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SINGAPORE

**STUDIES ON THE IMMUNOLOGICAL MECHANISMS
LEADING TO PROTECTION OR PATHOLOGY DURING
MALARIA INFECTION**

DANIEL RIBEIRO DA SILVA MCGUIRE

SCHOOL OF BIOLOGICAL SCIENCES

2019

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DANIEL RIBEIRO DA SILVA MCGUIRE

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement
for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Daniel Ribeiro da Silva McGuire

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Laurent Claude Rénia

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This thesis **does not** contain any materials from papers published in peer-reviewed journals or from papers accepted at conferences in which I am listed as an author.

3rd August 2019

A handwritten signature in dark ink, reading "Daniel McGuire". The signature is written in a cursive style, with the first name "Daniel" and the last name "McGuire" clearly legible. The "M" in "McGuire" is particularly prominent.

Daniel Ribeiro da Silva McGuire

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Summary

Malaria is a deadly disease caused by the *Plasmodium* parasite that affects over two hundred million people and results in more than four hundred thousand deaths a year, of which sixty percent are children under the age of five. This vulnerable population is also the one most likely to exhibit severe cases of kidney failure, acute respiratory distress and cerebral malaria, signs of the more extreme forms of this disease. The fight against malaria has proven a challenging one. Despite decades of research and a multitude of immunization strategies we still have not been able to produce a safe and efficacious vaccine. Despite various promising vaccine candidates based on immunodominant malaria antigens, subunit vaccination trials have been unable to consistently induce strong, long-lasting immunity. Nevertheless, trials with the most advanced vaccine to date, RTS,S, have demonstrated that small, incremental iterations such as the use of ideal adjuvants, could significantly bolster the effectiveness of promising candidates.

Thermogel polymers are hydrogels that reversibly transition from liquid to solid phase as the temperature increases. This defining trait, combined with the fact that their modular composition allows for fine tuning of their mechanical, thermoresponsive and biocompatible properties, makes them particularly attractive drug delivery systems in clinical use. Two novel biocompatible and biodegradable thermogels, capable of polymerizing at 37°C, were developed by the Institute of Materials Research and Engineering (IMRE, A*STAR). These two gels, Gel 3 and Gel 4, paired with the well characterized MSP1-19 protein, were tested for their ability to act as adjuvants and, through subunit vaccination, induce protective immunity to lethal *Plasmodium yoelii* YM (PyYM) challenge in BALB/cJ mice. While we demonstrated that both gels were capable of inducing a humoral IgG1 and IgG2a response and protect the mice from death, comparison of parasitemia curves and antibody titers revealed that this

protection was lower than that induced when using Freund's adjuvant. A total of three vaccination trials were carried out whereupon mice vaccinated with the gels consistently exhibited high peripheral parasitemia (above 40%) but variable survivability between trials. While a broad association between antibody titers and survival was observed in our model, we did not find correlation between survival and antibody-mediated mechanisms, such as inhibition of invasion or opsonic phagocytosis, normally associated with protection against MSP1-19. Nevertheless, the thermogels here explored show potential as adjuvants and should be further studied in subunit vaccination.

Recent epidemiological studies have shown that vaccination with BCG or against measles can result in innate cells displaying long-term memory-like changes in their functional program, leading to enhanced or diminished response to secondary stimuli. This memory-like mechanism, termed trained immunity, has also been observed after infection with parasitic and viral pathogens as well as in vaccination studies using live attenuated *Candida* strains.

The attenuated *C. albicans* strain R24 has been previously shown to induce protective innate immune responses against heterologous bacterial and fungal challenges. Using R24 as a live attenuated vaccine we evaluated the dynamics of this infection in BALB/cJ and C57BL/6J mouse strains before assessing its ability to protect against lethal malaria challenge. We discovered that viable R24 cells were not only detectable in brain cultures of both mouse strains until at least 21 days post vaccination but that mice exhibited abnormal neurological behaviors consistent with inflammation of the central nervous system. *Post hoc* histological analysis of C57BL/6J brains at day 21 confirmed that this strain was able to cross the blood brain barrier and induce localized inflammation even though it was genetically incapable of undergoing morphogenic switch to hyphal form.

While vaccination with R24 appeared ineffective against PyYM challenge, it protected C57BL/6J from experimental cerebral malaria (ECM) induced by *P. berhei* ANKA, although mice ended up succumbing to hyperparasitemia later on. Upon further study we were able to determine that protection against ECM was due to reduced cross-presentation by endothelial cells in the brain while the number of sequestered iRBC and parasite-specific CD8⁺T cells remained the same. Once we established that this reduced cross presentation was not due to differential levels of systemic or localized IFN- γ and IL-10 cytokines, we demonstrated that endothelial cell expression of adhesins ICAM-1 and VCAM-1 as well as MHC-I molecules remained unchanged.

In conclusion, we explored two novel mechanisms of inducing immunological responses against severe malaria: one directed towards the adaptive immune system, focused on the humoral response, and another geared towards the innate system.

Taken together, these findings demonstrate that if we are to develop effective vaccines against malaria, we must take into account not only both arms of the immune response but also potential effects that previous infections or vaccinations exert on the establishment of protection or susceptibility to malaria.

Chapter 1 - Introduction

1.1 General Introduction

1.1.1 Epidemiology of malaria

Malaria is a deadly disease caused by the *Plasmodium* parasite that to this day still results in more than four hundred thousand deaths a year, of which sixty percent are children under the age of five years. Although the number of reported malaria cases worldwide has been decreasing over the years, a staggering 219 million cases occurred in 2017 and this number has remained stagnant in the last few years (WHO 2018). The global burden of this disease occurs mainly in sub-Saharan Africa and India with nearly half of all malaria cases reported worldwide restricted to just five countries: Nigeria, Democratic Republic of Congo, Mozambique, India and Uganda.

In 2017 alone, US\$ 1.3 billion were invested in malaria control and elimination efforts, of which US\$ 588 million went to investments in malaria research and development. However, despite decades of research, there is no effective licensed vaccine to date, and the mechanisms underlying successful protective immunity remain ill-defined and poorly understood.

Clinical symptoms of the disease can be varied but usually include cyclical fever, chills, muscular ache, weakness, vomiting, cough, diarrhea and abdominal pain. In more severe cases organ failure can manifest in the kidneys, as acute renal failure; in the lungs, leading to pulmonary edema; in the brain, resulting in cerebral malaria. Convulsions, circulatory collapse, coma and death typically follow severe organ failure. Young children, pregnant women, the elderly and immunocompromised people are at most risk of developing severe symptoms (Kain & Keystone 1998, White 2018).

Human malaria is mainly caused by four protozoan species: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*. On occasion, zoonotic species such as *P. knowlesi*, *P. brasilianum*, *P. cynomolgi* and *P. simium* can cross over and infect human hosts (Lalremruata *et al.* 2015, Brasil *et al.* 2017, Imwong *et al.* 2019). Of all the malaria parasite species capable of infecting humans, *P. falciparum* is the most prevalent worldwide and the deadliest. The other *Plasmodium* species are rarely life-threatening.

1.1.2 Parasite life cycle

The complex life cycle of the *Plasmodium* parasite exhibits three distinct stages and involves two different hosts. The first part of the life cycle occurs within the *Anopheles* female mosquito vector and is responsible for the production of *Plasmodium* sporozoites, the invasive forms of the parasite. Concentrating in the salivary glands, sporozoites are transmitted to the mammalian host when the mosquito takes a blood meal (Ménard 2000). Once injected into the skin, the sporozoites enter the blood stream and migrate to the liver where they invade hepatocytes, undergo asexual multiplication and develop into hepatic schizonts. This first stage, commonly referred to as liver or pre-erythrocytic stage, is asymptomatic, can last anywhere from one to four weeks, depending on *Plasmodium* species, and encompasses all the events between the injection of the sporozoites into the skin and the release of the merozoites into the blood stream. For a comprehensive description of pre-erythrocytic stage please refer to the review by Vaughan, Aly and Kappe (Vaughan *et al.* 2008). As the parasite reaches maturity, hepatocytes release merozoites, vesicles containing thousands of merozoites (Sturm *et al.* 2006, Soulard *et al.* 2015), that migrate to the lungs. There, the merozoites are released into the bloodstream and within seconds invade red blood cells (RBCs) (Coquelin *et al.* 1999, Baer *et al.* 2007). This process is extremely efficient and extracellular exposure is reduced to a bare minimum as the antigens on the surface of the

merozoite are vulnerable to immune attack. Invasion of the RBC initiates the erythrocytic or blood-stage part of the malaria life cycle. Once inside the RBCs, merozoites develop through ring, trophozoite and schizont stages in a new asexual reproduction stage that results in production of 16 to 32 daughter merozoites. Release of these new merozoites into the blood stream leads to a new cycle of RBC invasion, asexual replication, RBC rupture and merozoite release (Cowman & Crabb 2006). The duration of the intra-erythrocytic cycle depends on the species of *Plasmodium* but can last between 24 hours, for *P. knowlesi*, to 72 hours for *P. malariae*. For *P. falciparum* the cycle lasts approximately 48 hours. As the parasite multiplies, an increasing number of RBCs are invaded and lysed releasing various parasite byproducts. The combination of egressing merozoites, parasite cycle byproducts and ruptured RBCs leads to an inflammatory response resulting in cyclic fever, sweating, chills and lethargy which, in the absence of treatment or immunity can build up to hemolytic anemia, multi-organ failure and death (Crompton *et al.* 2014). The blood-stage is then where clinical symptoms of malaria manifest.

Within the erythrocyte the parasite induces drastic remodeling of the RBC membrane. This process entails the surface expression of various parasite-encoded proteins, or variant surface antigens (VSAs), that serve numerous functions. Encoded by highly polymorphic subtelomeric gene families, VSAs play critical roles in virulence and immune evasion (Freitas-Junior *et al.* 2000). Through a process termed antigenic variation, the parasite is able to switch which allele expresses the surface antigen allowing it to evade the host immune response by rapidly cycling out targeted antigens (Scherf *et al.* 2008). VSAs can also act as receptors for various cell ligands thus mediating adherence of infected RBCs (iRBCs) to non-infected RBCs, resulting in rosetting, or to endothelial cells, a process known as cytoadherence. Sequestration of the iRBCs to microvascular endothelial cells allows the parasite to bypass splenic clearance by hiding in other organs (Frech & Chen 2013). Inevitably though, this leads to localized

inflammation resulting in more severe symptomology and higher likelihood of host death. A prime example of severe inflammation occurs when parasites sequester in the microvasculature of the brain resulting in cerebral malaria (Grau & Craig 2012). At a certain stage of the infection, some of the erythrocytic merozoites differentiate into male and female gametocytes which are then taken up by a mosquito during a blood meal. Once inside the vector's midgut, they differentiate into male and female gametes and fuse to form ookinetes. These sexual stage ookinetes undergo sporogonic development and differentiate into sporozoites which then proceed to the mosquito's salivary glands where they can infect a new host (Ménard *et al.* 2013). Figure 1-1 below sums up the malaria life cycle as well as some key immune responses observed during the liver and blood stages.

Figure 1-1 – *Plasmodium* life cycle. The *Plasmodium* life cycle involves two hosts. When the *Anopheles* female mosquito takes a blood meal from the host (a) it injects into the skin sporozoites which then quickly proceed through the blood to the liver (b) where they invade hepatocytes (c). Within the hepatocytes, sporozoites differentiate into hepatic schizonts which then release thousands of merozoites into the blood stream (d). Once there, the liver stage ends, and the blood stage of the malaria infection begins. Although the liver stage is asymptomatic and there is no natural immunity to it, vaccination using whole-parasite vaccines has been shown to induce sterilizing immunity based on CD4⁺ and CD8⁺ T cell response together with NK, NKT and $\gamma\delta$ T cells. Repetitive cycles of erythrocyte invasion, differentiation to schizonts and RBC rupture with new merozoite release (e) lead to the clinical symptoms typically associated with malaria. During its differentiation the parasite remodels the erythrocytic membrane inducing extracellular expression of variant surface antigens that can act as receptors for various cell ligands mediating adherence to endothelial cells (f), thus avoiding splenic clearance or to non-infected RBCs, resulting in rosetting (g). At a certain stage of the infection, some merozoites differentiate into gametocytes which are then taken up by a new mosquito as it takes a blood meal. Within the mosquito, the parasite undergoes sexual reproduction producing new sporozoites and thus renewing the cycle. Immune responses to the blood stage are mainly mediated by antibodies in conjunction with CD4⁺ T cells although innate immune effectors like NK, NKT, $\gamma\delta$ T cells and macrophages play a role. Adapted, with minor changes, from Crompton *et al.* 2014.

1.1.3 Complications derived from severe malaria infections

While most malarial infections are uncomplicated and tend to be resolved without serious pathological side-effects, in around 1% of cases the infection can become severe and life-threatening. The majority of severe forms of malaria are caused by *P. falciparum*. Major complications include cerebral malaria, pulmonary edema and acute respiratory distress syndrome, acute renal failure, severe anemia, generalized convulsions and circulatory collapse. Any of these can develop quickly and progress to death within days or even hours and it is quite frequent to see various complications developing concomitantly. Of all the complications of severe malaria, the deadliest and most frequent is cerebral malaria responsible for 20% of adult and 15% of childhood deaths (Luzolo & Ngoyi 2019). This neurological dysfunction can occur suddenly by way of a generalized convulsion or appear gradually with initial drowsiness and confusion which gradually worsens until patients enter coma. The clearest diagnosis is unrousable coma not attributable to any other cause accompanied by malaria infected peripheral blood erythrocytes. The deeper the coma the worse the prognosis (Trampuz *et al.* 2003).

1.1.4 Triggers and cascade events in cerebral malaria.

The mechanisms responsible for the onset and progression of cerebral malaria in humans (HCM) are not completely understood and data gathered from murine experimental cerebral malaria (ECM) doesn't always support epidemiological findings. Nevertheless, since human brain experimentation is restricted for ethical reasons, most of what is understood about the key players in this disease come from either post-mortem analysis or murine trials using the *P. berghei* ANKA (PbA) malaria parasite (Rest 1982, reviewed in Sierro & Grau 2019). Nevertheless, some common factors can be identified between these models. Early studies demonstrated, in mice (Baptista *et al.* 2010) and humans (Rowe *et al.* 2009), that infected

RBCs (iRBCs) circulating through the body either sequester or adhere to endothelial cells (ECs) of the brain microvasculature. In *P. falciparum* infection this cytoadherence mechanism is mostly restricted to late stage parasites and is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of iRBC (Grau & Craig 2012) binding to receptors expressed by the ECs (Pasternak & Dzikowski 2009). While there are differences between HCM in children and adults (Hawkes *et al.* 2013), sequestration of iRBC seems to be one of the first triggers. Parasite presence in the blood also leads to increased systemic levels of pro-inflammatory cytokines IFN- γ , TNF- α (TNF), TNF- β (lymphotoxin), IL-1 β (Grau *et al.* 1987). These pro-inflammatory cytokines, together with adhesion of the iRBC to the endothelium, lead to activation of the ECs (Jambou *et al.* 2010, El-Assaad *et al.* 2013) and increased expression levels of adhesion molecules such as CD36 (Franke-Fayard *et al.* 2005); intercellular adhesion molecule 1 (ICAM-1) (Avril *et al.* 2016); or vascular adhesion molecule 1 (VCAM-1) (El-Assaad *et al.* 2013) just to name a few. Increased expression of these adhesion molecules then contributes to more iRBC sequestering in the brain microvasculature, as well as platelets (Grau *et al.* 2003), further activating the ECs in a positive feedback loop. This repetitive amplification inevitably triggers the recruitment of leukocytes to the brain microvasculature.

Contrary to HCM, where the relative importance and roles that each leukocyte subset plays are not clearly defined, the ECM model exhibits a somewhat straightforward sequence of events that are easier to piece together.

In ECM, the accumulation of leukocytes, especially CD8⁺ T cells, is a hallmark trigger in the development of the disease that correlates with protection to or severity of the neurological signs (Belnoue *et al.* 2002, 2008, Engwerda *et al.* 2005, Poh *et al.* 2014). Mice without CD8⁺ T cells, RAG2^{-/-} or through depletion (Belnoue *et al.* 2002), don't display ECM while adoptive transfer of CD8⁺ T cells collected from mice with ECM restores the pathology (Nitchau *et al.*

2003). Phenotypic characterization of the recruited CD8⁺ T cells revealed that they express high levels of perforin and Granzyme B (Haque *et al.* 2011, Howland *et al.* 2013, Poh *et al.* 2014) and that expression of those cytotoxic proteins is essential for the onset of ECM (Nitcheu *et al.* 2003, Potter *et al.* 2006, Haque *et al.* 2011). Nevertheless, while the presence of parasite-specific CD8⁺ T cells in the brain microvasculature is vital, that factor is not enough to induce ECM. Previous work in our laboratory (Howland *et al.* 2013, 2015a, 2015b) demonstrated that, in order for cerebral neuropathy to ensue in mice, three key events had to occur: parasite iRBC had to adhere to endothelial cells in the microvasculature of the brain; functional parasite-specific previously primed CD8⁺ T cells had to be recruited to the brain; and the endothelial cells had to cross-present parasite antigens to the CD8⁺ T cells in a process that was IFN- γ dependent. It was postulated that this cross-presentation occurred via MHC-I presentation, possibly after parasite phagocytosis by the endothelial cells (Jambou *et al.* 2010, Howland *et al.* 2013, Wheway *et al.* 2013, 2014), although there is a possibility this phagocytosis is carried out by monocytes sequestered in the microvasculature (Sierro & Grau 2019). Upon cross-presentation, CD8⁺ T cells secreted granzyme and perforin, disrupting the blood brain barrier (BBB) integrity and resulting in lethal outcome (Nishanth & Schlüter 2019).

Other immune cellular subsets have also been found to play a role in ECM pathogenesis. Depletion of CD4⁺T cells has been associated with protection of malaria but only when this depletion occurred before or early in the infection (Claser *et al.* 2011, Hochman *et al.* 2015, Swanson *et al.* 2016) but not just before the onset of ECM (Belnoue *et al.* 2002). These results seem to point out that CD4⁺ T cells play more of a supportive role in ECM pathology, through the production of IFN- γ . The importance of CD4⁺ T cells as early sources of IFN- γ and its role in ECM was demonstrated when CD4⁺T cells from wild-type mice were adoptively transferred into IFN- γ deficient mice thus restoring ECM pathology (Villegas-Mendez *et al.* 2012).

The role of the innate immune branch in ECM is less defined but nevertheless some correlations have been established. Monocytes have been shown to accumulate in the microvasculature of the brain several days before the onset of any symptoms (Pai *et al.* 2014) and been associated with EC activation and increase in disease severity (Niewold *et al.* 2018). Depletion of these monocytes/macrophages has also been associated with amelioration of ECM symptoms in some studies (Schumak *et al.* 2015) although others (Claser *et al.* 2011) showed no effect. The role of NK cells is still unclear although, there is evidence they are required for induction of ECM through production of immunomodulatory cytokines (Burrack *et al.* 2018) or by influencing recruitment of T cells (Hansen *et al.* 2007).

1.1.5 Building up immunity against malaria

In regions of high endemicity, where people are exposed year-round, infection by malaria parasites does not necessarily lead to disease. Once past the period of high susceptibility in early childhood (WHO 2018), a buildup of immune protection can be directly correlated to the frequency of exposure. While never quite reaching sterile immunity, adults exhibit asymptomatic infections with low to very low levels of parasitemia. This naturally acquired protection is gradually obtained and maintained through repeated infections over the span of several years (White *et al.* 2014).

Variant-specific immunity

The evolutionary battle between the malaria parasite and its host's immune defenses has led to the parasite developing a multitude of self-preservation mechanisms. Within the complex multistage life cycle already described, we see that the majority of the host's responses are mainly geared towards the blood stage. There, selective pressures have induced the parasite to build up an extensive array of highly polymorphic genes encoding for immunodominant antigens expressed on the surface of iRBCs. As successive generations of haploid parasites

quickly multiply within the host, each population is subjected to a high frequency of mutations. Selective pressure from the host eliminates disadvantageous mutations while those conferring an advantage are quickly spread through the population. In *P. falciparum* each parasite carries between fifty and sixty *var* genes expressing a selection of variant surface antigens (VSAs) collectively termed erythrocyte membrane protein 1 (PfEMP1). Expressed on the extracellular domain of the iRBCs, these proteins can be targeted by the host's immune response. Each individual parasite only expresses one *var* gene at a time thus expressing only one variant on the surface of the iRBC, a transcriptionally regulated mechanism known as mutually exclusive expression. At each replication event, a small percentage of replicating merozoites switch to express an alternative *var* gene (Klein *et al.* 2014) thus presenting an ever-changing array of slightly different proteins, a process known as antigenic variation (Roberts *et al.* 1992). Evidence for the success of this antigenic variation as an immune evasion mechanism is clearly demonstrated by the limited efficacy of naturally acquired immunity (Recker *et al.* 2004). Children and adults in malaria endemic regions present an ever-growing array of variant-specific antibodies targeting PfEMP1 that must be constantly updated through repeated infections in order to maintain protection (Marsh & Howard 1986, Bull *et al.* 1998).

Besides PfEMP1, *P. falciparum* expresses a myriad of other VSAs during its life cycle organized into three other gene families. The *rif/stevor* multigene family, the largest, composed of over 235 genes; the *surfin* family, containing 10 genes; and the *Pfmc-2TM* family, containing 12 genes (Frech & Chen 2013). The RIFIN (*rif* genes), STEVOR (*stevor* genes) and PfEMP1 (*var* genes) proteins have been the most studied. These ligands, beyond playing a role in immune system evasion through antigenic variation, have been shown to influence sequestration and cytoadherence of late-stage iRBC (schizonts and trophozoites) in vascular endothelium (Lee *et al.* 2019). Sequestration allows the parasite to not only avoid splenic clearance (Gomes *et*

al. 2016) but has been associated with severe malaria (Mackintosh *et al.* 2004). These VSAs also exploit host components such as platelets and localized inflammation, which can lead to agglutination of iRBCs with uninfected RBCs, resulting in “rosettes” (Yam *et al.* 2017, Lee *et al.* 2019). Rosetting has been hypothesized to protect the parasites from the immune response by shielding the exposed VSAs on the iRBC behind a mask of seemingly innocuous RBCs. Beyond masking the iRBCs from host immunity, some RIFINs have been shown to inhibit activation of B cells and natural killer (NK cells) (Saito *et al.* 2017), while STEVOR proteins have been shown to protect released merozoites from host immunity (Niang *et al.* 2014).

Other *Plasmodium* parasites also express VSAs. *P. vivax* contains the large *vir* gene family (~300 genes) which is homologous to the *kir* gene family in *P. knowlesi* (~65 genes), the *yir* in *P. yoelii* (~800 genes), the *bir* in *P. berghei* (~100 genes) and *cir* in *P. chabaudi* (~200 genes). Together, these five families compose the largest *Plasmodium* gene family, the *pir* superfamily (Frech & Chen 2013). Contrary to the well-studied *P. falciparum* VSAs RIFIN STEVOR and PfEMP1, while there are numerous roles proposed for the PIR proteins, such as antigenic variation; immune evasion; and adhesion, direct evidence is still scarce (Yam & Preiser 2017).

Thus, these mechanisms not only delay the buildup of an effective immune response during the current infection but also guarantee that previously infected hosts don't possess adequate protection from reinfection. A consequence is then that naturally acquired immunity is highly stage and strain specific, short lived and slow to build (Doolan *et al.* 2009). Interestingly though, while protection from clinical symptoms of malaria appears to be a life-long task, cohort data from Sub-Saharan Africa has evidenced a reduction of severe malaria cases after just a couple of exposures (Gupta *et al.* 1999). It appears then that, immune response to the parasite presents a clear dichotomy in pathology whereupon immunity to

infection and mild disease is mediated by slow increments in variant-specific responses while resistance to severe malaria is rapidly acquired, long-lasting and strain-transcending.

On the host side, acquired protective immunity to the blood stage has been shown to involve a combination of antibody and cell-mediated responses with the antibodies carrying the lead role.

While the humoral branch is the main mediator of immunity in plasmodium's blood stage, innate and cell-mediated responses have been shown to play a supporting role (Good *et al.* 2004, 2005). Clinical studies, together with data gathered in mouse studies, point to innate immunity contributing to malaria infection control by attempting to limit parasite density (reviewed in Stevenson & Riley 2004). Observations in malaria-endemic regions have provided empirical evidence supporting the hypothesis that immune innate mechanisms are triggered when parasite densities reach a certain threshold (Bruce & Day 2003). During infection, iRBCs trigger innate immune cells such as monocytes, macrophages, dendritic cells (DC), natural killer (NK) cells and neutrophils. These cells then intervene directly by delaying the multiplication of the parasite through phagocytosis or indirectly through modulation of the adaptive immune response via cytokine production. Macrophages are essential for clearance of iRBC through phagocytosis (Couper *et al.* 2007) which can be induced directly, by binding of iRBC to CD36 (Patel *et al.* 2004, Ayi *et al.* 2005, Thylur *et al.* 2017), or via opsonization mediated by either complement (Su *et al.* 2002) or antibodies (Yoneto *et al.* 2001). NK cells have been shown to directly lyse iRBCs without need for prior sensitization or parasite-specific antibodies and are the earliest source of the pro-inflammatory cytokine IFN- γ (Artavanis-Tsakonas *et al.* 2003). NK cells are activated during early stages of infection through contact with monocytes and dendritic cells and at late stages by IL-2, IL-12 and IL-18 cytokines released by T cells (Newman *et al.* 2006). In early stages of infection they can contribute to controlling parasitemia by elimination of iRBCs through natural cytotoxicity and

antibody-dependent cellular cytotoxicity (ADCC) and regulate the adaptive immune response mainly through secretion of IFN- γ (Kitaguchi *et al.* 1996, Mohan *et al.* 1997, Burrack *et al.* 2019).

Dendritic cells are the gold standard antigen presenting cells (APCs). They are the primary activators of the adaptive immune response in the early stages of infection by capturing antigens at the periphery and delivering them to the secondary lymphoid organs where they are presented to the B and T cells (Yap *et al.* 2019). Neutrophils present an interesting conundrum. While high levels of circulating neutrophils have been correlated with inflammation and severe malaria (Tangteerawatana *et al.* 2014, Mahanta *et al.* 2015), adoptive transfer experiments between *P. berghei* resistant adult rats to young, susceptible ones was shown to provide partial protection against infection (Pierrot *et al.* 2007). Most studies seem to find though that activated neutrophils correlate with higher cytoadhesion levels of iRBC to endothelial cells in the brain (Feintuch *et al.* 2016), which could induce cerebral malaria, although their depletion during *P. berghei* infection in mice does not prevent ECM (Schumak *et al.* 2015).

It is well established that B cells and antibodies play a crucial role in immunity to blood-stage malaria in humans and mice. Passive transfer of immunoglobulins from protected individuals to naïve individuals has been documented to alleviate disease symptoms and reduce parasitemia levels in humans (Cohen *et al.* 1961, Edozien *et al.* 1962, Spencer Valero *et al.* 1998, Narum *et al.* 2000) while *in vitro* invasion inhibition assays demonstrated serum from protected mice reduced invasion of erythrocytes (de Koning-Ward *et al.* 2003, Bergmann-Leitner *et al.* 2006). Furthermore, B cell-deficient mice have been shown to exhibit no protection against blood-stage infection (von der Weid *et al.* 1996, Langhorne *et al.* 1998).

Despite the brief exposure of the merozoites to the extracellular domain, a high number of proteins expressed on their surface or apical organelles, like the rhoptries and micronemes, can be targeted by the host's immune responses. In *Plasmodium*-endemic areas, strong associations between humoral responses targeting merozoite proteins and protection from disease have been demonstrated (Osier *et al.* 2008). Antibodies targeting these antigens can interfere with the merozoite's ability to invade new RBC thus stopping the asexual replication cycle before it has even started (Dutta *et al.* 2005).

Contrary to sporozoites and iRBCs, in which the antibody response is mainly directed towards one dominant antigen (respectively Circumsporozoite protein (CSP) and PfEMP1), merozoites present dozens of immunogenic targets (Chan *et al.* 2012, Dups *et al.* 2014). Such a vast array of targetable antigens thus complicates the identification of key mediators of protection. Systematic reviews of the elicited humoral response have suggested that, during the course of an infection, the majority of merozoite surface proteins are targeted by antibodies (Richards *et al.* 2013, Ondigo *et al.* 2014). While not every merozoite antigen induces a protective response, a large number of them have shown enough correlation with protection in longitudinal studies that their potential as vaccine candidates has been considered (Fowkes *et al.* 2010, Richards & Beeson 2009, reviewed in Beeson *et al.* 2016). Interestingly, research by Chan and colleagues (Chan *et al.* 2017) found that when two longitudinal cohorts of children in Papua New Guinea, one with children between 1 and 4 years of age and one between 5 and 14, antibodies against merozoites were not associated with protection in the younger children. In that group, protection was mediated by antibodies targeting the iRBC surface. Older children showed association between protection and high antibody titers against both iRBC and merozoites. These results seem to indicate that the buildup of an effective antibody repertoire is not only influenced by exposure but age-dependent as well. These findings, and others, while as yet unsatisfactory in translating into effective vaccines,

have shown that identification of antibody targets and the mechanisms behind their acquisition and maintenance are valuable and essential tools not only as serological markers of exposure but as guidelines for future research (Elliott *et al.* 2014).

Antibodies against merozoites mediate protection through four main mechanisms. By blocking receptor-ligand interactions they can inhibit invasion of the iRBC by the merozoites; they can interact with Fc γ receptors of innate effector cells to induce phagocytosis or antibody dependent cellular cytotoxicity; interact with complement through recruitment of C1q and activation of the classical pathway; and prevent merozoite release by causing agglutination of merozoites in rupturing schizonts (Beeson *et al.* 2016). The functional properties of antibodies are determined by factors such as antibody subclass, epitope specificity and affinity which differ between antigens (Irani *et al.* 2015) and can be directed through vaccination by selective use of different adjuvants (Moyer *et al.* 2016).

Merozoite surface protein 1 (MSP1), expressed on the surface of merozoites, is the most abundant of the merozoite antigens and has been implicated in mediating initial contact with the RBC prior to invasion (Boyle *et al.* 2010). Just before schizont rupture, this 180 kDa polypeptide is processed by proteolytic cleavage into four fragments of 83 kDa, 28 kDa, 38 kDa and 42 kDa (Gilson *et al.* 2006). At the moment of merozoite release into the bloodstream only the 42 kDa fragment, GPI-anchored to the merozoite membrane, remains. At the moment of attachment to the RBC, a second proteolytic cleavage breaks down this peptide into a 33kDa fragment, which is released and a 19kDa fragment which will remain attached to the merozoite all through invasion (Blackman *et al.* 1990). This C-terminal fragment, known as MSP1-19, possesses two epidermal growth factor (EGF)-like domains that are highly conserved between *Plasmodium* species (Miller *et al.* 1993, Holder 2009) and has been shown to play an essential role in invasion. Attempts at disrupting MSP-1 function by genetic manipulation fail to yield viable results and monoclonal antibodies directed to MSP1-19 have

been shown to inhibit invasion of the RBC *in vitro* (Blackman *et al.* 1990), attesting to the vital importance this antigen plays in the parasite life cycle. The conformation of the EGF-like domains appears to be important in the establishment of protection. Studies with the murine parasite *Plasmodium yoelii* (Py) have demonstrated that immunization with MSP1, and specifically MSP1-19, elicits a strong protective immune response that is correlated with high antibody titers although the presence and correct conformation of the EGF-like domains is essential (Holder & Freeman 1981, Daly & Long 1993, Ling *et al.* 1994).

In humans, sera from malaria endemic regions exhibits high antibody titers targeting MSP-1 and, in certain cases, a correlation between protection and antibodies that target specific epitopes has been established (Egan *et al.* 1996, Fowkes *et al.* 2010b). Children not exhibiting IgG against MSP1-19 were shown to be ten times more susceptible to malaria infection (Branch *et al.* 1998) while infected but asymptomatic children were shown to have higher concentrations of antibodies targeting MSP1-42 than those exhibiting clinical signs (Riley *et al.* 1992). In West Africa, a longitudinal study demonstrated that the presence of maternally-derived antibodies against MSP1-19 protected infants from clinical malaria in the first year of life (Høgh *et al.* 1995). In 1999 Egan and colleagues showed that MSP1-19 antibodies from human sera were capable of inhibiting parasite growth *in vitro* (Egan *et al.* 1999).

Delving deeper into the nature of the protective humoral response in human blood revealed that while antibody responses targeting MSP1-19 are composed mainly by IgG1 and IgG3 subtypes, only IgG1 consistently correlated with protection (Shi *et al.* 1996, Cavanagh *et al.* 2001, Krettli *et al.* 2002, Stanisic *et al.* 2009). Yet, while many studies clearly demonstrated an association between high anti MSP1-19 titers and protection against clinical symptoms, not every study was able to show the same results (Dodoo *et al.* 1999, Riley *et al.* 2000, Ekala *et al.* 2002, Scopel *et al.* 2005). This discrepancy seems to indicate that high antibody titers against a specific, conserved target are not just a matter of ELISA titers. Specificity and

subtype of the immune response, as well as their functional efficiency, such as ability to inhibit parasite invasion of RBCs, seem to play a key role in protection. It is important then to keep this information in mind when defining correlates of protection. The design of effective vaccines against malaria relies not just on choosing the correct antigens but in pairing them with the correct adjuvants in order to modulate specific antibody subtypes that perform functional responses.

Induction of immunity to malaria has also been shown in experimental settings where studies have mostly focused on two major approaches: whole-parasite immunization and subunit vaccines (Draper *et al.* 2018, Goh *et al.* 2019).

Whole-parasite immunizations

Whole-parasite-immunization using radiation-attenuated sporozoites (RAS), genetically attenuated sporozoites (GAS) or unattenuated parasites under drug cover (infection-treatment-vaccination, ITV) have been shown to induce protection in humans (Carlton *et al.* 2002, Hoffman *et al.* 2002, Roestenberg *et al.* 2009, Seder *et al.* 2013) and in mice (Nussenzweig *et al.* 1967, Beaudoin *et al.* 1977, Belnoue *et al.* 2008, Keitany *et al.* 2014). RAS and GAS rely on inactivation or arrest of infectious sporozoites when they reach the blood-stage or just before. The main goal of these two vaccination approaches is to develop strong immunity to the pre-erythrocytic cycle, preventing infectious parasites from reaching the blood stage, and the clinical symptoms of malaria never manifest. While sterile immunity has been achieved, the attenuated parasite does not undergo its full life cycle and expresses only a subset of its genes. The induced immune response is therefore mostly restricted to pre-erythrocytic stages and strongest against homologous challenge (Belnoue *et al.* 2004, Nganou-Makamdop & Sauerwein 2013). ITV, on the other hand, employs immunization with wild type parasites that are allowed to undergo partial or full development within the blood

stage. Wild-type sporozoites or infected red blood cells are administered concurrently with anti-malarial drugs like chloroquine (Bijker *et al.* 2013), mefloquine (Bijker *et al.* 2014) or artemisinin derivatives (Peng *et al.* 2014), that target intra-erythrocytic blood-stage forms controlling parasitemia and preventing pathology. By allowing a full expression of the parasitic genome, the immune system is exposed to a much broader array of potential antigens resulting in a much stronger immune response, while also requiring a smaller number of injected parasites (Rénia *et al.* 2006, Nganou-Makamdop & Sauerwein 2013). Furthermore, ITV has been repeatedly shown to induce sterile, cross-stage immunity to homologous challenge in mice (Belnoue *et al.* 2004, 2008, Doll *et al.* 2014, Peng *et al.* 2014). Recently, Michael Good and collaborators have adapted the concept of whole-parasite vaccinations using sporozoites to the blood-stage and evaluated the feasibility of using chemically attenuated blood-staged parasites. Immunizations in mice with this novel whole-parasite vaccine showed it capable of inducing cross-species, stage-specific immunity with long term immunological memory (Good *et al.* 2013). Interestingly, when vaccinating with attenuated *P. yoelii* in BALB/cJ mice they observed that protection was not only dependent on both CD4⁺ T cells and B cells but that CD8⁺ T cells contributed to increased pathology in the absence of CD4⁺ T cells (Raja *et al.* 2016). Recently, a pilot study using attenuated *P. falciparum* asexual blood-stages documented observed proliferation of antigen-specific T cells with production of TNF- α and IFN- γ and, two cytokines important in protection against blood-stage parasites. Interestingly, no antigen-specific antibody responses were detected in this study although there was only a very small cohort involved and one single priming dose (Stanisic *et al.* 2018). Still, results are promising (Burns 2018).

While whole-parasite immunizations have shown consistent induction of protective immunity, they are not without disadvantages. Dependency on viable stabilates has implications on parasite growth and isolation, storage, transportation and inoculation

methodologies that make the cost of large-scale production prohibitive and mass distribution unviable (Butler *et al.* 2012).

Subunit vaccination

Rather than injecting the whole parasite, subunit vaccines are composed of key antigens carefully selected to induce a specific, targeted immune response. Since there is no requirement for a live parasite, they tend to be easier to manufacture, easier to administer and are generally considered safer.

The development of malaria subunit vaccines has focused on a limited number of promising antigen candidates shown to be associated with protection in immunoepidemiologic studies (Figure 1-2) (Draper *et al.* 2018). While these antigens have proven to be highly immunogenic (Orlandi-Pradines *et al.* 2006, Kirkman & Deitsch 2012), many of them, such as merozoite surface protein 1 (involved in the invasion of RBCs), are encoded by multiple alleles. The high genetic diversity of *Plasmodium* species and especially allelic polymorphism, has proven a difficult hurdle in the translation of these vaccines from laboratory to real-world application (Genton *et al.* 2002, Ogutu *et al.* 2009, Thera *et al.* 2011, Smith & Sack 2012). To that fact, all subunit vaccines based on immunodominant antigens have failed to elicit adequate levels of protection in regions of endemicity over sustained periods of time.

Figure 1-2 - Pre-erythrocytic and blood-stage *Pf* subunit vaccine candidates currently in development.

Represented are the vaccine candidates together with their corresponding clinical phase (in brackets). In the pre-erythrocytic stage, these vaccines are either directed towards the sporozoites (as is the case of RTS,S) through induction of antibody responses or towards the infected hepatocytes through engagement of T cell responses. The blood stage vaccines are geared towards induction of strong antibody responses as these are the main mediators of immunity. They can either block invasion of the RBCs by targeting merozoites or prevent iRBC-mediated pathology usually associated with severe malaria. Figure adapted from (Draper *et al.* 2018).

Moreover, in over 50 years of research only one vaccine candidate RTS,S/AS01, a CSP-based vaccine directed towards the erythrocytic stage of the parasite (Cohen *et al.* 2010), has ever progressed to Phase III trials. While this is the most protective sub-unit vaccine developed to date and has gone through various iterations to increase efficacy it still only provides around

34% protection in adults (Polhemus *et al.* 2009) and 23-30% protection in infants 6 to 12 months (Partnership 2015) against clinical disease.

To say that attempts at producing effective subunit vaccines have been disappointing is to understate the problem. While these antigens may certainly have uses as diagnostic tools, they come up short as vaccine candidates. If successful subunit vaccines are to be developed the focus must be placed on finding and understanding the determinants of immunogenicity (Mahanty *et al.* 2015).

Regardless of the targeted life-cycle stage, human malaria subunit vaccines share a common need to induce durable and/or functional responses that are efficacious and long lasting. The problem lies not just in finding the correct antigen(s) to target but also in promoting a strong, long term, antibody and/or cell mediated immunity in the body.

In 1920, Gaston Ramon discovered that addition of inflammatory substances coupled with desired antigens induced increased immunogenicity at the site of injection. From then on, adjuvants have been added to vaccine formulations with great effect (Pasquale *et al.* 2015). Over the years, subunit vaccine research has delved into the search for new adjuvants and the exploration of the mechanisms behind why and how they work (Moyer *et al.* 2016). Since their discovery, adjuvants have been shown to increase the duration of induced immune memory after vaccination; reduce the required amount of antigen needed to induce a strong response by activating and recruiting APCs; induce production of immunomodulatory cytokines; induce localized inflammation and recruit various cellular subsets (Cox & Coulter 1997, Coffman *et al.* 2010, Reed *et al.* 2013).

Adjuvants have come a long way since the discovery of the potential of aluminum salts (Glenny *et al.* 1926) and Freund's adjuvant (Opie & Freund 1937), two of the most used adjuvants to date. Used for over 80 years, aluminum salts such as aluminum hydroxide,

aluminum phosphate, and aluminum potassium sulfate (commonly known as alum) are the most used adjuvants in human vaccinations in the world (Kool *et al.* 2012, Pasquale *et al.* 2015).

Complete Freund's adjuvant (CFA) is a water-in-mineral oil emulsion containing heat-killed dried mycobacteria (*Mycobacterium tuberculosis* normally). Its incomplete form, IFA, does not contain mycobacteria. Both CFA and IFA are commonly used in murine trials to evaluate the immunogenicity of antigens by serving as delivery systems that ensure a continuous release of the antigen from the oily deposit. They've been shown to increase antigen lifetime and to induce innate immune responses such as enhancing phagocytosis, leukocyte infiltration and cytokine production (Müssener *et al.* 1995). Although undeniably strong immune activators, the use of CFA and IFA in human vaccinations is questionable due to the high induction of hazardous side-effects such as granulomas, abscesses and ulcerative necrosis.

While few adjuvants are currently licensed for human vaccination, several new formulations are being evaluated in animal and clinical trials (detailed list in Apostólico *et al.* 2016). Adjuvants can be classified according to their physicochemical properties or their mode of action (Table 1-1). Mode of action mechanisms are usually one of two types: they either work as delivery systems, guaranteeing the antigen is delivered to appropriate cells of the immune system where it will induce optimal stimulation; or they work as immune potentiators, employing early innate immune responses to aid in the generation of robust, long-lasting immune responses (Pashine *et al.* 2005).

Table 1-1 Classification of adjuvants based on physiochemical properties and action mechanisms.

Mucosal adjuvants work as both delivery systems and immune potentiators, so they are on a separate category. Adapted from Apostólico et al. 2016.

Type	Adjuvant/formulation
Delivery systems	
Mineral salts	Aluminum salts [alum] Calcium phosphate
Lipid particles	Incomplete Freund's adjuvant MF59 Cochleates
Microparticles	Virus-like particles Virosomes PLA (polylactic acid), PLG (poly[lactide-coglycolide])
Immune potentiators	dsRNA: Poly(I:C), Poly-IC:LC Monophosphoryl lipid A (MPL), LPS Flagellin Imidazoquinolines: imiquimod (R837), resiquimod (848) CpG oligodeoxynucleotides (ODN) Muramyl dipeptide (MDP) Saponins (QS-21)
Mucosal adjuvants	Cholera toxin (CT) Heat-labile enterotoxin (LTK3 and LTR72) Chitosan

A prime example of the importance of adjuvants in the effectiveness of a vaccine stems from the first trials using the current best vaccine against malaria, RTS,S. Initial formulations failed to provide protection in numerous trials. Since then, various iterations and development of new proprietary adjuvant platform have led to significant increases in immunogenicity and efficacy (Garçon *et al.* 2007, Kester *et al.* 2009).

The depot effect is the oldest and most widely described mechanism of action of adjuvants and usually the safest for use in human vaccination. The theory behind this effect is that antigens trapped in the adjuvant are kept at the site of injection for an extended period of

time and slowly released over time ensuring a steady input of immunogenic targets to stimulate the immune system. While the depot effect has been shown to impact both antibody responses and cell-mediated immunity, the link between duration of this effect and impact on immune response is not well established. Characteristics such as residence time, antigen geography (location of administration) and amount of antigen that actually reaches the adaptive immune cell receptors (T and B cells) are parameters determined by the depot effect that are key to a successful immune response (Zinkernagel *et al.* 1997). Despite this, very little is known about the optimal characteristics of these parameters, what their overall impact in vaccination is or even how they relate to one another (Schijns & Lavelle 2011). Nevertheless, the general consensus is that longer lasting depots induce stronger immune responses (Schijns & Lavelle 2011), a notion that is supported by Freund's early experiments (Freund 1951). Furthermore, many adjuvants previously considered to act primarily as depots or formulations (such as alum or emulsions) have been shown to trigger innate responses which are central to their adjuvant activity (Coffman *et al.* 2010).

1.2 Testing thermogel polymers as potential vaccine adjuvants

1.2.1 Definition of thermogel polymers

Thermogel polymers (thermogels) are hydrogels that undergo a transition from a liquid to a gel state within a specific temperature range. Their thermo-sensitivity is a defining trait that allows them to maintain a liquid state at low and room temperatures but undergo a reversible sol-gel transition (gelation) at body temperature (37°C). This means that bioactive agents can be incorporated into the liquid phase at low temperatures and then injected into the body where the higher temperature will lead to a solid gel matrix that traps the drug or peptide within (Lim *et al.* 2018). This biodegradable flexible reservoir can then function as a sustained,

controlled and long-term release mechanism as it is gradually broken down by the body by enzymes or through hydrolysis. One important aspect for this process to be considered safe is that the gel degradation products be small, non-toxic and easily excreted from the body (Lim *et al.* 2018). This can be achieved by the introduction of biodegradable linkages in the polymer backbone.

The majority of thermogelling systems currently in use are triblock copolymers (Figure 1-3). These copolymers are composed of elements (blocks) with different hydrophobicity. This means that, while at low temperatures, strong hydrogen bonds between water molecules and each block make the polymer hydrophilic enough to keep it in a solvated state. As the temperature is raised, the hydrogen bonds between the water and the polymer blocks are weakened leading to the dehydration of the polymer. Micellar aggregation occurs as hydrophobic blocks cluster together, thus forming a cross-linked network that induces the state change (gel formation). Reducing the temperature will return the thermogel polymer back to its liquid form. By adjusting the composition and ratio of each block polymer we can fine-tune the gelation temperature as well as modulate long term delivery of proteins and drugs (Wu *et al.* 2016) (Figure 1-3). In other words, they allow for a tailoring of the depot effect, fine tuning the release of incorporated proteins *in situ*.

Figure 1-3 - Example of the structure of a triblock copolymer thermogel polymer. The balance between hydrophilic blocks (PEG) and hydrophobic blocks (PPG) determine at which temperature these

compounds switch from the liquid to the gel state. The third compound can contribute to hydrophobicity as well as influence the hydrolytic degradability of the polymer determining the degradation rate.

These compounds have been exhaustively applied in cancer therapy research as reservoirs of chemotherapy drugs or as scaffolds in tissue engineering with promising. Some have been approved for human use by the FDA and trials using thermogels as controlled release depots of insulin in diabetic patients have shown some success (Loh *et al.* 2019). In another example, Wu and colleagues (Wu *et al.* 2016) demonstrated that use of these block copolymer thermogels formed biodegradable hydrogels that released bovine serum albumin (BSA) protein for as long as 80 days.

1.2.2 Developing thermogel polymers as potential vaccine carriers

In a collaboration between with the Soft Materials (SOF Department) of the Institute of Materials Research and Engineering (IMRE, A*STAR) and Florent Ginhoux's Laboratory (SigN, A*STAR) five novel biodegradable and biocompatible thermogelling polymers were investigated for their potential as injectable antigen-carriers. *In vitro* evaluation of immunological responses showed that these gels were capable of stimulating migratory DCs, resulting in expansion of the population, and that these cells were able to migrate into the thermogels. *In vivo* studies were carried out by injecting thermogels containing ovalbumin (OVA), the adjuvant ODN1826 and recombinant human Flt3 ligand (rhFlt3-L) subcutaneously into the flanks of C57BL/6 mice. ODN 1826, is a murine TLR9 agonist known to induce strong immunostimulatory effects. Flt3-L stimulates the recruitment of peripheral blood DCs. Results showed that three of the five gels elicited strong CD8⁺ T cell response against OVA and two of the gels elicited a six-fold higher response than a control using incomplete Freund's adjuvant (IFA). Immunizing the mice with thermogels containing influenza virus antigen also showed significantly higher CD4⁺ and CD8⁺ influenza-specific T cell proliferation in the same three gels

than the IFA control group. Furthermore, mice immunized with the same three gels containing OVA showed high IgG1 and IgG2a anti-ova antibody titers from day 10 through day 62 post immunization, although the titers were not significantly different than the IFA control. Interestingly, gels were observed to have drained completely from the injection after five days (Ginhoux F., unpublished data). After analysis of all the data, the five initial thermogel polymers were reduced to two: “Gel 3” (4:2:2.5wt% PEG:PPG:PCL) and “Gel4” (4:2:2.5wt% PEG:PPG:PTHF). Both gels are composed of blocks of polyethylene glycol (PEG) and polypropylene glycol (PPG) at a 2:1 ratio but Gel 3 possesses a polycaprolactone backbone (a biodegradable polyester) and Gel 4 possesses a polytetrahydrofuran, backbone (a polycarbonate). Both these gels were shown to be biocompatible and biodegradable.

One major advantage of the thermogel polymers as adjuvants is that they allow for a tailoring of the depot effect of adjuvants in a way that was not previously possible. They allow for a stable release of protein in a controllable way that can be fine-tuned to necessities and their biocompatibility and biodegradability mean that they are safe to use in human vaccinations.

To further the study of the immunogenic potential of these two gels as vaccine adjuvants, a decision was made to test their ability to protect BALB/cJ mice against lethal malaria blood-stage challenge using the *P. yoelii* YM (PyYM) murine parasite. Previous studies carried out in our lab (Rénia *et al.* 1997, Claser *et al.* 2017) have shown that naïve BALB/c mice succumb to fulminating infection between six to ten days after intraperitoneal injection of PyYM. Maintaining the same subcutaneous vaccination method as initially used to assess and narrow down the gels, we compared their immunogenic potential and ability to protect against lethal challenge to the commonly used Freund’s adjuvant (Opie & Freund 1937, Freund *et al.* 1945). The results of these trials compose Chapter 3 of this thesis.

1.3 Gut-evolved *Candida albicans* and broad-spectrum protection through innate immunity

Most current vaccines confer protection primarily through humoral immunity (Plotkin 2010) and studies tend to focus on the end-product of vaccination protocols, namely antibody titers; immunoglobulin subtypes and T and B cells induced. While this strategy has proven massively successful, there are large groups of the population for which current vaccines fail to induce the expected levels of protective antibodies.

The primary host response to a foreign antigen is the innate immune system. Innate responses are faster, more generalized and determine the character and downstream induction of adaptive immunity (Fearon & Locksley 1996, Iwasaki & Medzhitov 2010). This is true both in the context of infection and in vaccination. Antigen presenting cells, such as DCs, are found throughout the body, including common vaccination sites in humans (subcutaneous and intramuscular) and are the primary sentinels for antigen uptake and processing and thus responsible for the triggering signal of an immune response (Schijns & Lavelle 2011).

Over the last couple of decades our understanding of the cells, molecules and receptors that govern the innate response and how it interacts with the subsequent adaptive immunity has grown significantly. This knowledge has helped better understand the working mechanisms behind current adjuvants and to guide in the development of new ones. Most immunostimulatory adjuvants are either ligands for pattern recognition receptors (PRR) present in innate immune cells, provide immunomodulatory cytokines or directly stimulate activation pathways in innate cells. Depot adjuvants, primarily considered passive reservoirs have been found to trigger innate responses that are key to their adjuvant effect (Coffman *et*

al. 2010). Activation of innate immunity appears then to be key in inducing an effective response against both infection and vaccination. Unfortunately, innate immune responses tend to wane rapidly and typically do not lead to memory which means that a subsequent encounter with a microbial pattern (or antigen) does not produce a faster or more intense response than the first one.

Trained immunity denotes the enhanced response some previously triggered innate immune cells manifest when subjected to a secondary stimulus. First coined by Netea and colleagues (Netea *et al.* 2011), this term refers to an observed behavior that is defined by the following criteria: it involves innate immune cells such as monocytes, macrophages or NK cells; the secondary response is not specific to a particular pathogen; it is mediated through epigenetic reprogramming; and the altered functional state is short-lived (weeks to months) (Netea *et al.* 2016).

1.3.1 Gut-evolved *C. albicans* and the selection of attenuated strain R24

In an attempt to understand the evolutionary mechanisms that govern the dynamic equilibrium between host and pathogen, Tso and colleagues (Tso *et al.* 2018) investigated whether serial passages of wild-type *Candida albicans* (strain SC5314) through the murine GI tract could lead to an evolutionary selection of less pathogenic, more symbiotic microbes. This method is similar to the one used to produce “live-attenuated” vaccines. In the process, the authors discovered that not only did isolates lose pathogenicity but, when used in vaccination trials, were able to induce trained immunity.

The vast majority of microorganisms colonizing mammalian species reside within the gastrointestinal (GI) tract where they outnumber host cells by a factor of ten to one. In homeostasis, the nature of this relationship is precariously balanced between the individual

microorganism's pathogenic potential, the host's immune system and the need to maintain competitive fitness within the environment. As a result, most microorganisms develop a symbiotic or commensal relationship and contribute to various metabolic and immune functions that play a crucial role in the health of the host (Relman 2008).

Through establishment of an experimental system based on long-term GI tract colonization, Tso and colleagues (Tso *et al.* 2018) carried out fecal transplants from colonized antibiotic-treated C57BL/6 mice into naïve hosts. After 10 weeks of serial passages, clonal isolates were shown to display enhanced intra-GI competitive fitness. A more in-depth analysis revealed that this increase in fitness was due to irreversible loss of function mutations in *C. albicans* hyphal development. As hyphal morphogenesis is a key factor in virulence of *C. albicans* (Lo *et al.* 1997), the loss of this phenotype resulted in significantly reduced cytotoxicity when co-cultured with murine macrophages and human colon epithelial cells. Furthermore, while intravenous administration of wild-type *C. albicans* resulted in severe kidney pathology and death within three to four days, only mild to moderate kidney necrosis was observed with the gut-evolved strains. Intravenous priming with these gut-evolved strains was shown to protect from subsequent lethal systemic challenge with wild-type *C. albicans*. The same results were observed when challenge was repeated using the filamentous fungi *Aspergillus fumigatus*; the gram-negative bacterium *Pseudomonas aeruginosa*; and the gram-positive bacterium *Staphylococcus aureus*. Similar challenges in RAG-1 deficient mice revealed this protection to be driven by the innate branch of the immune system and to begin one day post priming and last approximately one month. One evolved strain in particular, named "R24", was shown to induce significantly higher levels of protection than the others. Analysis of the cytokine levels showed increased serum levels of the interleukin-6 (IL-6) at day 7 post priming that returned to physiological levels at day 28 but remained elevated in the kidneys throughout this time.

Ex-vivo stimulation of splenocytes at this time point induced production of both IL-6 and TNF- α .

Altogether, these findings reveal that gut-evolved *C. albicans*, and especially strain R24, is capable of inducing a non-specific, broad-spectrum, short-lived, protective immune response that is not dependent on T or B lymphocytes. The authors postulated that this immune response presented many of the hallmarks of the innate memory-like mechanism trained immunity.

1.3.2 Trained immunity and its potential role in malaria disease

Recent epidemiological studies have shown that, in addition to eliciting protection against the specific disease, vaccines using bacille Calmette-Guerin (BCG) or against measles can also elicit nonspecific effects on the immune system's ability to respond to heterologous infections (Benn *et al.* 2013, Goodridge *et al.* 2016). Research into the protection mechanisms suggests that the non-specific effects of these vaccines are due to innate immune training through epigenetic reprogramming and that these effects can last for an extended period of time (Saeed *et al.* 2014). For example, *in vitro* studies using monocytes from adults vaccinated with BCG collected 2 weeks and 3 months post vaccination found these cells produced higher levels of the pro-inflammatory cytokines IFN- γ , TNF- α and IL-1 β when presented with *C. albicans* or *S. aureus* (Kleinnijenhuis *et al.* 2012). Several *C. albicans* mutants exhibiting low virulence, mostly due to loss of ability to form hyphae, have been used as live attenuated vaccines to elicit T and B cell independent mechanisms that protected immunized mice from a subsequent lethal systemic infection (Bistoni *et al.* 1986, Fernández-Arenas *et al.* 2004, Saville *et al.* 2009). These studies indicate a need to shift the way we look at vaccination, the generalized impact it plays on the immune system and how it can be modulated by vaccines to impact overall resistance to a broad spectrum of diseases (Sánchez-Ramón *et al.* 2018).

Within the specific context of malaria, studies have pointed to the potential trained immunity can play in resolving or ameliorating malarial infections. It is well defined that resistance to malaria is age-related and dependent on previous exposure (Kurtis *et al.* 2001, Doolan *et al.* 2009) but cannot always be explained by our current understanding of the mechanisms of protection. It could be that these differences are better explained when we take into account potential induction of innate cell memory by previous exposure. In fact, some studies have shown that resistance or susceptibility between certain ethnic groups appeared to be influenced not by genetic variation but by differential patterns of epigenetic modifications or histone methylation in genes associated with resistance or susceptibility to malaria (Qidwai *et al.* 2014, Gupta *et al.* 2017, Quin *et al.* 2017). The parasite itself, beyond inducing the well described immunosuppressive tolerance (Boutlis *et al.* 2006, Shanks 2019) has also been shown to elicit responses reminiscent of trained immunity. Initial stimulation with *P. falciparum* iRBCs or hemozoin was shown to induce human PBMCs to hyper respond to subsequent ligation of TLR2 and this response correlated with epigenetic changes at important immune gene promoters. Furthermore, these modifications were also seen in Kenyan children naturally infected with malaria (Schrum *et al.* 2018). Peripheral blood mononuclear cells (PBMCs) from febrile patients displaying clinical signs of acute malaria exhibited an *in vitro* hyper response to TLR ligand stimulation that resulted in overproduction of proinflammatory cytokines (Franklin *et al.* 2009). The mechanisms interconnecting the difference between tolerance and trained immunity and their role in mediating inflammatory disease are still undefined. Human beings don't live in controlled sterile environments, they are subjected to various diseases throughout their lives which could modulate immune innate memory leading these cells to hyper or hypo-respond when subjected to the next heterologous challenge. Accumulation of sequestered monocytes in the brain microvasculature has been reported in both humans (Stanisic *et al.* 2014, Hochman *et al.* 2015) and mice (Grau *et al.* 1987) sometimes days before manifestation of the clinical signs

(Pai *et al.* 2014) of cerebral malaria. To disregard the role that these cells can play when they have been previously triggered is to ignore a potential key player in the onset, duration and severity of this pathology. If we are to fully understand what governs an effective protective response against malaria, we have to start taking into account the importance of trained immunity. The exploitation of the principles of trained immunity might very well guide the next generation of anti-malarial diseases.

In order to investigate the potential role that induced trained immunity could play in protection against malaria, we decided to test the effect that vaccination (priming) with the attenuated *C. albicans* R24 isolated by Tso and colleagues could elicit on subsequent malaria infections in mice. Results of these trials as well as a description of the used model compose Chapter 4 of this thesis.

1.4 Aims and scopes

In order to glean some further understanding into the mechanisms that govern resistance and pathology to malaria infections this thesis studies the effects that two different murine vaccination models have in inducing protection from lethal malaria challenge.

The first vaccination model is aimed at the adaptive immune system. By using the well-described C-terminal merozoite surface protein 1 (Ling *et al.* 1994) we test whether thermogel compounds can be used as vaccine carriers/adjuvants and how they compare to the same protein given under the same conditions emulsified in the strong adjuvant Freund's adjuvant. Through survival studies and analysis of serum collected from the mice prior to vaccination we try to understand how these compounds influence the antibody response and what impact it has on murine survival to lethal challenge.

The second vaccination model employs the use of a live attenuated *C. albicans* gut-derived strain to elicit innate immune responses previously equated to trained immunity. We describe what effects the priming has on two different mouse strains (BALB/cJ and C57BL/6J) and what impact it has on the survival to lethal challenge with malaria strains.

Chapter 2 - Materials and Methods

2.1 Vaccination with thermogel adjuvants

2.1.1 Adjuvant formulation and basic characterization (done by IMRE)

Synthesis, formulation and *in vitro* characterization of the thermogel polymers were carried out by Dr. Sing Shy Liow, Dr. Kun Xue and Dr. Kangyi Zhang at the Soft Materials (SOF Department) of the Institute of Materials Research and Engineering (IMRE, A*STAR) following a previously published methodology (Loh *et al.* 2007).

Synthesis of poly(PEG/PPG/PCL urethane) (Gel 3) and poly(PEG/PPG/PTHF carbonate urethane) (Gel 4)

All starting materials were used as received. Gel permeation chromatography (GPC) was measured with a Viscotek GPC max module with two phenogel columns (103 and 105Å) (size: 300 × 7.80 mm). Tetrahydrofuran (THF) with flow rate of 1.0 ml per min was utilized as eluent. ¹H NMR (proton nuclear magnetic resonance) analyses were carried out at room temperature using JEOL 500 MHz NMR spectrometer, deuterated chloroform (CDCl₃) was used as solvent.

Gel 3 was synthesized from PEG, PPG and PCL diol, using 1,6-hexamethylene diisocyanate (HMDI) as coupling agent, and dibutyltin dilaurate (DBT) as catalyst, as shown in Figure 2-1 below. The weight ratio of PEG to PPG was kept at 2:1 and 1 wt. % of PCL diol. The amount of HMDI added in the reaction mixture was set at [OH]:[NCO] 1:1. Briefly, to prepare Gel 3 (2:1, 1 wt. %), PEG and PPG were weighed into a 250 ml round bottom flask at a ratio of 2x g PEG to 1 g PPG. PCL diol corresponding to 1% weight of the previous polymers was added into the flask, followed by the addition of 30 ml of anhydrous toluene. The reaction mixture was sonicated for 15 min. Trace amount of water in the reaction mixture was removed through an azeotropic distillation by rotary evaporation until only a viscous solution remained in the

flask. This process was repeated twice. 30 ml of anhydrous toluene was added into the flask, before it was heated to 110 °C and 1.22 ml (7.6×10^{-3} mol) of HMDI plus two drops of DBT ($\sim 8 \times 10^{-3}$ g) were added sequentially. The reaction mixture was stirred at 110 °C under nitrogen atmosphere for 24 h. A total of 100 ml of anhydrous toluene was added into the flask during the reaction. The resultant copolymer was precipitated with excess diethyl ether.

Figure 2-1 - Polymerization procedure for the synthesis of poly(PEG/PPG/PCL urethane) (Gel 3)

Poly(PEG/PPG/PTHF carbonate urethane) (Gel 4) (2:1, 2.5 wt. %) was prepared using the same protocol, as shown in Figure 2-2, but PCL diol was replaced with PTHF carbonate diol at a concentration of 2.5%.

Figure 2-2 - Polymerization procedure for the synthesis of poly(PEG/PPG/PTHF carbonate urethane) (Gel 4)

For *in vivo* experiments, both gels were further purified using a dialysis method. The precipitated copolymer was dissolved in isopropanol (~150 - 200 ml, with heating to 50 °C)

and dialyzed against DI water. Molecular weight cut-off of the dialysis membranes used was 3-5 kDa. White floss copolymers were obtained after freeze-drying.

Weight ratio (%) based on ^1H NMR peak integration of characteristic peaks:

- Gel 3 (PEG-PPG-PCL 2:1, 1 wt. %) - $M_n \sim 12100 \text{ g.mol}^{-1}$, $M_w \sim 7700 \text{ g.mol}^{-1}$ and PDI ~ 1.6 obtained by GPC (in THF).
- Gel 4 (PEG-PPG-PTHF 2:1, 2.5 wt. %) - $M_n \sim 14900 \text{ g.mol}^{-1}$, $M_w \sim 9300 \text{ g.mol}^{-1}$ and PDI ~ 1.6 obtained by GPC (in THF)

Adjuvant cytotoxicity test

Thermogels (100 mg/ml) were incubated in a Transwell® (Costar) setup for 24 hrs to test for biocompatibility. Two cell types were used - human dermal fibroblasts (HDF) (ATCC®) and NIH/3T3 mouse fibroblasts (ATCC®). The cells were grown in the bottom of a 24 well plate (1 ml of 5E4 cells per well). After 2 hrs of cell adhesion, 400 μl of thermogel was added to the Transwell® insert and the insert was transferred to the cell plate. The polycarbonate inserts used were 6.5 mm diameter and 8.0 μm in pore size. After 24 hrs, CellTiter-Blue® (Promega) was used to determine the viability of cells incubated with the thermogel relative to those without thermogel exposure (positive control). HDF cell viability was determined to be above 90% while NIH/3T3 cell viability was determined to be above 85% viability after 24hrs (Sing Shy Liow, personal communication)

In vitro drug release

Polymer was dissolved in 10mg/ml BSA solution and left in the fridge overnight to form a 7 weight-percent solution. 1 ml of polymer solution was dispensed into a 20 ml vial and put into a 37°C water bath for 1h to form a gel containing BSA. Ten ml of preheated PBS was added in as buffer, and the release samples were incubated in a 37°C water bath.

The gel samples were assayed at regular time points to determine the amount of BSA released. At each time point, all the PBS buffer was removed and replaced with the same volume of preheated PBS. The collected samples were stored at -20 °C for further analysis by the Micro BCA™ Protein Assay Kit (Thermo Scientific™). Results showed that BSA was mostly released from the thermogel within the first 2 days following a diffusion-mediated release profile (Sing Shy Liow, personal communication).

Storage of the thermogel adjuvants prior to use in SIgN

One of the intrinsic characteristics of these thermogel polymers is their biodegradability as they slowly hydrolyze over time. It is important therefore to protect them from exposure to moisture.

The first batch of gels was stored at -20°C in 50 ml falcon tubes wrapped in parafilm. After discussion with Dr. Sing Shy Liow (IMRE), we decided to store the second batch of gels in 15 ml falcon tubes wrapped in parafilm at room temperature in a desiccator cabinet (Secador, Scienceware). Exposure of the thermogels to air moisture was minimized as much as possible.

2.1.2 Immunization

Animals

Female BALB/cJ mice 4-5 weeks old purchased from InVivos Pte Ltd were used in the adjuvant subunit vaccination trials 1 and 3. Subunit vaccination trial 2 used female BALB/cJ mice 4-5 weeks bred under specific pathogen-free conditions in the Biological Resource Centre (BRC). All mice were kept under specific pathogen-free conditions in the Biological Resource Centre (BRC), Agency for Science, Technology and Research, Singapore (A*STAR) and InVivos mice were acclimatized for a minimum period of 72hrs before any experiment. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC

#140968 and #181314) of the Agency for Science, Technology and Research, Singapore, in accordance with the guidelines of the Agri-Food and Veterinary Authority and the National Advisory Committee for Laboratory Animal Research of Singapore.

Antigens used for vaccination

GST-MSP1EGF or glutathione S-transferase-MSP1 combined EGF-like domains fusion is a recombinant protein containing the two C-terminal conserved epidermal growth factor (EGF)-like modules of the merozoite surface protein 1 (MSP-1) of PyYM (residues 1649 -1754) (Ling *et al.* 1994). Protein was kindly provided courtesy of Dr. Anthony Holder (Crick Institute, London UK). Proteins were kept at -20°C in aliquots and thawed when needed. Glutathione S transferase protein (GST) was prepared under and stored under the same conditions as GST-MSP1EGF.

Parasites

Studies were carried out using transgenic PyYM expressing luciferase (PyYM*luc*). Infected red blood cell (iRBC) stabilates used to initiate infections were free from other infectious agents and were prepared through *in vivo* passage in BALB/cJ mice, collected via orbital bleeding and stored in liquid nitrogen at 2×10^6 iRBC/ml in Alsever's solution. Parasites were thawed in ice and injected into the mice within 30 min of thawing. Parasites were kindly provided by Dr. Kami Kim (Mwakingwe *et al.* 2009).

Vaccine preparation

The day before administration of the vaccine, thermogel polymers were weighed and resuspended in PBS and left at 4°C in a shaker tube rotator until complete solubilization. Once that was achieved, the MSP protein was added, and tubes were left overnight at 4°C in the tube rotator. For the vaccines containing Freund's adjuvant, MSP or GST protein was

resuspended in PBS and left overnight at 4°C in the same tube rotator. Emulsification with the Freund's adjuvant was done at a 1:1 ratio on the day of vaccination.

Due to the hydrolyzing nature of the gels, each batch was tested before the first immunization to determine the lowest concentration at which the thermogel polymers could form a stable gel after overnight solubilization at 4°C followed by a 30 min incubation in a 37°C heating block. Gels 3 and 4 were weighted into various 1.5 ml Eppendorf tubes so as to achieve a final concentration between 3% and 11% (1% increments) in 200 µl of PBS. Eppendorf tubes were left overnight at 4°C in a shaker tube rotator. The following day, the solubilized gels were divided into two groups. Group one was placed at 37°C in a heating block for 30 min while group two was evaluated for ability to aspirate into the vaccination syringes while in liquid form. The lowest concentration at which the gel was still possible to aspirate and formed a stable matrix after incubation in the heating block was chosen.

Based on the previous protocol, the final concentration used for gels from batch 1 was 4% w/v for both gels and 10% w/v for Gel 3 and 6% w/v for Gel 4 in gels from batch 2.

All vaccine formulations were kept at 4°C or on ice until injection into the mice.

Mouse immunization protocol

Test groups (5 mice each) were administered 30 µg of the recombinant protein GST-MSP1EGF emulsified in complete Freund's adjuvant (CFA, Sigma) or in one the thermogels (Gel 3 or Gel 4). Subcutaneous injections were given first on the left rear flank. A boost with 30 µg of the same protein emulsified in incomplete Freund's adjuvant (IFA, Sigma) or in the gels was given 2 weeks and 3 weeks after the primary immunization alternating the mouse flank (following the protocol described in Rénia *et al.* 1997). Groups of control mice received just the GST portion of the protein emulsified in Freund's adjuvant, just Freund's adjuvant or just the gels.

During vaccination, mice were anesthetized with isoflurane and laid on a 37°C heating block for 3-5 min before and 3-5 min after the subcutaneous injection (s.c) of the vaccine. This was done to maximize polymerization speed of the thermogel polymers. Control groups were subjected to the same conditions.

A preliminary trial using Alum (Brenntag Alhydrogel® - Aluminum Hydroxide Gel 10mg/ml) was carried out concomitantly with trial 3. The recombinant protein was emulsified in Alum at a 1:1 concentration and vaccination was administered to groups of five female BALB/cJ mice under the same conditions as the other vaccines described above. Negative control consisted of vaccination with just Alum.

Challenge and parasitemia

One week after the last immunization mice were challenged with a lethal dose of 2×10^5 red blood cells infected with PyYM administered intra-peritoneally (i.p.). Peripheral parasitemia was monitored on blood drops obtained by tail bleed starting from day 3 or 4 until end of experiment following the protocol previously established by Malleret *et al.* 2011. Simply, 1 μ l of blood was resuspended in 100 μ l of PBS and stained for 20 min with 1 μ l of Hoechst 33342 (800 μ M, Sigma B2261), 0.5 μ l of dihydroethidium (1 μ g/ml, Sigma 127008) and 2 μ l of Rat IgG2a anti-mouse CD45-APC (clone 30F11.1, Miltenyi biotec). Four hundred μ l were added after incubation to stop the reaction. Samples were acquired using a BD FACSDiva software and analyzed using FlowJo software version 10. Data was plotted using GraphPad Prism version 8.

2.1.3 Serum characterization assays

Serum collection

Mice were anesthetized with isoflurane and blood was collected via retro-orbital bleeding and allowed to coagulate at room temperature for a 4-6 hours then spun down on a micro centrifuge (Beckman) 1 min at 10000 g, 4°C. Supernatant was transferred to a new microtube and spun down again (1 min, 10000 g, 4°C). Final supernatant was collected and stored at - 20°C until use.

For experiments where mice weren't euthanized on serum collection day, 100 µl of blood were collected at each time point. All other mice were terminally bled.

Serum from naive mice of the same background and age was also collected at each time point for background determination.

MSP1 protein ELISA

MAXISORP U96 NUNC- Immuno plates (Thermo Fisher Scientific, Denmark) were coated overnight at 4°C with 50µl of GST-MSP1EGF peptide diluted in carbonate-bicarbonate coating buffer (3.03g Na₂CO₃, 6.0g NaHCO₃, pH 9.6) at a 5 µg/ml. After three washes with 200µl of PBST (PBS + 0.05% Tween 20), wells were blocked with 100µl of 5% Bovine Serum Albumin in PBST, at room temperature, for 2 hours. After another three washes with PBST, 100µl fourfold serial dilutions of pooled sera from each group (5 mice each, starting at 1/30 dilution) were added to each well and incubated at room temperature. Each pooled serum was run in duplicate. After a 2 hour incubation, supernatant was discarded and wells washed four times with PBS. Anti-mouse IgM (Sigma A8786) and IgG (Promega W402B) HRP-conjugated antibodies diluted in PBS with 1% BSA were added to the respective wells (each well 100µl) and plates were incubated at room temperature for 2 hours. Plates were washed five times

with PBST and 100µl of TMB substrate (Sigma T8665) added to each well. Plates were then incubated at room temperature for 5 min in the dark and the reaction was stopped with 50µl of 1M HCL. OD was read at 450nm and 570nm using a Perkin Elmer Multimode Plate Reader (Enspire, Enspire Software version 4.13.3005.1482). Results were analyzed using Microsoft Excel and Graphpad Prism.

Final OD value was determined after subtracting OD570nm from OD450nm to correct for background noise. Endpoint titer was defined as the highest dilution of serum at which the final OD was greater than the mean of all the control groups plus 3 times the standard deviation. The control groups were pooled naïve serum, pooled serum from mice vaccinated with Gel 3, pooled serum from mice vaccinated with Gel 4 and pooled serum from mice vaccinated with CFA/IFA. GST + CFA mouse serum was not used as a control for estimation of the endpoint titers.

Parasite lysate ELISA.

BALB/cJ mice (5-6 weeks old) were infected with PyYM as mentioned above (**Challenge and parasitemia**). When mice reached above 30% parasitemia each were terminally bled into a separate 50 ml falcon tube containing 30 ml of PBS. Tubes were centrifuged 1500g for 10 min and the supernatant discarded. The RBC pellet was resuspended in 1 ml of Alsever buffer for each 150µl of blood collected and 500µl aliquots were stored at -80°C until use.

96-well costar flat bottom tissue culture plates (Corning, NY USA) were coated overnight at 4°C with 75µl of Poly-L-lysine P4832 (Mw 150 kDa – 300kDa, Sigma-Aldrich) diluted 1 to 2 in PBS. Next day, plates were washed with distilled water and allowed to dry. One 500µl iRBC vial (corresponding to approximately 150µl of blood) was thawed per 96-well plate used. Cells in the iRBC vials were pelleted and washed with 1 ml of PBS. The washed pellet was resuspended in 10 ml of PBS and 100 µl distributed into each previously coated well. Plates

were centrifuged at 1600g for 5 min and the supernatant removed with pipette. Cells were then fixed with 100µl of cold methanol for 10min at room temperature, spun down again as before and washed three times with PBS. 100µl of blocking buffer (PBS + 10% Fetal Bovine Serum) were added to each well and plates were incubated at room temperature for 2 hours. The rest of the protocol is as described in (**MSP1 protein ELISA**) except all washes were done with PBS and secondary antibodies were diluted in PBS + 10% FBS.

Secondary antibodies used: IgM and IgG as mentioned above, IgG1 HRP conjugated (Invitrogen PA1-7442), IgG2a HRP conjugated (Santa Cruz Biotechnology sc-2061), IgG2b HRP conjugated (Santa Cruz Biotechnology sc-2062), IgG3 HRP conjugated (Invitrogen M32607).

CHO transient transfection and serum recognition of ectopically expressed proteins

Transient transfection and surface expression of malaria proteins of interest on Chinese Hamster Ovarian Cells (CHO) was carried out by slight modification of the protocol developed in our lab by Dr. Wan Ni Chia (Chia *et al.* 2014).

pDisplay™ vectors (recombinant plasmids) containing the MSP1 C-terminal fragment (residues 1648 – 1754, MSP1-19) or a Circumsporozoite Surface Protein (CSP) fragment (amino acid position 52 to 1209). Both recombinant vectors were created by Dr. Chia and kept at -20°C until use. Endofectin CHO (Genecopoeia) was used to transfect the recombinant plasmids into CHO cells. The night before transfection 6.5x10⁵ CHO cells were seeded into 6cm tissue culture petri dishes and incubated overnight in F12 Complete Medium (F12 Medium Gibco, 1% Pen Strep, 10% Hi FBS) at 37°C with 5% CO₂. Next day the Endofectin CHO transfection mix was made and added to the cells according to manufacturer's instructions. Standard ratio of Endofectin to DNA was 2:1 and dilutions were carried out in Opti-MEM I (Thermo Fisher Scientific). Medium was changed to F12 complete medium the next day and cells were allowed to stabilize for 48h before testing.

Transiently transfected cells were lifted with PBS containing 2mM EDTA, washed once with PBS with 10% FBS (staining buffer) and resuspended in staining buffer. Live cell concentration was determined by hemocytometer using Trypan blue (1:1), and 200 000 cells were seeded per well of a 96 well v-bottom plate (Nunc). After washing cells were incubated with either pooled sera (5 mice each group), rabbit anti-myc (Millipore) or rabbit anti HA (Sigma) antibodies diluted 1/100.

After incubation, cells were washed three times and incubated with AlexaFluor488-conjugated anti-rabbit (Molecular probes), anti-mouse IgM (Molecular Probes) or anti-mouse IgG (Molecular Probes) diluted 1/500. Propidium iodide diluted to 1µg/ml was mixed with the secondary antibody solution to stain for dead cells. Cells were incubated for 30 min at 4°C with shaking, washed three times with wash buffer and resuspended in wash buffer. After staining, cells were analyzed using a BD Accuri™ using BD Accuri™ C6 Plus software (BD Biosciences). Non-transfected cells were concomitantly stained under the same conditions. All washes were done with PBS + 5% FBS and antibodies and pooled sera were diluted in PBS + 10% FBS. All data was exported and analyzed using FlowJo v10 (FlowJo, LLC).

The same gating strategy was applied for transfection efficiency and serum recognition. After removal of debris (SSC-A/FSC-A) and isolation of single cells (FSC-H/ FSC-A and SSC-H/ SSC-A), samples were analyzed on a Propidium Iodide (PI) / AF 488 dot plot. Transfection efficiency was determined by gating on non-transfected cells (Figure 2-3). Serum response was determined by gating on naïve serum incubated with cells expressing each of the antigens (Figure 2-4).

Figure 2-3 - Gating strategy employed for determination of transfection efficiency. Quadrant gates are drawn on non-transfected cells. Propidium iodide negative, AlexaFluor488 (AF488) positive cells are considered positively transfected and expressing the antigen of interest on the surface. Intensity of signal is equated with more plasmids transfected into cells not higher transfection efficiency. Number in the bottom right quadrant represents the percentage of cells expressing the malaria antigen (as determined by anti-myc or anti HA recognition).

Figure 2-4 - Gating strategy employed for determination of serum response and examples of low and high serum response. Quadrant gates are drawn on naïve serum response incubated with transfected cells. Propidium iodide negative, AlexaFluor488 (AF488) positive populations are considered as a positive response to the antigen expressed on the cells. Number in the bottom right quadrant represents the percentage of cells recognized by the pooled sera (as determined by anti-IgM or anti-IgG recognition). Pictures are representative only.

All data was then exported to Excel (Microsoft®) for statistical analysis. Plots were drawn using GraphPad Prism v8.

Transfection efficiency was determined as the percentage value of cells detected after gating on non-transfected cells and removing the background.

For each serum testing, protein expression was quantified by the highest value observed for either myc or HA tags in that particular condition. This value was thus considered to be the percentage value of cells expressing the protein (Transfected cells %). Serum recognition was defined as the percentage of AF488 positive cells with test serum (Serum recon cells %) minus the percentage of AF488 positive cells with naïve serum (Naïve recon cells %) divided by the total percentage of cells expressing the protein as represented in the equation below:

$$\text{Serum Response} = \frac{\text{Serum recon cells (\%)} - \text{Naïve recon cells (\%)}}{\text{Transfected cells (\%)}}$$

Each serum was tested in duplicate and final serum recognition value represents the average of both experiments.

Invasion inhibition assay

Merozoite inhibition assay was carried out as described in Cho *et al.* 2016 with some slight modifications.

Briefly, BALB/cJ mice (5-6 weeks old) were infected with PyYM as mentioned above (**Challenge and parasitemia**). When mice reached above 30% parasitemia each were terminally bled into a separate 15 ml falcon tubes containing 5 ml of sterile PBS with heparin. Concomitantly, blood from naïve BALB/cJ mice (5-6 weeks old) was similarly collected. Leukocytes were removed by filtrating twice through Non-woven fiber filter columns (NWF columns) and filtered blood was spun down (10 min, 1500g, Room Temperature (RT)) and washed with 5 ml RPMI 1640 (Gibco) with 10%FBS. Blood from all the infected mice was joined into one falcon tube, and the pellet resuspended to 50% haematocrit (by volume) with

RPMI medium. Suspension was overlaid on a differential 45% - 65% Percoll (diluted in RPMI) column and centrifuged for 20min at 1450g RT without brake to separate schizont-stage parasites. Schizont layer was collected, washed with RPMI + 10% FBS and kept at room temperature for no more than 30 min before use. Naïve mouse RBC were stained with carboxyfluorescein succinimidyl ester (CFSE, Sigma) at 75µM in PBS RT for 30 min before inactivating with pure FBS and washing with 5 ml of complete RPMI 1640 (10% FBS, 1% PenStrep). CFSE-stained RBC were mixed with isolated schizonts at 6% parasitemia (proportion of infected RBC to non-infected RBC) and a final hematocrit of 2%. Invasion assay was carried out in 96-well microtiter plates (Beckman Dickinson) at a final volume of 200µl. To each well, pooled sera from vaccinated (4 groups of n=5) or naïve was added for a final dilution of 1/30, 1/120 or 1/480. At T0h, 20µl of the final suspension were collected and stained with 1µL of Hoechst stain (Hoechst 33342, Sigma) for 20min at room temperature. To terminate the reaction, 400µl of PBS were added and samples were analyzed by flow cytometry using a BD LSRII cytometer (BD Sciences) and FlowJo version 10. After a 14-hour incubation (T14h), 20µl of the remaining suspension were collected and similarly stained and analyzed. Newly invaded RBCs were defined as Hoechst positive and CFSE positive RBCs.

Efficiency of invasion was determined as the percentage of newly invaded RBC after the incubation period (T14h) minus the percentage of newly invaded RBC at the beginning of the trial (T0h).

The inhibition of invasion was calculated using the ratio between the efficiency of invasion on the test well and the efficiency of invasion on a well incubated with naïve serum (negative control) according to the following formula:

$$\text{Inhibition of invasion \%} = 100 - \left(\frac{\text{Efficiency of invasion in test well}}{\text{Efficiency of invasion in negative control}} \right) \times 100$$

The cysteine protease inhibitor E64 (Sigma) was used at 100 μ M as a positive control for inhibition because it inhibits schizont rupture, thus preventing merozoite release.

Each sample was run in triplicate. Data was plotted using GraphPad Prism version 8.

Phagocytosis assay

For the phagocytosis assay, isolation of the mature stages of the parasite was carried out as described in **Invasion inhibition assay** until the end of the Percoll column step.

After the 20-minute centrifugation step with the differential Percoll column, layers containing schizonts were collected and stained with CFSE as described previously (**Mouse immunization protocol**) for naïve RBC, washed twice with 10 ml of RPMI 1640 and resuspended in 3 ml of RPMI. Using a 5 ml syringe, schizonts were ruptured by forcing the suspension through a 1.2 μ m membrane pre-wetted with RPMI. Another 3 ml of fresh RPMI were passed through the membrane to maximize recovery and the filtrate was passed through a magnetic sorter (MACS, Miltenyi) using LS columns pre-equilibrated with 3 ml of RPMI. Flow-through was collected into 15 ml falcon tubes and centrifuged at 4000 g for 10 min. The supernatant was discarded, and the pellet resuspended in 200-300 μ l RPMI (depending on pellet size). Giemsa stained smears were carried out before and after filtration to assess purity of the samples and presence of merozoites. To determine merozoite count, 5 μ l of the final merozoite suspension was transferred to an Eppendorf tubes containing 194 μ l of PBS and 1 μ l of Hoechst stain. Samples were stained for 20 min at room temperature and 5 μ l of CountBright Absolute counting beads (Invitrogen) added. Samples were analyzed by flow cytometry (LSR II) using BD FACSDiva software and the concentration of merozoites determined as defined in the CountBright beads protocol.

CFSE-stained merozoites were opsonized between 1/50 to 1/5000 serum dilution using individual mouse sera from vaccinated groups, respective adjuvant controls and naïve mice. As a positive control for opsonization a hyperimmune serum was used. This serum originated from BALB/cJ mice, vaccinated with MSP + Gel 4 (Hyperimmune serum A) or MSP + Gel 3 (Hyperimmune serum B), challenged with PyYM, allowed to recover from challenge, challenged again with PyYM immediately after and the blood collected after clearance (*data not shown*).

After a 30 min opsonization window carried out at room temperature, the opsonized merozoite solutions were added to the Raw 264.7 macrophages at a 10:1 target to phagocyte ratio. A sixty-minute incubation at 37°C was carried out before cells were twice washed with cold PBS, scraped from the wells and transferred to Micronic tubes (Micronic, Netherlands) to assess by flow cytometry (BD LSR II, FlowJo).

For the phagocytosis assay (Figure 2-5), 1×10^4 Raw 264.7 murine macrophages were seeded on a P96 well flat-bottom tissue culture plates at least two to four hours before the start of the assay. Samples were tested in duplicated and opsonization was carried in the same tube before adding the merozoites to the Raw264.7 cells.

All data was analyzed using FlowJo v10 (FlowJo, LLC) and statistics were carried out using GraphPad Prism version 8.

Figure 2-5 - Gating strategy employed in phagocytosis assay. a) Gating for estimation of merozoites numbers. Shown are the gates for counting CFSE-stained (FITC-positive) merozoites and corresponding number of beads. A minimum of 1000 beads were acquired for each estimation. **b)** Gating strategy employed in the estimation of Raw 264.7 macrophages capable of phagocytosing opsonized merozoites. Merozoites are stained with CFSE so, CFSE positive macrophages have phagocytosed the merozoites. Indicated are examples of the histograms obtained with (left to right order) just Raw 264.7 macrophages with no merozoites; Raw 264.7 macrophages with stained, non-opsonized merozoites at 10:1 ratio; Raw 264.7 macrophages with stained merozoites at 10:1 ratio opsonized with naïve serum

and Raw 264.7 macrophages with stained merozoites at 10:1 ratio opsonized with HyperImmune serum.

2.1.4 CD4⁺ T cell depletion

At days 0, 2 and 4 post PyYM challenge, rat anti-mouse CD4 InVivo monoclonal antibody (BioXcell, clone GK1.5) was used to deplete the CD4⁺ T cells. Groups of 5 mice vaccinated with either MSP + CFA or MSP + Gel 3, as described previously, were given 0.5mg of depleting antibodies or purified rat IgG isotype control (Sigma) intraperitoneally. An equal number of non-vaccinated mice, but likewise challenged, were used as controls for depletion and non-depletion of CD4⁺ T cells. Depletion efficiency was verified to be above 95% by flow cytometry 24 hours after the first injection (day 0) using 20µl of blood collected from the tail. Tail blood was collected into tubes containing PBS with 10µM EDTA and the red blood cells were lysed using ACK lysing buffer (1000 ml H₂O, 8.29g NH₄Cl, 1.0g KHCO₃, 0.074g EDTA, pH 7.4). Samples were then washed with FACS buffer (PBS 1X, FBS 1%, NaN₃ 0,01%). Lysis step and wash step were repeated to ensure no RBC remained and, after pelleting (500g, 5 min) samples were resuspended in 50µl of blocking buffer (PBS 1X, FBS 1% NaN₃ 0,01%, 10% Rat serum) and stained anti-mouse CD4 APC-conjugated (eBioscience, clone GK1.5) for 20 min at room temperature. After staining, samples were washed again with FACS buffer, resuspended in FACS buffer and acquired with a BD LSRII cytometer (BD Sciences) using BD FACSDiva software and analyzed using FlowJo software. Gating strategy employed consisted of removal of debris in an SSC-A vs FSC-A plot, selection of singlets in FSC-W vs FSC-A plot and gating on the APC-positive population in an SSC-A vs APC plot. Percentage of depletion was calculated as the percentage of APC positive cells in each CD4⁺ T cell depleted mouse divided by the average percentage of APC positive cells in naïve non-depleted mice.

2.2 *Candida albicans* immunization experiments

2.2.1 Animals

BALB/cJ and C57BL/6J mice 5-6 weeks old age and sex matched were used in the *Candida albicans* trials. All mice were bred and kept under specific pathogen-free conditions in the Biological Resource Centre (BRC), Agency for Science, Technology and Research, Singapore (A*STAR). All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC: 140968, IACUC: 181314) of the Agency for Science, Technology and Research, Singapore, in accordance with the guidelines of the Agri-Food and Veterinary Authority and the National Advisory Committee for Laboratory Animal Research of Singapore.

2.2.2 Microbial stocks

Candida albicans R24: attenuated strain adapted from *Candida albicans* SC5314 through 10 successive weekly serial passages as fecal transplant in antibiotic-treated wild-typed C57BL/6J mice. This evolved strain contains a recessive mutation in *FLO8*, which encodes a transcription factor required for hyphal development and is therefore unable to form hyphae. Strain was evolved and isolated by Tso and colleagues (SigN, A*STAR Singapore; Tso *et al.* 2018). *Candida* strain was isolated and kept in glycerol stock in liquid nitrogen.

PbA-luc: Transgenic PbA strain (231c11) expressing luciferase under control of *ef1-a*. Strain used here was kindly provided by Dr. Christian Engwerda (Queensland Institute of Medical Research, Brisbane, Australia; Franke-Fayard *et al.* 2005). Infected Red Blood Cell (iRBC) stabilates used to initiate infections were free from other infectious agents and were prepared through *in vivo* passage in C57BL/6J mice, collected via orbital bleeding and stored in liquid nitrogen at 1×10^7 iRBC/ml in Alsever's solution. Parasites were thawed in ice and injected into the mice within 30 min of thawing.

PyYM-luc: Transgenic PyYM strain expressing luciferase. Same strain mentioned in **Parasites**.

2.2.3 Characterization of *C. albicans* R24 infection

Intravenous administration of attenuated R24 strain

Candida albicans R24 were collected by scraping the original glycerol stock isolated by Tso and colleagues (Tso *et al.* 2018), thawed by streaking on YPDA (2% Peptone, 1% yeast extract, 2% glucose, 1.5% agar, 0.5% PenStrep) plates and incubated at 37°C overnight. Plates were then parafilm and kept stored at 4°C for up to one week. The day before the experiment, the *Candida* was cultured in Liquid YPD (2% Peptone, 1% yeast extract, 2% glucose, 0.5% PenStrep), overnight at 37°C, washed with PBS and concentration determined by counting in a Neubauer chamber (C-Chip DHC-N01, InCyto). Concentration was then adjusted with PBS and 5×10^5 CFU in 200 µl were injected intravenously (i.v) in the right lateral tail vein of each challenged mouse. Control groups were injected with just PBS.

Tissue burden

For determination of tissue burdens in blood, groups of three to four mice (C57BL/6J and BALB/cJ) were bled 5 min, 20 min, 5 hours, 24 hours, 72 hours, 5 days, 7 days, 10 days, 14 days and 21 days after *i.v.* injection of *C. albicans* R24. At each time point, 10 µl of blood were collected from the tail tip into 1.5 ml Eppendorf tubes containing 90 µl of sterile PBS. Serial 1/10 dilutions were then carried out and 90 µL of blood were plated on YEPDA plates. Plates were incubated for 48 hours at 37°C and number of colonies counted. As a measure of control, 10 µl of the inoculum given to the mice were also collected and plated in the same manner. At each time point one naïve mouse was equally bled for control.

For determination of tissue burdens in individual organs the brain, spleen, kidneys, liver, heart and lungs were collected from C57BL/6J mice at days 7, 14 and 21 post *C. albicans* injection

and from BALB/cJ mice at days 7, 14 and 28 post *Candida*. At each time point, 3 BALB/cJ mice or 5 to 9 C57BL/6J mice were terminally bled retro-orbitally and terminated via cervical dislocation. Individual mice were sprayed with 70% ethanol and each organ was collected to previously tared 2.0 ml Eppendorf tubes containing 1 ml of PBS. All tools were wiped with Clidox between each organ. On processing, all organs and PBS, except the heart, were transferred to a 40µm cell strainer placed in a 6 well plate and homogenized using the back of a 3 ml syringe plunger. Hearts were transferred to gentleMACS M tubes (Miltenyi Biotec) and homogenized in a Dispomix® Drive homogenizer (Xiril AG, Switzerland) at a gradual increase to 14000 rpm for 40 sec. Organ homogenates were transferred back to original tared tube, vortex and serial 1/10 dilutions were plated in YEPDA plates. Plates were incubated for 48 hours at 37°C. The kidneys were harvest and processed together so, CFU values represented refer to both kidneys. Blood collected by orbital bleed was plated as described above. At each time point, a naïve mouse was similarly sacrificed, and its organs plated for contamination control. All tissue burden samples were kept at 4°C before plating. Blood tissue burdens were expressed as $\log_{10}$ CFU ml⁻¹ tissue while organ tissue burdens are expressed in CFU g⁻¹. The detection threshold for each organ was determined as the minimum CFU/g needed to detect 1 colony in 100µl of plated non-diluted homogenate. This value is calculated based on the average weight measured for each organ at all time points and the volume of the final homogenate suspension (1.5 ml for Liver, 1 ml for all other organs). Thresholds determined for each mouse were, in CFU/g, as follows:

Table 2-1 – CFU minimum detection thresholds per organ, per mouse.

Mouse strain	CFU detection thresholds per organ (CFU/g)						Blood threshold (log ₁₀ CFU/ml)
	Brain	Heart	Kidneys	Liver	Lungs	Spleen	
C57BL/6J	23	79	38	13	66	85	2.0
BALB/cJ	25	79	28	13	64	75	

Behavior scores and mouse weight

Mouse weight was assessed every 2 to 3 days from one day before *Candida* administration until end of experiment. A simple scoring system for the assessment of the mouse behaviors was created. Mice were observed every 2 days by 2 independent observers. Observed behavior was given an individual yes or no scored based on whether the Helicoptering, Head Twist or Twitching behavior was observed at the time. When in doubt, behavior was given a no score. Conflicting data by the two observers was considered as a no. Head twist side was documented. Behaviors were analyzed separately and were represented as the percentage ratio of the mice showing that particular behavior over the total number of *Candida*-infected mice observed on that day. A negative control group of at least 3 naïve mice were kept and assessed together with test groups to rule out spontaneous occurrence of neurological behaviors. No naïve mice exhibited any neurological symptoms at any point in the study.

2.2.4 Characterization of co-infection models

Priming and challenges

For the malaria blood-stage challenge, groups of ten C57BL/6J mice 5 to 6 weeks old were either primed by i.v. injection of R24 or PBS as described before and, after a 14-day recovery window challenged i.p. with 2×10^5 iRBC of PyYM. Groups of five BALB/cJ mice 5 to 6 weeks

old were primed and challenged as above but given either a 14-day, a 28-day or 42-day window before challenge. For the experimental cerebral malaria (ECM) studies, groups of ten C57BL/6J mice 5 to 6 weeks old were both primed with R24 or PBS and challenged with 1×10^6 iRBC of PbA-luc i.p. after a 14-day window. In all studies, parasitemia was followed, as described in section **Challenge and parasitemia**, every day from day 3 post malaria challenge until day 15 and every other day after that until mice died or there was no detectable parasitemia.

Mice with non-detectable parasitemia until day 6 post challenge were considered failed infections and excluded from the study.

For the ECM studies, C57BL/6J mice were monitored for neurological symptoms typical for cerebral malaria. Mice exhibiting paralysis, ataxia, deviation of the head, convulsion and/or coma who died during the ECM window (6 to 12 days post PbA challenge) were considered as having died of experimental cerebral malaria. R24-injected mice displaying neurological symptoms *a priori* to PbA challenge that died during the ECM window were considered to have died of ECM.

Ex-vivo brain imaging in ECM mice

D-luciferin potassium salt (Caliper Life Sciences), the substrate for luciferase, was dissolved in PBS at a concentration of 5 mg/ml. At day 7 post PbA challenge, mice were anesthetized with ketamine/xylazine (100 μ l per 100 mg) and D-luciferin was injected i.p and allowed to circulate for 2 min. Mice were then perfused with PBS until cleared of blood. Brains were extracted, washed with PBS and placed on a petri dish. A couple of drops of D-luciferin were dripped onto the brains and bioluminescence was measured using an IVIS Spectrum (Perkin Elmer). Bioluminescence imaging was acquired with a 10 cm field of view (FOV) and exposure times of 10 sec, 20 sec, 40 sec, 80 sec followed by a 5-minute delay and further 80 second

exposure. Naïve mouse brains were used for as control for measuring background luminescence. Results were analyzed using the software Living Image 3.0 and bioluminescence was quantified for the whole brain as the highest average radiance (p/s/cm²/sr) measured within the detection range.

Profiling of brain leukocyte populations in ECM mice

At day 7 post PbA challenge, mice were anesthetized with ketamine/xylazine (100µl per 100mg) and perfused with PBS until full blood clearance. Whole brains, including the 2 olfactory bulbs and the brain stem, were collected. Brains were placed in a 40µm cell strainer half submerged in 5 ml of cold complete RPMI medium (10% FBS, 1% PenStrep) inside a small petri dish and crushed using a syringe plunger. After complete maceration, 5 ml of digestion solution (1X PBS, 20 µg/ml DNase, 1 mg/ml Collagenase IV) was added to the homogenate and samples were incubated for 30 min at room temperature with agitation. After incubation, cell suspension was passed through a 40 µm cell strainer and centrifuged at 500 g for 1 minute to pellet large debris. Supernatant was overlaid on 10 ml of cold 30% v/v Percoll (Sigma) and centrifuged at 1800g for 10 min. Supernatant and fats at the interface were discarded and the pellet resuspended in 1X ACK lysing buffer (1000 ml H₂O, 8.29g NH₄Cl, 1g KHCO₃, 0.074g EDTA, pH 7.4) for RBC lysis. Cells were washed, resuspended in 1 ml of complete RPMI and transferred to 1.5 ml Eppendorf tubes. 2 µl of the 1 ml suspension was counted in a Neubauer chamber (C-Chip, Incyto) and the total number of leukocytes estimated. Samples were spun down for 5 min at 500 g, supernatant was discarded, and the cells stained with 1/1000 dilution Live/Dead dye (Aqua Fluorescent reactive dye, Invitrogen) in PBS for 30 min at room temperature. After washing, cells were resuspended in 50µl of blocking buffer (PBS 1X, FBS 1% NaN₃ 0,01%, 10% Rat serum). 1µl of tetramer H-2Db – Pb1 PE tetramer (Howland *et al.* 2013) was added and samples incubated on ice for 15 min. Samples were further stained with a master mix of antibodies for anti-mouse CD45 (BD Bioscience clone 30-F11) , CD3 (BD

Bioscience clone 145-2C11), CD8 (Biolegend clone 53-6.7), CD11a (eBioscience clone M17/4) for 30 min on ice. Samples were then washed with FACS buffer (PBS 1X, FBS 1%, NaN₃ 0,01%), fixed with PFA 1%, washed and resuspended in FACS buffer and kept at 4°C until analysis. Samples were acquired using a BD LSRII cytometer (BD Sciences) using BD FACSDiva software and analyzed using FlowJo software version 10. Data was plotted using GraphPad Prism version 8.

Cross presentation of the P. berghei-specific CD8⁺ T cell epitope SQLLNAYL by brain microvessels

The cross presentation was performed as previously described by Howland and colleagues (Howland *et al.* 2013). Briefly, on day 7 post challenge, mice terminally bled retro-orbitally and the brains (without meninges or brain stem) harvested and finely minced into RPMI complete. Samples were homogenized using 3 ml syringe attached to 23-gauge needle, passing up and down the needle 3 times. After, the homogenized brain was mixed with an equal volume of 30% dextran (MW 70000, Sigma) in PBS. After a 15-minute centrifugation at 10,000g at 4°C, the supernatant and fatty layer were discarded. The pellet was resuspended in PBS and passed through a 40µm cell strainer, which retains the microvessels. After washing the cell strainer with PBS, the microvessels were dislodged by backflushing with 2 ml of PBS into a 6-well plate containing 40µl of FBS (2%), 2µl of DNaseI (10µg/ml) and 40µl of collagenase IV (1mg/ml) in each well. Plates were incubated with shaking at room temperature for 90mins after which the content in each well was transferred into a 15 ml falcon tube, with 5 ml complete medium. After pelleting (1500 rpm, 5 min), the supernatant was discarded, and the pellet resuspended in 500 µl of complete medium. Each brain was equally divided into 5 wells of a filter plate (PALL 8029) and incubated with 3x10⁴ LR-BS8.4a cells overnight at 37°C. Next day, wells were stained with X-gal and the degree of cross-

presentation determined by counting the number of blue spots using an ImmunoSpot 5.0 Analyzer Professional DC (Cellular technology Ltd).

IFN- γ and IL-10 cytokine extraction and quantification

Organs were extracted and homogenized in 1 ml RIPA buffer (Thermo Scientific) with 1x complete™ Protease Inhibitor Cocktail (Roche; cat no. 11873580001) using gentleMACS M tubes (Miltenyi Biotec). Homogenization was carried out in a Dispomix® Drive Homogenizer (Xiril AG) at 2 cycles of 15 sec at 4000 rpm. Final samples were pelleted (1500 rpm for 1min) and supernatant was transferred to 1.5 ml Eppendorf tubes and stored at -80°C until use.

IFN- γ (Quantikine® ELISA MIF00, R&D systems) and IL-10 (Quantikine® ELISA M1000B, R&D systems) ELISA were performed according to manufacturer protocol and results acquired using a Perkin Elmer Multimode Plate Reader (Enspire, Software version 4.13.3005.1482). Results were analyzed using Microsoft Excel and GraphPad Prism. The limits of detection for IFN- γ were 2 pg/ml and for IL-10 were 5.22 pg/ml.

Blood was collected into 2 ml Eppendorf tubes and allowed to coagulate at RT for 4-6hrs. Tubes were spun down twice for 1 min at 10000 g, with supernatant collected to new tube after each spin. Final supernatant was aliquoted and stored at -80°C until use.

Expression of ICAM-1 and VCAM-1 in brain endothelial cells

Brains were harvested on day 7 post PbA challenge and brain microvessels were extracted and isolated as described in section **Cross presentation of the *P. berghei*-specific CD8+ T cell epitope SQLLNAKYL by brain microvessels**. The final microvessel suspension was pelleted and stained with 1/1000 dilution Live/Dead dye (Aqua Fluorescent reactive dye, Invitrogen) in PBS for 30 min at room temperature. After washing, microvessels were resuspended in 50µl of blocking buffer (PBS 1X, FBS 1% NaN₃ 0,01%, 10% Rat serum) containing anti-mouse

CD45 (Miltenyi #130-102-783), CD31 (Biolegend #102418), ICAM-1/CD54 (R&D Systems FAB796A), VCAM-1/CD106 (Biolegend Clone 429) and H-2D^b (Biolegend #111505) and stained for 20 min. After washing in FACS buffer, samples were fixed with 1% paraformaldehyde, washed and resuspended in FACS buffer for acquisition. Samples were acquired using a BD FACSDiva software and analyzed using FlowJo software version 10. Data were plotted using GraphPad Prism version 8.

Preparation of samples for histological analysis

At day 7 post PbA challenge, mice were anesthetized with ketamine/xylazine (100µl per 100mg) and perfused with 4% paraformaldehyde (PFA) until full blood clearance. Left brain hemisphere, left kidney and spleen were extracted, placed in falcon tubes containing 4 ml of 4% PFA and sent for histopathological analysis at the Advanced Molecular Pathology Laboratory (IMCB, A*STAR)

Chapter 3 - Results for Thermogel Adjuvants

3.1 Thermogel polymers as possible adjuvants in vaccination against blood-stage malaria

As mentioned in chapter **1.4 Aims and scopes**, the first aim of this thesis is to evaluate the potential use of thermogel polymers as adjuvants in subunit vaccination. For this purpose, the well characterized MSP1-19 fragment (Daly & Long 1995a) was used as the antigen and mice were inoculated using either of the thermogelling polymers or CFA/IFA as adjuvants before being subjected to lethal challenge.

3.1.1 Vaccination and survival studies

To determine whether the thermogelling polymers could be used as adjuvants against a blood-stage malaria challenge, groups of 3- to 4 -week-old female BALB/cJ mice were immunized via subcutaneous flank injection. Test groups were administered 30 µg of recombinant protein GST-MSP1EGF (hereafter referred to as MSP) emulsified in complete Freund's adjuvant (CFA) or in one the thermogels (Gel 3, Gel 4). A boost with 30 µg of the same protein emulsified in incomplete Freund's adjuvant (IFA) or in the gels was given 2 and 3 weeks after the primary immunization (Figure 3-1a). Groups of control mice received GST with Freund's adjuvant, just Freund's adjuvant or just the gels. Mice were then challenged with a lethal dose of 2×10^5 red blood cells infected with PyYM administered intra-peritoneally (i.p). Figure 3-1b details the vaccination groups and respective controls used in this study.

Figure 3-1- Immunization protocol and control groups employed in this study. a) Vaccination protocol and b) mouse groups employed in the study. Female BALB/cJ mice in test groups were immunized by subcutaneous injection 3 times with 30µg of GST-MSP1EGF (hereafter referred to as MSP) emulsified in either complete (priming) or incomplete (boosts) Freund's adjuvant (1:1), thermogel polymer 3 (4% w/v) or thermogel polymer 4 (4% w/v). Negative controls were immunized by just administering the same adjuvant with no added protein. A negative control group for GST protein (GST + CFA) was also included and mice in this group were immunized the same way as the MSP Freund's adjuvant group (MSP + CFA). After initial priming, 2 boosts were carried out after 2 weeks and 3 weeks, and blood was collected by retro-orbital bleeding 1 week after each boost (indicated as serum collection). Challenge with PyYM was carried out by intra-peritoneal (i.p) injection of infected red blood cells (iRBC) at the lethal dose of 2×10^5 iRBC. Parasitemia was measured from day 4 post-infection to clearance (around day 30 post challenge). CFA – Complete Freund's adjuvant, IFA- Incomplete Freund's adjuvant. /// - no protein was given, just PBS.

Immunization of the mice with either GST+CFA/IFA or just the adjuvant (CFA/IFA, Gel 3 or Gel 4) failed to protect the mice from lethal challenge and all mice died between days 7 and 9 post challenge (Figure 3-2). One exception was one mouse in the group immunized with just Gel 3 that showed no detectable parasitemia until day 6 and was excluded from the study. By contrast, three out of the five mice in the group vaccinated with MSP + Gel 4 and four out of

the five mice vaccinated with MSP + Gel 3 and MSP + CFA/IFA (MSP + CFA) survived the lethal challenge (Figure 3-2).

Figure 3-2 – Vaccination with MSP using thermogel adjuvants protects mice from lethal PyYM challenge. Represented are the individual parasitemia curves contrasting vaccinated groups **a)** MSP+CFA (n=5), **d)** MSP + Gel 3 (n=5), **g)** MSP + Gel 4 (n=5) with their respective control groups **b)** GST + CFA (n=5), **e)** Gel 3 (n=4), **h)** Gel 4 (n=5). Mortality curves comparing each vaccine formulation and respective controls are also represented in **c)**, **i)** and **f)**. One mouse from Gel 3 control was removed from the study as it did not show detectable parasitemia until day 6 post infection. Parasitemia and mortality curves for CFA/IFA control were the same as those for GST+CFA so are not included here.

Vaccination with the recombinant protein in Freund's adjuvant showed the strongest protection, with the four surviving mice showing a delay on the onset of parasitemia, earlier

clearance and lower parasitemia levels (average peak parasitemia of $1.17\% \pm 0.46\%$) (Figure 3-2). These results are consistent with results documented previously (Ling *et al.* 1997) and validate the protocol employed.

Groups vaccinated with the recombinant protein in the gels showed lower levels of protection with parasitemia levels reaching dangerously high percentages that persisted until days 17-18 post infection. Average peak parasitemia in these groups was very similar at $44.8\% \pm 19\%$ and $47.6\% \pm 19.5\%$ for MSP + Gel 3 and MSP + Gel 4 respectively (Figure 3-2). Overall, the parasitemia curves between both these groups show similar trend, peak and duration and while one more mouse died in the MSP + Gel 4 vaccinated group than in the others, groups were too small to infer any significant differences.

3.1.2 Characterization of mouse serum post vaccination.

Before challenge, serum samples were collected via retro-orbital bleed on days 22 and 29 after first vaccine dose corresponding to one week after the administration of each boost (Figure 3-1a). Sera were pooled from samples of the same group and total IgM and IgG antibody levels against the MSP-1 protein used in the vaccines were assayed by ELISA to evaluate differences in immunogenicity against the protein in each of the protein-adjuvant combinations.

As can be seen in Figure 3-3, after the first boost, the level of antibody titers is very high for all groups immunized with both the recombinant protein and with just the GST protein.

Figure 3-3 – Vaccinated mice produce antibodies targeting the protein used in the vaccine. Total IgM and IgG ELISA titers against GST-MSP1EGF protein for each group measured in sera collected 1 week after each boost. Sera was pooled and quantified by ELISA against 250ng of the recombinant protein used for immunization. Antibody titers were defined as the highest dilution yielding an $OD_{450nm} - OD_{570nm} = 0.1$ interpolated from the plot after subtracting the average value of pooled naïve serum tested concomitantly under the same conditions. All dilutions were tested in duplicate and results shown are expressed as average $\log_{10}$ values $\pm$ standard deviation of duplicates. ND – not detected

MSP + CFA (in blue) exhibited the highest titration levels for both IgM and IgG even before the administration of Boost 2. Overall, the greatest differences between the test groups seems to lie in the high IgM response observed for MSP + CFA and GST + CFA that is not seen in the thermogel adjuvant groups. Curiously, previous studies using this particular recombinant protein (Ling *et al.* 1997) also observed high titers of MSP1-specific IgM not only in groups using CFA/IFA but also with other adjuvants under evaluation for human vaccination (QS21, formulations SBAS2.1 or SBAS2 and liposomes containing monophosphoryl lipid A).

Considering the high IgM and IgG titration values observed for GST + CFA after boost 2 we decided to assess if the test groups were indeed producing antibodies that target the MSP1EGF portion of the recombinant protein and not the fused GST subunit portion. We therefore tested the specificity of the IgG and IgM antibodies induced in the test groups after boost 2 against the C-terminal portion of MSP1 (residues 1648 – 1754) expressed on the surface of Chinese Hamster Ovarian (CHO) cells (Figure 3-4). These cells are transiently transfected with a pDisplay vector carrying the gene sequence for the protein of interest, in

this case the MSP fragment, flanked by 2 epitope markers (hemagglutinin and myc). pDisplayTM is a 5.3 kb mammalian ectopic expression vector that allows high level expression and display of proteins on the cell surface. Expressed proteins are fused at the N-terminus to a transmembrane domain, which directs the protein to the extracellular side and contain the hemagglutinin A (HA) and myc epitopes. Determination of protein transfection efficiency is determined by detection of myc and HA epitopes using fluorescent antibodies (Figure 3-4).

Figure 3-4 - Simplified protocol for transfection and expression of MSP1 protein fragment in CHO cells. The C-terminal portion of MSP1 (residues 1648 – 1754), here marked in green as MSP, is inserted in a pDisplay vector and flanked by 2 epitope markers, hemagglutinin (HA) and myc (in orange). The plasmid contains a N-terminal cell surface targeting signal (in blue) and a C-terminal transmembrane anchoring domain (in purple). When transiently transfected into Chinese Hamster Ovarian Cells (CHO), the plasmid induces the cells to produce and express the protein fragment on the cell surface together with the myc and HA epitopes. The ectopic (extracellular) expression of the protein allows antibodies to easily target it and the myc and HA epitopes allow for detection of protein expression.

As can be seen on Figure 3-5 below, all test groups show strong IgG response against MSP1-19 thus corroborating the IgG response observed in the ELISA assay against the protein. The trend also seems to be similar to the results shown in Figure 3-3 whereupon the MSP + CFA group expressed the highest levels of MSP1 recognition ($82\% \pm 3\%$).

Figure 3-5 - Specificity of IgM and IgG antibodies induced after second boost against MSP1 fragment expressed in CHO cells. Vaccinated mice produce IgG targeting the MSP protein used in the vaccination. By cloning the MSP1 C-terminal fragment (residues 1648 – 1754) into a pDisplay vector that is then transfected into CHO cells we induced these cells to produce and ectopically express the protein. Antibodies that target this protein fragment can be considered specific to the MSP1EGF portion of the recombinant protein used in the vaccination trials. Serum response was calculated by determining the number of cells recognized by IgG or IgM in the serum divided by the number of cells expressing the protein of interest (as detected by HA and myc tags co-expressed with and flanking the MSP1 fragment). The cutoff line of 20% was defined as a threshold for positive response based on serum response to CHO cells expressing a non blood-stage malaria protein (CSP protein fragment). Sera from each group (n=5 mice each) were pooled and results are expressed as average values of serum response $\pm$ standard deviation of duplicates.

Interestingly, MSP + Gel 4 sera shows an IgG response to MSP1-19 $\approx$ 1.2 times greater than MSP + Gel 3 sera ($69\% \pm 1\%$ and $58\% \pm 4\%$, respectively), a point that appears to mirror the findings in the ELISA against the protein whereupon MSP + Gel 4 showed IgG titration values $\approx$ 1.1 times greater than MSP + Gel 3 ($\log_{10} 4.54 \pm 0.08$ and $\log_{10} 4.43 \pm 0.01$ for Gel 4 and Gel 3 respectively).

Curiously, the IgM response to MSP1-19 was low across all groups. This result is expected in pooled sera from MSP + Gel 3 and MSP + Gel 4 groups where no IgM titers were detected but

in MSP + CFA it brings into question if the IgM response observed in these mice might not be the result of either unspecific binding or targeted towards the GST portion of the protein.

Together this data shows that the chosen immunization protocol is capable of inducing high antibody titers, specifically IgG, directed against the parasite fragment of the GST-MSP1EGF protein. Moreover, these levels are very high after only one boost and remain at similarly high levels after boost 2 indicating the enormous immunogenic potential of this protein even after just 2 vaccine doses.

To further characterize the immune response induced in these groups we decided to test the specific response of the pooled sera against PyYM lysate. Total IgM and IgG were measured by ELISA using PyYM infected red blood cells (iRBC) as targets for the pooled sera. Contrary to ELISA measured against just the protein, this method allows the detection of an antibody response directly targeted towards the parasite that recognizes the proteins in their natural conformations.

As shown on Figure 3-6, the immunized groups exhibit the same pattern as previously measured by ELISA against the GST-MSP1EGF protein. High IgM antibody titers were only detected in the MSP + CFA vaccinated group (in blue) and dropped dramatically from boost 1 to boost 2 indicative of immunoglobulin class switch. This pattern again mirrors results shown in Figure 3-3 and Figure 3-5 leading to the belief that IgM in the MSP + CFA group may be a result of unspecific binding while in the GST + CFA group it is targeted towards the GST tag.

From boost 1 to boost 2 there is a marked increase in IgG expression in all groups for all detected antibody subclasses (Figure 3-6). The mouse group immunized with MSP + CFA exhibits the highest antibody titers with total IgG values over 20 times higher after boost 2 than MSP + Gel 3 and 7 times higher than MSP + Gel 4. As before (Figure 3-3), Gel 4 appears to induce higher antibody titers than Gel 3.

Looking into the IgG subclasses we clearly observe that the antibody profile is geared towards a very strong IgG1 response coupled with slightly lower IgG2a titers. IgG2b antibodies were only detected in the group vaccinated with MPS + CFA no parasite-specific IgG3 antibodies were detected in any of the groups at the lowest serum dilution tested (1/30).

Figure 3-6 - Total IgM, total IgG and subtypes IgG1, IgG2a and IgG2b titrated against PyYM infected red blood cells (PyYM iRBC). ELISA plates were coated with PyYM iRBC lysate and incubated with pooled sera from each test group. Endpoint titer was defined as the highest dilution of serum at which the final OD was greater than the mean of all the control groups plus 3 times the standard deviation. All dilutions were tested in duplicated and results shown are expressed as average $\log_{10}$ values $\pm$ standard deviation of duplicates. Dotted line represents the minimum threshold corresponding to $\log_{10}$ of the lowest dilution analysed (1/30).

Taking into account the antibody titer results shown above, together with the observed parasitemia curves shown in Figure 3-2, results suggest that induction of high levels of IgG, and specifically IgG1 and IgG2a, are key for protection against lethal PyYM challenge. Similar results were shown by Ling and collaborators (Ling *et al.* 1997) whereupon a strong IgG1 and a lower IgG2a response were observed after vaccination with the GST-MSP1EGF protein. Interestingly, mice vaccinated with Gel 4 as adjuvant produced higher antibody titer across

the board than those vaccinated with Gel 3, yet these differences did not translate in reduced parasitemia or higher survival rates.

3.1.3 Current thermogel vaccination protocol does not consistently protect from death

During the course of this study a total of three vaccination trials were carried out in the lab, yielding variable outcomes regarding the survival of the mice. The first two trials were done using the original batches of gels and only the MSP + CFA mice survived both challenges. As seen in Table 3-1 and Figure 3-8, the second trial with the first batch of the thermogels (Trial 2) did not yield the same results as previously observed. In fact, parasitemia and survival curves did not differ from the mice vaccinated with just the adjuvants (*data not shown*) and no mice survived the challenge. Upon further investigation we came to the conclusion that the thermogel polymers had suffered hydrolysis and were no longer reliably behaving as before. These gels are designed to be biocompatible and biodegradable and to hydrolyse over time when injected into the body. Due to their nature, ambient moisture affects the quality of the gels and shelf-life, in the best storage conditions, is at most six months. The fact that the BALB/cJ mice used in Trial 2 were obtained from in-house stock instead of ordered from InVivos could also have played a role in the observed differences. While the genetic background, sex and age of the mice is the same, intrinsic breeding factors could have also contributed to the observed results as the MSP + CFA group also higher parasitemia values. A new batch of both gels was therefore made to continue the studies and all further studies were carried out using InVivos mice. As a further measure of control and to better ascertain the quality of our vaccination protocol, we decided to include in Trial 3 a group vaccinated with MSP emulsified in alum (Alhydrogel), an antigen commonly used in human vaccination (Table 3-1, Figure 3-7).

Table 3-1 - Synopsis of the vaccination trials detailing survival percentages. Represented are the main observations from the three vaccination trials carried out in this study. Trials 1 and 2 were carried out using the same batch (Batch 1) with gel concentration used at 4% w/v. Trial 3 was carried out using gel batch 2 at a different concentration (10% w/v for Gel 3 and 6% w/v for Gel 4). Trial 2 was carried out using mice bred in-house while trials 1 and 3 used mice purchased from InVivos. Age and sex of mice is the same in all trials. Detailed are the numbers of vaccinated, surviving and dead mice for each adjuvant in each trial. % of Survival refers to number of surviving mice over total of vaccinated mice. Trial 3 included a further vaccination control group where mice were vaccinated with the MSP protein emulsified in alum. For details on the different concentrations of the thermogel adjuvants refer to **Vaccine preparation**.

Vaccination Trial	Vaccine formulation	Adjuvant concentration	Vaccinated	% Survival
1	MSP + CFA	1:1	5	80 %
	MSP + Gel 3 (Batch 1)	4% w/v	5	80 %
	MSP + Gel 4 (Batch 1)	4% w/v	5	60 %
2	MSP + CFA	1:1	5	80 %
	MSP + Gel 3 (Batch 1)	4% w/v	5	0 %
	MSP + Gel 4 (Batch 1)	4% w/v	5	0 %
3	MSP + CFA	1:1	5	100 %
	MSP + Gel 3 (Batch 2)	10% w/v	5	60 %
	MSP + Gel 4 (Batch 2)	6% w/v	5	0 %
	MSP + Alum	1:1	5	60%

Interestingly, Trial 3 also did not yield the same results as previously observed and mice vaccinated with MSP + Gel 4 did not survive the challenge although two of the mice survived until day 21 and 24 respectively (Figure 3-8h). Mice vaccinated with MSP + CFA maintained their consistency throughout all the trials and, although there were some small variabilities in peak parasitemia, with Trial 2 showing higher and more variable parasitemia levels (median = 17.9%, mean $36.5 \pm 37\%$) between mice, survival was at least 80% for all trials. As for mice vaccinated with MSP + Alum (Figure 3-7a), parasitemia and survival curves present

comparable trend, peak and duration to those observed for mice vaccinated with thermogels 3 and 4 in trial 1 and thermogel 3 in trial 3. In the test group, four out of the five challenged mice reached peak parasitemia above 45% (average $62.3\% \pm 15.6\%$) and only one mouse showed low levels of parasitemia (1.3% peak parasitemia). High parasitemia on two of the three surviving mice persisted until days 16 to 18 post infection after which complete clearance of the parasite was observed. Similar to previously observed, all mice vaccinated with adjuvant control died between days 7 and 12 post-challenge (Figure 3-7b).

Interesting point of note is the similarity in mouse survival and parasitemia curves observed between the thermogels and alum (Figure 3-2, Figure 3-8, Figure 3-7). As vaccinations using MSP1-19 using alum as adjuvant have been shown to protect mice against *P. yoelii* challenge (Daly & Long 1996), we expected a much stronger level of protection than the one observed.

Figure 3-7 – Vaccination with MSP emulsified in Alum yields similar protection to vaccination with thermogels upon lethal PyYM challenge. Represented are the individual parasitemia curves for mice vaccinated with **a)** MSP + Alum (n=5) and **b)** respective control group Alum (n=5) as well as **c)** mortality curves for both groups.

Figure 3-8 – Different vaccination trials show variances in survival to challenge when using the thermogels as adjuvants. Represented are the individual parasitemia curves contrasting vaccinated groups in Trial 2 (gel batch 1) **a)** MSP + CFA (n=5), **d)** MSP + Gel (n=5), **g)** MSP + Gel 4 (n=5) and those in Trial 3 (gel batch 2) **b)** MSP + CFA (n=5), **e)** MSP + Gel 3 (n=5), **h)** MSP + Gel 4 (n=5). Mortality curves comparing both trials for each adjuvant are represented **c)**, **i)** and **f)**. Log rank (Mantel-Cox) test did not indicate significant difference between curves (Gel 3 $p=0.0802$; Gel 4 $p=0.0545$; CFA $p=0.2636$)

Comparing the individual antibody titers after boost 2 for IgG1 (the main antibody detected after vaccination) and IgG2a, shown in other studies to be induced in vaccination with MSP1-19 and to play a role in protection (White et al. 1991, Ling et al. 1997) revealed some differences (Figure 3-9, Table 3-2).

Figure 3-9 – Comparing antibody titers after vaccination reveals differences between trials.

Comparison of individual antibody titers after boost 2 for two key antibodies **a) IgG1** **b) IgG2a** for all three vaccination trials. Values represent individual titers for each mouse ($n=5$) in each group (MSP + CFA in blue, MSP + Gel 3 in red, MSP + Gel 4 in green, MSP + Alum in grey) tested against PyYM lysate. MSP + Alum trial was concomitant with Trial 3. No statistically significant difference was found between MSP + Alum and trial 3 samples. Endpoint titer was defined as the highest dilution of serum at which the final OD was greater than the mean of three naïve sera plus 3 times the standard deviation. All dilutions were tested in duplicated and results shown are expressed as average $\log_{10}$ values. Bars represent the standard deviation of each group. The dotted line represents the threshold of detection corresponding to 1/120 serum dilution, the lowest dilution used in this trial. Statistical analysis was carried out using a Kruskal-Wallis test with Dunn's post correction (** $p<0.01$).

Table 3-2 – Comparison of mean IgG1 and IgG2a antibody titers observed between subunit vaccination trials. Values represent $\log_{10}$ mean $\pm$ standard deviation (top) and mean $\pm$ standard deviation (bottom) for individual antibody titers after boost 2. Average of 5 mice in each group (MSP + CFA, MSP + Gel 3, MSP + Gel 4, MSP + Alum) tested against PyYM lysate. Endpoint titer was defined as the highest dilution of serum at which the final OD was greater than the mean of three naïve sera plus 3 times the standard deviation. All dilutions were tested in duplicated and results shown are expressed as average $\log_{10}$ values (top) or antilog values (bottom). //// - not present in trial

	IgG1			IgG2a		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
MSP + CFA	5.65 $\pm$ 0.20	4.77 $\pm$ 0.62	5.12 $\pm$ 0.68	4.12 $\pm$ 0.26	3.30 $\pm$ 0.51	3.14 $\pm$ 0.85
MSP + Gel 3	4.34 $\pm$ 0.57	3.14 $\pm$ 0.78	4.97 $\pm$ 0.26	2.44 $\pm$ 0.47	0.56 $\pm$ 1.27	2.90 $\pm$ 3.25
MSP + Gel 4	5.00 $\pm$ 0.58	4.40 $\pm$ 0.76	4.49 $\pm$ 0.95	2.55 $\pm$ 1.53	0.96 $\pm$ 1.33	0.67 $\pm$ 2.44
MSP + Alum	////	////	4.83 $\pm$ 0.41	////	////	2.35 $\pm$ 2.70
MSP + CFA	479746 $\pm$ 215246	115537 $\pm$ 126723	296230 $\pm$ 356161	15009 $\pm$ 7452	3282 $\pm$ 3546	3791 $\pm$ 4034
MSP + Gel 3	39078 $\pm$ 38572	6622 $\pm$ 13114	104448 $\pm$ 41215	437.6 $\pm$ 450.2	141.8 $\pm$ 314.8	2098 $\pm$ 1366
MSP + Gel 4	199708 $\pm$ 222628	82698 $\pm$ 139274	130944 $\pm$ 207344	2498 $\pm$ 3520	125.1 $\pm$ 214.5	383.3 $\pm$ 492.7
MSP + Alum	////	////	85494 $\pm$ 47278	////	////	549.3 $\pm$ 306.7

IgG1 titers (Table 3-2) for mice vaccinated with MSP + CFA were, on average, over 4 times higher in Trial 1 than in Trial 2 and over 1.5 times higher in Trial 1 than in Trial 3, which could explain the difference in parasitemia levels observed in Figure 3-8. Mice vaccinated with MSP + Gel 3 exhibited IgG1 levels, on average, almost 6 times higher on Trial 1 than on Trial 2 but lower than on Trial 3 (2.6 times less). Mice vaccinated with MSP + Gel 4 had IgG1 titers over 2.4 times those observed in Trial 2 and over 1.5 times those observed in Trial 3 which could explain the differences in survival (Figure 3-2, Figure 3-8) between trials. One interesting point of note for mice vaccinated with MSP + Gel 4 is that, contrary to mice in the other groups, the antibody titers for individual mice after Trial 1 are less clustered together (higher standard deviation in relation to mean) and exhibiting more spread which is indicative of higher differences within the group. On average, IgG1 titers for MSP + Alum, were the lowest observed when compared with other Trial 3 titers. Titers were almost 3.5 times lower than those for MSP + CFA, 1.2 times lower than those for MSP + Gel 3 and 1.5 times lower than

those for MSP + Gel 4. Interestingly, although the average IgG1 titers for MSP + Alum are lower than MSP + Gel 4, mean values for MSP + Alum (100781) are actually higher than those for MSP + Gel 4 (30720) indicating that overall, this vaccination group is composed of mice centered around higher titers (more consistent results).

Regarding IgG2a, antibody titers (Table 3-2) for mice vaccinated with MSP + CFA were, on average, 4.6 times higher between Trial 1 and Trial 2 and 4 times higher in Trial 1 than on Trial 3. This differs from the results observed for the IgG1 titers where Trials 1 and 3 showed more similar results. As for mice vaccinated with MSP + Gel 3, titers were 3 times higher in Trial 1 than in Trial 2 but 4.8 times lower than in Trial 3. This is more consistent with the IgG1 titers observed for this group. MSP + Gel 4 on the other hand, exhibited antibody titers 20 times higher in Trial 1 than in Trial 2 and 6.5 times higher than in Trial 3; a much greater difference than that observed for IgG1. The MSP + Alum group displayed IgG2a titers that were 6.9 times, 3.8 times lower than CFA and Gel 3 groups, respectively. Titers were 1.5 times higher than for Gel 4 group.

Interestingly, the only statistically significant difference observed was between Gel 3 mice in Trials 2 and 3 which can mean that either the vaccinated groups are too low to accurately represent meaningful differences in titers or the difference in protection cannot be explained by antibody titers measured this way.

Pooling together serum titers measured for all vaccination trials for each adjuvant and contrasting them with mouse survival status (Figure 3-10) revealed statistically significant differences in both IgG1 and IgG2a titers between mice that survived and mice that died but only in groups vaccinated with MSP + Gel 4. While both CFA and Alum vaccinated groups don't have enough representation for an accurate evaluation, the lack in measurable differences

observed in the Gel 3 group brings to question whether, in this group, antibody titers correlate with survival.

Figure 3-10 – Comparing pooled antibody titers against PyYM lysate reveals differences in survival status only in mice vaccinated with MSP + Gel 4. Comparison of pooled antibody titers after boost 2 for two key antibodies **a) IgG1 b) IgG2a** for all three vaccination trials. Values from all vaccination trials have been clustered according to whether mice survived (closed circle) or succumbed (cross) to challenge. MSP + CFA in blue, MSP + Gel 3 in red, MSP + Gel 4 in green, $n=15$ in each group; and MSP + Alum in grey, $n=5$. Endpoint titer was defined as the highest dilution of serum at which the final OD was greater than the mean of three naïve sera plus 3 times the standard deviation. All dilutions were tested in duplicated and results shown are expressed as average $\log_{10}$ values for each mouse. Bars represent the standard deviation of each group. The dotted line represents the threshold of detection corresponding to 1/120 serum dilution, the lowest dilution used in ELISAs. Statistical analysis was carried out using Unpaired t test with Welch's correction (* $p<0.1$, ** $p<0.01$).

As the trial with the highest antibody titers and survival rates overall, Trial 1 was considered the most successful. The following assays were carried to characterize functional characteristics of antibody response that could explain the observed differences in the best-case scenario.

3.1.4 Invasion inhibition assay

Merozoite invasion of the RBC is an essential step in the asexual reproduction cycle of *P.* parasites during the blood-stage and a key factor in the onset and progression of Malaria disease symptoms. The glycosylphosphatidylinositol (GPI)-anchored merozoite surface protein 1 (MSP1) and more specifically, its C-terminal fragment MSP1-19, has been shown time and again to play a critical role in invasion and antibodies targeting this protein are known to block invasion of the RBC by merozoites (Blackman *et al.* 1990, 1994)

As such, we decided to investigate if the antibodies induced in the vaccination could inhibit invasion of RBC *in vitro*. Figure 3-11 illustrates a simplified depiction of the invasion assay protocol. Simply put, red blood cells infected with PyYM schizonts are incubated together with red blood cells stained with the fluorescent dye, CFSE. When the schizonts burst and release the merozoites, these will invade the CFSE-stained RBC. After a 14-hour incubation, the majority of the schizonts have burst and invaded RBC should exhibit at least the early stages of infection, namely ring forms. As RBC do not contain nuclei, staining the CFSE-positive cells with the DNA dye Hoechst allows the differentiation between cells that have been invaded by the parasite from those that have not. Efficiency of invasion is determined then by the percentage of invaded CFSE-stained RBC after the incubation period minus the percentage of invaded CFSE-stained RBC at the beginning of the trial (T=0h).

Figure 3-11 - Schematic flowchart of the invasion assay protocol with representative flow-cytometry dot-plots. At T0h, red blood cells containing *P.yoelii* YM schizonts (iRBC inoculum) are cultured with carboxyfluorescein succinimidyl ester-stained RBC (CFSE⁺ RBC target). After a 14-hour incubation period, CFSE⁺RBC invaded by the parasite are determined by Hoechst stain of the parasite DNA inside the RBC. Invasion efficiency is determined as the percentage of invaded CFSE⁺RBC at T14h minus the percentage of invaded CFSE⁺RBC at T0h.

The inhibition of invasion can be estimated using the invasion efficiency assay described in Figure 3-11 but adding mouse sera at the beginning of the trial. Inhibition can thus occur by one of two forms, either the antibodies in the sera prevent the schizonts from bursting and releasing the merozoites or they prevent the merozoites from entering the new RBC. Either way, if the sera collected after boost 2 in the previously vaccinated mice contains any antibodies that inhibit invasion, the assay will show a significant difference between the pooled sera from the vaccinated mice and their respective controls under the same conditions. We decided to test the pooled sera at 3 serial dilutions 1/30, 1/120 and 1/480. As a positive control for inhibition we used E64 at 100 μ M as it inhibits schizont rupture. Inhibition of invasion values for wells incubated with E64 were above 90%.

Inhibition of invasion assay results are depicted in Figure 3-12.

Figure 3-12 – Pooled sera from vaccination have low inhibitory capacity of invasion *in vitro*. Inhibition efficiency of pooled sera from MSP + CFA (solid blue), MSP + Gel 3 (solid red) and MSP + Gel 4 (solid green) mice ($n=5$ each) collected after boost 2. Results depicted represent the average efficiency of inhibition calculated in 5 wells (data pooled from 2 independent experiments) at 3 serum dilutions (1/30, 1/120 and 1/480). Efficiency was calculated using the average invasion efficiency of naïve serum at 1/480 dilution using the following formula: $\text{Inhibition efficiency} = 100 - [(\% \text{ invaded CFSE}^+ \text{ RBC with test serum}) / (\% \text{ invaded CFSE}^+ \text{ RBC with naive serum diluted 1/480})] \times 100$. Above the solid bars is the statistical significance between vaccinated group and respective control group at the same dilution. Data was analyzed using two-tailed paired T test (* $p < 0.05$, ** $p < 0.01$, ns not significant). Threshold was arbitrarily set as the average of the inhibition efficiency of all control groups (CFA, Gel 3, Gel 4) at the lowest serum dilution. Threshold value – 7.31%. Average inhibition of invasion in samples incubated with E64 was $93.5\% \pm 0.7$. Mean inhibition values and standard deviation are represented in supplementary Table S1.

As is evident above (Figure 3-12), vaccinated sera do not exhibit high inhibition activity *in vitro*. The highest level of inhibition observed, situated around 30%, was with the pooled sera of mice vaccinated with MSP + Gel 4 (solid green). Of note is also the fact that sera from the control group vaccinated only with Gel 4 (green line) exhibit inhibition of invasion levels similar to those observed for mice vaccinated with MSP in CFA or Gel 3. Statistical differences between test groups and their respective controls at the same concentration showed low

significance although variability between wells was quite high. The only exception was for the mice vaccinated with MSP + Gel 4 mice at the highest serum concentration. All together these results do not explain the differences in survival and parasitemia curves observed in Figure 3-2.

3.1.5 Phagocytosis assay

As the MSP1-19 protein used in vaccination is a merozoite surface protein, we decided to isolate merozoites to test whether the antibodies induced by vaccination can opsonize the parasite and induce phagocytosis *in vitro*. The employed method for isolation of the merozoites was like that employed for the phagocytosis assay in that the filtrate was suspended on a differential Percoll column to isolate the late stage parasites. As seen in Figure 3-13a, the top two layers of the column contained schizont-stage parasites and were collected for further processing to maximize the final merozoite yield. The pellet (bottom layer) contained only non-infected RBC and early stage parasite forms and was discarded. The collected layers were then stained with CFSE and schizonts were ruptured by forcing the suspension through a 1.2 μm membrane. Resulting fraction, when observed in thin smear with Giemsa stain, showed that the resultant filtrate contained merozoites, some unburst cells and high concentration of debris and hemozoin. Since free hemozoin has been shown to form aggregates with merozoites and interfere with phagocytosis (Hill *et al.* 2014) the suspension was purified by passage through a magnetic MACS filter. The final suspension (Figure 3-13b) showed some unburst cells, merozoites and still some hemozoin although at a much-reduced concentration from before. Although further filtration might have yielded a more purified result, it would come at a cost in merozoite yield so we decided to maintain the current number of purification steps.

Figure 3-13 – Giemsa thin smears of the some key steps in the isolation of the merozoites for the phagocytosis assay. a) Percoll gradient column separation of PyYM parasites shows presence of schizonts in top and middle layers when observed with Giemsa stain **b)** Final merozoite suspension shows presence of some unburst early stage parasite forms (blue arrows) with a majority of merozoites (red arrows). Filtration through MACS filter removed most of the hemozoin, but some still remains (yellow arrow).

After successful isolation of the merozoites we decided to test what the optimal merozoite to phagocyte ratio would be to use in the evaluation of the vaccinated mouse sera. Keeping the highest concentration of serum constant (1/50) we incubated Raw264.7 cells with increasing numbers of merozoites (see Figure 3-14a). Tests were performed using two different hyperimmune sera in comparison with naïve serum. The hyperimmune sera were collected from a BALB/cJ mouse vaccinated with MSP + Gel4 (HyperImmune A) and a BALB/cJ mouse vaccinated with MSP + Gel 3 (HyperImmune B) that survived the challenge with PyYM and were subsequently re-challenged with the same parasite, at the same dose. Both mice survived the re-challenge and showed lower parasitemia and faster recovery rate (*data not shown*). One day after parasitemia levels were below detection limits both mice were terminally bled, and the serum stored at -20°C to use as controls. As can be seen in Figure 3-14a, the percentage of Raw264.7 cells that phagocytose merozoites increases concurrently with the ratio of merozoite to phagocyte. This finding is valid for both hyperimmune sera and for naïve sera where at the 100:1 ratio, differences between opsonizing sera are very low

(93% for Naïve sera versus 99.3% and 99.1% for HyperImmune A and B, respectively). The average percentage of cells positive for phagocytosis with naïve sera is $93.0\% \pm 7.139$, compared to $97.93\% \pm 2.454$ and $98.87\% \pm 4.131$ for HyperImmune A and HyperImmune B (Figure 3-14a). Taking into account the limited yield in merozoite numbers and the significant difference between the hyperimmune sera and naïve sera at a 10:1 merozoite to phagocyte ratio, this was the ratio selected to evaluate the opsonizing potential of the vaccinated sera. As can be seen in Figure 3-14b, at the chosen ratio, the sera did not show any difference either between themselves or to the naïve sera at the majority of the dilutions. The graph represented depicts the values registered when opsonizing merozoites with sera at three different dilutions 1/100, 1/500 and 1/5000. The decrease in the opsonization effect as serum dilution increases is clearly visible in the positive control (▲) and naïve groups (X) but has a smaller effect in the vaccinated groups (●). Similar to the invasion inhibition studies, the induced effect is quite low in all mice and does not explain the differences observed in parasitemia curves observed in Figure 3-2.

To examine whether CD4⁺ T cells contribute to the survival of the mice in our vaccination model we decided to deplete the CD4⁺ T cells at the beginning of the challenge. Groups of 5 mice were vaccinated as before and on day 0, 2 and 4 post challenge they were either administered an anti-CD4⁺ T cell depletion antibody or an isotype control. Non vaccinated mice were challenged in conjunction with the vaccinated groups and underwent the same procedure. Taking into account that no mice survived challenge in vaccinations with gel 4 using the latest batch, a decision was made to only focus on thermogel 3.

As seen in Figure 3-15, similar to results seen in trial 2 vaccinations, the isotype control group for mice vaccinated with MSP + Gel 3 did not survive the challenge and parasitemia and survival curves show no difference between the CD4-depleted mice and respective controls or even the naïve mice. Within these groups all the mice ended up dying of hyperparasitemia between days 9 and 14 making it impossible to infer what role, if any, CD4⁺ T cells play in mice vaccinated with MSP + Gel 3. The mice vaccinated with MSP + CFA, on the other hand, showed a clear distinction between the CD4-depleted and the isotype control group (Figure 3-15g-i). Firstly though, it is important to note that, in this trial, isotype control mice vaccinated with MSP + CFA exhibited higher parasitemia values, on average, than previous trials (median= 59.5%, average $44.9\% \pm 38.2\%$). This leads us to believe that, the depletion protocol employed here might have negatively impacted the immune response of the mice leading to higher level parasitemia in all the mice. This phenomenon might be the reason why the MSP + Gel 3 group did not survive this trial.

Comparing the CD4⁺ T cell depleted group to its respective control (Figure 3-15), we observed that, although mice reached the first peak in parasitemia around the same time (day 10-12 post challenge), four out of the five CD4-depleted mice were unable to clear infection and maintained high parasitemia values until all mice succumbed of hyperparasitemia (above 80%) between days 25 and 45. The one surviving mouse in this group, never reached 1%

parasitemia, meaning that either initial antibody levels were able to control the parasite before it multiplied exponentially or this result is a product of error during the infection process resulting in what is essentially a failed infection.

Figure 3-15 - Parasitemia and survival curves for $CD4^+$ T cell depletion assay. Represented are the parasitemia curves after PyYM challenge in $CD4^+$ T cell depleted mice b), e), h) and respective isotype controls a), d), g). Groups of 5 mice each were either not vaccinated (black graphs); vaccinated with MSP + Gel 3 (red graphs) or vaccinated with MSP + CFA (blue graphs) before being challenged with PyYM i.p. Survival curves contrast $CD4^+$ T cell depleted mice and corresponding isotype control.

When looking more closely at the progression of the infection in these two groups we can clearly see that the parasitemia values are quite similar in the beginning of the infection

(Figure 3-16) and from day 15 they start to differentiate as the isotype control group is able to control infection while the CD4-depleted group is not.

Figure 3-16 – Contrasting parasitemia levels with and without CD4⁺ T cells in MSP + CFA vaccinated mice during PyYM challenge. Isotype control mice (given IgG isotype control) and mice depleted of CD4⁺ T cells in the first four days post challenge (in open circles) show very similar parasitemia levels until days 10 to 15 post PyYM challenge. After day 15 parasitemia levels start to differentiate as isotype control mice (n=5) start clearing parasites but CD4-depleted mice (n=4) do not. Represented are individual levels of parasitemia at selected days post challenge. One mouse from CD4-depletion group was excluded as its parasitemia levels never reached 1%. Statistics done using unpaired Mann-Whitney test (*p<0.05).

In the CD4⁺ depleted group, parasitemia just continues increasing and mice eventually reach parasitemia levels above 80%. Similar findings are described by Daly and Long (Daly & Long 1995a). From these findings we can conclude that, in our vaccine model using the CFA/IFA adjuvant, CD4⁺ T cells play a role in control and clearance of the parasite towards the later stages of the infection.

3.2 Discussion

In this chapter we looked at the potential use of two thermogel polymers developed by IMRE institute (A*STAR) as vaccine adjuvants in protection against malaria blood-stage challenge. We chose a well-studied immunodominant parasite blood-stage protein, MSP1-19, and an immunization method that has been shown to induce strong antibody-mediated immunity (Daly & Long 1993, Ling *et al.* 1997, Rénia *et al.* 1997). MSP1-19 was then emulsified into Freund's adjuvant, alum (alhydrogel) or either of the gels at their lowest gelation concentration and three vaccine doses were given to groups of 5 BALB/cJ mice. Each antigen/adjuvant combination was accompanied by a group vaccinated in the same way but only using adjuvant. Mice were then subjected to lethal challenge with PyYM, a murine parasite well characterized for rapid growth in the blood stage resulting in severe and lethal anemia between 6 and 12 days after challenge (Claser *et al.* 2017).

Initial results (Trial 1) were promising, 80% of the mice vaccinated with the thermogel 3 and 60% of the mice vaccinated with thermogel 4 survived lethal challenge and were able to completely clear the parasite within 30 days of challenge. Yet, despite the relatively high survival rates in both these groups, peripheral parasitemia in the majority of the mice reached dangerously high levels (above 40%) and mice displayed signs of severe anemia. Peripheral parasitemia peaked at around day 10 post challenge and remained high until day 23-26 after which it quickly subsided until full clearance. This result, when contrasted with mice vaccinated with the same protein emulsified in Freund's adjuvant (where the highest observed parasitemia for surviving mice was around 1% in our first trial) showed that the protection elicited by these gels was less effective. In this control group, peak parasitemia was reached at day 9 and subsided by day 12 with only the occasional recrudescence. Control groups vaccinated with just the adjuvants succumbed to infection between 6 and 10 days after challenge exhibiting high parasitemia (above 50%).

Characterization of the induced antibody isotypes revealed that all groups vaccinated with the protein produced MSP1-19 specific capable of recognizing the protein both when expressed by a heterologous system using CHO cells and in fixed parasite lysate. A comparison of the antibody titers in blood collected after boost 1 and boost 2 showed that only the group vaccinated with MSP1-19 in Freund's adjuvant (MSP + CFA) had any detectable IgM antibodies against the parasite and that these levels decreased between the first and second boosts. On the other hand, a marked increase between boost 1 and boost 2 in both IgG1 and IgG2a was detectable in all groups, with IgG1 antibodies being the most predominant. MSP+CFA mice showed the highest antibody titers in all subtypes at all points of comparison followed by Gel 4 and finally Gel 3. Induction of IgG1 and IgG2a has been previously described in when using this particular protein in subunit vaccination in mice (Hirunpetcharat *et al.* 1997, Ling *et al.* 1997). Moreover, correlation between high IgG1 and IgG2a titers against this protein and survival to PyYM challenge has been repeatedly established (Hirunpetcharat *et al.* 1997, 1998, Jeamwattanalert *et al.* 2007). Data from our successive trials seems to support these findings. Although no specific titer threshold for survival was determined, mice vaccinated with MSP + CFA showed higher parasitemia in the trials where the antibody titers were lowest even though mice still survived the challenge. As for mice vaccinated with the thermogels, mice in these groups showed lower antibody titers and higher parasitemia in all trials. This fact is clearly demonstrated by the decrease in antibody titers between Trial 1 and Trial 2 whereupon all mice vaccinated with the thermogels succumbed to challenge. A more detailed look at the antibody titers in mice vaccinated with MSP + Gel 4 offers a good example to support this hypothesis. In Trial 1, where this group exhibits the highest titers for IgG1 and IgG2a, three out the five mice survived the challenge. In Trial 2, where these mice succumbed to challenge within the first ten days, the group exhibited 2.4 times lower IgG1 titers and 20 times lower IgG2a titers. In Trial 3, although all the mice succumbed to the challenge, two mice survived until day 21 post challenge. Antibody titers were 1.5 times lower for IgG1 and

6.5 times lower for IgG2a titers (Table 3-2). Pooling antibody titers from all trials (Figure 3-10) revealed a statistically significant difference between survival and death associated with antibody titers in both isotypes. In contrast, Gel 3 mice showed no such difference for either isotype bringing into question whether antibody titers measured here can be used as correlates of protection for vaccinations using this gel.

Furthermore, in the first trial, mice vaccinated with MSP + Gel 4 showed over 5 times higher IgG1 and IgG2a antibody titers than MSP + Gel 3 although one less mouse survived (3 out of 5 versus 4 out of 5, respectively; Figure 3-2).

If it was just a question of numbers, we would expect that above a certain threshold, mice should exhibit similar survival curves regardless of adjuvant used. Nevertheless, it is interesting to point out that, specifically for mice vaccinated with Gel 4, the trials where the mice did not survive were those where the average IgG2a titers were lowest. This finding points to the possibility that IgG2a might play an important role in survival of mice vaccinated with Gel 4. Similar finding was shown by White and colleagues (White *et al.* 1991) whereupon they determined that IgG2a played an important role in controlling parasitemia in lethal *P. yoelii* challenge.

This point is further reinforced by the inclusion of the alum vaccination group in Trial 3. Comparison of antibody titers within the trial shows that this group exhibits, on average, lower IgG1 titers than the other groups although parasitemia curves and mortality rate are similar to those observed for Gel 3 in this trial (Trial Figure 3-8, Figure 3-7). As with Gel 3, comparison of average antibody titers between Alum and Gel 4 mice reveal that, on average, Gel 4 have lower IgG2a titers.

Taken together, these results point to the possibility that antibody fine specificity or function could play key roles depending on type of adjuvant used and that antibody subtypes, while

similarly induced, might have different protective effects depending on adjuvant used. Information that is not determined by ELISA alone.

The role by which antibodies targeting the MSP1-19 protein protect against infection is not clear. Passive transfer studies (Daly & Long 1995a, Ling *et al.* 1997, Spencer Valero *et al.* 1998) and vaccination studies (Daly & Long 1996, Rotman *et al.* 1998, Hirunpetcharat *et al.* 2003, Jeamwattanaalert *et al.* 2007) in mice challenged with *P. yoelii* point to inhibition of invasion as the main mechanism while studies in *P. berghei* and humans point to opsonization as an important immune mediator (Hill *et al.* 2017).

For this purpose, we investigated the potential for the sera collected from mice to inhibit invasion *in vitro* (Figure 3-12) and to induce phagocytosis in murine macrophages (Figure 3-14).

Results for the inhibition studies did not match up with our parasitemia curves (Figure 3-2). As the group showing the lowest levels of parasitemia and the highest titers, MSP + CFA should exhibit the highest inhibition of invasion in our studies *in vitro*. This was not the case. In our studies, mice vaccinated with MSP + Gel 4 showed higher inhibition of invasion at the highest concentration tested although similarly high inhibition was also observed in its respective control group. Diluting the sera from 1/30 to 1/120 abrogated this effect in the control group while the vaccinated group still exhibited relatively higher inhibition. While it is possible that protection induced by our vaccination model is mediated by mechanisms other than inhibition of invasion, these baffling results should be further clarified before we can confidently assert this.

Regarding the phagocytosis assay, while there was a clear dilution effect in induced phagocytosis when using the Hyperimmune serum a much smaller effect was observed in sera from our vaccinated mice. As Figure 3-14a clearly demonstrates, the ratio of merozoite to

phagocyte can have a clear impact on the level of phagocytosis regardless of the sera used. As such, taking into account the very small difference observed between the test sera and naïve sera it is possible that the 10:1 ratio might have been too low to show any clear differences, increasing the ratio to 25:1 or 50:1 might yield more discriminating results.

To determine whether protection was mediated solely through antibodies in our model, we depleted CD4⁺ T cells in mice vaccinated with MSP + CFA and MSP + Gel 3. Both isotype and antibody-depleted mice in the MSP + Gel 3 group did not survive challenge, exhibiting similar parasitemia curves to the naïve mice used as control. This effect might have been due to the effects of bleeding the mice during challenge to control for CD4⁺ T cell depletion. While attempts were made to keep bleeding to a minimum, multiple infections with this parasite in our laboratory (data not shown) have demonstrated that the slightest intervention can sometimes result in increased parasitemia and mouse death both in C57BL/6 and BALB/c mice. The fact that parasitemia levels were the highest in this trial for MSP + CFA mice seems to support this. Regarding CD4⁺ T cell depletion, the MSP + CFA group showed a differential response between the CD4⁺ T cell-depleted mice and the isotype control group. In the former, parasitemia just continued increasing and mice eventually reached parasitemia levels above 80%, eventually succumbing to the challenge 30 days later. Similar findings were described by Daly and Long (Daly & Long 1995a) and Hirunpetcharat (Hirunpetcharat *et al.* 1997, 1999) whereupon it was determined that unless there is an overwhelming quantity of inhibiting antibodies present in the sera, there is a requirement for involvement of CD4⁺T cells towards later stages of infection possibly through cytokine release. From these findings we can conclude that, in our vaccine model using the CFA/IFA adjuvant, CD4⁺ T cells play a role in control and clearance of the parasite towards the later stages of the infection.

One important factor at play in our vaccination model is the consistency of the gels at the time of vaccination. Trial 2's lower antibody results might be explained by hydrolytic

degradation of our original batch of gels after long term storage (we were informed after we started our experiments that these gels had a shelf-life of 6 months). One important fact to mention is that mice in Trial 2, were acquired in-house and not ordered from InVivos. While the genetic background is the same, as are both age and sex, and mice were kept in exactly the same conditions; this factor should be taken into account. Hydrolytic degradation of the gels most certainly played an important role in the difference in immune response observed in Trial 2 mice. Nevertheless, the higher parasitemia levels and lower antibody titers observed for control mice vaccinated with CFA in Trial 2 could be indicative of influences stemming from the different origins of the mice as alterations in the living conditions of mice can affect the cellular composition of the innate and adaptive immune systems (Beura *et al.* 2016).

As such we commissioned a new batch of the same gels for further studies and restricted experiments to InVivos mice. Unfortunately, while the new batch of Gel 3 exhibited similar results to our successful first trial, we were unable to obtain similar results with Gel 4 and none of the mice survived the challenge. One interesting key point to note was that we had to increase the critical gelation concentration at which we used gels from batch 2 in order to achieve the same gelation results we observed in the beginning. This indicates an inconsistency between batches and, taking into account the high parasitemia we observed in mice vaccinated with the thermogels, could be the difference between survival and death. Unfortunately, lack of understanding of the *in vivo* performance of these gels (Loh *et al.* 2019) means that we without further study we cannot establish what impact differences in concentration between batches of the same gels have in the establishment of protective immunity. What particular effect this change can elicit in antibody titers, fine specificity and overall protection is unclear and requires further study.

One final point lies with our vaccination protocol. In our trials, our vaccination protocol consisted of three vaccine doses given over the course of one month followed by challenge

with the parasite at the end of the month. While, the majority of our mice vaccinated with MSP + CFA survived, we never really achieved sterile immunity and parasitemia levels varied between trials (reaching as high as 30% in some cases). Trials by Hirunpetcharat and colleagues (Hirunpetcharat *et al.* 1997) have demonstrated that the number of vaccines and method of administration play a major role in the final results, namely the induction of sterile immunity and other studies have shown that the site of immunization (s.c or i.p) can play a role in antibody production, as supported by the vaccination using alum (Near *et al.* 2002).

To this point, we were never able to establish what antibody titers must be reached to achieve full protection (sterile immunity) and that might have limited subsequent characterization of the collected serum. The other limitation was that we hypothesized the gels would behave similarly to Freund's adjuvant and within the same time frame. These gels are designed to form a mechanically stable matrix within the body that slowly releases protein or chemical of interest as it is broken down through hydrolysis. It is quite possible that the full dose of protein was not presented to the immune system and a full response was not given time to build up before challenge. Different composition of the gels also influences their hydrolytic degradation (Liow *et al.* 2017, Loh *et al.* 2019) bringing into question whether the release of the protein within the animal's body follows the same pattern between both gels and between batches. The dynamics of the protein release by these gels inside the host is a factor that should be better evaluated not only hydrolyzation of the gels within the host body, but also mechanical stress induced by movement. Considering the mice vaccinated in this study were both healthy and young, this factor should not be disregarded and deserves further study. Nevertheless, these gels show potential as vaccine adjuvants and should be studied further.

Chapter 4 - Results for studies of *C. albicans* R24 as a co-infection model for protection against malaria challenge in mice.

The second aim of this thesis focuses on studying the effects of inducing trained immunity in mice by use of a live attenuated strain of *Candida albicans*. This strain, named R24, was isolated by Dr. Norman Pavelka's group in SigN (Tso *et al.* 2018). Initial studies found that not only is this strain not pathogenic when mice are subjected to systemic challenge at levels proven to be lethal in wild-type *Candida albicans* (5×10^5 CFU per mouse; MacCallum & Odds 2005) but it induces broad-range, non-specific, T and B cell independent, short-lived protection against homologous and heterologous challenge. This mechanism is reminiscent of the previously described trained immunity (Netea *et al.* 2011) whereupon innate immune cells develop a memory-like mechanism (through epigenetic reprogramming) after an initial challenge that leads to an enhanced, non-specific response to a secondary stimulus.

The following chapters begin by characterizing the vaccination or priming model of *C. albicans* R24 in mice. We track the progress of i.v. injection of R24 in two mice models, C57BL/6J and BALB/cJ, by looking at clearance rates, tissue burdens and side effects. The later chapters look at the effect that priming with R24 has in protection against two lethal malaria parasites PyYM and PbA.

4.1 Characterization of intravenous administration of *C. albicans* R24 in BALB/cJ and C57BL/6J mice

4.1.1 Body weight changes in mice administered *C. albicans* R24

Weight loss has long been a marker of murine response to infectious agents (Olfert & Godson 2000, Ray *et al.* 2010, Trammell & Toth 2011) . A typical side effect of challenging mice intravenously with *C. albicans* is the drastic decrease in mouse body weight during the first days of infections. As can be seen in Figure 4-1 both BALB/cJ and C57BL/6J mice exhibit similar trend starting with a steady and rapid decrease in body weight from the time of I.V. administration of *C. albicans* R24 until day 7 when they reach peak bottom (C57BL/6J average weight $\approx$ -23.6% $\pm$ 7.2% ; BALB/cJ average weight $\approx$ -26% $\pm$ 6.5%). After that, C57BL/6J mice show faster body weight recovery, reaching basal levels around day 21 post R24 priming while BALB/cJ mice only reach basal levels around day 28 post R24. Naïve mice in both strains show normal increase in weight during the trial.

Figure 4-1 - Changes in body weight as percentages in relation to baseline weight in C57BL/6J and BALB/c mice administered 5×10^5 CFU of *C. albicans* R24 intravenously in contrast to naïve mice given intra-venous PBS at the same time. Mice are age and sex-matched. C57BL/6J mice ($n=15$ mice from 3 different experiments) were followed for 21 days and BALB/c mice ($n= 20$ mice from 2 separate experiments) followed for 28 days. Graphs depict individual curves with measurements taken every 2-3 days. a) C57BL/6J mice plus R24 b) C57BL/6J mice plus 200ul PBS c) BALB/cJ mice plus R24 d) BALB/cJ mice plus 200ul PBS.

4.1.2 Behavioral changes in infected mice (“Twitch”, “Twist” and “Helicoptering”)

During the course of *C. albicans* R24 infection, 3 distinct and unusual behaviors were detected in some of the mice. We named these “Helicoptering”, “Head Twist” and “Twitching”. “Helicoptering” consisted of the mice spinning in elliptical fashion when lifted and held by the tail for 20 sec, “Head twist” consisted of the mice exhibiting a prolonged and continuous shift

of the head angle towards one side with some mice exhibiting clear balance problems in response to this and “Twitching” was the characteristic by which some mice showed head spasms and were unable to maintain the head still, similar to muscle spasms exhibited by people with Parkinson’s disease. “Twitching” behavior was more noticeable when the mice moved around the cage but still discernible when the mice were standing still. None of these side-effect behaviors were lethal and the mice appeared otherwise healthy.

To document these behaviors, we devised a binary scoring system and every 2-3 days starting from R24 administration we assessed the mice (Figure 4-2). As is clear from the figure, behaviors were only detected from day 10 post R24 administration but their incidence increased as time progressed.

The most common behavior was the “Head Twist” with over 40% of the mice afflicted by this condition towards the later endpoints of the study. Mice exhibiting this behavior displayed a clear head turn towards one side (left or right) and, although the head twist side could occasionally switch sides, it was a rare occurrence. The majority of the mice that exhibited “Head Twist” also exhibited “Helicoptering” and it is probable that “Helicoptering” was a side effect of the imbalance associated with the “Head Twist” phenotype although it was occasionally observed in mice with no other behaviors. The “Twitching” behavior was the least common and tended to appear isolated from the other symptoms. While a disturbing phenotype to observe it appeared to be restricted to the head and had no discernible impact on mouse balance or overall health.

Figure 4-2 - Increasing trend of mice exhibiting at least one of the 3 neurological symptoms (behaviors) observed after I.V. administration of *C. albicans* R24. A growing number of mice exhibited distinct and unusual behaviors consistent with inflammation and/or neurological damage to the central nervous system. The behaviors were termed “Head Twist” “Helicoptering” and “Twitching” and started appearing in both mouse strains only after day 10 post R24 injection with more and more mice showing one of the symptoms as the study progressed. Represented are the ratio of mice showing the specific symptom at that particular time point and the total mice. Mice may exhibit more than one symptom at each time point and although symptoms tend to be persistent if the behavior was not clearly observed, the mice were scored as not exhibiting the behavior at that time point. Results represent pooled data from 3 separate experiments for each strain and dotted lines are placed at key time-points: day 7 – peak weight loss; day 14 – midway point of weight recovery and start of symptoms on BALB/cJ mice; and day 21 – baseline recovery point for C57BL/6J. a) C57BL/6J mice (n=18 mice) were followed for 21 days b) BALB/cJ mice (n= 15 mice) followed for at least 28 days.

The date of onset of detectable behaviors was different between both mouse strains as BALB/cJ mice only started exhibiting changes from day 14 onwards instead of day 10 as observed for C57BL/6J mice. Comparing day 21, we observed that a similar percentage of BALB/cJ and C57BL/6J mice exhibit these behaviors at this time point but the trend points to an increase in ratio as time progresses. At day 28 over 50% of the BALB/cJ mice are showing one or more symptoms.

4.1.3 Time course of *C. albicans* R24 in C57BL/6J and BALB/cJ mice. Clearance from blood and tissue burden.

In order to determine the dynamics of *C. albicans* R24 in both mouse models we analyzed the tissue burdens at different time points after 5×10^5 CFU were injected into the tail vein of each mouse. As is clearly demonstrated in Figure 4-3, viable *C. albicans* cells are rapidly cleared from the bloodstream within the first 5 hours after injection and drop at least one $\log_{10}$ value within the first 20 min. Interestingly while C57BL/6J mice appear to clear the *Candida* at a much faster rate than BALB/cJ mice, although no statistically significant difference was found at timepoints T=5min and T=20min. After 5hrs, samples dropped to below detection levels and, except for the occasional CFU, remained at undetectable levels up to 28 days after injection.

Figure 4-3 – Rapid blood clearance of *Candida albicans* R24 in BALB/cJ and C57BL/6J mice after I.V. injection. C57BL/6J and BALB/cJ mice were injected with 5×10^5 CFU of *C. albicans* R24 (R24) via tail vein and 10 μ l of blood were collected via tail bleed at indicated timepoints (log 2 scale). Data represents values measured from 2 separate experiments for each mouse strain with pooled data from 8 BALB/c mice and 9 C57BL/6J mice. Dashed line indicates the limit of detection (defined as 1 CFU in 10 μ l of blood) and all zero values were arbitrarily changed to $1.5 \log_{10}$ for representation purposes. Above the graph are representative pictures of the colonies observed after 24hr incubation of blood collected at various timepoints (minutes, hours or days written underneath) in YEPDA agar plates. The inoculum picture corresponds to non-diluted plating of 10 μ l of the inoculum used. CFU – colony forming unit

When looking at the tissue burdens of R24 in individual organs of both mouse strains at three key timepoints (Figure 4-4) we observe a similar pattern. While all organs exhibit presence of viable *Candida* cells at day 7 there is a clear difference in the distribution of the *Candida* cells. Generally, kidneys exhibit the highest relative count per gram followed by the brain. This pattern is present in both mouse strains at all studied timepoints and, except for the slight drop in brain CFUs at Day 28 for BALB/c mice (Figure 4-4 f), burden values remain consistent for both these organs. As for the other organs, heart, liver, spleen and lungs we observe a decline in detected CFU values over time as they rapidly approach detection limits. At day 21 in C57BL/6J (Figure 4-4 c) mice, no *Candida* was detected in any of the plated lung samples

and only one liver sample was positive. Another point of notice is that, while consistently near the threshold of detection heart samples still showed positive detection. While all mice were exsanguinated via orbital bleed prior to organ harvest and no blood samples showed positive after plating at these timepoints it is possible that the resulting CFU could be resultant from viable cells circulating in the blood and not specifically in the heart.

Comparing BALB/cJ mice with C57BL/6J mice, organ burdens in BALB/cJ, similarly to what was observed in the blood (Figure 4-3), appear to be higher than burdens in C57BL/6J for the majority of the same organs at day 7. The only organ that shows no difference at day 7 (Figure 4-4 a and d) is the brain which seems to indicate that C57BL/6J have a harder time clearing the *Candida* from this organ than from other organs.

Figure 4-4 - Tissue burdens of *Candida albicans* R24 at 3 key timepoints after I.V. injection. Organs from C57BL/6J mice (n=5-9) and BALB/cJ mice (n=3) were collected at specific time points, macerated, plated at various dilutions in YEPDA plates and allowed to grow for 48hrs. Data shown represents estimated number of CFUs per gram of organ taking into account the average weight of the organs at that time point. **a)** C57BL/6J mice at day 7 post injection **b)** C57BL/6J mice at day 14 post injection **c)** C57BL/6J mice at day 21 post injection **d)** BALB/cJ mice at day 7 post injection **e)** BALB/cJ mice at day 14 post injection **f)** BALB/cJ mice at day 28 post injection. The grey dashed lines represent the threshold of detection for that particular organ. The threshold is calculated as the minimum CFU/g needed to detect 1 colony in 100 μ l of non-diluted organ suspension. This value is calculated based on the average weight measured for each organ and therefore varies depending on organ. Both kidneys were macerated together. For representation purposes, in days containing negative cultures, an arbitrary

number of 0.1 was added to all values for that day. Statistical analysis was carried out by ordinary one-way ANOVA with Tukey's correction test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Due to the high number of negative cultures, in days with organs showing null value points (zero CFUs), raw data was converted to $\text{Log}(x+1)$ before applying the statistical test. As most organs showed normal distribution for the majority of days, all data was assumed to be normally distributed. # refers to statistical significance when compared to kidneys. CFU – colony forming unit.

4.2 Attenuated *Candida albicans* R24 as co-infection model of protection against blood-stage *Plasmodium* sp.

4.2.1 Introduction

Previous studies using *C. albicans* R24 have demonstrated the ability of this attenuated strain to confer hosts a broad, cross-protective innate immunity against a wide range of pathogens (Tso *et al.* 2018). Based on the findings of Tso *et al.* we decided to investigate whether co-infection with *C. albicans* R24 can elicit protection against blood-stage malaria. Based on their published protocol, we administered R24 to 5-6-week-old mice by intravenous injection of 5×10^5 CFU via tail vein and allowed a recovery window of at least 14 days before challenging with the murine malaria parasites.

4.2.2 Priming with attenuated *Candida* sp. R24 has no impact on parasitemia levels of C57BL/6J mice infected with PyYM

In order to evaluate the impact of R24 on overall parasitemia levels and parasite clearance in a malaria blood-stage challenge, C57BL/6J mice were challenged with PyYM 14 days after R24 priming. PyYM is characterized by a rapid multiplication cycle in the blood easily reaching high parasitemia levels (above %50) within the first 7-9 days after challenge. While severe anemia is a given side effect of this hyperparasitemia, naïve C57BL/6J mice do not die from infection and recover from challenge 25-30 days later (Claser *et al.* 2017).

Figure 4-5 – Priming with *C. albicans* R24 has no impact on overall parasitemia levels or parasite clearance rates in C57BL/6J mice challenged with PyYM. a) C57BL/6J mice were challenged with PyYM 14 days after intravenous injection of R24 and mice were followed until complete clearance of the parasite (non-detectable parasitemia) around day 36 post challenge was observed **b)** Parasitemia curves are represented as the mean $\pm$ SD. Values at each timepoint were analyzed using Unpaired two-tailed t test (* $p < 0.05$ and ** $p < 0.01$). **c)** Mortality curves were analyzed by Log-rank (Mantel-Cox) test and no significance found.

As seen in Figure 4-5b, priming by R24 appears to have little to no detectable effect on overall PyYM parasitemia levels, multiplication rates or clearance in C57BL/6J mice. Except for a small difference in parasitemia levels at days 4 and 5 post challenge, both R24-primed mice and naïve mice exhibit similar parasitemia curves and reach the same high parasitemia around the same time (first peak at day 9-10) (Figure 4-5 b). Peak parasitemia for all but one naïve mouse was above 60% infected red blood cells and parasite clearance only started around day 17 with the majority of the mice showing levels below 0.5% parasitemia at day 25 post infection. Some of the R24-primed mice (4 out of 9) exhibited a small recrudescence around day 31 but by day 36 parasitemia levels were not detectable. It is important to point out that, although PyYM is normally non-lethal in C57BL/6J mice, four PyYM-infected mice and one R24-primed

mouse did not survive the challenge (Figure 4-5c). Two of the infected control mice and the one R24-primed mouse that died exhibited uncontrollable parasitemia (above 60%) at day 7 post challenge and eventually succumbed at days 8 and 9 (naïve mice) and day 14 (R24 mouse). The remaining two PyYM-challenged mice died at day 21 exhibiting similar parasitemia levels to the rest of the group.

4.2.3 Priming with attenuated *Candida sp. R24* does not protect BALB/cJ mice from lethal PyYM challenge

Co-infection studies with C57BL/6J mice and PyYM showed no difference in parasitemia levels and clearance rates between infected control and R24-primed mice yet some mice still succumbed to what was a supposedly non-lethal challenge. To investigate the potential effects of R24 in protection from lethal challenge we decided to repeat the previous challenge in BALB/cJ mice (Figure 4-6a). Contrary to C57BL/6J, BALB/cJ mice do not survive a PyYM challenge and mice succumb within 6-12 days after challenge exhibiting hyperparasitemia and severe anemia (Figure 4-6 b,c,d). BALB/cJ mice were thus challenged under the same conditions as those employed for C57BL/6J. As seen in Figure 4-6b, despite some small differences in parasitemia levels at day 5 post challenge, the overall trend is the same and all but one of the infected mice died at days 8 and 9. The exception was one R24-primed mouse that survived until day 27 post challenge. This mouse consistently experienced lower parasitemia levels than the other mice, never reaching 40% parasitemia, while the other mice died with 70-90% infected red blood cells.

Figure 4-6 - Priming with *C. albicans* R24 does not protect BALB/cJ mice from lethal Malaria challenge. **a)** BALB/cJ mice were administered 5×10^5 CFU of *C. albicans* R24 (R24) intravenously via tail

vein and subsequently challenged with a lethal dose of PyYM. In each mouse, 2×10^5 infected Red Blood Cells (iRBC) were injected intraperitoneally (i.p.) after **b**) 14 days, **c**) 28 days or **d**) 42 days and parasitemia followed from day 3 (**c**) & (**d**) or day 5 (**b**) post challenge. PyYM mice were injected 100-200 μ l of sterile PBS via tail vein instead of *C. albicans* R24 and challenged with the same iRBC dose. Next to each parasitemia curve is the corresponding survival curve for that challenge. Numbers in brackets represent the number of mice in each group and data are expressed as mean $\pm$ SD. Data used in (**c**) correspond to pooled data from two independent studies. Mortality curves were analyzed by Log-rank (Mantel-Cox) test and no significance found. Parasitemia values at each timepoint were analyzed using Unpaired two-tailed t test (* $p < 0.05$ and ** $p < 0.01$). Note: Mice with no detectable parasitemia until day 9 post-infection were excluded from the study.

Considering that all previous studies had been carried out in C57BL/6J mice and taking into account the differential response in terms of weight loss recovery (Figure 4-1), onset of neurological behaviors (Figure 4-2) and *C. albicans* tissue burdens (Figure 4-4) in BALB/cJ mice, we decided to gauge the effects of a longer recovery window. BALB/cJ mice were thus challenged with the same PyYM dose 28 days (Figure 4-6c) and 42 days (Figure 4-6d) post R24 priming.

As is demonstrated in the parasitemia curves, although there is a small difference in parasitemia levels during days 4-6 after challenge (R24-primed mice show lower parasitemia levels), parasitemia growth curves follow the same pattern as for day 14 and mice succumbed to challenge after 9 to 10 days.

4.2.4 Priming with attenuated *Candida* sp. R24 protects C57BL/6J mice from experimental cerebral malaria.

In Chapter 4.1 we demonstrated that *C. albicans* R24 is not only present in the brain of the mice, but tissue burdens remain high and, over time, a growing number of mice exhibit phenotypical traits (behaviors) associated with neurological inflammation. Taking these observations into account we decided to study the potential effects that R24 might have on an experimental cerebral malaria (ECM) model. PbA is a well-established murine model for

ECM. In C57BL/6J mice, an inoculation of PbA-infected red blood cells typically leads to over 70% of susceptible mice developing neurological symptoms (ataxia, paralysis, balance disorders, convulsions) at around day 5 and culminates in coma and death between days 6 and 12 (Rénia *et al.* 2012).

Following the previously employed protocol for C57BL/6J mice, we challenged R24-primed mice with PbA after a 14-day recovery period. Interestingly, while having no impact in overall parasitemia levels (as observed before with PyYM challenge), R24-primed mice survived well beyond the ECM window (6-12 days post challenge) only succumbing between days 24 to 26 of hyperparasitemia (Figure 4-7).

Figure 4-7 - Priming with *C. albicans* R24 protects mice from experimental cerebral malaria (ECM) in PbA challenge but has no effect on parasitemia levels. **a)** C57BL/6J mice were challenged with PbA 14 days after intravenous injection of R24 and mice were followed until death, 24 to 26 days post challenge. **b)** Peripheral parasitemia curves and **c)** mortality curves. Shaded area represents the experimental cerebral malaria time window. Parasitemia curves are represented as the mean $\pm$ SD and. Values at each timepoint were analyzed using Unpaired two-tailed *t* test and no significance found. Mortality curves were analyzed by Log-rank (Mantel-Cox) test (**** $p < 0.0001$). Data depicted are pooled from two independent experiments.

4.2.5 ECM protection due to reduced brain microvessel cross-presentation, not difference in parasite sequestration or number of CD8⁺ T cells

There are 3 key parameters necessary for ECM to occur: the parasite must sequester or adhere to the microvasculature of the brain (Baptista *et al.* 2010); there has to be a recruitment, migration and activation of parasite-specific CD8⁺ T cells to the brain (Shaw *et al.* 2015); and the endothelial cells of the brain microvasculature must cross present parasite epitopes to the CD8⁺ T cells in order to induce secretion of granzymes and perforins thus inducing disruption of the blood brain barrier (BBB) (Howland *et al.* 2013, 2015b).

Since the sequestration of iRBC in the brain is the primary trigger in the cascade of events resulting in ECM, we used ex-vivo bioluminescent imaging to assess parasite load in the brains of mice at day 7 post challenge, a point at which the majority of mice are exhibiting the classical signs of this disease. As the PbA used in these studies is luciferase-tagged, by injecting luciferin into the mice before extraction of the brain, we can measure the average radiance and thus assess the parasite load present in the brain.

As can be seen in Figure 4-8, R24-primed and naïve mice challenged with PbA showed no difference in the number of parasites sequestered in the brain microvasculature.

Figure 4-8 – Priming with *C. albicans* R24 does not affect parasite sequestration in the brain. a) Ex-vivo imaging of the brain of PbA-infected C57BL/6J mice on day 7 post challenge show no differences in parasite load. Representative pseudo-images of the naïve, PbA ($n=7$) and R24 + PbA ($n=6$) are depicted. **b)** Peripheral parasitemia values for the same mice depicted in a) show no differences in peripheral parasitemia. All values were analyzed using Unpaired two-tailed *t* test and no significance found.

As parasite sequestration showed no difference between our groups, we investigated whether the difference could lie in the CD8⁺ T cell population targeting the brain. Previous studies have shown that the presence of cytotoxic CD8⁺ T cells specific to blood-stage epitopes conserved in murine malaria parasites are a necessary component for the ensuing pathology in ECM (Howland *et al.* 2013, Poh *et al.* 2014).

As depicted on Figure 4-9, both groups challenged with PbA exhibit, at day 7, an increased number of activated, CD11a (LFA1⁺) CD8⁺ T cells and, although the R24 + PbA mice show a slightly higher number of CD8⁺ T cells in the brain, the number of CD8⁺ T cells targeting the *P. berghei*-derived Pb1 epitope (SQLLNAYL, from the GAP50 protein) show no statistically significant differences between both groups.

Figure 4-9 - Protection from ECM by priming with *C. albicans* R24 is not due to differences in Pb1-specific CD8 T cells in the brain. Total CD8⁺ T cells (a), LFA1⁺ CD8⁺ T cells and parasite-specific (Pb1 epitope) LFA1⁺ CD8⁺ T cells (c) in mouse brains at day 7 post PbA challenge. Naïve and R24 control groups (n=4), PbA-challenged group (n= 6) and R24-primed PbA challenged group (n=7). Data analyzed by Mann-Whitney two-tailed analysis (* $p < 0.5$, ** $p < 0.01$). Bars represent the mean $\pm$ SD.

Once it was established that the observed resistance to ECM was not due to differences in the number of sequestered parasites nor the number of Pb1-specific CD8⁺ T cells in the brain we looked into potential differences in cross-presentation levels of the Pb1 epitope by the endothelial cells present in the microvessels of the brain. Data revealed that, at day 7 post PbA challenge, microvessel fragments exhibited significantly lower cross-presentation levels in our R24-primed mice than in mice not co-infected with *Candida* (Figure 4-10).

Figure 4-10 – Priming with *C. albicans* R24 reduces cross presentation of SQLLNAYL (Pb1 epitope) by brain microvessels in PbA-challenged mice. Brain microvessel cross-presentation results from naïve ($n=10$), R24 control ($n=10$), PbA ($n=16$) and R24 + PbA ($n=17$) groups on day 7 post challenge. Unpaired two-tailed t test on $\log_{10}$ transformed data. Data shown is pooled from 2 independent experiments (**** $p < 0.0001$, ** $p < 0.01$).

From analysis of the previous data we can conclude that ECM protection induced by priming with *C. albicans* R24 derives from reduced cross-presentation of the Pb1 epitope by the endothelial cells present in the microvasculature of the brain while sequestered iRBC and parasite-specific CD8⁺ T cell numbers remain the same.

4.2.6 ECM protection is not explained by differences in IFN- γ and IL-10.

The pro-inflammatory cytokine IFN- γ is essential for ECM to occur, in part by facilitating cross-presentation of parasite antigens by endothelial cells present brain microvessels (Amani *et al.* 2000, Belnoue *et al.* 2002, Howland *et al.* 2015b). Additionally, IL-10 is an anti-inflammatory cytokine known to play a role in immunoregulation and inflammation that has been shown to downregulate the expression of Th1 cytokines like IFN- γ (Kumar *et al.* 2019). IL-10 has also

been shown to suppress the antigen-presentation capacity of antigen presenting cells and to protect against ECM (Eckwalanga *et al.* 1994, Kossodo *et al.* 1997).

To investigate whether these two cytokines, IFN- γ and IL-10, could be involved in the protection against ECM elicited by R24 we compared their levels in brain, spleen, lungs, kidneys and blood at peak ECM. Figure 4-11 shows that, although IFN- γ levels were consistently higher in all the organs of mice challenged with the PbA malaria parasite, PbA and R24 + PbA mouse groups do not show any difference between them that could explain the reduction in cross-presentation reported previously. Analysis of the IFN- γ levels in the brain of mice primed with R24 showed slightly higher levels of this cytokine at day 7 post *Candida* infection when compared with day 14 and day 21 (corresponding to day 7 post PbA challenge) but no significant difference was observed (*data not shown*).

Figure 4-11 - Malaria challenge leads to increased IFN- γ levels but shows no difference between R24 + PbA and PbA groups. Only kidneys show slight difference in IFN- γ levels. Unpaired two-tailed t test on $\log_{10}$ -transformed data (** $p < 0.0001$, *** $p < 0.001$, * $p < 0.1$) # - represents statistical comparison to naive.**

As for the expression levels of IL-10, as seen in Figure 4-12, except in the kidneys results show no difference between both PbA-challenged groups. Interestingly, the brain (Figure 4-12 a) is the only one of the studied organs where we did not observe a difference in IL-10 levels between any of the groups.

Figure 4-12 - Malaria challenge leads to increased IL-10 levels in some organs but shows no difference between R24 + PbA and PbA groups Unpaired two-tailed t test on log₁₀-transformed data (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$) # - represents statistical comparison to naive. Brain samples showed no statistical significance.

4.2.7 ECM protection is not explained by differences in expression of MHC-I or adhesion molecules ICAM and VCAM in endothelial cells from brain microvessels.

The onset of ECM in mice follows a fairly straightforward mechanism. Parasites sequester in the microvasculature of the brain, triggering activation of the endothelial cells which increase surface expression of adhesion molecules. These adhesion molecules have been shown to facilitate iRBC sequestration as well as recruitment and adhesion of leukocytes within the microvasculature thus contributing to the onset of cerebral malaria pathology (Ockenhouse *et al.* 1992). Vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) are two adhesion molecules expressed by endothelial cells whose levels have been

shown to increase during PbA infection (El-Assaad *et al.* 2013, Howland *et al.* 2015b, Freeman *et al.* 2016). Parasites sequestered to the endothelial cells are then phagocytosed by them and cross presented via the major histocompatibility complex through class I molecules (MHC-I) (Razakandrainibe *et al.* 2012, Howland *et al.* 2015b).

Since the observed differences in endothelia cell cross-presentation levels between our groups (Figure 4-10) were not explained by differences in the inflammatory cytokine IFN- γ (Figure 4-11) or the anti-inflammatory cytokine IL-10 (Figure 4-12) in any of the organs analyzed (except the kidneys), we hypothesized whether the effect could be due to either a reduced expression of MHC-I or adhesion molecules ICAM-1 and VCAM-1 by the endothelial cells. As is demonstrated in Figure 4-13, we found no difference in the expression of MHC-I (a), ICAM-1 (b) or VCAM-1 (c); between PbA and R24 + PbA groups. On an interesting note, mice infected with R24 alone exhibited similar levels of endothelial cell adhesion expression to the naïve indicating that *Candida albicans* R24 was not inducing endothelial cell activation 21 days post infection.

Figure 4-13 - Brain endothelial cells don't show differences in MHC-I expression or adhesins. Data values represent geometric mean fluorescence intensity as measured by flow cytometry. Unpaired two-tailed t test on $\log_{10}$ -transformed data (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$). R24 shows no statistical difference from naïve group.

These results (Figure 4-13) clearly show that the differences in cross-presentation, and through that, the protection against ECM, is not due to lower expression of MHC-I or the adhesins analyzed.

4.2.8 Histological differences between groups at day 7 post PbA challenge

During the course of the characterization of the coinfection model undertaken in this thesis, two organs were observed to display clear physiological differences between R24-primed and non-primed mice after PbA challenge. Upon visual comparison, it was clear that coinfecting mice exhibited a more pronounced splenomegaly (as well as increased weight) and a more severe pathology of the kidneys (Figure 4-14). In order to better characterize the inflammatory effects that R24 or PbA, singly and together, could be exerting in these organs, we decided to send them for histological analysis. Organs from naïve, R24, R24 + PbA and PbA groups were harvested 21 days post R24 infection, which corresponds to 7 days post PbA challenge.

Figure 4-14 – Coinfected mice show increased spleen weight and more severe pathology of the kidneys. a) Comparison of the registered weights of the spleens extracted from mice at day 7 post PbA challenge show spleens from R24 + PbA group (n = 9) to have a significantly higher mass than spleens from PbA (n=7) or R24 (n=9) which in turn exhibit higher organ mass than naïve mice (n=5). Values

*represent pooled data from 2 separate experiments. Statistical analysis carried out by ordinary one-way ANOVA with Bonferroni multiple comparison test (** $p < 0.005$, *** $p < 0.001$) # denotes comparison to naïve. No statistically significant difference found between R24 and PbA groups. b) Representative pictures contrasting the differences in visible damage observed in kidneys from different groups. Although the PbA group does present discoloration of the kidneys the clear necrosis of the kidneys in the Candida groups (and especially R24 + PbA) denotes the severe pathology in these organs.*

In blood-stage malarial infections, the spleen plays a major role in the host's defenses against *Plasmodium* spp. Not only does it reduce parasite burden through filtration and removal of iRBCs but it is essential in mounting both innate and adaptive immune responses to infection (White 2017).

Histological analysis of the spleens from the different groups revealed splenic lesions, increased extramedullary hematopoiesis for leukocyte production in response to infection and lymphoid hyperplasia consistent with antigenic stimulation in all groups except the naïve (Figure 4-15 top panels). Both groups challenged with PbA showed more significant structural changes in splenic architecture due to increased presence of macrophagic infiltrates containing cytoplasmic hemozoin consistent with iRBC phagocytosis and high lymphocytic turnover as evidenced by cell necrosis/apoptosis (Figure 4-15 bottom panels).

Figure 4-15 - Histological analysis of the spleen reveals increase in splenic white pulp associated with infiltrating macrophages and high leukocyte turnover. Representative histological H&E sections from spleens of naive, R24-primed, R24-primed challenged with PbA and challenged with just PbA groups collected at day 7 post PbA challenge (day 21 post R24 injection). Top panels – R24 + PbA and PbA groups show marked structural changes in splenic architecture with clear increase in size of lympho-follicular units (scale: 500µm). Bottom panels – magnification of lympho-follicular unit reveals high presence of necrosis/apoptosis events (red arrows) in PbA an R24 + PbA spleens (scale: 100µm).

Interestingly, the histopathology report indicated the splenic inflammation to be most significant in the R24 + PbA group followed by the PbA group and finally the R24. This finding coincides with the observed differences in spleen weight at the same time (Figure 4-14 and Figure 4-15).

In *C. albicans* infections in mice, there is a near linear correlation between kidney CFU burdens and mouse median survival times (Hurtrel *et al.* 1980). Acute renal failure is also one the possible complications derived from severe malaria, presenting as severe tubular necrosis due to capillary obstruction by iRBC and increased levels of localized inflammatory cytokines (Silva Junior *et al.* 2017). Decrease in kidney function has been associated with higher parasitemia in Pb-challenged mice (Abreu *et al.* 2014). During the course of infection, high *Candida* CFU counts were consistently found in the kidneys of R24-primed mice, even as the

fungus gradually cleared from all other organs (Figure 4-4). Although the kidneys in the PbA group showed some signs of discoloration (Figure 4-14b) when compared to the naïve group, the evident necrosis observed in the *Candida* groups is a clear sign of the extent of the pathology in these groups. Interestingly, visual comparison of the R24 + PbA and R24 kidneys appeared to show a more pronounced effect in the coinfecting group. Kidneys obtained from mice in this group appeared more discoloured, smaller and exhibited a wrinkled aspect (resembling a raisin).

Histologic analysis of kidneys of R24 and R24 + PbA groups revealed multiple renal cortex cicatrization areas of variable severity. Within these areas were clear signs of interstitial fibrosis as well as poly and mononuclear cellular infiltrates, renal tube degeneration and necrosis (Figure 4-16). Glomeruli and renal tubules exhibited atrophied epithelium and the presence of necrotic debris consisting of diseased or dead cells, namely neutrophils and eosinophils. *Candida* organisms were clearly identified among the inflammatory cells and within the tubular lumen. In groups challenged with PbA, presence of iRBCs were observed. Taken together we can conclude from these observations that *C. albicans* R24 not only causes kidney disease in these mice, but the effect appears to be exacerbated when coupled with PbA challenge.

Figure 4-16 - H & E stains representing renal pathology in *Candida* injected groups at day 7 post PbA challenge. a) representative histopathological images contrasting the difference in renal pathology between groups. *Candida*-injected mice clearly showing renal inflammation (red arrow), fibrosis (yellow arrow) and tubular damage (blue arrow) (scale 200μm).

Since we were unable to ascertain the reason for the observed reduction in cross-presentation in R24-primed mice and considering the presence of R24 tissue burdens in the brain of both C57BL/6J and BALB/cJ mice at all the observed time points (Figure 4-4), and their apparent association with the observed neurological behaviors, we decided to also send the brains of C57BL/6J for histopathological analysis.

The pathology report findings indicated distinct differences between groups R24 + PbA, R24 and PbA, which could be clearly separated into ECM associated factors and *Candida* associated factors (Table 4-1). Comparing the sum of scores between these groups revealed that the coinfecting group displayed lower ECM associated traits, on average, than the PbA group (Figure 4-17a) and lower *Candida* associated traits, on average, than the R24 group (Figure 4-17b).

Brain sections of animals from the R24 and the R24 + PbA groups exhibited the presence of mononuclear and polymorphonuclear cells in the meninges (Table 4-1). The presence of glial granulomas was also observed and in some of the granulomas ovoid non-staining cells that were identified as the yeast forms of *C. albicans* could be clearly distinguished (Figure 4-18a). As expected from the attenuated strain R24, no hyphae were observed. Both groups challenged with PbA showed multifocal areas of hemorrhage at the meninges and neural parenchyma as well as increased Virchow-Robin spaces (perivascular space) and expansion of intramyelinic spaces consistent with brain edema (Figure 4-18 b,c). Samples from the PbA group exhibited infiltrating leukocytes containing hemozoin pigments present at vascular leakage and hemorrhage points (Table 4-1).

Table 4-1 - Histopathological findings and associated pathology report score from brains of mice at day 7 post PbA challenge. Findings were separated into two major groups – ECM associated factors (in red), consisting of hemorrhage, edema and infiltrating leukocytes containing hemozoin pigments and *Candida*-associated factors (in blue) consisting of meningeal inflammation (mono and polynuclear) and fungal-induced glial granuloma. Scores present are copied from the IMCB Advance Molecular Pathology Laboratory Report (number 0603-19). Scoring grades 0 – no abnormalities detected, 1- minimal, 2- mild, 3- moderate, 4 – marked, 5 – severe, NP – not present P – present; n=3 for each group.

		Naïve			PbA			R24 + PbA			R24		
		Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 3	Mouse 1	Mouse 2	Mouse 3
ECM Associated Factors	Hemorrhage	0	0	0	3	1	0	1	0	1	0	0	0
	Edema	0	0	0	2	1	0	1	0	0	0	0	0
	Infiltrating leukocytes with hemozoin pigments	0	0	0	3	1	0	0	0	0	0	0	0
	Sum of scores	0	0	0	8	3	0	2	0	1	0	0	0
	Group Average	0			3.67			1			0		
Candida Associated Factors	Meningeal inflammation (polymorphonuclear)	0	0	0	0	0	0	0	0	0	1	0	1
	Meningeal inflammation (mononuclear)	0	0	0	0	0	0	1	0	0	2	0	1
	Fungal-induced glial granuloma (FIGG)	0	0	0	0	0	0	1	1	0	1	2	1
	<i>Candida</i> cell within fungal-induced glial granuloma (FIGG)	NP	NP	NP	NP	NP	NP	NP	P	NP	NP	NP	P
	Sum of scores	0	0	0	0	0	0	2	1	0	4	2	3
	Group Average	0			0			1			3		

a) ECM traits brain pathology score
(Hemorrhage, edema, infiltrating leukocytes)

b) Candida traits brain pathology score
(Meningeal inflammation, FIGG)

Figure 4-17 - R24 + PbA mice tend to show lower brain pathology scores than mice infected with just PbA or just R24 at day 7 post PbA challenge. **a)** brain pathology sum score for ECM-associated factors in R24 + PbA and PbA mice as indicated in Table 4-1 **b)** brain pathology sum score for *Candida*-associated factors in R24 and R24 + PbA mice as indicated in Table 4-1. A two-tailed unpaired t test was carried out in samples shown in **a)** and **b)** but no statistical significance was found ($p=0.451$ and $p=0.1072$, respectively).

Figure 4-18 – R24 + PbA mice tend to show lower brain pathology than mice infected with just PbA or just R24 at day 7 post PbA challenge. a) Representative H&E staining of histological brain sections from R24 and R24+PbA showing the presence of Fungal-induced glial granuloma (FIGG). Green arrows indicate *Candida* sp. cells. **b)** Representative H&E staining of histological brain sections from naïve, R24, R24+PbA and PbA groups showing signs of edema (R24+PbA and PbA) and hemorrhage (PbA). Yellow arrows indicate hemorrhage, blue arrows indicate expansion of intramyelinic spaces (edema). **c)** Representative H&E staining of histological brain sections from R24 + PbA and PbA groups showing Virchow Robin spaces. Minimal for R24 + PbA, mild in PbA, indicated by the blue arrows.

At day 7 post challenge mice are within the ECM window of the infection and brain damage is to be expected. Mice challenged with PbA (both groups) corroborate this by showing more severe pathology and heavier signs of damage to the brain than do the mice in the R24 control group (Table 4-1). The R24 + PbA mice seem to exhibit a lower pathology sum score than the PbA mice when it comes to the severity of hemorrhage and edema (Figure 4-18b). It is interesting to note that one PbA mouse (mouse 3) did not evidence any signs of pathology in the brain and presents like a naïve mouse. Our records indicate that in the assessment of the individual pathology progression this mouse was evidencing signs of balance problems, slight convulsions and hunched posture from as early as day 6, all traits associated with the onset of ECM.

It is of importance to note that the presence of *Candida albicans* yeast forms were detected inside the brain tissue of not only PbA challenged mice but also the R24 control group, without evidence of hyphal forms. Similar findings have been previously reported by Fraga-Silva and colleagues (Fraga-Silva *et al.* 2015) where they also observed the presence of *Candida albicans* in the brain without evidence of hyphal forms. Our results show that, not only were we able to detect the R24 in the brain but we did so 21 days after injecting the CFUs into the blood stream and our attenuated strain is able to cross the blood brain barrier.

4.3 Discussion

In this chapter we demonstrated that vaccination with the attenuated *C. albicans* strain R24 could protect C57BL/6J mice from experimental cerebral malaria (ECM) induced by PbA although it had no effect on parasitemia levels, as R24-primed mice ended up dying of hyperparasitemia 24 to 26 days post challenge (Figure 4-7). These findings were supported by challenges using PyYM whereupon we demonstrated that R24-primed C57BL/6J mice exhibited similar peripheral parasitemia curves to challenged naïve mice (Figure 4-5). Furthermore, R24-primed BALB/cJ mice succumbed within 8 to 10 days post PyYM challenge regardless of the time window allotted between priming and malaria challenge (Figure 4-6). Although comparison of parasitemia levels in the first 6 days showed some differences, with R24-primed BALB/cJ mice exhibiting lower parasitemia, all mice inevitably reached similar parasitemia levels to naïve-challenged mice and succumbed within the same time period. While these differences might be due to an enhanced innate immune response elicited by the vaccination (trained immunity), they were altogether insufficient to significantly affect parasite kinetics or change the end result. Innate immune responses have been shown to control parasitemia during initial stages (first 6 days) of Py challenge (Couper *et al.* 2007) however, and especially for the YM strain, successful clearance requires the involvement of CD4⁺ T cells (Claser *et al.* 2017). Although there is a possibility that challenges using the non-lethal variant *P. yoelii* 17XNL, might yield different results, we can state that vaccination with R24 does not affect parasite kinetics in C57BL/6J or protect BALB/cJ mice from PyYM blood-stage challenge.

Weight loss is a common side effect of the inflammatory response in mice after an infectious challenge (Olfert & Godson 2000, Ray *et al.* 2010, Trammell & Toth 2011) and some correlations between over 20% relative body-weight loss and survival have been established (MacCallum & Odds 2005) in challenges with wild-type *Candida albicans*. In our vaccination

model we observed that, while both mouse strains displayed rapid decline in relative body weight, resulting in above 20% loss on day 7 (Figure 4-1), over 99% of the mice recovered from primary infection (data not shown) and returned to baseline levels. Interestingly C57BL/6J mice recovered earlier, reaching baseline levels at day 21 post infection, versus BALB/cJ mice that only reached this point at day 28 (Figure 4-1). This finding conforms to previously published data where BALB/cJ mice were shown to exhibit higher systemic inflammation derived from an exaggerated innate immune response (Watanabe *et al.* 2004).

Characterization of the progression of systemic R24 infection after vaccination showed that, similarly to previous studies (MacCallum & Odds 2005), both mouse strains were able to quickly clear the *Candida* from the blood within the first five hours (Figure 4-3) and, except for the occasional CFU, values remained undetectable until 28 days post infection. Consistent with rapid clearance, we observed splenomegaly in R24-primed mice as early as day 7 (data not shown). Furthermore, we observed that, during PbA challenge, R24-primed mice exhibited a more pronounced splenomegaly than singly infected mice (Figure 4-14a) a phenomenon also described in other coinfection models (Teo *et al.* 2018).

Tissue burden analysis revealed that at day 7 post R24 infection, viable *Candida* cells were detected in the liver, lungs, spleen, heart, brain and kidneys. Kidneys showed the highest level of infection, as is to be expected in murine infections with this fungus (Rogers & Balish 1976, Hurtrel *et al.* 1980). Also consistent with published findings (Lionakis *et al.* 2011a) were the signs of pathology observed in the kidneys (Figure 4-14b, Figure 4-16). Interestingly though, we observed that the coinfection model exhibited more severe renal pathology, a possible consequence of increased inflammation caused by higher IFN- γ levels (Figure 4-11) and lower IL-10 levels (Figure 4-12), cytokines shown to mediate disease in these organs (MacCallum *et al.* 2009). A high number of *Candida* cells were also detected in the brain at day 7 post infection. In fact, *Candida* numbers in kidneys and brain remained high until at least day 21

while all other organs exhibited a decline in tissue burdens (Figure 4-4). Together with these findings, we observed that mice began displaying abnormal behaviors consistent with neurological inflammation and/or damage. Three distinct behaviors were observed of which twisting of the head (dubbed “Head Twist”) was the most frequent. Bibliographical research of the observed symptoms revealed them to most likely belong to the dyskinesia movement disorder group, as they exhibit several similarities to choreathetosis (Ledoux 2005). Evidence of these behaviors was detectable from day 10 post R24 infection for C57BL/6J mice and from day 14 for BALB/cJ mice, and the number of mice exhibiting at least one behavior increased over time, reaching at least 40% of the mice by day 21 (Figure 4-2). Histological analysis of brain sections at day 21 post R24 infection revealed the presence of fungal cells inside the brain in granuloma-type structures (fungus-induced glial granuloma or FIGG) as well as meningeal inflammation and presence of mononuclear and polymorphonuclear cells (Figure 4-17, Figure 4-18). Other studies have shown the presence of *C. albicans* in mouse brains (MacCallum & Odds 2005), demonstrated their ability to interact with endothelial cells *in vitro* (Grubb *et al.* 2009) and, more recently, confirmed their ability to infiltrate the brain and cause cognitive impairment (Wu *et al.* 2019). Nevertheless, to our knowledge, this is the first study that demonstrated unequivocally that *Candida albicans* does not require hyphal morphogenesis to cross the blood brain barrier and that systemic fungal infection can induce long-term neurological effects leading to altered behavior.

Vaccination with R24 did not significantly protect mice from PyYM challenge, C57BL/6J mice showed no differences in peripheral parasitemia levels while BALB/cJ mice succumbed to hyperparasitemia within 6 to 12 days post challenge.

In PbA challenges, we observed that 60% of the mice were protected from ECM (Figure 4-7), surviving until day 24 post challenge. During a normal course of infection, C57BL/6J mice die within 6 to 12 days (ECM window) post PbA challenge exhibiting distinct neurological

symptoms (Rénia *et al.* 2012). Although R24-primed mice exhibited mild signs of early stage cerebral malaria (balance problems, rapid breathing, mild ataxia, partial paralysis and convulsions), together with the afore mentioned neurological behaviors (head twist, twitching and helicoptering), the majority survived past the ECM window and succumbed only at day 24 post challenge due to hyperparasitemia. Interestingly, while naïve PbA-challenged mice exhibit a clear set of symptoms whose severity allow for a straightforward tracking of the progression of ECM disease, the R24-primed mice proved more complicated. The combination of R24-induced symptoms together with the PbA ECM symptoms led to a synergistic enhancement of observed symptomatology that many times misled us with regard to the severity of the disease in these mice. Nevertheless, histopathological analysis of brain sections of R24 + PbA mice showed lower levels of edema, hemorrhage and infiltrating leukocytes when compared with PbA challenged mice and consistent with the observed protection (Figure 4-17, Figure 4-18). Upon further study we were able to determine that this protection was due to reduced cross-presentation by endothelial cells in the brain (Figure 4-10) although microvascular numbers of sequestered iRBC (Figure 4-8) and parasite-specific CD8⁺ T cells (Figure 4-9) remained the same. Moreover, we were able to determine that this reduction in levels of cross-presentation was not due to differential levels of systemic or localized inflammatory cytokine IFN- γ (Figure 4-11) nor of the anti-inflammatory cytokine IL-10 (Figure 4-12), two cytokines associated with ECM (Kossodo *et al.* 1997, Amani *et al.* 2000, Belnoue *et al.* 2002, Howland *et al.* 2015b, Kumar *et al.* 2019). To our surprise, analysis of endothelial cell expression of adhesins ICAM-1 and VCAM-1 were likewise unaffected as was the expression of MHC-I molecules, essential in cross-presentation. While we were able to clearly demonstrate a reduction in cross-presentation by brain endothelial cells in R24-primed mice during peak ECM, none of the factors we analyzed explained why this occurred. This brings to mind some further possible explanatory mechanisms. Endothelial cells must phagocytose, process and efficiently present the malaria pathogen epitopes through MHC-I

for activation of the parasite-specific cytotoxic CD8 T cells to occur (Howland *et al.* 2015b). While we showed that the activated endothelial cells in the brain microvasculature of R24-primed PbA-challenged mice exhibit MHC-I molecules at similar levels to the PbA-challenged mice we did not demonstrate that those MHC-I molecules were loaded with malaria antigens. Since the cross-presentation assay used in this thesis measures microvascular fragments specifically expressing the Pb1 epitope, one possible explanation could lie in the processing mechanism of the endothelial cells. We hypothesize that R24-primed PbA-challenged mice might have an impaired functionality of the phagosome-to-cytosol route involved in the processing and presentation of the malaria antigen, as this has been demonstrated a key component in ECM pathology (Howland *et al.* 2015b). An alternative hypothesis implies competition between *C. albicans* R24 and malaria parasites. We observed that *C. albicans* R24 is present in the brain before (Figure 4-4) and throughout PbA challenge (Figure S1). Furthermore, not only has *C. albicans* been shown to be able to adhere to endothelial cells of the microvasculature (Phan *et al.* 2000, Grubb *et al.* 2008, 2009) but endothelial cells have been shown able to phagocytose the fungus (Filler *et al.* 1995, Shintaku *et al.* 2013). It might well be that the observed reduction in endothelial cells cross-presenting malarial antigen was due to reduced phagocytosis of malaria parasites by unavailability of endothelial cells already phagocytosing the *Candida*.

Naturally, the role of innate immune cells in protection against ECM cannot be disregarded. Monocytes (Niewold *et al.* 2018), macrophages (Oakley *et al.* 2018) and neutrophils (Chen *et al.* 2000) have all been implicated in the pathophysiology of ECM. Further studies into these innate subsets might shed more light into the protective mechanisms behind vaccination with R24.

On a final note, while it is interesting to note that at day 21 we observed no differences between the naïve mice and our R24 singly-infected mice in the majority of the pro-

inflammatory signals (Figure 4-11, Figure 4-12, Figure 4-13) studied, this timepoint might be too delayed to observe the direct immunomodulatory effects of vaccination with this strain. Although we do observe signs of inflammation in the histopathological analysis of brains in this group (Figure 4-17, Figure 4-18) we do not know when the onset of this response began or even when *C. albicans* R24 crossed into the brain. If we are to understand the mechanisms by which this attenuated vaccine works, study of earlier timepoints is essential.

Chapter 5 - Conclusions and future work

Over the course of this thesis we have explored two distinct vaccination methods in order to ascertain immunomodulatory mechanisms pertaining to protection from lethal malaria challenge in mice.

The first vaccination method focused on the adaptive immune system by evaluating the potential use of two new thermogel polymers (Gel 3 and Gel 4) as adjuvants in subunit vaccination. Thermogel polymers are hydrogels that reversibly transition from liquid to solid phase as the temperature increases. This defining trait, combined with the fact that their modular composition allows for fine tuning of their mechanical, thermoresponsive and biocompatible properties, makes them particularly attractive drug delivery systems in clinical use. Pairing these two gels with the well characterized MSP1-19 protein, we compared their ability to, through subunit vaccination, induce protective immunity to lethal PyYM challenge in BALB/cJ mice. While we clearly demonstrated that both gels were capable of inducing a humoral IgG1 and IgG2a response that protected the mice from death, comparison of parasitemia curves and antibody titers revealed that this protection, while similar in type, was lower than that induced by the same vaccination using Freund's adjuvant. On the other hand, when compared to mice vaccinated with the same protein in conjunction with alum (alhydrogel), a more clinically approved adjuvant, the differences were negligible.

While we observed a broad association between protection from death and high antibody titers, we were unable to define a minimum threshold of protection whereupon mice did not succumb from challenge. For further studies to proceed, this criterion, together with the minimum threshold from which mice exhibit sterile immunity, must be clearly established from the beginning. Numerous studies in murine immunization with MSP1-19 have demonstrated that antibodies, and specifically IgG, need to be present at high titers at the

time of *P. yoelii* challenge (Daly & Long 1995b, Hirunpetcharat *et al.* 1997, 2003, Wipasa *et al.* 2002, Jeamwattanalert *et al.* 2007). Passive transfer experiments into nude and SCID mice confirm that antibodies alone are sufficient to control parasitemia and induce sterile protection if given in sufficient quantity.

The low level of protection observed in mice vaccinated with alum brings into question the vaccination protocol employed. While administering three subcutaneous doses is enough to elicit a strong level of protection when using Freund's adjuvant, the same cannot be said of the remaining adjuvants. Increasing the number of vaccine doses, allowing more time between vaccination and challenge and exploring alternative sites of injection (such as intraperitoneally) could be used to achieve this goal. Differences in level of antibody production have been shown to vary depending on site of administration even when using the same adjuvant (Daly & Long 1996, Near *et al.* 2002) and the majority of vaccination trials employ both subcutaneous and intraperitoneal injections during immunization. The next steps in exploring the potential of the thermogels as adjuvants against malaria should be to increase the number of inoculations from three to four and incorporate i.p. injections.

Freund's adjuvant is a water-in-oil emulsion that works by depot effect through prolonged and continuous release of the antigen from the oily deposit. Not only does this prolong antigen lifetime but the released oil droplets have been shown to induce stimulation of localized immunity by enhancing phagocytosis, leukocyte infiltration and cytokine production (Apostólico *et al.* 2016). Incorporation of heat-killed *Mycobacterium tuberculosis* or *M. butryicum* into the mix dictates the difference between complete and incomplete Freund's adjuvant. This combination results in a localized inflammation resulting in strong stimulation of both cellular and humoral immunity (Coffman *et al.* 2010). Unfortunately, resultant inflammation frequently leads to painful ulcers, making it unusable in human vaccination.

Incorporation of the Toll-like receptor agonist GPG ODN 1826 in the thermogels should also be considered as CPG can be used in human vaccination and has proven to enhance antibody response and promote long term parasite-specific memory B cells in vaccination (Hirunpetcharat *et al.* 2003, Kumar *et al.* 2004, Jeamwattanalert *et al.* 2007, Ellis *et al.* 2010). Furthermore, as the effects of GPG are enhanced by formulation with other depot adjuvants, this combination could result in a significant increase in the immunogenic response to the antigen.

The variability between batches and stability of the gels proved a challenge when attempting to reproduce initial results, hydrolysis of the gels leads to reduced molecular weight which affects the duration (stability) of the gels inside the body, affecting the depot effect. While the association between duration of depot effect and induction of immune responses is not well understood, the general consensus is that longer lasting depots allow longer exposure of the antigen to antigen presenting cells, resulting in stronger immune responses (Schijns & Lavelle 2011).

If these gels are to be used, more rigorous criteria than minimum gelling concentration need to be defined and applied before each vaccination trial. Furthermore, evaluation of the effects of long-term storage in the induction of protective immune responses should be carried out in order to define the maximum storage time and ideal conditions under which these gels can be reliably used. Characterization of molecular weight loss in the hydrogels during storage should better define the criteria for consistent use of the gels. Additionally, before every vaccination trial, in vitro protein release needs to be characterized using the in vitro drug release method described in Materials and Methods. As mechanical stress is another factor influencing the biodegradability of these gels, methods of evaluating gel duration and consistency within the mouse body should be devised and put into use. Considering these gels solidify into a visible stable matrix after subcutaneous injection, daily measurements of

the size of the inoculum under the skin could be a simple initial method of evaluation. Excision of the polymerized gel injected in the mouse should also be carried at specific time points and characterization and quantification of infiltrated cell populations (namely DCs) as well as remaining antigen should be carried out to ascertain *in vivo* protein release. The single greatest advantage of the thermogels is the possibility of modulating quantity and duration of release of emulsified antigens. Unfortunately, this cannot happen until we understand how these gels behave when inserted into the body.

In regard to the evaluation of the induced immune response by this vaccination model, we were unable to establish by which mechanism antibodies mediated protection. While survival or death of mice vaccinated with Gel 4 showed some association with IgG1 and IgG2a titers, the parasitemia levels observed for both gels were dangerously high to the point where minor environmental factors meant the difference between life and death.

Differences in parasitemia between the gels and CFA were not explained by opsonic phagocytosis assay using RAW264.6 cells nor *in vitro* inhibition of invasion bringing into question by what method do antibodies induced here control parasitemia levels

Other antibody-dependent mechanisms can play a role in protection. The role of complement in the serum, antibody dependent cellular inhibition (ADCI) and antibody dependent respiratory burst (ADRB) should also be assessed to differentiate between an induced immune response and a protective one.

Contrary to other findings (Hirunpetcharat *et al.* 2003), antibody titers were not sufficient to protect mice from challenge following depletion of CD4⁺ T cells. In mice vaccinated with CFA, depletion resulted in delayed onset of parasitemia followed by rapid growth that culminated in death of the mice as they exhausted their available repertoire of protective antibodies. This

points to the need for an active immune response post challenge in the absence of sufficiently high antibody levels.

Further work needs to be carried out with these gels before we can consider them for use in human vaccination.

The second vaccination method studied the use of the live attenuated *Candida albicans* strain R24 in protection against lethal PyYM and PbA challenges in mice. After characterizing the model in BALB/cJ and C57BL/6J mouse strains we discovered that viable R24 cells were detectable in brain cultures of both mouse strains until at least 21 days post primary infection and postulate this might be the reason for the observed abnormal neurological behaviors. Moreover, histological analysis of C57BL/6J brains at day 21 confirmed that this strain was able to cross the BBB and induce localized inflammation even though it was unable to undergo morphogenic switch to hyphal form.

Vaccination with R24 appeared ineffective against PyYM challenge as we observed no significant differences in parasitemia curves or survival in our models. Nevertheless, the potential role of trained immunity in protection against malaria shouldn't be so quickly dismissed. Mice vaccinated with BCG exhibited partial protection (lower parasitemia levels) when challenged two months later with *P. yoelii* 17XNL. Protection was associated with induced expression of innate immune molecules. Furthermore, BCG vaccination abrogated malaria-induced immunosuppression of genes encoding antimicrobial compounds (Parra *et al.* 2013). While the authors did not establish if the described effects were associated with trained immunity, BCG vaccination has been shown to induce trained immunity (Kleinnijenhuis *et al.* 2014). Furthermore BCG vaccination can induce trained immunity that affects the clinical course of *P. falciparum* (Walk *et al.* 2019), and even the *Plasmodium* parasite has been shown to induce trained immunity (Schrum *et al.* 2018). As such,

challenging C57BL/6J and BALB/cJ mice with Py17XNL after vaccination with R24 could reveal important differences not seen when using the much more aggressive PyYM strain.

One point of notice is that we did not evaluate whether R24 induces trained immunity in BALB/cJ or whether it was similar to the one observed in C57BL/6J (Tso *et al.* 2018). A strong point can be made that BALB/cJ and C57BL/6J mice have different innate immune responses, with BALB/cJ mice skewing more towards a Th2 phenotype (Watanabe *et al.* 2004). These mice have been shown to produce lower levels of type I cytokines, namely IFN- γ , at early stages of infection which would make them less protected against blood stage malaria (De Souza *et al.* 1997, van der Heyde *et al.* 1997). Interestingly, it was in BALB/cJ mice that we observed the only differences (although minor) in parasitemia between R24-vaccinated and non-vaccinated mice.

Vaccination with R24 protected C57BL/6J from ECM although mice ended up succumbing to hyperparasitemia later on. Upon further study we were able to determine that this protection was due to reduced cross-presentation by endothelial cells in the brain while the number of sequestered iRBC and parasite-specific CD8⁺ T cells remained unaffected. Once we established that this reduced cross-presentation was not due to differential levels of systemic or localized IFN- γ and IL-10 cytokines, we demonstrated that endothelial cell expression of adhesins ICAM-1 and VCAM-1 as well as MHC-I molecules remained unchanged. Two main hypotheses were put forth to explain the observed effect, either the mechanism by which the endothelial cells process and present antigens is affected or, endothelial cells are non-selectively phagocytosing either *Candida* or PbA present in the microvasculature and lower levels of cross-presentation are simply a side-effect of dilution by over-availability of targets. Proving that brain endothelial cells can phagocytose R24 *in vitro* would provide an important first clue to the viability of the competition model and is a necessary next step in the characterization of this model.

Histopathological analysis of the brain at peak ECM also revealed some important differences in the severity of the disease between groups. While differences in cross-presentation at the endothelial cells of the brain microvasculature can explain this, it might not be the only factor at play. There is no documented direct link between trained immunity and ECM (Sierro & Grau 2019) and we did not establish one here. In fact, based on our current data we cannot state whether trained immunity played a role in our malaria challenges at all. Yet until we carry out a characterization of the innate immunity population present in the brain microvasculature between infection with PbA and onset of ECM symptoms we cannot rule them out.

The association between neurological symptoms and the presence of *Candida* inside the brain also needs to be further characterized with a specific focus on microglia. These cells can phagocytose and kill *Candida*, have been shown to protect against lethal challenge and can ameliorate tissue damage in infection (Lionakis *et al.* 2011b). The connection between *Candida*, microglia and severity of ECM symptoms should also be taken into consideration. While the manifestation of the behaviors was only detectable from day 10 onward, we do not know if these manifest as a direct reaction to R24 crossing the BBB or if they evolve due to extended inflammation. Studying earlier timepoints in the infection would shed some light on the issue. Also, a more thorough characterization of the inflammatory response in the brain is needed. Considering the similarities between our findings and those recently published by Wu and colleagues (Wu *et al.* 2019), it would be important to determine if our inflammatory response is also governed by the same mechanisms.

The induction of neurological behaviors in mice by vaccination with the R24 strain while interesting, complicates the prognosis of ECM by adding additional symptoms to the very straightforward severity signs normally induced by PbA. Furthermore, the *Candida*'s ability to cross the blood-brain-barrier invalidates its use in human experimentation. Still, the

surprising discovery of its protective effects against ECM should not be disregarded and needs to be further explored.

Chapter 6 - Supplementary information

Table S1 – Mean inhibition efficiency values for samples represented in “Figure 3-12 – Pooled sera from vaccination have low inhibitory capacity of invasion in vitro”. Values indicated represent the mean and standard deviation (SD). Results shown are pooled from two independent experiments (n=5 wells each).

	Dilution	Mean $\pm$ SD
MSP + CFA	1/30	17.16 $\pm$ 9.76
	1/120	16.71 $\pm$ 4.16
	1/480	17.16 $\pm$ 9.76
MSP + Gel 3	1/30	14.68 $\pm$ 8.52
	1/120	11.99 $\pm$ 3.35
	1/480	17.94 $\pm$ 4.37
MSP + Gel 4	1/30	32.88 $\pm$ 1.46
	1/120	17.82 $\pm$ 7.17
	1/480	22.97 $\pm$ 3.07
CFA	1/30	8.91 $\pm$ 10.73
	1/120	5.94 $\pm$ 7.94
	1/480	7.00 $\pm$ 5.97
Gel 3	1/30	7.37 $\pm$ 7.77
	1/120	5.31 $\pm$ 4.52
	1/480	6.13 $\pm$ 5.54
Gel 4	1/30	20.26 $\pm$ 5.35
	1/120	6.86 $\pm$ 7.01
	1/480	8.63 $\pm$ 5.63

Figure S1 - Influence of PbA on tissue burdens of *Candida albicans* R24 in mouse organs at day 21 post R24 infection (peak ECM). Number of colony forming units (CFUs) appear to increase in most organs in co-infection mice, R24 + PbA ($n = 5-9$ mice), when compared to mice infected with just R24 ($n = 5-8$ mice). The only organ exhibiting decrease in CFUs is the brain. R24 $n = 5-8$. Values represented are pooled from 3 different experiments. Data analyzed by Mann-Whitney two-tailed analysis (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$). Bars represent the mean $\pm$ SD.

Chapter 7 - References

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