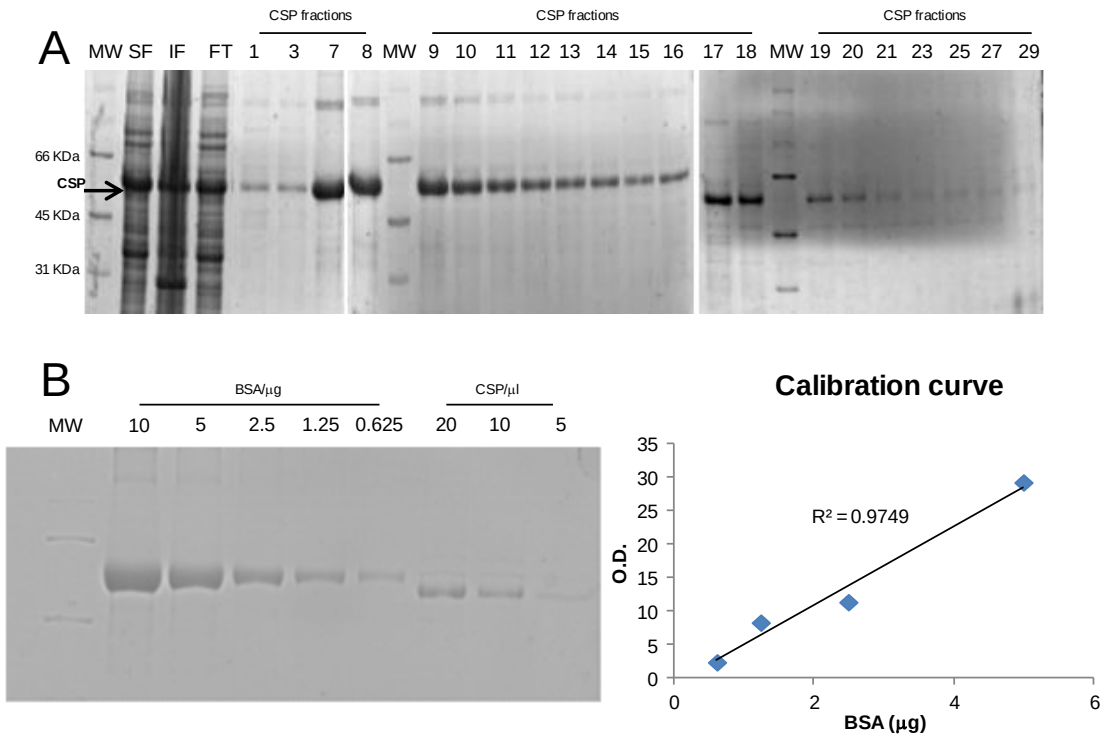

Figure 11 | Purification and quantification of recombinant CSP. (A) SDS-PAGE of several fractions obtained during CSP purification (MW – molecular weight; SF – soluble fraction; IF – insoluble fraction; FT – bacterial lysate flow through; 1, 3, 7 - 21, 23, 25, 27, 29 – eluted fractions of CSP). (B) Semi-quantitative SDS-PAGE of CSP, using BSA as standard and the respective calibration curve. The SDS-PAGE gels were stained with Coomassie Blue.

2. Characterization of humoral response

6 to 8 week-old female Balb/C mice were immunized with 5 μg of CSP alone or with adjuvants A or B (CSP+Adj.A or CSP+Adj.B). Mice immunized with saline or adjuvants alone served as a control. Animals were immunized at week 0 and at week 2 and blood was collected every two weeks until week 12, and then the collections became monthly. The *figure 12* shows a diagram of the experimental setup. Two independent experiments were performed. In order to characterize the humoral response, plasma was tested to CSP specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 as well as total IgG and IgM (CSP non-specific), by ELISA.

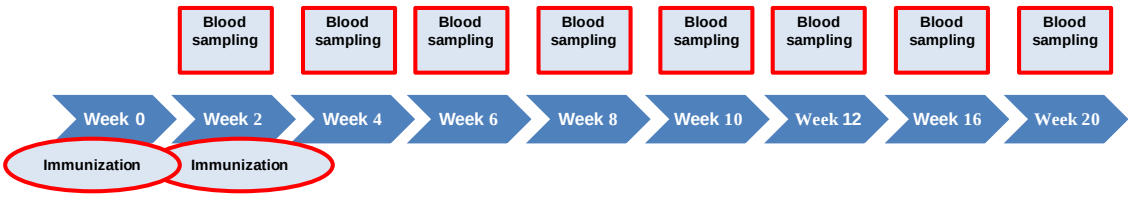

Figure 12 | Diagram of the experimental setup for the characterization of humoral immune response.

The mean total IgM titer between the immunized and control groups was similar at 2 and 4 weeks after the first immunization (*figure 13A*). At 2 weeks, all the mean total IgM titers were between 20 000 and 35 000. For immunized groups, the mean total IgM titer (CSP non-specific) decreased 4 weeks after the first immunization. This decrease was statistically significant for groups immunized with CSP plus adjuvants A or B and for two controls, namely PBS and adjuvant A.

As expected, no IgM CSP specific antibodies were detected in the plasma of mice immunized with saline or adjuvants (controls) between 2 and 10 weeks post-immunization (*figure 13C*). On the other hand (*figure 13B*), two weeks after the first immunization, the mean of CSP specific IgM titer for groups immunized with CSP plus adjuvants A or B was higher (1578+1070 and 1133+970, respectively) when compared with groups immunized with CSP only (722+543). Four weeks after the first immunization, the mean CSP specific IgM titer for groups immunized with CSP plus adjuvant A remained approximately with same value (1556+1028) but for other two groups (CSP only and CSP plus adjuvant B) slightly decreased (from 722+543 to 533+200 and from 1133+970 to 633+469, respectively) but overall was not statistically significant. In the end, the mean CSP specific IgM titer for immunized groups decreased for values approximately equal to controls at 10 weeks (*figure 13C*).

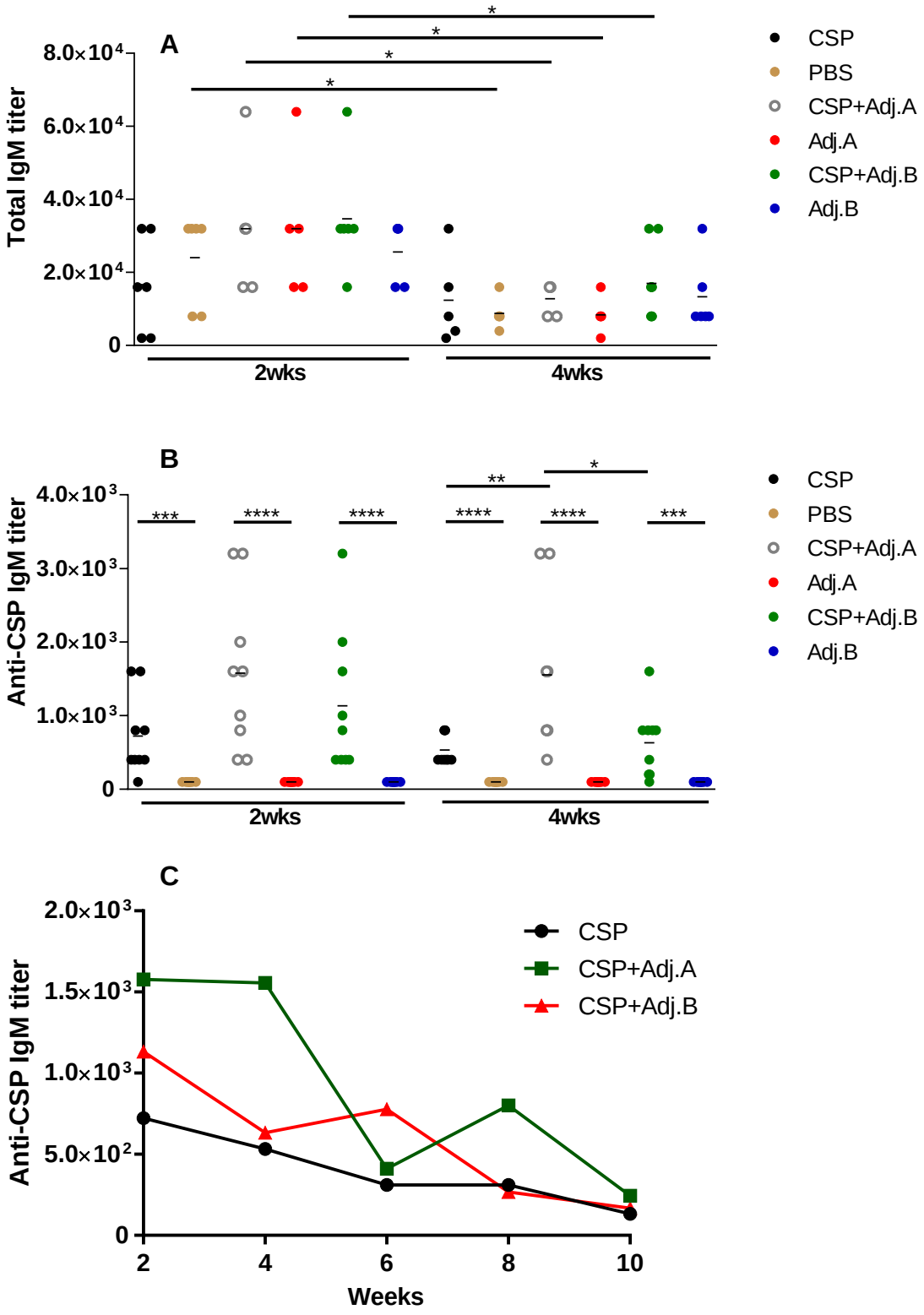

Figure 13 | Characterization of the host humoral response: IgM. (A) Total IgM and (B) anti-CSP IgM titers of controls, CSP, CSP+Adj.A and CSP+Adj.B groups at 2 and 4 weeks. (C) Kinetics of anti-CSP IgM mean titers of CSP, CSP+Adj.A and CSP+Adj.B groups between 2 and 10 weeks. The results represented were from a total of 9 animals, from 2 independent experiments. In the graphics A and B, antibody titers from each individual animal are represented. Symbols may overlap for animals with similar titers. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001.

The mean total IgG titers (CSP non-specific) between immunized and control groups was similar 2 weeks after the first immunization and remained between ~12 500 and ~30 000 (*figure 14A*). A significant increase and peak on total IgG levels was detected at 4 weeks after the first immunization in the groups of mice immunized with CSP plus adjuvant A (from 20 000+14 142 to 148 000+112 783) or B (from 30 000+11 547 to 210 000+136 137). For animals receiving only PBS, CSP, adjuvants A or B, no major changes on total IgG levels were observed at 4 and 6 weeks after the first immunization.

When it comes to CSP specific IgG antibodies, as expected, none of those were detected in the plasma of mice immunized with saline or adjuvants (controls) between 2 and 20 weeks. At 4 weeks after first immunization, the mean CSP specific IgG titers for immunized groups increased (*figure 14B*): from 344+309 to 3900+4745 for mice immunized with CSP alone; from 2889+2408 to 192 222+190 380 for groups immunized with CSP plus adjuvant A; and from 4222+4919 to 72 222+56 960 for groups immunized with CSP plus adjuvant B. This peak at 4 weeks was statistically significant for all groups immunized. It was observed, at 2, 4 and 6 weeks, that the mean CSP specific IgG titers for groups immunized with CSP alone is lower when compared with groups immunized with CSP plus adjuvants A or B. The mean CSP specific IgG titers of groups immunized with CSP plus adjuvants A or B were similar at 2, 4 and 6 weeks, since those are not statistically different. Looking to the kinetics of the CSP specific IgG titers (*figure 14C*), it was possible to observe that the mean CSP specific IgG titer maintained during time until 20 weeks, for all groups immunized, after the significant increase mentioned before at 4 weeks.

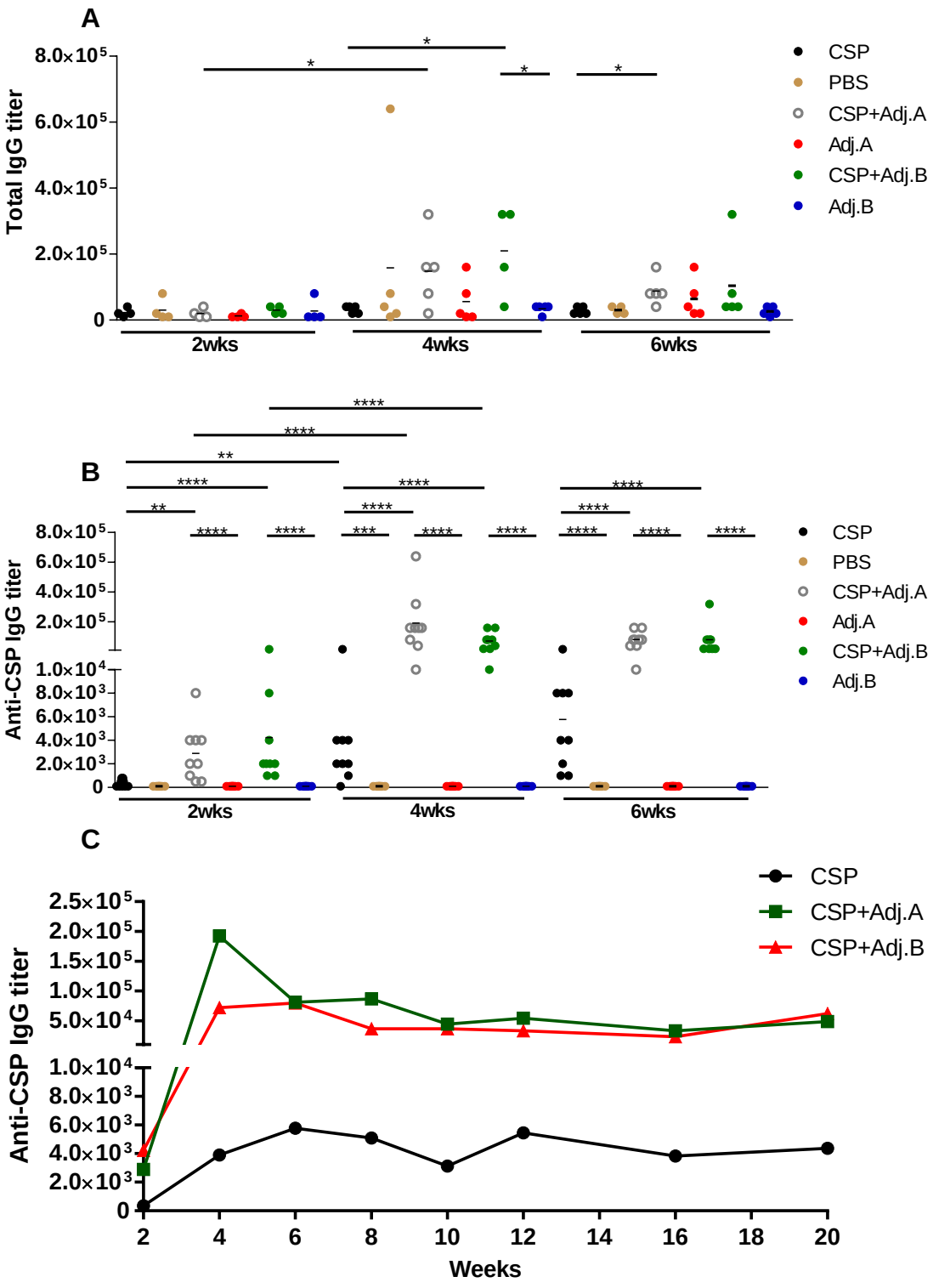

Figure 14 | Characterization of the host humoral response: IgG. (A) Total IgG and (B) anti-CSP IgG titers of controls, CSP, CSP+Adj.A and CSP+Adj.B groups at 2, 4 and 6 weeks. (C) Kinetics from 2 to 10 weeks showing the mean value of anti-CSP IgG titers from CSP, CSP+Adj.A and CSP+Adj.B. The results represented were from a total of 9 animals, from 2 independent experiments. In the graphics A and B, antibody titers from each individual animal are represented. Symbols may overlap for animals with similar titers. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001.

In order to investigate the immune profiles induced, the titers of antibody subclasses IgG1, IgG2a, IgG3 and IgG2b were analysed at 2, 4 and 6 weeks (*figures 15 and 16*) and during time, until 5 months (*figures 17* and *18*).

For animals immunized with CSP alone, it was observed a peak at 4 weeks for mean CSP specific IgG2a (178+233), IgG2b (456+650) and IgG3 (756+650) titers and a significant increase at 6 weeks for mean CSP specific IgG1 titer (12 944+11 555). For the animals immunized with CSP plus adjuvant A, the peak for all isotypes was detected at 4 weeks: 147 222+131 983, 56 667+101 581, 20 556+15 701 and 10778+9497 for IgG1, IgG2a, IgG2b and IgG3, respectively. For groups immunized with CSP plus adjuvant B, it was observed a peak at 4 weeks for mean CSP specific IgG2b (19 444+25 550) and IgG3 (9222+6360) titers and a significant increase at 6 weeks for mean CSP specific IgG1 (82 222+49 441) and IgG2a (35 556+27 437) titers.

In this study, the most abundant anti-CSP isotype was IgG1 for all immunized groups (*figure 17*). IgG2a was the second most abundant anti-CSP isotype detected in the animals immunized with CSP plus adjuvants A or B, while for animals immunized with CSP alone was IgG3. Important IgG2b levels were also detected in the animals immunized with CSP plus adjuvants A or B (*table 2*).

Analysing the kinetics of the CSP specific IgG isotypes titers, it was possible to observe that the mean CSP specific IgG isotypes titers were still high at 20 weeks, for all groups immunized, with an exception: the IgG3 titer for groups immunized with CSP alone, was quite similar to the controls (133+50).

Between 2 and 20 weeks after the first immunization, the mean CSP specific IgG isotypes titers were higher for groups immunized with CSP plus adjuvants A or B, when compared with groups immunized with CSP alone (*figure 18*). For example, the IgG1 titers are ~20 and ~10 fold higher, at 4 weeks, for groups immunized with CSP plus adjuvants A or B, respectively. Some differences in the titers were observed but in general, the mean CSP specific IgG isotypes titers were quite similar between the groups immunized with CSP plus adjuvants A and B.

Finally, the ratio IgG1/IgG2a (*table 2*) was strongly higher at 4 and 6 weeks for groups immunized with CSP alone, since IgG1 is the most predominant IgG isotype, as mentioned before. For the groups immunized with CSP plus adjuvants A or B, the ratio IgG1/IgG2a was lower and maintained until 20 weeks.

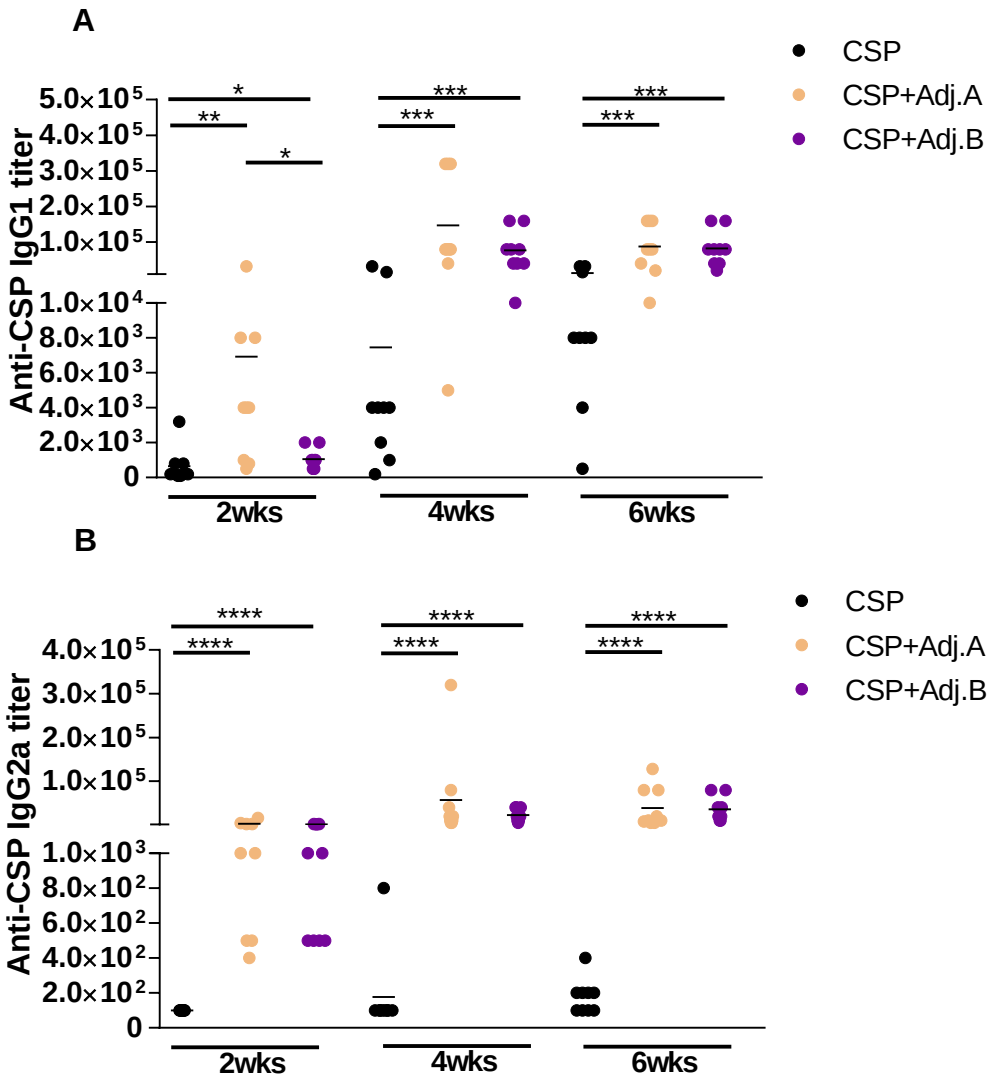

Figure 15 | Characterization of the host humoral response: IgG1and IgG2a. Anti-CSP IgG1 (A) and IgG2a (B) titers of controls, CSP, CSP+Adj.A and CSP+Adj.B groups at 2, 4 and 6 weeks. Antibody titers from each individual animal are represented and are from a total of 9 animals, from 2 independent experiments. Symbols may overlap for animals with similar titers. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<0.001; **** - P<0.0001.

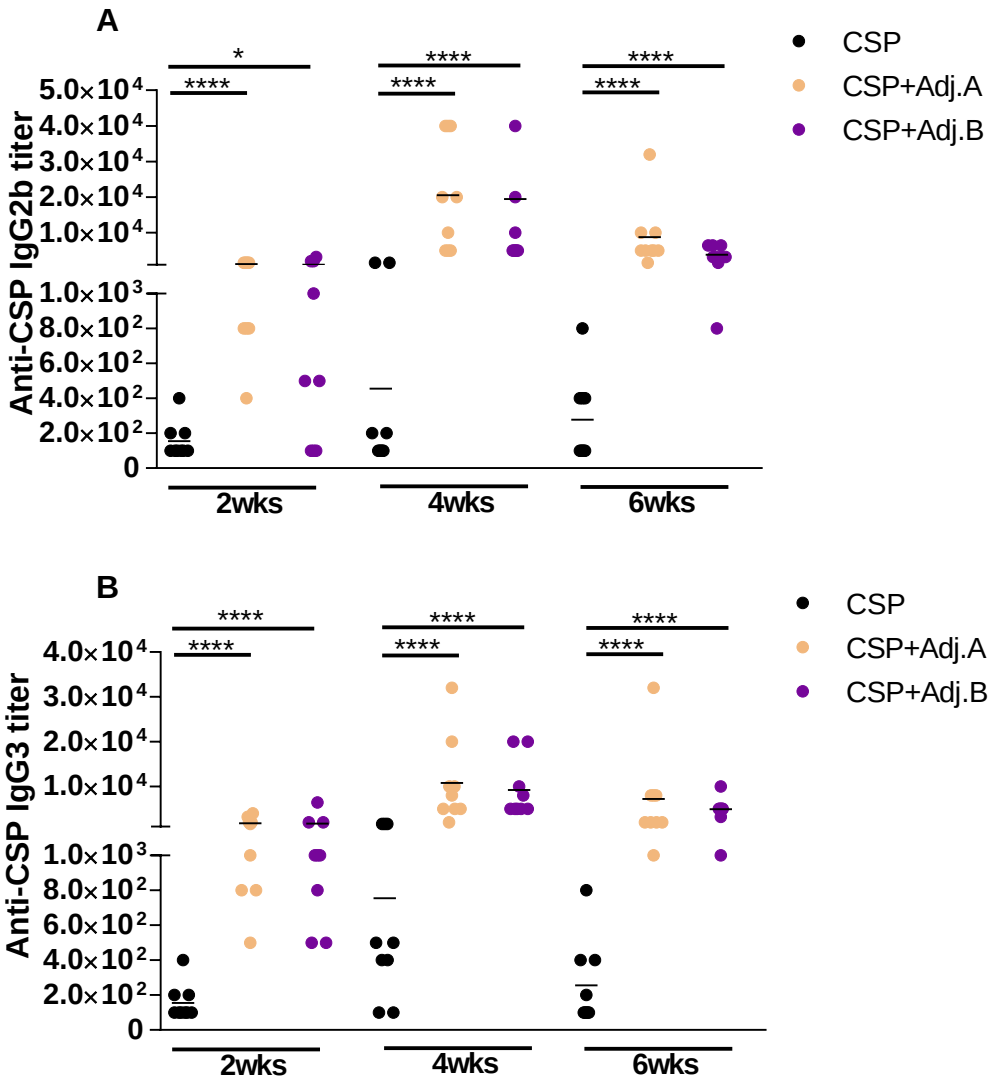

Figure 16 | Characterization of the host humoral response: IgG2b and IgG3. Anti-CSP IgG2b (A) and IgG3 (B) titers of controls, CSP, CSP+Adj.A and CSP+Adj.B groups at 2, 4 and 6 weeks. Antibody titers from each individual animal are represented and are from a total of 9 animals, from 2 independent experiments. Symbols may overlap for animals with similar titers. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<.0.001; **** - P<0.0001.

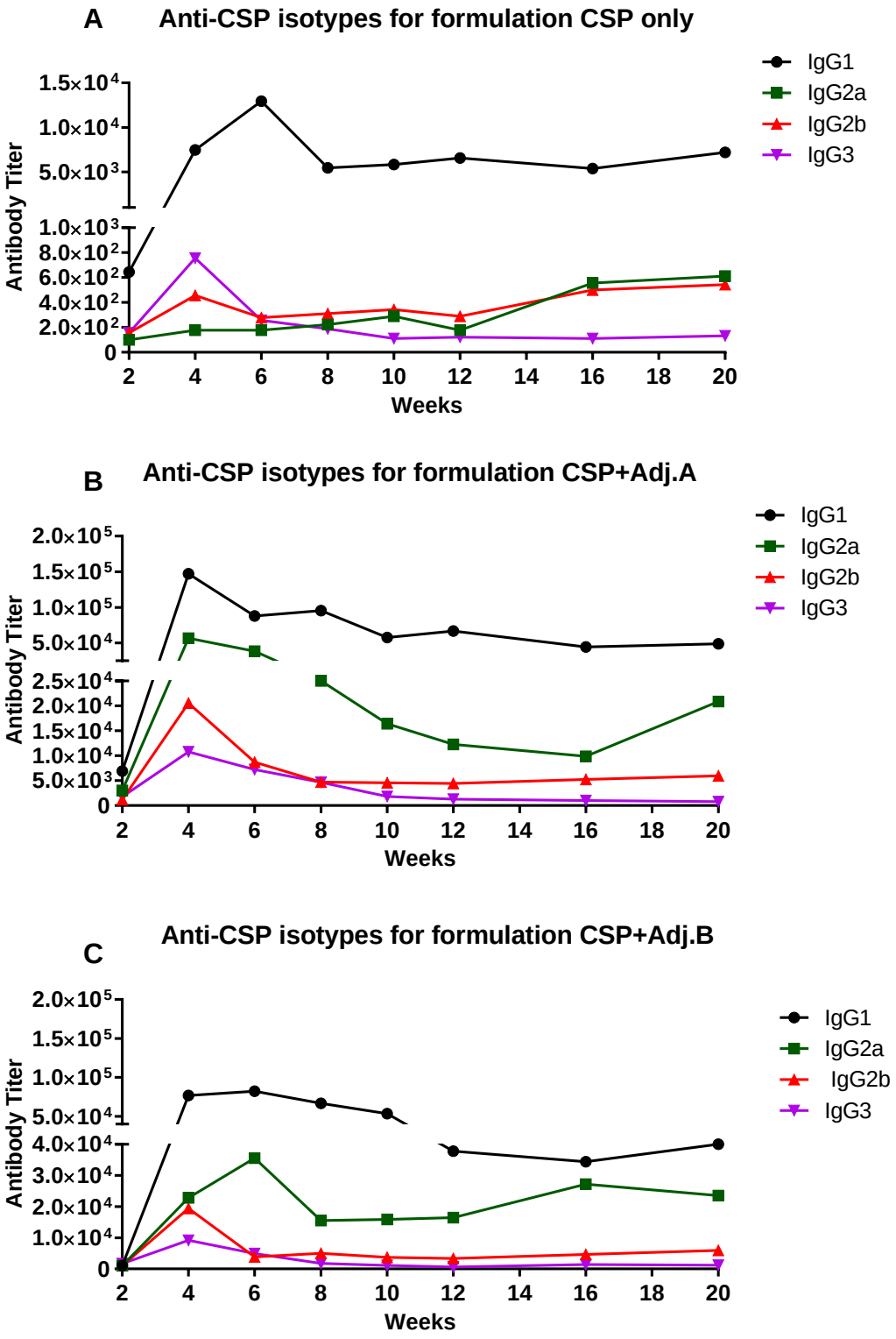

Figure 17 | Characterization of the host humoral response: isotypes kinetics. Kinetics from 2 to 10 weeks showing the mean value of anti-CSP IgG1, IgG2a, IgG2b or IgG3 titers from mice immunized with (A) CSP alone, (B) CSP+Adj. A and (C) CSP+Adj.B. The results represented were from a total of 9 animals, from 2 independent experiments. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<.0.001; **** - P<0.0001.

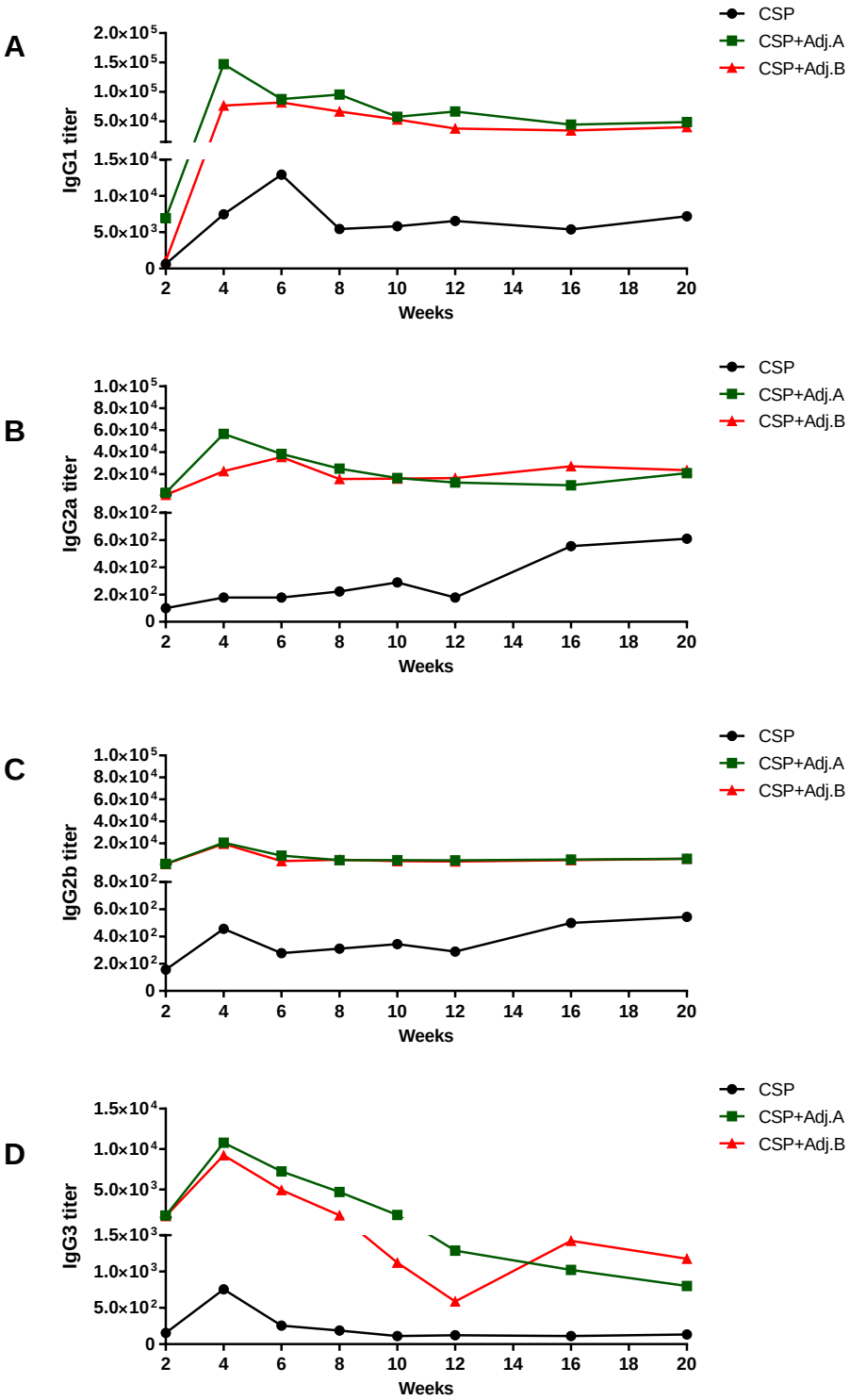

Figure 18 | Characterization of the host humoral response: isotypes kinetics. Kinetics showing the mean titers of anti-CSP IgG1 (A) IgG2a (B) IgG2b (C) and IgG3 (D) of mice immunized with CSP alone, CSP+Adj.A or CSP+Adj.B. The results represented were from a total of 9 animals, from 2 independent experiments. Lines represent the average for each group at each time point. Statistical analysis consisted of a Mann-Whitney test: * - P<0.05; ** - P<0.01; *** - P<.0.001; **** - P<0.0001.

Mean titer for groups immunized with CSP only				
Isotype	4 weeks	6 weeks	16 weeks	20 weeks
IgG1	7467+10 291	12 944+11 555	5389+5036	7200+5455
IgG2a	178+233	178+97	556+629	611+454
IgG2b	456+650	278+244	500+490	544+467
IgG3	756+650	256+240	111+33	133+50
IgG1/IgG2a	42.0	72.7	9.7	11.8
Mean titer for groups immunized with CSP+Adj.A				
Isotype	4 weeks	6 weeks	16 weeks	20 weeks
IgG1	147 222+131 983	87 778+59 954	44 444+29 202	48 889+24 721
IgG2a	56 667+101 581	38 444+45 547	9867+4468	20 889+9062
IgG2b	20 556+15 701	8733+9118	5244+3602	5956+3420
IgG3	10 778+9497	7222+9770	1022+977	800+624
IgG1/IgG2a	2.6	2.3	4.5	2.3
Mean titer for groups immunized with CSP+Adj.B				
Isotype	4 weeks	6 weeks	16 weeks	20 weeks
IgG1	76 667+52 915	82 222+49 441	34 444+11 304	40 000+17 321
IgG2a	22 778+13 944	35 556+27 437	27 222+49 974	23 556+8819
IgG2b	19 444+25 550	3822+2108	4622+7897	5978+5177
IgG3	9222+6360	4911+2350	1422+1930	1178+960
IgG1/IgG2a	3.4	2.3	1.3	1.7

Table 2. Mean plus standard deviation for CSP specific IgG isotypes titers; and ratio Th2/Th1 (IgG1/IgG2a), at 4, 6, 16 and 20 weeks, for groups immunized with CSP alone, CSP+Adj.A and CSP+Adj.B.

3. Characterization of cellular response

6 to 8 week-old female Balb/C mice were immunized with 5 μg of CSP, with or without adjuvant A (CSP+Adj. A) or B (CSP+Adj. B). Mice immunized with saline (PBS) or adjuvants A or B alone served as a control. Animals were immunized, two times, at weeks 0 and 2. At 6 weeks, after first immunization, mice were sacrificed and their spleens were removed for cellular immune studies. The *figure 19* shows a diagram of the experimental setup. The cells from individual mice spleens were isolated and cell population's analyzed.

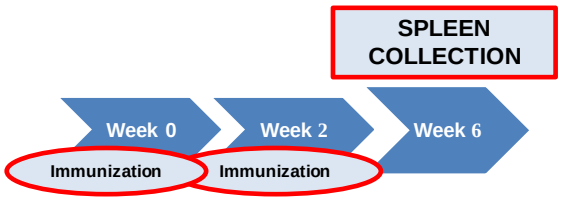

Figure 19 | Diagram of the experimental setup of the characterization of cellular immune response.

3.1. T and B cell populations

For T cell populations study, cells were stained with PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD8 and for B cell populations study, cells were stained with FITC-conjugated anti-mouse IgM. Dead cells were excluded by propidium iodide incorporation and cells were analysed by flow cytometry.

No significant differences were found between the percentage of B, CD4+ T and CD8+ T cells, between control and immunized groups (*figures 20A*, *B* and *C*, respectively). However, a statistical significant reduction on the total number of splenocytes from mice immunized with CSP compared to mice that received only PBS was detected (*figure 20D*). As a consequence, these mice had reduced number of total CD4+ T, CD8+ T and B cells compared to control group.

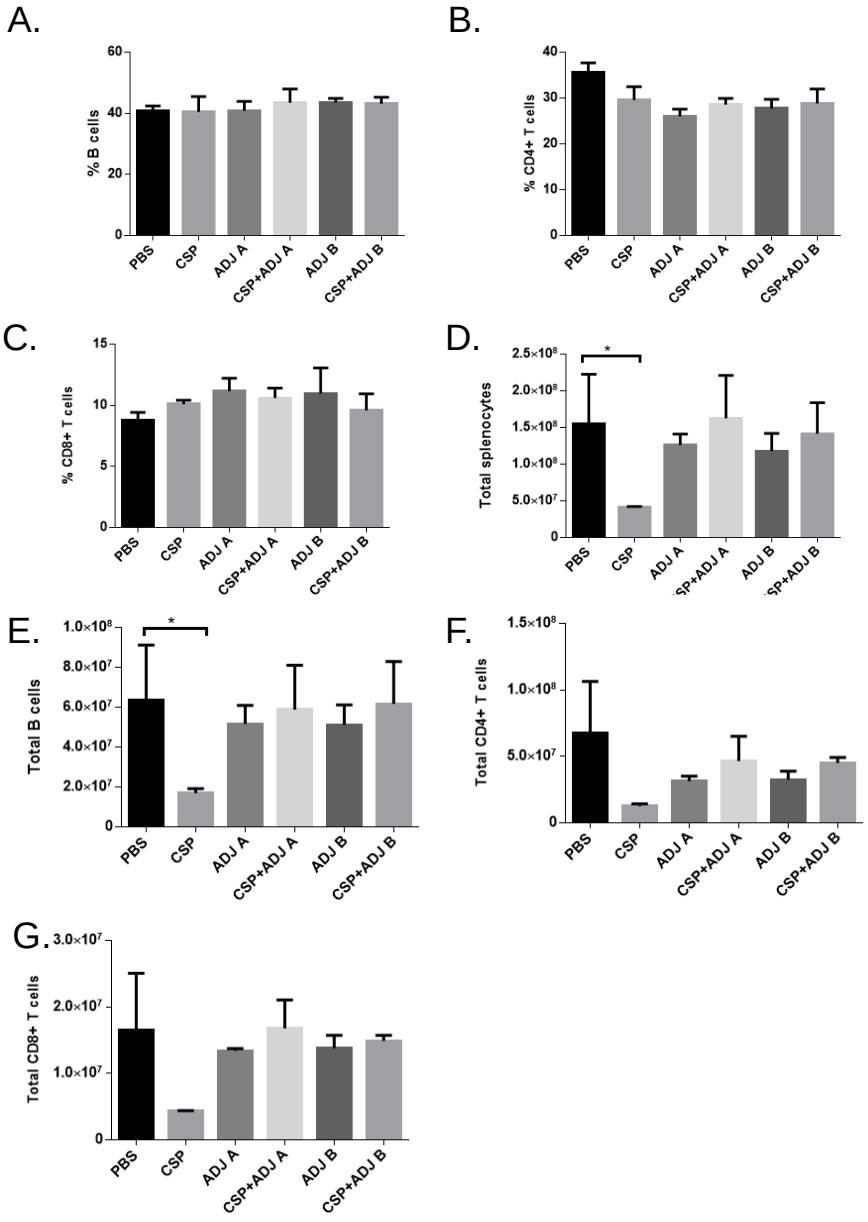

Figure 20 | Spleen cell populations. (A) Percentage of: B cells (B) CD4+ T cells (C) and CD8+ T cells (D) Total: splenocytes (E) ı B cells (F) CD4+ T cells (G) CD8+ T cells, for groups that received PBS, CSP, adjuvant A, CSP+adjuvant A, adjuvant B and CSP+adjuvant B. Data represents the average plus standard deviation, for each group. For each group n=4. Statistical analysis consisted of an ANOVA test: * - P<0.05.

3.2. Memory T cell populations

For memory T cell populations study, spleen cells were stained with PERCP-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD44 and APC-conjugated anti-mouse CD62L. After staining, cells were analyzed by flow cytometry. Naïve T cells were CD3+, CD44+ and $CD62L^{high}$; effector memory (EM) T cells were CD3+, CD44+ and $CD62L^{low}$; and central memory (CM) T cells were CD3+, $CD44^{high}$ and $CD62L^{high}$, as represented in *figure 21A*.

The percentage of naïve, EM and CM CD3+ T cells, between control and immunized groups, were similar and no statistically significant differences were found (*figures 21B*, *D* and *F*, respectively). As a consequence of the reduction in total number of splenocytes animals immunized with CSP alone presented a statistically significant reduction in the numbers of naïve, EM and CM CD3+ T cells when compared to the animals that just received PBS (*figures 21C*, *E* and *G*, respectively).

3.3. Intracellular cytokines

To investigate the cytokine profile of the splenocytes from the different experimental groups, cells were stimulated ex-vivo with PMA and ionomycin and subsequently incubated with brefeldin A. T cells were labeled with PE-cy7-conjugated anti-mouse CD3. Following fixation and permeabilization cells were stained with FITC-conjugated anti-mouse IFN-γ, Pacific Blue-conjugated anti-mouse IL-2, PE-conjugated anti-mouse IL-6, APC-conjugated anti-mouse IL-10, PERCP-conjugated anti-mouse TNF-α, statistically significant higher frequencies (~3 fold) of CD3+T cells producing IL-6 were found in splenocytes of mice immunized with CSP and adjuvant B when compared with animals that only received adjuvant B (figure 18G). No differences were found in the frequencies of IFN-γ, TNF-α, IL-2, or IL-10 producing CD3+T cells between the different groups (*figures 22A, C, E* and *I*). On the other hand, a reduction in the absolute number of CD3+T cells producing IFN-γ, TNF-α, IL-2, IL-6 and IL-10 was detected in the animals immunized with CSP when compared to the respective control group as consequence of the decrease in the number of total splenocytes (*figures 22B*, *D*, *F H* and *J*, respectively).

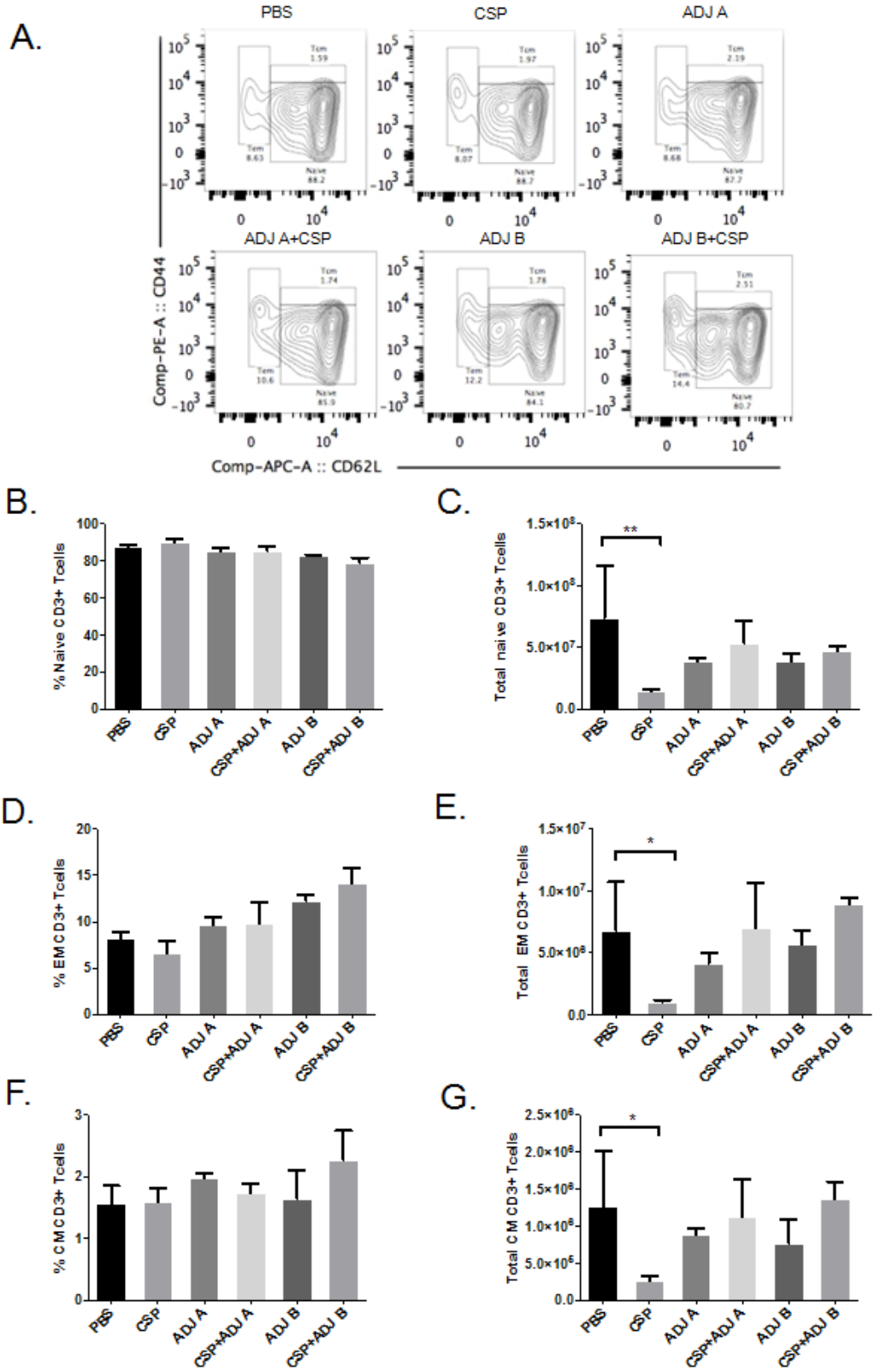

Figure 21 | Memory T cell populations study. (A) Representative percentage of naïve ($CD44^{+}$, $CD62L^{high}$), EM ($CD44^{+}$, $CD62L^{low}$) and CM ($CD44^{high}$, $CD62L^{high}$) CD3+ T cell populations. (B) Percentage of naïve CD3+ T cells (C) Total naïve CD3+ T cells (D) Percentage of EM CD3+ T cells (E) Total EM CD3+ T cells (F) Percentage of CM CD3+ T cells (G) Total CM CD3+ T cells of groups that received PBS, CSP, adjuvant A, CSP+adjuvant A, adjuvant B and CSP+adjuvant B. Data represents the average plus standard deviation, for each group. For each group n=4. Statistical analysis consisted of an ANOVA test: * - P<0.05; ** - P<0.01.

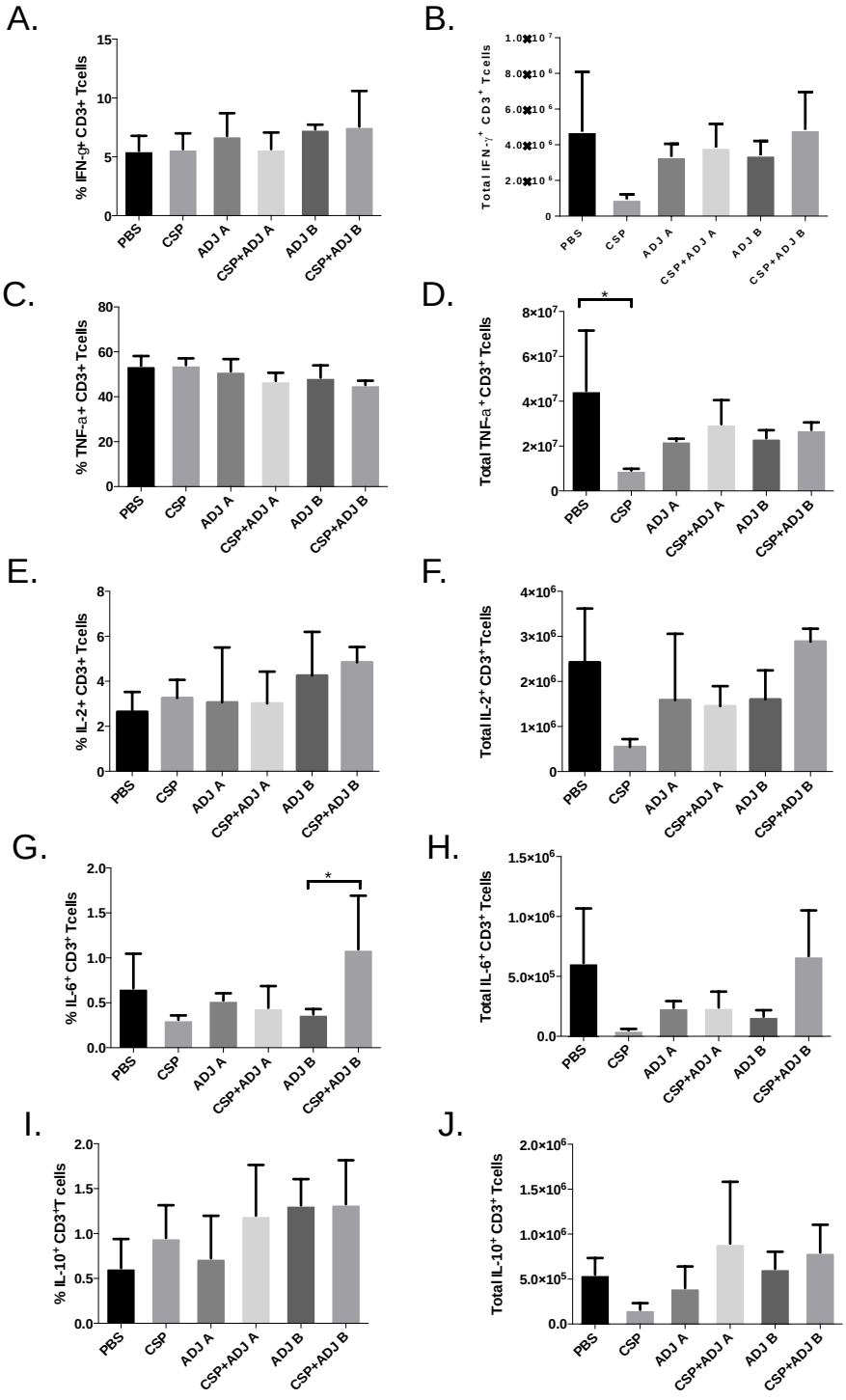

Figure 22 | Intracellular cytokines study. Percentage and total numbers of CD3+ T splenocytes producing: (A-B) IFN-γ+; (C-D) TNF-α; (E-F) IL-2+; (G-H) IL-6+ and (I-J) IL-10+ from animals that received PBS, CSP, adjuvant A, CSP+adjuvant A, adjuvant B and CSP+adjuvant B upon stimulation with PMA and ionomycin. Data represents the average plus standard deviation, for each group. For each group n=4. Statistical analysis consisted of an ANOVA test: * - P<0.05.

IV. DISCUSSION AND CONCLUSIONS

In this study we have characterized the immune response induced upon immunization of mice with recombinant *P. yoelli* CSP administered alone or in combination with adjuvants. An important aspect when using these new CSP formulations compared to RTS,S vaccine, is the fact that we are using a full length CSP. As expected, immunization with CSP alone or with adjuvants induces the production of specific antibodies. However, animals immunized with CSP in combination with adjuvants A or B presented higher titers of specific IgGs compared to animals that just received CSP. Moreover, important differences were found in the immunoglobulin subclasses between the groups immunized with CSP alone or when in combination with adjuvants. The subclass of immunoglobulin's induced after immunization is an indirect measure of the relative contribution of Th2-type cytokines versus Th1-type cytokines (Finkelman *et al.*,1990). More specifically, the production of IgG1-type antibodies is primarily induced by Th2-type cytokines, whereas production of IgG2a-type antibodies reflects the involvement of Th1-type cytokines. Both Th1 and Th2 responses seem to be involved in protective immune response against malaria (Carcaboso *et al.*, 2004; Hu, 2013).

An imbalanced Th2:Th1 ratio ranging from ~42 to ~72 at 4 and 6 weeks respectively, was found in the animals immunized with CSP alone. A similar result was previously reported in mice immunized with a plasmid encoding for CSP lacking the GPI anchor (Scheiblhofer S. et al., 2001). Importantly, both adjuvants, A or B, were capable of modulating the immune response to CSP by inducing the production of specific IgG2a antibodies (the second most abundant isotype) and consequently balanced the Th2:Th1 ratio to ~2-3. Immunization with CSP in combination with adjuvants A or B also induced the production of important levels of specific IgG2b and IgG3. Despite, no statistically significant differences in the titers of specific immunoglobulins between animals immunized with CSP in combination with adjuvants A or B were found, higher titers of specific IgG1 and IgG2a were detected at 4 weeks in the groups immunized with CSP plus adjuvant A. The protective role of CSP-specific antibodies in the pre-erythrocytic phase has been linked to sporozoites immobilization and consequent inhibition of parasites progression from the skin to the liver (Yoshida et al., 1980; Stewart et al., 1986; Schofield et al., 1987; Weiss et al., 1988; Romero et al., 1989; Vanderberg *et al.*, 2004). Therefore it's logical to think that a vaccine against sporozoites should elicit a strong and long-lasting humoral response. This was indeed

observed in animals immunized with CSP in combination with adjuvants A or B, in which the high titers of specific imunoglobulins were maintained at least during 20 weeks. Whether protection is dependent on the immunoglobulin subclass remains to be elucidated, since antibodies can be used in vaccine strategies against malaria (Pleass et al., 2005). In clinically immune individuals, the naturally acquired antibodies are mainly from the IgG1 and IgG3 subclasses; this association is, however, quite inconsistent when considering antibody responses to single malaria antigens. Additionally, there is little correlation between the levels of these antibodies and resistance to infection or disease (Ndungu et al., 2002; Oluwasogo et al., 2012; Pleass et al., 2005).

Mice immunized with CSP in combination with the adjuvant B showed a high frequency of CD3+ splenocytes producing IL-6. These results, despite preliminary and awaiting further confirmation and characterization, are very exciting since these cells may contribute to eliminate liver stage parasites. Work in rodents and cultured hepatocytes showed that several host cytokines such, IFN-γ, TNF- α, IL-1 and IL-6 strongly inhibit parasite development inside hepatocytes (Schofield *et al.*, 1987; Maheshwari, 1990; Pied *et al.*, 1992; Vreden *et al.*, 1992). Both TNF-α-induced (Vreden *et al.*, 1992) and IL1-induced (Nussler *et al.*, 1991) inhibition of liver-stage development are mediated by IL-6. In contrast, recombinant IL-6 is sufficient to reduce liver-stage development inside cultured hepatocytes (Pied *et al.*, 1992) exerting its action throughout liver-stage maturation (Nussler *et al.*, 1991). One of the mechanisms by which IL-6 might control parasite liver infection is by regulating iron homeostasis through hepcidin (Nemeth *et al.*, 2004), which limits *Plasmodium* development inside hepatocytes (Portugal *et al.*, 2011). Additional work is required to characterize whether other cell types in addition to T cells also produce IL-6 and not only in the spleen but also in the liver.

In conclusion, we would like to propose that high titers of specific antibodies generated in the animals immunized with CSP in combination with adjuvants A or B may equally prevent sporozoites to reach the liver. However, the sterile protection observed in the animals immunized with CSP in combination with adjuvant B might be related with the production of IL-6 that will prevent the development in the liver of sporozoites that eventually were not blocked by the antibodies.

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