An *in vitro* assay to measure antibody-mediated inhibition of malaria sporozoite infection

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Declaration

I, Ana Rodríguez Galán, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

A large research effort is currently underway to find an effective and affordable malaria vaccine. Tools that allow the evaluation of the host immune response are essential to vaccine development. This thesis focuses on an assay to evaluate antibody-mediated inhibition of sporozoite infection: the Inhibition of Sporozoite Invasion assay (ISI). ISI measures antibody capacity to prevent sporozoite infection in an *in vitro* system that reflects the actual infection context. This assay was first described in 1982 by Hollingdale et al., but the assay has been affected by many limitations, particularly time-consuming and subjective microscopy readouts. The recent development of transgenic parasites detectable by flow cytometry (due to the expression of reporter proteins such as GFP) provides a readout which highly increases the throuphut of the technique. The objective of this thesis is to further develop the ISI assay and adapt it to evaluate humoral responses in candidate screening and clinical studies. Chapter 1 describes the process to optimise a protocol for a flow cytometry based ISI assay. In Chapter 2, mouse, macaque, and human serum samples (obtained after vaccination with a viral vector vaccine expressing a *P. falciparum* antigen) were tested using *P. berghei* transgenic parasites expressing *P. falciparum* antigens. In studies with mouse samples, the assay proved to be useful comparing the functional capacity of antibodies generated against different antigens and with different vaccination strategies, such as the adjuvant or vaccination dose. A positive increase of antibody functionality after vaccination was shown for some mouse, macaque, and human volunteer samples, and interesting correlations were found for ISI assay data and other antibody assays and clinical data.

Acknowledgements

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List of Abbreviations

aa	Ammino acid
Ab	Antibody
ACT	Artemisin Combination Therapy
ADCC	Antibody-dependent cellular cytotoxicity
AMA1	Apical Membrane Antigen 1
APCs	Antigen Presenting Cells
BCR	B Cell Receptor
BF	Bright field
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CDS	Coding DNA Sequence
CelTOS	Cell-Traversal protein for Ookinetes and Sporozoites
ChAd63	Chimpanzee Adenovirus serotype 63
CS	CircumSporozoite Protein
CSP	CircumSporozoite Protein
CTLs	CytoToxic Lymphocytes
DAMPs	Damage-Associated Molecular Patterns
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium
DNA	DeoxyriboNucleic Acid
EBA-175	Erythrocytic binding protein
EEFs	Exo-Erythrocytic Forms
ELISA	Enzyme-Linked Immunosorber assay
ELISPOT	Enzyme-Linked ImmunoSpot assay
EMA	European Medicines Agency
ETRAMP5	Early TRAnscribed Membrane Protein 5
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
GAPs	Genetically Arrested Parasites
GFP	Green Fluorescent Protein
GIMO	Gene insertion/marker out
GPI	Glycosylphosphatidylinisotol
HBsAg	Hepatitis B surface antigen
HEK 293	Human Embryonic Kidney cells 293
HI	Heat-Inactivated
HT	Hexose Transporter
ICAM-1	Intercellular Adhesion Molecule-1
ICS	Intracellular cytokine staining
IFAT	Immunofluorescence antibody test
IFN-ɣ	Interferon-ɣ
ifu	Infectious Unit
Ig	Immunoglobulin
IL	Interleukin
ILSDA assay	Inhibition of Liver Stage Development Assay
IPAT	Immunoperoxidase antibody test

iRBCs	infected Red Blood Cells
IRS	Indoor Residual Spraying
ISI assay	Inhibition of Sporozoite Invasion assay
ITNs	Insecticide-Treated mosquito Nets
LIPS	Luminescence ImmunoPrecipitation System
LSA1	Liver-Stage Antigen 1
LSA3	Liver-Stage antigen 3
LSAP1	Liver-Stage-Associated Protein-1
LSAP2	Liver-Stage-Associated Protein-2
LSEC	Liver Sinusoidal Endothelial Cells
mAb-*Pb*CS	monoclonal antibody against *Pb* CSP (3D11 Fab)
mAb-*Pf*CS	monoclonal antibody against *Pf* CSP (2A10)
Mda5	Melanoma Differentiation-Associated protein 5
ME	Multiple Epitope
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	minute(s)
MSP1-9	Merozoite Surface Protein 1-9
MVA	Modified Vaccinia virus Ankara
NK cells	Natural Killer
ºC	Celsius degrees
OD	Optical Density
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
PAMPs	Pathogen-Associated Molecular Patterns
Pb	*Plasmodium berghei*
*Pb*CS	*Plasmodium berghei* Cicumsporozoite Protein
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PFA	Paraformaldehyde
*Pf*CS	*Plasmodium falciparum* Circumsporozoite Protein
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein-1
PfRH	*P. falciparum Reticulocyte binding Homologue*
pfu	Plaque-Forming Unit
PRRs	Pattern Recognition Receptors
qRT-PCR	quantitative Reverse Transcription Polymerase Chain Reaction
R21	VLP vaccine contains CSP regions fused to HBsAg
RBCs	Red Blood Cells
RNA	RiboNucleic Acid
RP-L3	Ribosomal Protein L3
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute medium - 1640
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SFC	Spot-Forming Cell
SPECT1	Sporozoite microneme Protein Essential for Cell Traversal 1
SPECT2	Sporozoite microneme Protein Essential for Cell Traversal 2

T regs	T regulatory cells
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th	T helper cells
TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor-α
TRAP	Thrombospondin-Related Adhesive Protein
UIS3	Upregulated in Infective Sporozoites 3
UIS4	Upregulated in Infective Sporozoites 4
UTRs	UnTranslated Regions
VCAM1	Vascular Cell Adhesion Molecule-1
VLP	Virus Like Particle
WHO	World Health Organization
WT	Wild type

INTRODUCTION

Malaria impact and control measures

Malaria is a parasitic infectious disease which affects mainly tropical and subtropical regions of the world [FigureI.1], in particular Sub-Saharan Africa, India and Southeast Asia. According to the 2014 World Malaria report published by the WHO (1), around 198 million clinical episodes occurred in 2013 leading to 584000 deaths from malaria which took place mainly in African countries, affecting mostly children under 5 years of age and pregnant women. Malaria mainly affects poor populations with limited access to health care and prevention measures. In addition to the high morbidity and mortality, malaria has extremely negative consequences on the economic growth of malaria endemic countries (2).
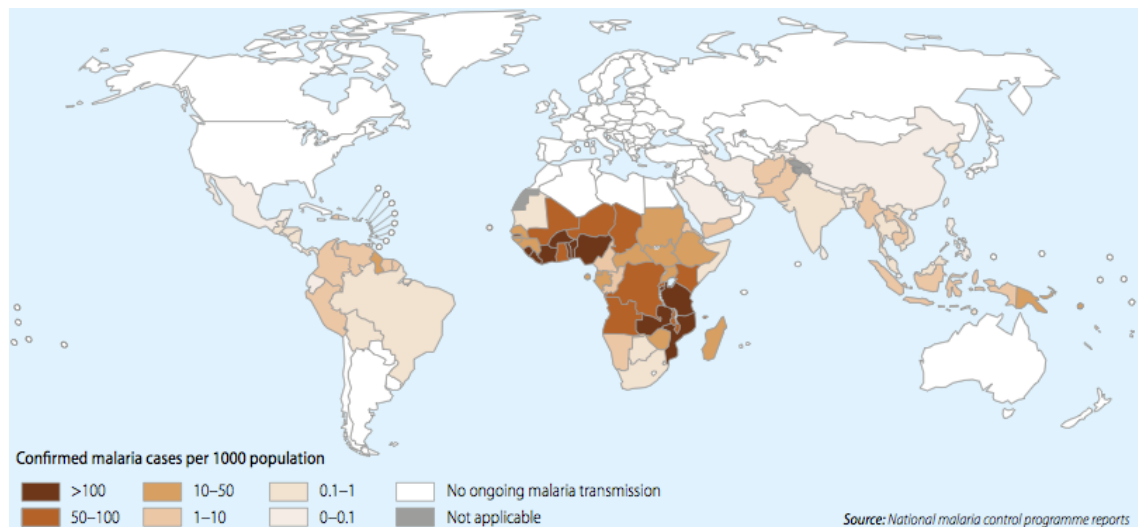
Figure I1. Countries with ongoing malaria transmission. Taken from 2014 World Malaria report, WHO (1).

Malaria is caused by a protozoa (unicellular eukaryotic parasite) of the genus *Plasmodium* and is transmitted from one person to the other by the bite of an infected female Anopheles mosquito. *Plasmodium* species belong to the phylum of the apicomplexans, which are obligate intracellular parasites. There are several *Plasmodium* species that differ in their host species and their ability to cause disease. Five species can infect humans *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi,* which is also a macaque monkey parasite. *Plasmodium berghei*, *Plasmodium yoelii*, and *Plasmodium chabaudi* parasitize rodents and are important models in malaria research. *Plasmodium falciparum* is responsible for up to 90% of human malaria infections (1) and for most of malarial deaths (3). *Plasmodium vivax* can also cause human deaths

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(4), but presents a lower risk of infection (since it requires the presence of Duffy gene which is absent in many populations) and causes a more benign disease since infected red blood cells do not attach to the vascular endothelium (5,6).

Control strategies to target this public health challenge have been very effective in recent years (7–9), in fact, according to the WHO, malaria mortality rates have decreased by 47% between 2000 and 2013 (1). These interventions target malaria patients, exposed individuals and the mosquito vector. Vector control strategies focus on reducing malaria transmission mainly through the use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). ITNs have been shown to reduce mortality rates (by around 55% in children in Sub-Saharan Africa (10), and to decrease in about 50% the number of malaria cases in various regions (11). IRS has been described to have a similar impact than ITNs reducing malaria incidence (12). Exposed individuals could also be protected through administration of a preventive drug treatment during periods of higher risk, which would particularly benefit pregnant women and children living in regions with seasonal malaria (13,14). Regarding infected individuals, early diagnosis (usually rapid diagnostic test or observation of a blood smear with a microscope) facilitates an appropriated and more effective treatment. Artemisin combination therapy (ACT) is the treatment for uncomplicated *P. falciparum* malaria recommended by WHO (15). Anti-malarial drugs from the artemisins family are very effective against blood stages and reduce malaria transmission (16–19). Chloroquine and sulphadoxine-pyrimethamine were employed previously as treatments, but parasites have developed resistance against these drugs. An important threat to malaria control is parasite resistance to chemotherapy, due to the large genetic plasticity of malaria parasites. In fact, resistance to artemisin has been reported in various countries (20–22). Important efforts need to be made to discover not only new anti-malarial drugs, but also new insecticides since mosquitoes also develop resistance (23).

Malaria life cycle and associated pathogenesis

Plasmodium parasite presents a complex life cycle, which involves both vertebrate and mosquito hosts and transformation through multiple developmental stages with their own antigenic variety [FigureI.2]. The cycle is initiated when the vertebrate host is bitten by an infected Anopheles mosquito, which transfers sporozoites that grow asexually first in hepatocytes and later within erythrocytes. Gametocytes are formed in the vertebrate host and their fusion and further development and propagation occurs in the mosquito.
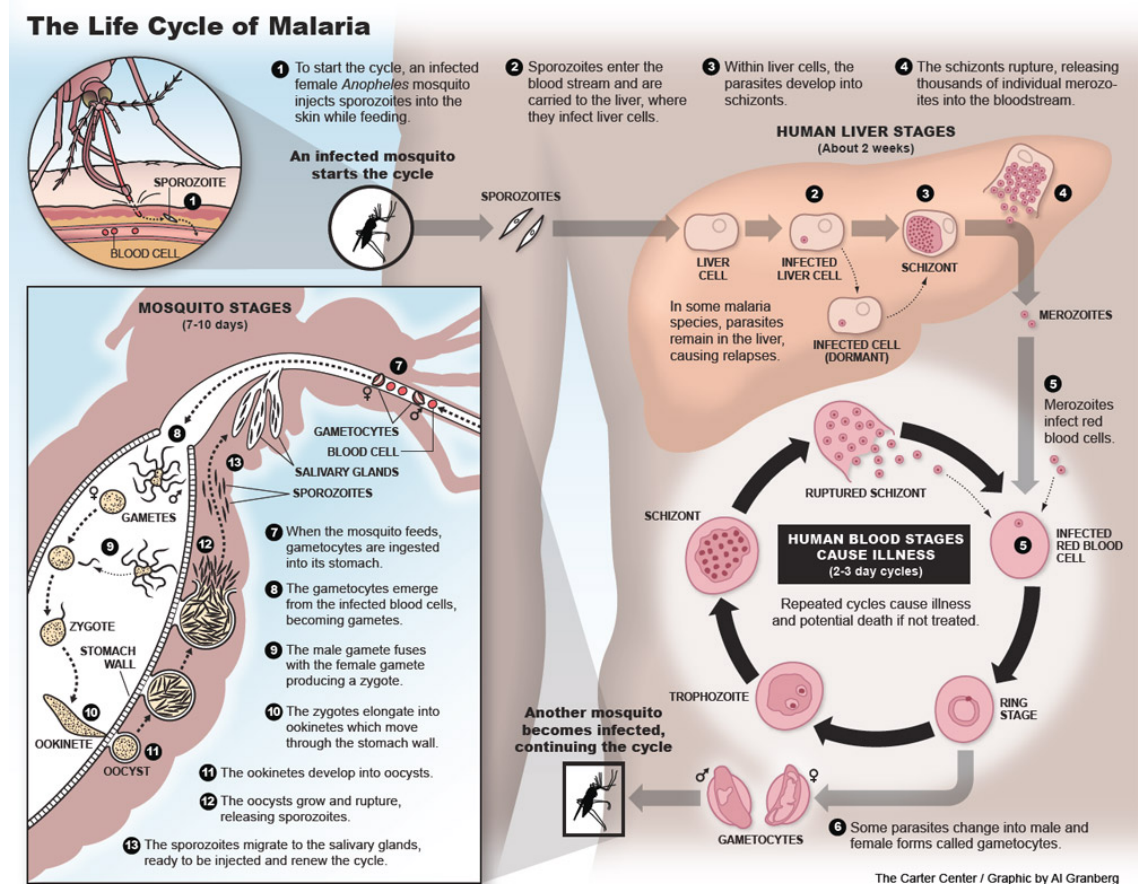
Figure I.2. Malaria life cycle. Taken from The Carter Center (24).

Pre-erythrocytic stages

Plasmodium parasites are transmitted to the vertebrate host through the bite of an infected *Anopheles* mosquito. Sporozoites are released from the mosquito salivary glands and injected in the host skin (25,26) while the mosquito is taking a blood meal. Only female mosquitoes are vectors of the disease since they need a blood meal for laying their eggs. Sporozoites are able to reach capillaries in the skin entering the circulatory system and migrate to the liver, within the first 15 minutes to few hours of infection (27,28). In addition, a proportion of sporozoites are drained to a proximal

lymph node where most are degraded and some achieve a partial development into exo-erythrocytic forms (26). Those sporozoites which reach the liver can cross the sinusoidal barrier through either Kupffer cells (resident macrophages in the liver) or endothelial cells (29,30). Sporozoites pass through several hepatocytes and finally invade one, where a vacuole is formed around the sporozoite as a result of the hepatocyte plasma membrane invagination (31).

Migration through tissues and invasion of host cells is possible due to a sporozoite form of locomotion known as gliding motility, which is powered by an actin-myosin motor (32,33). TRAP is a protein essential for this movement, since it is a transmembrane protein with a cytoplasmic tail that is fixed to actin filaments and also counts with extracellular adhesive domains. The extracellular portion of TRAP binds to host proteins and TRAP is translocated to the posterior end on the sporozoite, enabling a forward parasite movement (34). Using their locomotion machinery, sporozoites are able of disrupting host cell membranes (cell traversal capacity has been described on macrophages, epithelial cells and hepatocytes (31,35)) or enter host cells surrounded by the host cell plasma membrane that becomes part of the parasitophorous vacuole (36,37).

CSP is an abundant protein on the sporozoite surface, which binds to heparin sulfate proteoglycans present on hepatocytes providing an attachment to host target cells and activating sporozoites for host cell invasion (38–40). Parasites develop into exo-erythrocytic forms (EEFs) within the hepatocyte, undergoing asexual replication inside the parasite vacuole. This process lasts between 5 and 7 days for *Plasmodium falciparum* (human parasite) and 42 hours for *Plasmodium berghei* (rodent parasite), and ends with the release of 20000 to 30000 merozoites (41). Merozoites leave hepatocytes within hepatocyte-derived vesicles called merosomes (42).

Malaria liver stage is an ideal target for vaccination and drug discovery since this stage is clinically silent and the number of parasites present in the vertebrate host is very low compared to blood-stages.

Erythrocyte stages

In the blood stream, individual merozoites invade red blood cells (RBCs) where they undergo asexual replication inside a parasitophorous vacuole. RBC invasion begins with the interaction between receptors on the host cell and proteins expressed on the merozoite surface (such as MSP1-9, AMA-1 or *Pf*RH family), followed by proteolysis of some of these proteins (43–45). Once inside the RBC, the parasite loses

the apical complex (invasion organelles) and adopts a ring shape known as trophozoite, which then matures and grows forming an erythrocytic schizont. This process is completed within 48 hours for *P. falciparum* (between 24 and 72 hours, depending on the species) and results in the iRBC rupture and release of approximately 20 new merozoites ready to infect erythrocytes. Parasitaemia levels can be higher than 50000 infected RBCs per microliter of blood (46).

This cycle of RBC invasion, multiplication and rupture causes malaria pathology. Symptoms such as fever, headaches, fatigue and vomiting, coincide with erythrocyte rupture, due to the inflammation caused by parasite products released upon iRBCs lysis. In addition parasites are able to export some proteins to the RBC membrane, for example *Pf*EMP1 (47). This protein mediates infected RBCs sequestration in tissues by binding to molecules on endothelial cells (such as ICAM-1, upregulated during inflammation), and in this way parasites avoid entering the spleen where they could be destroyed (47) and complicate diagnosis since sequestrated iRBCs can not be detected in peripheral blood smears. Moreover, *Pf*EMP1 is also involved in binding to other RBC causing rosetting (48,49), which make the symptoms associated to erythrocyte sequestration worse and has been associated with clinical forms of severe malaria (50,51). iRBCs are sequestrated in a variety of organs including liver, heart, kidney, lung, brain and placenta, impeding normal tissue perfusion which is aggravated by the destruction of RBCs that further limits oxygen delivery. Peripheral tissues (under hypoxia conditions) and parasites produce lactid acid, leading to metabolic acidosis and respiratory distress (hyperventilation to expel more carbon dioxide) (52). Sequestration of iRBCs in the placenta is associated with reduced birth weight, early delivery and increase mortality in the newborn and the mother (53). The worst outcome of iRBCs sequestration occurs when iRBCs adhere to the brain microcapillaries causing vascular occlusion, local hypoxia and damage of the blood-brain barrier (54). Other severe malaria manifestations are anaemia, coma and renal problems. *P. falciparum* is responsible for most clinical cases of severe malaria and malaria deaths. The main difference between *P. falciparum* and *P. vivax,* which also cause severe symptoms, is that *P. falciparum* is able to invade a larger number of RBCs and presents a higher cytoadherence capacity which causes parasite sequestration (55).

Sexual stage

Some merozoites differentiate into gamatocytes (male and female) which are ingested by a mosquito while taking up a blood meal. Male and female gametes fuse and fertilise in the mosquito midgut forming a zygote which transform into an ookinete

with motile capacity to cross the midgut epithelium and reach the basal lamina where the development into oocyst occurs. The oocyst grows and undergoes multiple nuclear divisions, becoming a mature oocyst, which eventually bursts and releases thousands of sporozoites. Finally, sporozoites migrate towards the salivary glands where they mature and are stored for 1 to 2 months ready to be injected in a vertebrate host during a mosquito bite.

Gametocytes present in the vertebrate host blood have not been associated with malarial clinical symptoms.

Immune response to malaria

The knowledge of the immune response to malaria is essential in order to develop a protective vaccine and effective therapies to treat the disease. Animal models (56) and longitudinal studies following volunteers with natural or experimental malaria infection have provided insight in the host immune response to the malaria parasite. In endemic areas with seasonal malaria transmission, cohorts of volunteers can be monitored before, during and after malaria infection, allowing gene-expression analysis and antibody profiling which can be correlated with clinical and parasitology data (57). Controlled malaria infections have been performed to analyse immune responses and evaluate vaccine candidates. These infections are achieved by exposing volunteers to mosquito bites or to the injection of infected red blood cells, and can be treated with available drugs, such as chloroquine (58–60).

Individuals living in endemic areas develop an immune response that prevents the appearance of severe malaria symptoms (61,62) but they still suffer parasite infections (63,64). However, sterilizing immunity has been achieved through the administration of radiation-attenuated sporozoites (which do not achieve complete development within the hepatocyte) in studies with mice (65), non-human primates (66) and humans (67). The same effect has been reproduced with sporozoite injections together chloroquine treatment (which kills blood-stages) (58) and using transgenic parasites (which have been modified to avoid progression to blood stages) (68). The weak natural immune response elicited by malaria could be related with the immune-regulatory environment of skin and liver and the low numbers of injected sporozoites or to sporozoite mechanisms to avoid the immune system (46). In addition, the complex parasite life cycle with intracellular stages that facilitates hiding from the host immune

system and its high antigen variability (due to frequent mutations), also contributes to the challenge of mounting strong immune responses.

Both innate and adaptive immune systems contribute to the host immune response to *Plasmodium* parasites.

Innate immune response

The innate immune system provides a first line of defence through soluble components (e.g. complement system) and cellular components such as epithelial barriers (with epithelial cells providing a physical barrier and producing antimicrobial substances; and including intraepithelial T lymphocytes), antigen presenting cells (DCs, Macrophages and B cells) or NK cells (able to kill infected cells and activate the killing of phagocytosed parasites). The most relevant innate immune response to malaria is the stimulation of adaptive immune responses and inflammation.

Antigen presenting cells (DCs, B cells and macrophages) internalise parasite components, which are degraded and presented as peptides on MHC molecules on the cell surface. $CD4^+$ and $CD8^+$ T cells can recognize the pathogen peptide on MHC through their TCR and a co-receptor. DCs obtained from lymph nodes of malaria-infected mice or cultivated *in vitro* in the presence of sporozoites have been shown to present parasites epitopes to T cells (69–71). Antigen presentated to T cells, together with cytokines released by APCs has can activate T cells responses.

During the inflammatory response, leukocytes (mainly neutrophils and monocites) and plasma proteins (e.g. complement and antibodies) are recruited to control and clear the infection, although it can also lead to pathogenesis. Inflammation is induced by cytokines (e.g. IL-1, IL-2, IL-6, IL-12, IFN-ɣ, TNF-α) released by cells such as macrophages and DCs in response to parasite recognition, and stimulate the expression of endothelial adhesion molecules (e.g.ICAM-1, VCAM-1) leading to leukocytes infiltration in tissues. Pro-inflammatory cytokines play an important role stimulating adaptive responses and controlling parasite growth, but they are also associated with malaria symptoms such as fever and iRBC are sequestrated through binding to endothelial adhesion molecules (whose expression is stimulated by inflammatory cytokines). Inflammatory responses are curtailed by activation of T regulatory cells (72,73). DCs also release anti-inflammatory cytokines at later stages of infection (74). The balance between anti and pro-inflammatory cytokines determines the extent of infection and pathogenesis (75,76). The inflammatory response takes place against erythrocytic stages and appears to be absent in pre-erythrocytic malaria

stages, since both the skin and the liver present an immunoregulatory environment (with populations of macrophages and regulatory T cells producing anti-inflammatory cytokines, mainly IL-10 and TGF-β).

Certain pathogen structures (pathogen-associated molecular patterns, PAMPs) and host molecules produced during damage (damage-associated molecular patterns, DAMPs) can be recognised by the innate immune system (through pattern recognition receptors, PRRs, such as TLRs or complement proteins). Several examples of *Plasmodium* parasite components have been described to be recognised by the innate immune system such as *Plasmodium* RNA from liver stage; and GPI, AT-rich DNA or *Pf*EMP1 from blood stages. Liehl et al. found that *Plasmodium* RNA is recognised as a pathogen-associated molecular pattern (PAMP) by the cytosolic receptor Mda5 in hepatocytes, initiating a type I interferon response which can trigger myeloid cells resulting in a reduction of the parasite burden and a delay in the parasite release into the blood (77). GPI, a glycolipid which serves as an anchor for membrane proteins (e. g. merozoite surface protein-1, MSP1), is recognised by TLR2 and TLR4 on macrophages, stimulating the production of pro-inflammatory cytokines and nitric oxide (78,79). Parasitic protein-DNA complex is recognised by TLR9 on DCs, which in turn activate NK cells to produce IFN-γ (80). AT-rich DNA from *P. falciparum* has been shown to activate a type I interferon response (81). *Pf*EMP1 (*P. falciparum* erythrocyte membrane protein) binds CD36 on DCs which respond by producing IL-10, IL-12 and IL-18 (82). On the contrary, it has been shown that parasite adherence through *Pf*EMP to dendritic cells prevents dendritic cell maturation and the subsequent T cell activation (83), which would be a mechanism of immune evasion.

Other example of the innate immune system action was described by Qingfeng et al who found that NK cells killed iRBCs in a contact-dependent manner, using humanised mouse models infected with *Plasmodium falciparum* (84).

Adaptive immune response

The adaptive immune system can provide long-lasting immune responses to a wider range of antigens than the innate immune system and is able to generate memory cells. There are two classes of adaptive immune responses: cellular immunity which is carried out by T cells (e.g. CD4$^+$, CD8$^+$, Tregs) and humoral immunity, generated by B cells producing antibodies.

a) Humoral responses

Humoral immunity is generated by B cells producing antibodies. B cells are able to bind parasite antigens through the BCR expressed on their surface and internalize the pathogens components which are degraded within the cell (85). Resulting peptides are loaded on MHC II molecules ready to be recognised by CD4$^+$ T cells, which are able to activate the B cell in a contact-dependent manner and through cytokine secretion (86). Activated B cells undergo clonal expansion and after secreting IgM antibodies the antibody isotype they produce switches to IgG, IgE or IgA. Firstly after antigen encounter short-lived plasma cells secrete antibodies, and afterwards, memory B-cells and long-lived plasma cells are the populations responsible for the antibody production (87,88).

Antibodies have been shown to play an important role in the immune response to malaria. Passive transfer of purified antibodies from individuals with certain immunity to malaria to infected volunteers, has been shown to decrease parasitaemia (89–91). In addition, a positive correlation between protection and antibody titres has been observed in clinical studies where volunteers where challenged after immunisation with a CSP subunit vaccine or irradiated sporozoites (67,92–94). However, some of the protected individuals had low antibody titres, and not all the volunteers with high antibody titres were protected, indicating the presence of other mechanisms contributing to immunity. Mouse models have also provided evidence for the relevance of antibodies in the malaria response both neutralizing parasites and helping to eliminate them (95,96).

The main limitation of humoral responses is that parasites are exposed to antibodies for a very short amount of time, since malaria parasites are predominately present as intracellular forms and parasites travel quickly between host cells. Inoculated sporozoites can reach the liver within a few minutes (although this migration can last hours) (97) and merozoites released from an infected red blood cell can infect other one in minutes or less (98). Therefore, high antibody concentration and avidity would be required to mount an effective immune response at the moment of infection.

Antibodies prevent hepatocyte and merozoite invasion (99), facilitate parasite clearance enhancing phagocytosis and opsonize and agglutinate infected erythrocytes avoiding their sequestration in blood vessels. Antibodies carry out their function through several mechanisms, either acting on their own (e. g. neutralization, agglutination) or mediating a cellular function through opsonisation (which enhances phagocytosis) or antibody-dependent cellular cytotoxicity (ADCC). Neutralization avoids host cell invasion (100,101) due to the presence of antibodies impeding normal

function or the interaction of relevant parasite proteins with hepatocytes or red blood cells. For instance, antibodies targeting CSP on sporozoite surface have been described to inhibit cell traversal activity (102), antibodies against TRAP (protein located in sporozoite micronemes) inhibit sporozoite gliding motility (34) and antibodies specific for *P. falciparum* erythrocytic binding protein (EBA-175) block invasion (103). Antibodies bound to the parasite can recruit complement proteins leading to parasite lysis, for instance, antibodies present in human sera targeting MSP1 and MSP2 can trigger complement deposition on merozoites avoiding erythrocyte invasion (104). Antibody-dependant complement lysis has also been described to have an effect on sporozoites (105) and on gametes (106). Opsonisation occurs when antibodies cover sporozoite or merozoite surface facilitating uptake by macrophages and dendritic cells, leading to parasite clearance (107–109). Schwenk et al., in a study where volunteers were vaccinated with RTS,S and later infected, described a higher sporozoite endocytic activity in the sera of protected individuals than in volunteers susceptible to infection (110). Antibody-dependent cellular cytotoxicity has been described in malaria mainly with monocytes (111–113). Antibodies have also described to affect parasite development in pre-erythrocytic (114), erythrocytic (115) and sporogonic stages (116,117).

Antibodies can be raised with specificity for antigens present in different parasite development stages, and also against parasite proteins present on the surface of iRBCs. CSP (circumsporozoite protein) has been extensively described as a sporozoite antigen targeted by humoral responses, in particular a region (NANP) which contains peptide repeats and is shared by all *Plasmodium* species (118). Later, studies in naturally infected humans have found antibodies against CSP with different specificities (119), and also against other pre-erythrocytic antigens such as LSA1 (liver-stage antigen 1) and TRAP (thrombospondin-related adhesive protein) (120). High antibody titres targeting these sporozoite proteins have shown a positive correlation with protection in endemic areas (120–122).

Blood stage antigens are also targeted by antibodies, and humoral responses are particularly relevant in blood stages since iRBC can not express MHC class molecules (which can be recognized by TCR on the T cell surface). Antibodies against MSP2 (merozoite surface protein-2) have been associated with protection in Papua New Guinea (123). *Pf*EMP also has been described to be targeted by protective antibodies in endemic areas (109), antibodies against this protein on the iRBCs would opsonize and agglutinate infected erythrocytes avoiding the sequestration in blood vessels.

In addition to the antibody antigen specificity, the kind of antibody isotype has also been regarded as important for the immune response, with studies describing that IgG1 and IgG3 (which have high affinity for Fc receptors and fix complement proteins) are the antibody subclasses more relevant to achieve protection (124–126).

b) Cellular responses

T lymphocytes express on their surface a TCR receptor and either co-receptor CD4 or CD8. The TCR recognises a peptide presented in the context of an MHC molecule and initiates a signaling cascade with the aid of co-receptors. MHC class I molecules are expressed on the surface of all nucleated cells, while MHC class II molecules can be found on APCs (B cells, dedritic cells and macrophages). Peptides are generated through the degradation of endogenous or exogenous proteins (pathogen proteins that are inside the cell due to infection or because they have been internalised) and loaded on MHC molecules inside the cell forming complexes (MHC-peptide) which are exported to the plasma membrane. This antigen presentation process provides a system to show signs of potential infection on the cell surface. CD4$^+$ T cells recognise MHC class II molecules on APCs and CD8$^+$ T cells recognise MHC class I molecules on all nucleated cells (127).

CD4$^+$ T cells act mainly by recruiting and activating other immune cells and can be divided into different subtypes according to phenotypic markers and functionality, for example Th1, Th2, Th17 and T regs. Th1 cells secrete cytokines such as IFN-ɣ and TNF-α which mediate pro-inflammatory responses. In addition, Th1 cells support B cell and CD8$^+$ T cell responses through cytokines and contact-dependent interactions, which elicit activation, proliferation and isotype switching for B cells. Th1 cells can trigger apoptosis in infected cells through the interaction between their Fas ligand (CD178) and Fas (CD95) on a target cell. Th2 cells, which secrete mainly IL4, IL5 and IL13, play an important role in immunity against extracellular pathogens. T regs, which are identified by high levels of CD25 and transcription factor FoxP3, down-regulate the immune response secreting anti-inflammatory cytokines such as IL-10 and TGF-β. In malaria, T regs may act in preventing immunopathology, however they can also have a negative effect inhibiting a protective Th1 response (72).

CD8$^+$ T cells (cytotoxic lymphocytes, CTLs), upon recognition of a specific MHC class I-peptide (128,129), are able to act as effector cells eliciting apoptosis in target cells through cytokine and cytotoxic granules secretion or through interaction between Fas and Fas ligand. CD8$^+$ T cells also secrete IFN-ɣ which can activate innate cells

(e.g. macrophages), up-regulate expression of components involved in antigen presentation (e. g. MHC molecules) and stimulate the production of effector molecules such as NO.

Liver-stage

The liver is an autonomous priming site where antigen presentation can take place without the infiltration of APCs. Numerous cell types such as liver-resident dendritic cells, Kupffer cells (liver-resident macrophages), hepatic stellate cells, liver sinusoidal endothelial cells (LSEC) and infected hepatocytes have been described to present antigens to T cells (130–133). T cells are abundant in the liver, particularly CD8$^+$ T cells (134,135). Bertolino et al. showed that hepatocytes can activate naive CD8$^+$ T cells (133). Nevertheless, the liver presents an immunoregulatory environment (136,137), for instance, Kupffer cells (138) and liver dendritic cells (139) have been reported to secrete IL-10, inducing T cell tolerance. Both CD8$^+$ and CD4$^+$ (mainly Th1) T cells produce IFN-ɣ which inhibits sporozoite development into merozoites inside hepatocytes (140). CD8$^+$ and CD4$^+$ T cells specific for pre-erythrocytic *Plasmodium* antigens can also kill parasites through direct cytolisis. Studies with mice immunised with irradiated sporozoites, have shown that CD4$^+$ and CD8$^+$ T cells play an important role in the immune response elicited (96,141–143). Protection from clinical malaria has been associated with T cell responses against pre-erythrocytic antigens, for example: lymphocyte proliferation responses to LSA1 in Gabonese children (144) and CD4$^+$ T cell responses to CSP in The Gambia (145).

Blood-stages

Erythrocytes and free merozoites lack MHC expression; therefore, they can not activate T cells through interaction with TCR. However, Pombo et al. described that volunteers challenged with iRBCs and cured, developed proliferative cellular immune responses against subsequent challenges (146). In protected volunteers, they detected CD4$^+$ and CD8$^+$ IFN-ɣ-producing cells and high levels of nitric oxide synthase in PBMCs. In addition, Riley et al. found T cell proliferative responses against MSP1 (blood-stage antigen) associated with protection from severe malaria in children living in West Africa (147). Moorman et al. also showed an association between IFN-ɣ responses to MSP1 and delayed time to reinfection in western Kenya (148). Carvalho et al. measured higher *in vitro* cellular responses to MSP1 in PBMCs from indivuals who have been more exposed to malaria in Brazil (149).

Vaccine development and current status

A malaria vaccine would be the most cost-effective control measure against malaria. The feasibility of a malaria vaccine is supported on the knowledge that natural immunity is adquiered over the years after repeated malaria infections, and that it is possible to achieve sterile protection immunising with radiation-attenuated sporozoites (RAS) (67). However, in spite of the large research efforts in the last decades, a licensed vaccine is not still available. The leading malaria vaccine is RTS,S with an efficacy of 30-50% which decreases after one year (150–152). Once phase 3 clinical trials were completed, the EMA (European Medicines Agency) gave a positive scientific opinion to the vaccine on July 2015 (153) and WHO's recommendations on RTS,S are expected by November 2015. Afterwards, countries where the vaccine will be used will decide on licensing the vaccine. The partial efficacy of RTS,S will prevent a large number of malaria cases, but research continues to search for a second generation malaria vaccine that will elicit higher protection, in addition to be safe, affordable and easy to transport and deliver in endemic countries.

Malaria vaccine candidates target pre-erythrocytic, erythrocytic and mosquito stages. The pre-erythrocytic stage has been regarded as the ideal target for malaria vaccination since it is clinically silent and it is the first step of infection with low parasite numbers and a duration of about one week (for *P. falciparum*) allowing enough time for the immune system to fight the infection at an early stage. Pre-erythrocytic stage vaccines aim to avoid sporozoite invasion of hepatocytes through protective antibodies or to eliminate infected hepatocytes by inducing $CD8^+$ and $CD4^+$ effector T cells. Blood-stages vaccines attempt to reduce parasite levels in the blood by inhibiting merozoite invasion of RBC through humoral responses and stimulating $CD4^+$ T cell responses. Inducing the production of antibodies that prevent iRBC sequestration is also helpful to avoid severe malaria symptoms. Lastly, transmission-blocking vaccines target parasite proteins involved in the development within the mosquito gut, eliciting antibodies that block the *Plasmodium* cycle at the mosquito stage and avoid the subsequent transmission to other vertebrate host. Transmission-blocking vaccines do not provide a direct benefit to those vaccinated, but generate protection at a population level (herd immunity).

According to their composition, potential vaccines can be classified into those using the whole parasite or a subunit strategy. Whole parasite vaccines are prepared with parasites whose development is arrested during liver stages avoiding the clinical symptoms, but exposing the parasite antigens to the immune system. Sterile immunity

has been achieved immunising with irradiated sporozoites in both rodents and volunteers (65,67,154,155). Irradiate sporozoites invade hepatocytes, but can not complete their development and progress further in the life cycle. An important limitation for these vaccination approach is the large number of bites required (using over 1000 infected mosquitoes) to achieve immunity, making vaccine delivery in endemic countries complicated, and there is a risk of a breakthrough infection. The biotechnology company Sanaria is testing a vaccine consisting on purified and cryopreserved irradiated *P. falciparum* sporozoites, which are administered intradermally, subcutaneously or intravenously, and protects 90% of vaccinated volunteers (156–159). Other approaches to immunise with sporozoites that can not complete their development in the vertebrate host are heat-killed sporozoites (which can not invade hepatocytes), genetically arrested parasites (GAPs, which lack an esential gene involved in host cell invasion or development) and administration of sporozoites under drug treatment (e.g. chloroquine, primaquine) (68,160). Whole parasite vaccines have been described to elicit immune responses mainly mediated by CD8^{+} T cells (161–164).

Subunit vaccines, which deliver a component of the pathogen, are safer and easier to produce and administrate than whole parasite vaccines. Most efforts on subunit vaccines have been focused on the protein CSP which is abundant on the sporozoite surface, but other pre-erythrocytic antigens have also been targeted such as TRAP, LSA1 and Exp1. Blood stage subunit candidate vaccines have been prepared mainly against MSP1 and AMA1. Subunit vaccines can be based on a peptide, a protein or even a DNA sequence. When the vaccine is a protein, adjuvants (e.g. aluminum salts, liposomes) have an important role enhancing immune responses, mainly activating the innate immune system which subsequently stimulates the adaptive response. RTS,S, the most advanced malaria vaccine, is an example of subunit vaccine which comprises a region of *P. falciparum* CSP fused to the hepatitis B virus antigen administered with a liposomal adjuvant (AS01) (165). Adjuvant formulation in RTS,S has been optimised and shown to be essential to induce an immune response (94,166). RTS,S elicits protection mainly through antibodies against CSP which prevent sporozoite entry into hepatocytes (167).

Instead of a protein, vaccines can be based on a DNA sequence, which upon injection enters the host cells where the DNA encoded protein is synthesized. The pathogen protein expressed in the host cells can be degraded into peptides which are loaded on MHC Class I molecules to present the pathogen peptide on the cell surface to CD8^{+} T cells. A method of DNA delivery described to elicit strong immune responses

are viral vectors, such as poxvirus or adenovirus, that can carry a foreign DNA sequence and also have an adjuvant effect themselves (168). The serum samples tested in this thesis were collected after vaccination with chimpanzee adenovirus serotype 63 (ChAd63) and MVA enconding various antigens. MVA (Modified Vaccinia virus Ankara) is a Poxvirus vector that was obtained by serial passaging until it lost its replication capacity (169). Replication of adenoviruses can be avoided through the deletion of a region (named E3) in their genome. The vector ChAd63 is a simian adenovirus, which is used to avoid the possibility of interference with pre-existent immunity against human adenovirus (170). Combination of viral vectors can be administered as a heterologous prime-boost vaccination regimen, in particular, ChAd63 prime followed 8 weeks later by MVA boost has been the strategy followed in the laboratory and has been shown to elicit high titre protective antibodies and strong T cell responses (171), and protection in endemic areas (172). The most advanced malaria vaccine targeting cellular immunity consist of ChAd63 and MVA vectors encoding ME-TRAP, a string of multiple epitope (ME) fused to TRAP. Phase IIb field clinical studies recently conducted with Kenyan adults, have shown that the vaccine induces partial protection against *P. falciparum* which was associated with T cell responses to TRAP (173). RTS,S/AS01 and viral vector ME-TRAP vaccines are being administered in combination in an ongoing study (already in Phase I/IIa trials) with the objective of improving efficacy and durability (171).

Tools in malaria research: mouse models, *in vitro* cultures and transgenic parasites.

Plasmodium falciparum, the causative agent of most malaria deaths, only infects humans and some primates such as Aoutus monkeys (which are of great value for malaria research, particularly for evaluation of drugs and vaccines for human use) (174,175). For this reason, *P. berghei* and *P. yoelii*, have been widely used as models to study malaria, since their life cycle can be reproduced in a laboratory using mice as vertebrate host and *Anopheles stephensi* mosquitoes. These rodent species can induce a range of pathologies that can be cured spontaneouly or even cause death depending on the mouse strain (176,177).

Research on liver-stages is particularly difficult, since sporozoites need to be freshly collected by dissection of mosquito salivary glands for mouse infections and *in vitro* cultures; and studies with volunteers are not possible due to obvious ethical

reasons. The main tools to study pre-erythrocytic stages are animal models and cell cultures of primary hepatocytes or hepatoma cell lines. The establishment of hepatoma cell lines has supported *in vitro* studies of liver stage invasion using *Plasmodium* parasites. Common cell lines used are HepG2 and Huh7 which support complete liver-stage development of *P. berghei*; and HC04, where not only *P. berghei* but also *P. falciparum* can develop (178). On the contrary, blood stages can be obtained from frozen infected blood and from peripheral blood of volunteers, and *in vitro* cultures of *P. falciparum* erythrocytic stages using RBCs are widely used (179–181).

Mouse models have provided important knowledge about the biology of the parasite, the pathogenesis of the disease and the host immune response (56). In addition, they are essential in vaccine development and drug discovery, since they have given the possibility of screening pre-clinical malaria antigens, and gaining a better understanding on the immune responses elicited by vaccines.

The development of transgenic parasites through targeting a particular gene or cloning a protein into the parasite genome is giving a new insight in parasite biology and an important tool in vaccination development. This technique has allowed the generation of parasites that express GFP (green fluorescent protein) (182,183) and can be tracked by flow cytometry, enabling the quantification of infected cells in a cell culture or blood sample (183–186). Genetic manipulation has also been used to obtain knock-out parasites in which a relevant protein has been deleted in order to study how its absence affects the behaviour of the parasite (187–189). In addition, transgenic parasites allow pre-clinical evaluation in mouse models of *P. falciparum* antigens that are absent in rodent parasites, since the *falciparum* protein can be expressed in transgenic parasites. In fact, transgenic parasites expressing *falciparum* antigens have been used to challenge vaccinated mice whose post-vaccination sera have been evaluated in this thesis (190).

Evaluation of humoral responses:
Inhibition of Sporozoite Invasion assay (ISI).

Immune responses to malaria have been evaluated mainly by measuring levels of antibodies, cytokines and IFNγ using ELISA (Enzyme-Linked Immunosorbent assay), ELISPOT (Enzyme-Linked ImmunoSpot assay) and ICS (intracellular cytokine

staining) techniques. A unique immune correlate of protection has not been identified in the context of malaria, probably due to the diversity of the immune responses elicited by different vaccination strategies and to the presence of several immune mechanisms providing protection. Therefore, gathering the maximum amount of information about the immune response measured with different assays is extremely helpful to gain a better knowledge on the host immune response induced through vaccination.

As it has been previously described, antibody responses play an important role in pre-erythrocytic malaria stage, in fact, RTS,S elicits protection mainly through humoral responses (167). This thesis focuses on the evaluation of post-vaccination antibody responses using the ISI assay, which measures antibody capacity to inhibit sporozoite invasion. ISI uses and *in vitro* system where sporozoites are co-cultured with hepatocytes to reflect the actual infection context, with the objective of measuring functionality of antibody responses rather than antibody concentration. ISI assay has evolved from time-consuming microscopic readouts to flow cytometric measures that make it possible to incorportate this assay into a routine of vaccine candidate evaluation. The following lines review how this assay was firstly described and subsequently improved.

In 1982, Hollingdale et al. described an assay to evaluate the capacity of antibodies to prevent *P. berghei* sporozoites infection *in vitro* (191). They used human embryonic lung cells (WI38), since in previous studies (36) they showed this cell line could support *P. berghei* sporozoite infection and development with releasing of merozoites. At different incubation times after the addition of sporozoites, the cells were fixed with methanol and stained with Giemsa or an immunofluorescent antibody, allowing detection of sporozoites by microscopy. In the immunofluorescent test, serum from mice that had been injected with sporozoites was used as a primary antibody and a goat anti-mouse antibody labeled with fluorescein, as secondary antibody. In this first description of the ISI assay (191), a monoclonal antibody against *Pb*44 (former name given to the sporzoite surface antigen CSP) and its Fab fragment were shown to prevent the entry of sporozoites in WI38 cells, while sporozoites non-treated with antibodies could be seen inside the cells eight hours after infection. These results were consistent with previous studies where mice that had received a passive transfer of the same monoclonal antibody were protected against sporozoite challenge (192). The fact that this assay was able to reflect a functional effect of an antibody against a sporozoite antigen already seen *in vivo*, supported the idea of using this *in vitro* assay to evaluate the effect of antibodies and drugs aimed to prevent the sporozoite infection.

Following on from this work, the *in vitro* culture system was improved by substituting the target cells for HepG2, a liver cell line, as it had been shown to support the complete development of *P. berghei* to the point of merozoite release (193). HepG2 cells were isolated from a child with primary hepatoblastoma and hepatocellular carcinoma (194) and shown to have surface receptors similar to those present in primary hepatocytes (195). A higher rate of infected cells was observed when using HepG2 instead of WI38 (196) and, most importantly, this cell line provided the opportunity to study the interaction between sporozoites and hepatocytes (their natural host cells). An immunoperoxidase-based technique (197) was found to offer a better resolution to identify early stages of parasite development, when used for the staining. In this case, the secondary antibody was a rabbit anti-mouse conjugated with horseradish peroxidase whose substrate was 3,3'-Diaminobenzidine.

Hollingdale et al. and Leland et al. extended their studies using other *Plasmodium* parasites: *falciparum* and *vivax*, which also invaded HepG2 cells *in vitro*; testing the inhibitory capacity of different monoclonal antibodies, human sera from endemic areas and sera from volunteers immunised with irradiated sporozoites (198,199). *P. falciparum* and *P. vivax* did not achieve a complete development within HepG2 cells (198). Mazier et al. showed that *P. falciparum* could accomplish complete development in primary hepatocytes (174) and they used this system to study the effect of sera from mice immunised with CSP peptides on the parasite invasion and intracellular development (200).

In all the studies described so far, the cell cultures were analyzed with microscopy after staining with a primary antibody specific for parasite antigens and a secondary antibody (bound to a fluorescent dye -fluorescein- or conjugated with and enzyme -peroxidease- that processes a substrate), or with Giemsa which is a dye able to stain *Plasmodium* chromatin (201). Microscopy readouts were not only time-consuming, but also associated to the subjectivity of the observer's criteria assessing whether the parasites were outside or inside the cells, or which stage of development they had reached. With the aim of using a more objective method to quantify the outcome of these *in vitro* assays, Zavala proposed an immunoradiometric assay in 1985 (202). The two-site immunoradiometric assay (IRMA) quantified the amount of CSP on hepatocytes as a measure of sporozoite infectivity and relied on the presence of at least two identical epitopes (binding sites) in each CSP molecule. An extract of infected cell culture was added on a well coated with monoclonal antibody (3D11) against *P. berghei* CSP, after incubation and washing, the same monoclonal antibody

labelled with ^{125}I was used in order to generate a signal in a gamma counter proportional to the amount of CSP antigen inside infected hepatocytes.

As an improvement to the staining techniques for microscopy readouts, which allowed observing the location and development of the parasites (unlike the immunoradiometric assay), Rénia et al. described a double staining method in 1988 (203). With the aim of increasing the objectivity when distinguishing between intracellular and extracellular sporozoites (until then, this distinction had been made using morphological criteria), they proposed a two step protocol combining two staining techniques that had been commonly used in the ISI assay. Each step employed the same primary antibody (a monoclonal antibody against antigens on the sporozoite surface), but different secondary antibodies. First, the cell cultures were fixed with paraformaldehyde, which did not allow the entrance of antibodies into the cells. As a result, the primary antibody was bound only to extracellular sporozoites, identified with a secondary antibody conjugated to peroxidase (Immunoperoxidase antibody test, IPAT). Secondly, the cell cultures were treated with ethanol, so the intracellular parasites were accessible to the primary antibody. In this step, an anti-mouse immunoglobulin antibody labeled with fluorescein was used (Immunofluorescent antibody test, IFAT).

In 1998, Sinnis et al. described a method to quantify sporozoite invasion combining a gamma-counter readout with two different kinds of fixation (204). Each sample was tested in duplicate, fixing with paraformaldehide after incubation and adding methanol in only one of the replicates. In samples where methanol had been added, monoclonal antibody against CSP (3D11, labeled with ^{125}I) could access both intracellular and extracellular parasites, while when methanol was not added only extracellular parasites were detected. By substracting the amount of extracellular parasites from the total number of parasites, a measure of hepatocyte invasion was given.

RT-PCR (which had been previously used to quantify malaria liver stages (205)) was introduced by Kumar et al. (206) in 2004 as a quantitative readout of a sporozoite neutralizing assay using *P. berghei* transgenic sporozoites expressing *P. falciparum* CSP repeats (207). RT-PCR was performed amplifying the *P. berghei* 18S rRNA, and, with normalization purposes, the HepG2 beta-actin gene.

Natarajan et al. in 2001 and Tarun et al. in 2006 used FACS (Fluorescence-activated cell sorting) to isolate hepatocytes infected with sporozoites (*P. berghei* and *P. yoelii*, respectively) expressing GFP (183,184). Prudencio et al. used flow cytometry

in 2008 to quantify sporozoite invasion of hepatocytes, and to obtain other information relevant for the malaria liver stage, such as cell traversal capacity of sporozoites or parasite development within liver cells (208). The assay was performed adding *P. berghei* GFP sporozoites to Huh7 cells and incubating the co-culture for 2 hours. GFP signal within infected hepatocytes was detected when acquiring the samples on a flow cytometer, allowing measuring the percentage of infected hepatocytes. It was found that the intensity of the GFP signal was higher at longer times of incubation (12-36h), and this increase correlated with the number of parasites copies found by PCR, indicating a possible method to measure parasite development within liver cells. In 2012, Kaushansky et al. also described a flow cytometry method to evaluate sporozoite infection (209). This study used *P. falciparum* sporozoites and HC04 cells, which are susceptible to infection by this *Plasmodium* species. In order to detect infected cells, 90 min after incubation cells were fixed, permeabilized and stained with anti-CSP sera and a secondary antibody conjugated with Alexa-fluor 488.

Flow cytometry provides a readout method for sporozoite inhibition of invasion assays, which highly increases the throughput of the technique and overcomes the bias associated to the traditional microscopy quantifications. qRT-PCR can also provide a quantitative readout with higher throughput than traditionally microscopy techniques, but flow cytometry is even less expensive and time-consuming than PCR, and gives information at a single cell level.

According to the time of incubation of sporozoites with host cells, the antibody inhibition assay has been named ISI (Inhibition of Sporozoite Invasion Assay) when incubation lasts 1 to 24 hours and ILSDA (Inhibition of Liver Stage Development Assay) when incubations are extended between 1 or 2 days up to 6 days (210,211). Although in this thesis the antibody inhibition assay has been referred as ISI, it is actually in the limit between ISI and ILSDA, since sporozoite incubations lasts around 24 hours, an endpoint which was selected to detect the maximum percentage of infected cells on the flow cytometer.

Although these antibody inhibition assays have limitations, mainly the difficulty in obtaining fresh sporozoites and biological parasite variability, a flow cytometric readout enables larger numbers of samples to be analysed more easily. This assay can therefore be very helpful in evaluation of antibody responses in vaccine studies and in the development of drugs targeting sporozoites and liver-stage parasites.

Thesis aims and outline

A large research effort is currently underway to find an effective and affordable malaria vaccine. Tools that allow the evaluation of the host immune response are essential to vaccine development, since, together with challenge studies and clinical data, they provide a criteria to select the best vaccine candidates. In malaria, there is not clear correlate of immune protection; both humoral and cellular responses have been described to play a role in host immunity. Therefore, a complete description of the immune response elicited by vaccination requires various assays to measure antibody and effector cell responses. This thesis focuses on an assay to evaluate antibody-mediated inhibition of malaria sporozoite infection: the Inhibition of Sporozoite Invasion assay (ISI). This assay measures antibody capacity to prevent sporozoite infection and was first described in 1982 by Hollingdale et al. Since then, different host cells, sporozoite species and readouts have been used, but the assay has not been systematically employed to test large numbers of samples. The recent development of transgenic parasites detectable by flow cytometry provides a readout which highly increases the throughput of the technique and overcomes the subjectivity of the person performing a microscopy readout. The assay still presents many limitations: the complication of working with live sporozoites (the variability of infectivity achieved by different batches of sporozoites and the requirement to obtain enough freshly dissected sporozoites to perform the assay), the non-specific inhibition effect of naive serum and lack of standardisation and reproducibility. In spite of the difficulties associated, the ISI assay provides an *in vitro* system that reflects the actual infection context. Therefore, the measure of antibody-mediated prevention of sporozoite invasion would be a better reflection of antibody functionality and protection *in vivo* than measures of antibody concentration such as ELISA.

The objective of this thesis is to further develop the ISI assay and adapt it to test a large number of samples. This work has been developed in collaboration with the Jenner Institute where an important part of the research conducted consists on evaluation of pre-clinical and clinical malaria candidate vaccines. So far, humoral responses in vaccine studies have been evaluated mainly using ELISA. This project aims to support the incorporation of a new protocol to evaluate humoral responses in candidate screening and clinical studies.

Aims:

-To find the conditions where the infectivity rates may be higher with the objective of making the best use of sporozoites, which are a limited resource. To accomplish this objective different hepatoma cell lines, cell culture media, incubation times and cell:sporozoite ratio will be tested.

-To optimise the number of host cells and sporozoites to use in each replicate in order to obtain reliable data while testing a large number of samples. To find adequate controls to normalise background effect and variability in infectivity associated to different sporozoite batches.

-To adapt the assay for the use of transgenic parasites currently available at the Jenner Institute.

-To evaluate whether the reporter protein MFI (Mean Fluorescence Intensity) measured by flow cytometry could be an indicator of parasite development and, consequently, be a potential measure of antibody effect on sporozoite development inside hepatocytes.

-To use the assay to evaluate serum samples from mouse and macaque pre-clinical studies and clinical trials, collected in studies performed at the Jenner Institute, in order to obtain relevant information about antibody responses to the candidate vaccines of interest and validate the optimised assay.

-To find whether there are associations between the data obtained with the ISI assay and other clinical and immunological data available for the samples tested.

MATERIALS & METHODS

Cell lines & Culture media

Four different hepatocyte cell lines have been used as host cells for sporozoites: a mouse cell line (BNL) and three different human cell lines (HepG2, Huh7 and HC04). The HepG2 cell line was established from a liver biopsy of a 15-year-old caucasian male from Argentina who had a hepatocellular carcinoma (194,212). The Huh7 cell line was established from hepatocellular carcinoma tissue removed from a 57-year-old Japanese male (213). HC04 was obtained from normal liver tissue taken from a hepatoma patient in Thailand (214). In our laboratory, HC04 cell line obtained from ATCC is genetically identical to HepG2 but grows differently. These cell lines have morphology characteristics that resemble those of liver parenchyma cells.

DMEM (Dulbecco's Modified Eagle's Medium) [Sigma Aldrich] and RPMI (RPMI-1640, Roswell Park Memorial Institute medium) [Sigma Aldrich] were used as culture media with the addition of 10% heat inactivated FCS [Sigma Aldrich], 100U/ml penicillin, 100μg/ml streptomycin [Sigma Aldrich] and 2mM L-glutamine [Sigma Aldrich]. Cells were cultured at 37ºC and 5% CO_2. TrypLE Express Enzyme [Life Technologies] was used to detach hepatocytes from culture plates or flasks.

Chimeric parasites

All the experiments were performed using chimeric *P. berghei* sporozoites expressing GFP as a reporter gene. Sporozoites expressing GFP or GFP-luciferase (both under eef1α promoter) were used in chapter 1 to find the optimal conditions to perform the ISI assay. In chapter 2, in order to evaluate the capacity of post-vaccination serum samples to reduce hepatocyte invasion, chimeric *P. berghei* parasites expressing GFP-luciferase and the same *P. falciparum* antigen expressed in the vaccine. These chimeric *P. berghei* parasites provided the possibility of testing *P. falciparum* antigens without the hazard of working with *P. falciparum* parasites and could be produced at the laboratory insectary.

These chimeric parasites, kindly provided by DPhil student Ahmed Salman, were generated through GIMO ('Gene insertion/marker out') transfection (215). Chimeric parasites contain a construct, inserted in the genomic silent locus 230p, which includes a *P. falciparum* gene under *Pb* UIS4 promoter and a GFP-luciferase cassette under the control of *Pb* eef1α promoter.

P. berghei chimeric parasite	*P. falciparum* antigen	*P. berghei* homologue antigen	Reporter
'WT'	-	-	GFP
	-	-	GFP luciferase
Replacement CS	CSP	CSP *berghei* deleted	GFP luciferase
Addition parasite including the *P. falciparum* antigen added	CSP	*P. berghei* homologue present in chimeric parasites	GFP luciferase
	TRAP		
	CelTOS		
	UIS3		
	Falstatin		
	SPECT1		
	SPECT2		
	HT		
	RP-L3		
	ETRAMP5	No *P. berghei* homologue	
	LSA1		
	LSA3		
	LSAP1		
	LSAP2		

Table 1. Chimeric *Plasmodium berghei* sporozoites used. Parasites were generated and kindly provided by Ahmed Salman (DPhil student).

Chimeric parasites used included one of the *P. falciparum* antigens: CelTOS, LSA1, LSA3, LSAP1, LSAP2, UIS3, ETRAMP5, Falstatin, SPECT1, SPECT2, RP-L3, HT, CSP and TRAP [Table1]. CelTOS, UIS3, Falstatin, SPECT1, SPECT2, RP-L3, HT, CSP and TRAP are present both in *P. falciparum* and *P. berghei*, while the rest of the *P. falciparum* antigens tested do not have a *P. berghei* homologue. The *P. falciparum* antigen was expressed under UIS4 promoter as an additional copy ('addition parasites'). This strategy allows the generation of transgenic parasites even when a *P. berghei* homologue is not present. In this work, some experiments were performed with CSP 'replacement parasites' in which *P. berghei* CSP had been deleted and *P.*

falciparum CSP is expressed under the control of the *P. berghei* CSP promoter. This chimeric parasite was generated through the GIMO method, replacing the *P. berghei* CSP by the *P. falciparum* CSP. *Pf* CSP replacement parasites express GFP-luciferase under *Pb* eef1α promoter in 230p, as 'addition transgenic parasites'.

Both the genotype and phenotype analysis of the transgenic parasites were performed by Ahmed Salman [Ahmed Salman, DPhil thesis]. The correct integration of the construct in the chimeric parasites was demonstrated through southern analysis and PCR. The expression of the *P. falciparum* antigen was probed by IFAT (Immunofluorescence antibody test) using monoclonal antibodies whenever available or sera collected from vaccinated animals.

Serum samples & Monoclonal antibodies

Serum samples used in this work were provided by various researchers at the Jenner Institute (Oxford University) and had been obtained in pre-clinical vaccine studies, performed with mice and macaques, and in clinical trials [Table 2].

All serum samples collected counted with all the necessary ethical approvals for the study. Mice work was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under the project license number 30/2414 granted by the UK Home Office. University of Wisconsin-Madison IACUC (Animal Care and Use Committee) granted ethical approval for use of male rhesus macaques. With regard to clinical trials, approvals for VAC045 (CS and TRAP samples) were granted by the United Kingdom National Research Ethics Service (reference 12/SC/0037) and the United Kingdom Medicines and Healthcare Products Regulatory Agency (reference 21584/0293/001-0001). Clinical trial MAL034 (TRAP samples), was approved by the Oxfordshire Research Ethics Committee (OXREC A 09/HO604/9) and the Medicines and Healthcare products Reglatory Agency (EudraCT 2008-006804-46) . All volunteers gave informed consent prior to participation.

Species	Antigens	Vaccination regime	Time of serum collection	Year	Samples provided by
Mouse samples	CSP TRAP	$1 \cdot 10^8$ Ad, $1 \cdot 10^7$ MVA (8 weeks interval)	8-14 days after MVA	2013	Alexandra Spencer
	Falstatin CelTOS UIS3 LSAP1 LSAP2 LSA1 LSA3 ETRAMP5	$1 \cdot 10^8$ Ad, $1 \cdot 10^6$ MVA (8 weeks interval) $1 \cdot 10^8$ Ad, $1 \cdot 10^7$ MVA (8 weeks interval)	two weeks after MVA	2012	Rhea Longley Ahmed Salman
	SPECT1 SPECT2 HT RP-L3	$1 \cdot 10^8$ Ad, $1 \cdot 10^7$ MVA (8 weeks interval)	8-14 days after MVA	2014	Ahmed Salman
	R21 RTS,S	0.5-5 µg R21 / RTS,S (Virus Like Particles) 2 doses (8wk) 3 doses (3wk)	11-21 days after vaccination	2013 2014	Katharine Collins
Macaque samples	TRAP	$5 \cdot 10^7$ Ad, $8 \cdot 10^7$ (8 weeks interval)	one week after MVA	2012	Alexandra Spencer
Clinical samples	CSP	$5 \cdot 10^{10}$ Ad, $2 \cdot 10^8$ MVA (8 weeks interval)	two weeks after MVA	2012	Katie Ewer Georgina Bowyer
	TRAP	$5 \cdot 10^{10}$ Ad, $2 \cdot 10^8$ MVA (8 weeks interval)	two-three weeks after MVA	2009 2012	Katie Ewer Georgina Bowyer

Table 2. Serum samples tested with ISI assay in chapter 2. Post-vaccination serum was obtained from mice, macaques and volunteers that had been vaccinated against a *P. falciparum* antigen.

Two monoclonal antibodies have been used in the work described here, both of which target the repeat region of CSP protein: 3D11 (fab fragment) [mAb-*Pb*CS], which is specific for *P. berghei* CSP and 2A10 [mAb-*Pf*CS], specific for *P. falciparum* CSP. Both antibodies were available at the laboratory.

Mosquito dissection

After being fed on an infected mouse, *A. stephensi* mosquitoes were kept at the Jenner Insectary at room temperature (to promote sporozoite growth, (216)) for three weeks. Just prior to dissection, infected pots of mosquitoes were chilled at 4ºC, to reduce mosquito movement, and they were transferred to a petri dish, which was kept on ice during mosquito dissection. To isolate the salivary glands, the heads of mosquitoes were removed with the aid of insulin syringes [Nu-care Products] and forceps and salivary glands teased from the body of the mosquito [Figure.1.B1]. Ideally six per mosquito were collected, transferred into a homogenizer and kept in cold media to preserve their infectivity (217). As illustrated in [Figure.1.A] the salivary glands collected [Figure1.B1] were disrupted using a homogeniser, in order to release the sporozoites. GFP signal from chimeric sporozoites could be detected in salivary glands [Figure.1.B2].
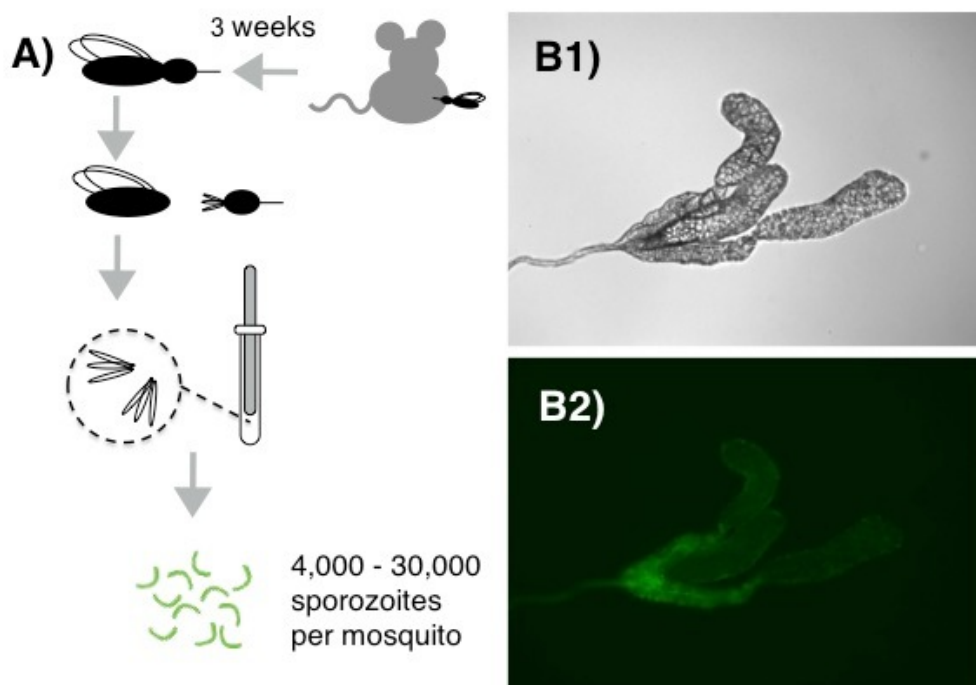
Figure 1. A) Schematic representation of sporozoite dissection. Mosquitoes were dissected under a microscope, pulling out the head with insulin syringes, to obtain the salivary glands. B) Image of three salivary glands obtained in a LEICA DM1300B microscope: B1) bright field, B2) GFP+ signal.

Infection of liver cell cultures

Hepatocytes from the cell line BNL, HepG2, HC04 or Huh7 were seeded on a 96 well-plate. The standard number of cells used per well was 30000. Cells were plated at least 6 hours prior to sporozoite addition. Once salivary glands had been homogenised

and sporozoites counted using a disposable counting slide [Immune systems], sporozoites were diluted in culture media. Sporozoites were mixed on ice with serum samples or monoclonal antibody diluted in media, or just media. Culture media was removed from hepatocyte cultures, and 100 μL of sporozoite preparation was added to each well. Usually, 15000 sporozoites were used per well (together with the amount of cells, these numbers varied in optimisation experiments in Chapter 1). Plates were centrifugated (at 1600 rpm, 5 min, 4ºC) to enhance sporozoite entry into hepatocytes (210,218,219) and placed in the incubator at 37ºC.

Flow cytometry

Cells were harvested after overnight incubation when using GFP *P. berghei*, but with GFP-luciferase *P. berghei* at least 20 hours of incubation were needed to obtain GFP signal, cells were acquired after 24 hours of incubation for optimal results.

Culture media was removed from each well and 30 μL of trypsin [TrypLE Express Enzyme, Life Technologyies] added and incubated for 10-15 minutes, since hepatoma cells used were very resistant to this treatment. The liver cell lines used tend to aggregate which could block the flow cytometer, for this reason trypsin was not washed away from the cells. Trypsin not only detached hepatocytes from the plate, but also served to remove non-infectious sporozoites that may have attached to the hepatocyte surface avoiding potential false positives (208). Cells were resuspended in 1%BSA [PAA Laboratories] in PBS [Sigma Aldrich] and transferred to a FACS tube or to a 96-well U bottom plate, to be acquired manually or using a plate reader, respectively. DAPI (1µg/ml final concentration added just prior to acquisition) [Sigma Aldrich] was used as a live-death staining, as it is excreted from live cells while remains in larger amounts in death cells. Samples were acquired with a LSR II[TM] flow cytometer [BD Biosciences] using FACSDIVA[TM] software V 6.2 [BD Biosciences].

Hepatocyte population was selected using forward and side scatter, and then single and live cells were selected [Figure2. Gating strategy]. The population of infected cells (GFP+ cells) was selected in a diagram were the GFP signal recorded in the Alexa Fluor 488 channel was represented against the signal recorded in the PE-A channel. Hepatocytes show high autofluorescence, which could be confused with GFP signal. Cell autofluorescence was detected both in PE-A and Alexa Fluor 488 channels and single live hepatocytes can be shown forming a diagonal when PE-A signal is plotted against Alexa Fluor 488 signal. The population of infected cells was selected as those that presented a greater signal in Alexa Fluor 488, standing out of the diagonal.

Using this method, cells with high autofluorescence were not included as false positives.
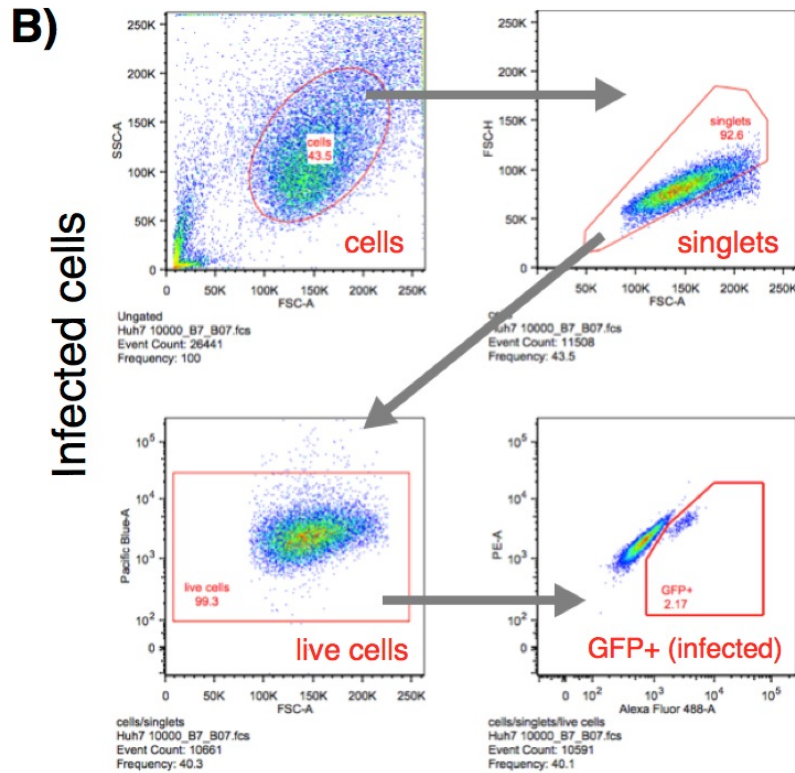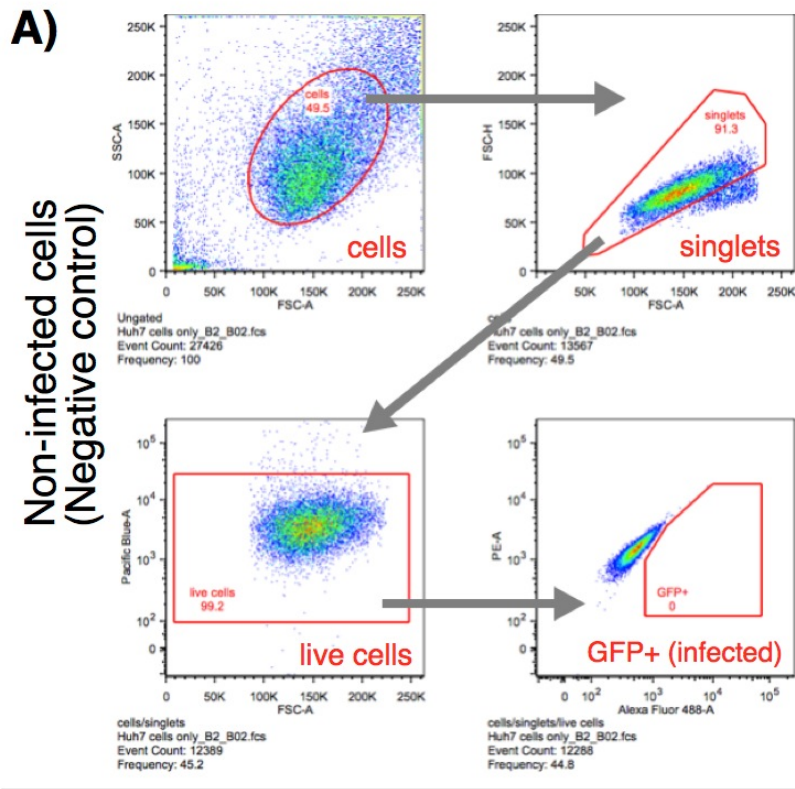
Figure 2. Gating strategy.
A) Non-infected cells (negative control). B) Infected cells.

Immunofluorescence Antibody Test (IFAT)

To prepare IFAT slides, 0.01% poly-lysine (20 μL) was added to each well of a 8-well slide and left until dried (overnight incubation). 20000 sporozoites in RPMI media (20-50 μL) were added to each well of the poly-lysine coated slide, after 20 minutes on ice (to allow sporozoites enough time to reach the bottom of the slide and interact with the poly-lysine) the liquid was removed and samples were fixed using 4% PFA in PBS (50-60 μL) at room temperature for 20 minutes incubation. Afterwards, each well was washed twice with PBS and slides were stored at 4ºC in sterile PBS (two slides in a 50 mL Falcon).

With the purpose of detecting post-vaccination antibodies binding to sporozoites antigens, mouse sera (targeting a *P. falciparum* antigen) were used to stain IFAT slides. Each well containing fixed sporozoites was washed three times with PBS. Samples were blocked using PBS containing 1% BSA and 10% FCS (20 μL) which was incubated for 30 minutes at room temperature. After removing blocking media, the primary antibody was added (1:10 and 1:50 post-vaccination serum dilution in blocking media) and incubated for 1 to 2 hours at room temperature. After primary antibody removal and three washes with PBS, the secondary antibody: Alexa Fluor 488 goat anti-mouse IgG [Life Technologies], was added (1:800 dilution in blocking media), incubated at room temperature for 1 hour and washed three times with PBS. In order to stain sporozoite nuclei, Hoechst 1:50 [Cell Signaling Technology] was added and incubated for 10 minutes at room temperature before washing twice with PBS and leaving slides until the remaining liquid had almost dried. Finally, a drop of Fluorescence Mounting Medium [Dako] was placed on each well and a coverslip was laid over the 8-well slide. Once mounting medium was dry, images were taken using a DMI-300B Leica fluorescence microscope, recording green signal (GFP: antibody bound to sporozoite antigens) for 7 seconds, and blue signal (Hoechst: nuclei staining) for 3 or 4 seconds. The signal was recorded over the same exposure time for every image, in order to compare the levels of antibody binding. For the same reason, brightness and contrast were adjusted in the same proportion using ImageJ software.

Confocal microscopy

Hepatocyte cultures infected with *P. berghei* sporozoites were incubated for 45 hours at 37ºC. 24 well-plates were used, containing 60000 Huh7 cells seeded on circular coverslips (12mm) [neuVitro], laid on the bottom of each well, and 20000 sporozoites added per well. After incubation, culture media was removed and each well washed three times with PBS (200 μL). Cells were fixed with 4% PFA + 4% sucrose in PBS (200 μL) for 30 minutes at room temperature. Fixed cells were washed twice with PBS (1 mL), and quenched with 50 mM NH_4Cl in PBS (300 μL) for 15 minutes at room temperature before washing again with PBS (1 mL). Cells were permeabilised with 0.1% Triton in PBS (300 μL) for 5 minutes at room temperature. After two washes with PBS (1 mL), cells were blocked using 1% BSA in PBS (300 μL) which was incubated for 15 minutes. Alexa Fluor 594 goat anti-mouse IgG [Invitrogen] diluted 1:200 in 1% BSA in PBS was added (100 μL per well) and incubated for 30 minutes at room temperature. Samples were washed two times with PBS and the hepatocyte nuclei stained by addition of DAPI 1:5000 (100 μL) for 5 minutes. Coverslips were mounted on microscope slides using Fluorescence Mounting Medium [Dako] and left to dry at 4ºC. Images were acquired using a LSM 710 Carl Zeiss confocal microscope.

Data management and statistical analysis

Cytometer acquisitions files (.fcs) were analysed with FlowJo.V 9.7.6 [Tree Star]. Blocking values were calculated with the percentage of infected cells measured in the presence or absence of the serum or monoclonal antibody of interest [Figure 3].

$$\text{\% Infected cells (GFP+)} = 100 \cdot \frac{\text{number of infected cells}}{\text{number of single live hepatocytes}}$$

$$\text{\% Infection blocked} = 100 \cdot \frac{\text{\% infected cells (spz) - \% infected cells (spz + Ab)}}{\text{\% infected cells (spz)}}$$

Figure 3. Gating strategy. % Infected cells and % Infection blocked formulae

Graphs were plotted using GraphPad Prism 6.0, where statistical analyses were also performed. A potential correlation between two parameters was evaluated using a nonparametric Spearman correlation test (r value) and linear regression (R^2; p-value

Symbol	p-value
*	≤0.05
**	≤0.01
***	≤0.001
****	≤0.0001

[Table 3]. Symbols for statistical significance on figures.

from F test: is the slope significantly different than zero?). When comparing the average blocking response between various groups, Kruskal-Wallis test with Dunn's multiple comparisons test were used. With macaque and human samples, to evaluate whether there was an effect of vaccination, blocking values pre- and post-vaccination (paired data) were analysed with a Wilcoxon matched-pairs signed rank test (two tailed). P-values lower than 0.05 were considered to be significant. [Table 3] shows how the levels of statistical significance have been represented on graphs, where p-values over 0.05 and under 0.3 were stated.

Chapter 1:
Optimising a flow cytometry based Inhibition of Sporozoite Invasion assay

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1.1. Introduction

Sporozoite neutralisation assays are relevant techniques to determine the functionality of antibodies in their ability to inhibit parasite invasion and/or impede the development of malaria liver stages. As described in the introduction, these assays have been typically performed using microscopy as the readout method, causing the technique to be time-consuming and associated to the bias of the person analysing the sample. More recently, new approaches to measure the percentage of infected cells through PCR (206), and by flow cytometry (183,208) have made it suitable for the analysis of a high quantity of samples.
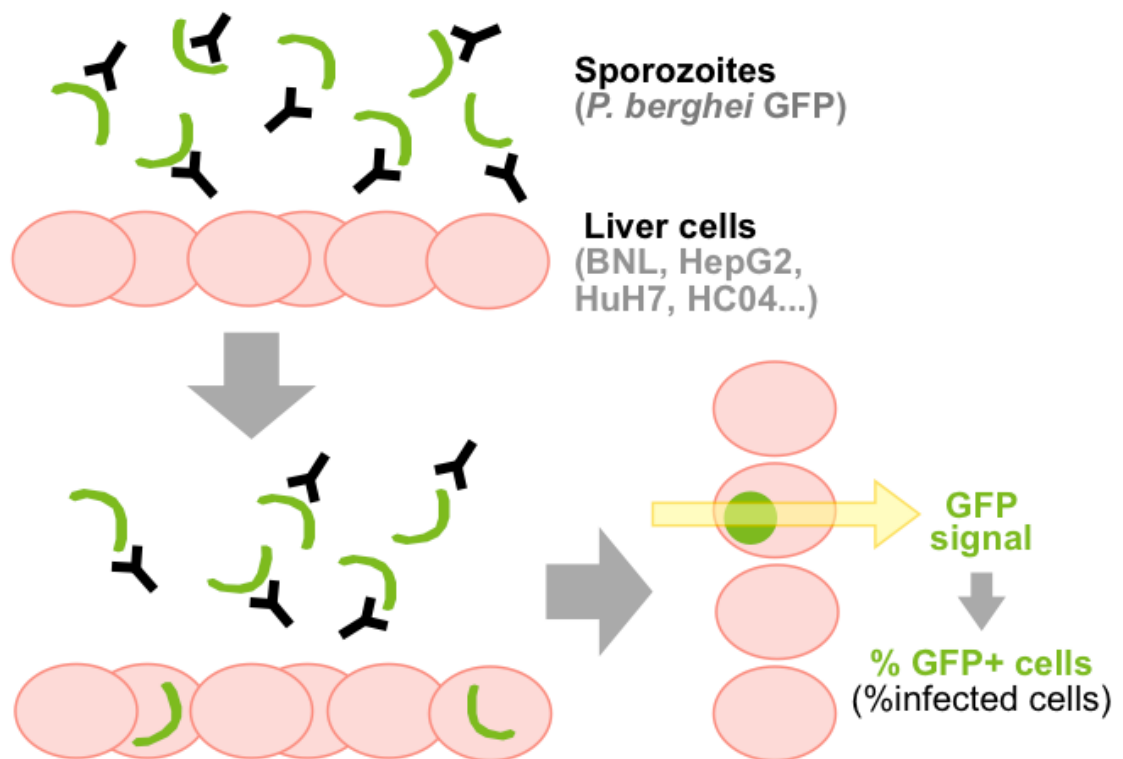
Figure 1.0. Schematic representation of the Inhibition of Sporozoite Invasion (ISI) assay. GFP expressing *P. berghei* sporozoites enter into a hepatocyte and develop within it. Infected cells can be detected by flow cytometry as cells with positive GFP signal. The blocking capacity of an antibody is calculated knowing the percentage of infected cells measured in the absence and presence of the antibody.

This chapter describes a series of experiments that were performed to optimise a flow cytometry based Inhibition of Sporozoite Invasion (ISI) assay. The protocol has been developed to make it suitable for the study of post-vaccination serum from vaccine pre-clinical and clinical studies, at a research centre with a limited capacity of sporozoite production and the need to test a large number of samples. The most limiting factor in these experiments was sporozoite availability. Therefore, one of the main objectives was to use the minimum amount of sporozoites to test each serum sample. A number of experiments described in this chapter were performed to find the conditions (e.g. host cell line, sporozoite:cell ratio, cell culture media, temperature of the first hour of incubation and time of incubation) where the infection rates were higher and where the antibodies could best show their blocking capacity (e.g. serum concentration and pre-incubation with sporozoites).

Four different cell lines were tested in this chapter as potential hosts for sporozoites: a mouse cell line (BNL) and three human cell lines (HepG2, HuH-7 and HC-04). HepG2 is a cell line established from a liver biopsy of a 15-year-old caucasian male from Argentina with hepatocellular carcinoma (194,220). HuH-7 cell line was established from hepatoma tissue with hepatocellular carcinoma removed from a 57-year-old Japanese male (213). HC-04 was obtained from normal liver tissue obtained from a hematoma patient in Thailand (214). These cell lines have morphology characteristics that resemble those of liver parenchyma cells.

Two monoclonal antibodies were used in this work: 2A10 and 3D11. Both antibodies recognize the repeat region of CSP, either of *P. falciparum* (2A10) (221) or *P. berghei* (3D11) (222). 2A10 antibody has been described to inhibit *P. falciparum* sporozoite invasion of HepG2 cells (198). Similarly, 3D11 (previously described as anti-*Pb*44) antibody and its Fab fragments prevented *P. berghei* entry into WI38 cells (191).

P. berghei chimeric parasites expressing GFP or GFP-luciferase (both under eef1α promoter) were used to find the optimal conditions to perform the assay. In addition *P. berghei* parasites expressing both GFP-luciferase and *P. falciparum* CSP were used to test 2A10, the monoclonal antibody against *P. falciparum* CSP (mAb-*Pf*CS). This experiment was performed initially with 'addition parasites' that express *P. falciparum* CSP (under UIS4 promoter) as an additional copy. Afterwards, 'replacement parasites' expressing *P. falciparum* CSP (under *P. berghei* CSP promoter) in which *P. berghei* CSP had been deleted, were also tested. Ahmed Salman (DPhil student) generated all the chimeric parasites through GIMO ('Gene insertion/marker out' (215)). Eventually, the chimeric sporozoites used to evaluate efficacy in pre-clinical studies (described in chapter 2) were *P. berghei* sporozoites expressing a *P. falciparum*

antigen of interest and GFP-luciferase, which was taken into account when optimising the time of readout in this chapter. GFP-luciferase was chosen over GFP as a reporter since the fused protein allows not only flow cytometry detection (GFP signal) and but also live-imaging microscopy (luciferase signal). All the transgenic parasites used presented the same behaviour in the ISI assay (no differences where found in their infectivity capacity).

1.2 Results

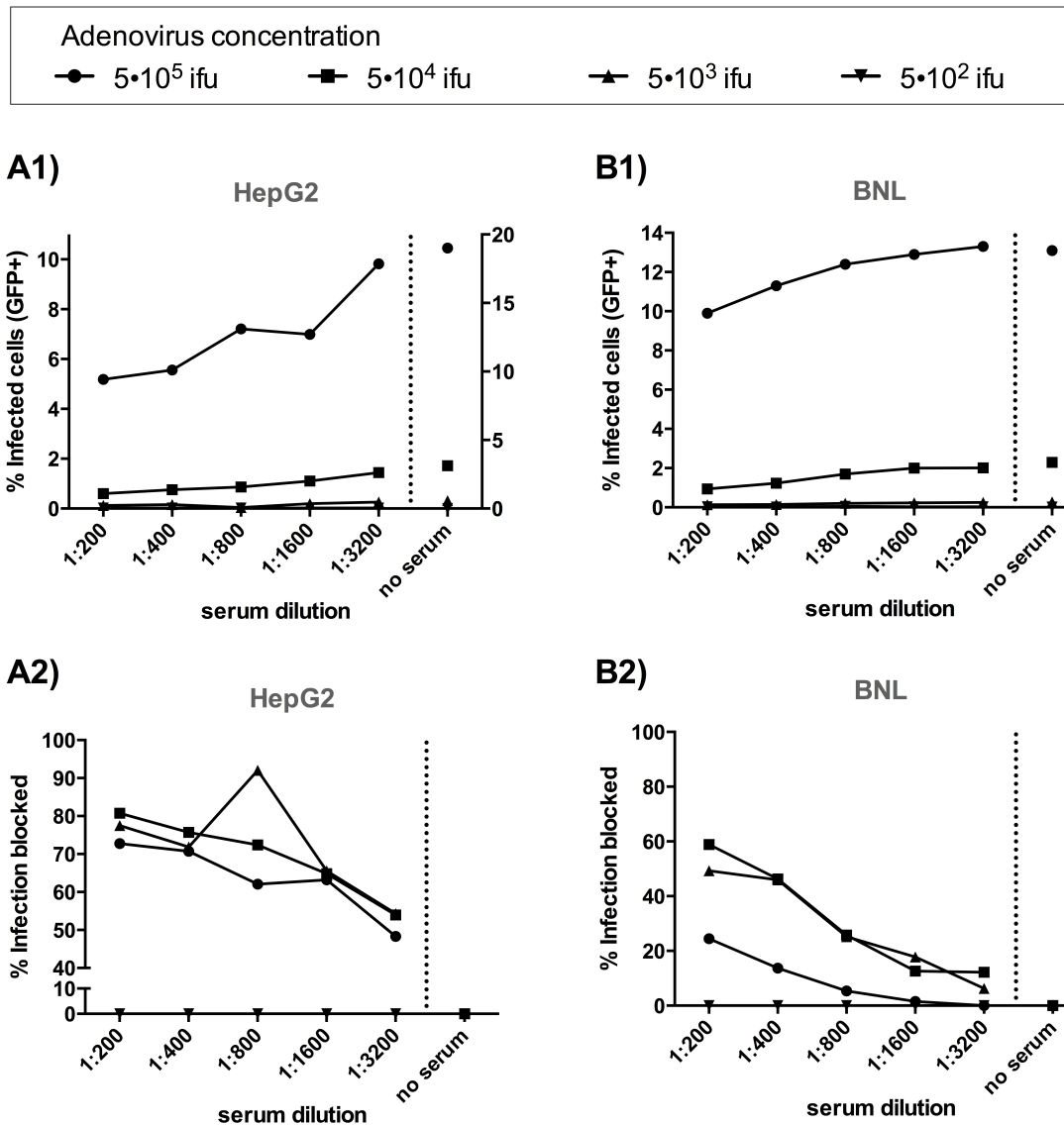## 1.2.1. Adenovirus infection of HepG2 cells and BNL cells

To determine whether small changes in the number of cells expressing GFP could be detected by flow cytometry, a preliminary experiment was carried out whereby liver cell lines were infected with adenovirus expressing GFP in combination with post-vaccination serum (from mice that have been vaccinated with the same adenovirus).

Two lines of liver cell lines, HepG2 cells (human derived) and BNL (mouse derived), were infected with different concentrations of adenovirus expressing GFP (AdC63 cmvLP eGFP). A range of dilutions of post-vaccination serum were also mixed with the adenovirus preparations and incubated for one hour at 37ºC. The different samples were added to each well (containing 50000 cells, in a 96 well-plate) and cells cultured overnight, prior to harvesting and acquisition on a flow cytometer to measure the proportion of cells expressing GFP (infected cells).

When $5x10^5$ ifu of adenovirus were added to the cells, 19% of HepG2 cells [Figure 1.1.A1] and 13% of BNL cells [Figure 1.1.B1] were infected. $5x10^4$ ifu generated 3% of infected HepG2 [Figure 1.1.A1] and 2% of infected BNL [Figure 1.1.B1]. The presence of serum was found to block the infection in a concentration-dependent manner [Figure 1.1.A2, B2].

For HepG2 cells, serum dilutions from 1:200 to 1:3200 prevented 80% to 50% of the infection [Figure 1.1.A2]. The percentage of infection blocked by the addition of serum appeared to be slightly lower when a higher concentration of virus was used and could be related to a limitation of antibody availability. Similar blocking curves were observed for HepG2 infected with $5x10^3$ ifu, $5x10^4$ ifu and $5x10^5$ ifu [Figure 1.1.A2], although the variation of the data was larger for $5x10^3$ ifu as the percentages of infected cells ranged from 0 to 0.6% and the error associated with the experiment represents an important part of the measure. The amount of infection blocked was lower for BNL cells (60% to 0%) [Figure 1.1.B2] and the percentage of blocking observed when adding $5x10^5$ ifu of virus, was approximately three times smaller than observed with $5x10^4$ ifu of virus. This decrease in the percentage of the blocking associated with the use of a higher virus concentration, was hardly present in HepG2.

In general, higher concentrations of serum were needed for BNL cells to achieve a similar reduction in infection than observed for HepG2 cells with less serum. As HepG2 cells are more permissive to infection, it could be expected that they would require a higher concentration of serum to prevent the infection. However, this was not observed, and the fact that BNL cells require higher amounts of serum to block the infection could be due to antibodies with reduced neutralization effect of the virus on the surface of BNL cells in comparison to the surface of HepG2 cells.

Figure 1.1. Inhibition of GFP adenovirus (AdC63 cmvLP eGFP) infection of the liver cell lines HepG2 and BNL using post-vaccination mouse serum. 50000 HepG2 [A] or BNL [B] cells per well were infected with $5 \cdot 10^2$ to $5 \cdot 10^5$ ifu of adenovirus which had been pre-incubated for 1 hour with serum concentrations ranging from 1:200 to 1:3200. Graphs represent the percentage of infected cells (GFP+) [A1 and B1] or the percentage of infection blocked [A2 and B2].

1.2.2. Infection of liver cell lines with *P. berghei* GFP sporozoites

HepG2 cells and BNL cells were infected with transgenic *Plasmodium berghei* sporozoites expressing GFP. 5000, 10000, 20000 or 40000 sporozoites were added to 50000 cells seeded to each well of a 96 well-plate. Plates were incubated overnight with cells harvested and run on a flow cytometer the following day. An infectivity of 1% to 5% was found for HepG2 cells [Figure 1.2.A], while the percentage of GFP+ cells was always lower than 0.5% for BNL cells [Figure 1.2.B]. The differences in infectivity could be due to the distinctive repertoire of surface receptors expressed on the hepatoma cell line. In both cases, the percentage of infected cells increased proportionally to the amount of sporozoites added, which also indicates that the method used is a valid one to evaluate the amount of infected cells. Due to the low level of infectivity achieved with BNL cells, we decided not to use this cell line with the purpose of the ISI assay.
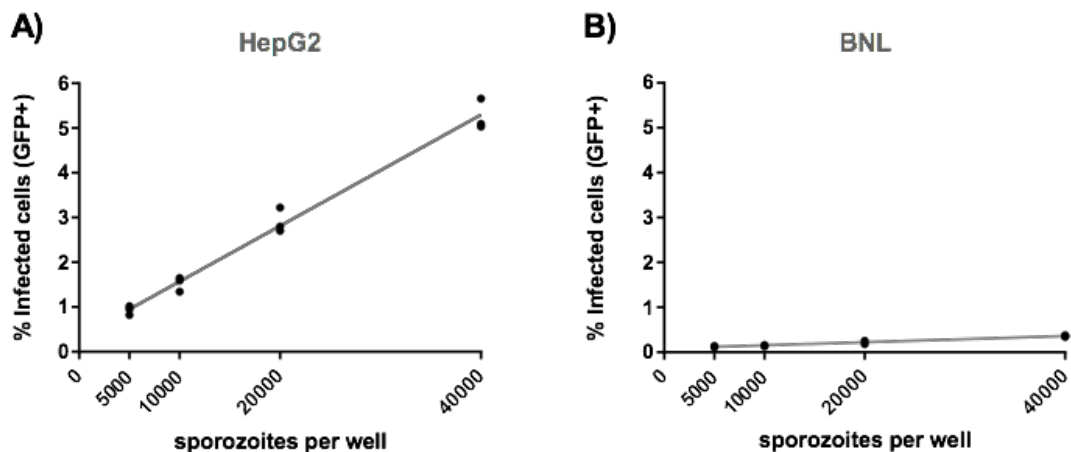
Figure 1.2. Infection of the liver cell lines HepG2 and BNL with *P. berghei* GFP sporozoites. Percentage of infected cells when 50000 HepG2 [A] or BNL [B] were infected with different amounts of sporozoites (5000 to 40000).

With the aim of increasing the percentage of infected cells, the effect of plating a different number of cells per well was assessed. Plating 30000 cells per well led to higher percentages of infected cells compared to wells with 50000 cells [Figure 1.3.A], this was true for any amount of sporozoites added. The same percentage of infected cells (over 3%) was achieved using 30000 sporozoites and 50000 cells than with 20000 sporozoites and 30000 cells. When a smaller number of cells were seeded, the population of infected cells represented a larger proportion of the total population of cells. Nevertheless, the total numbers of positive GFP events [Figure 1.3.B] acquired on the flow cytometer were within a similar range regardless the number of cells plated

per well. Using fewer cells did not have a negative effect in the total number of positive events recorded. Interestingly, when using lower number of sporozoites to infect the cells the variability in the percentage of infected cells measured between replicates seemed to be smaller, which will be helpful for the ISI assay.
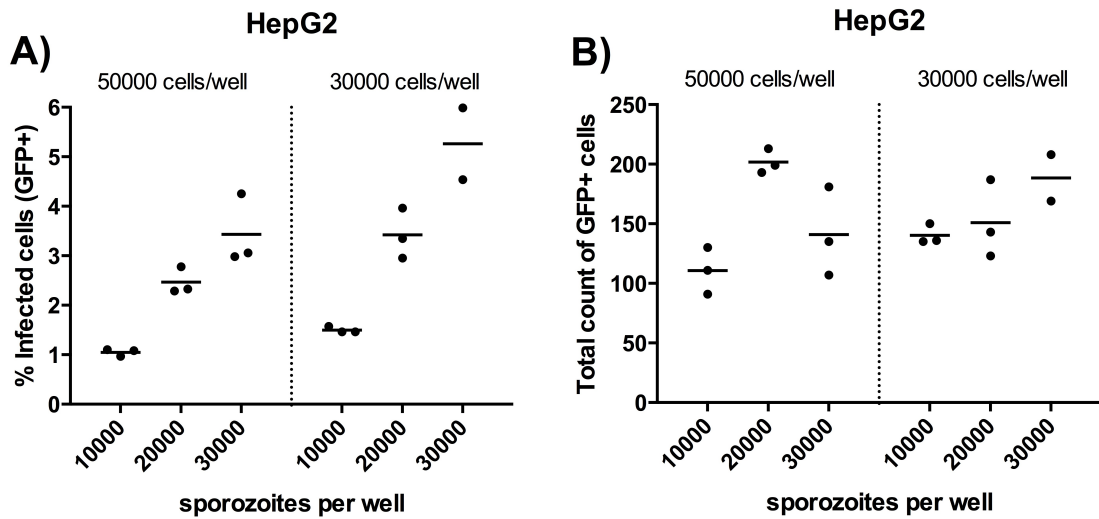
Figure 1.3. Effect of the number of cells per well on the percentage of infected cells. 50000 or 30000 HepG2 cells were plated per well and infected with 10000, 20000 or 30000 *P. berghei* GFP sporozoites. A) Percentage of infected cells for different amount of cells and sporozoites per well. B) Total number of GFP positive cells detected for each sample on the flow cytometer.

The number of sporozoites obtained per mosquito varied between batches of infected mosquitoes, usually between 4000 and 30000. The typical yield of mosquitoes, produced at the insectary while this work was performed, was around 10000 sporozoites per mosquito. Therefore, large numbers of mosquitoes need to be dissected for these *in vitro* studies, which could involve a long time of dissection, especially when the mosquitoes presented low yields. The limiting factor determining the size of each experiment (the number of conditions and samples to test) was the number of sporozoites obtained. For these reason, it is desirable to adjust the ISI assay to be performed using a low amount of sporozoites. This may involve a higher risk to find less positive events, but can be minimized through the use of replicate wells.

1.2.3. Effect of culture media on sporozoite infectivity

The media used for cell culture was also regarded as a factor that could influence cell infectivity. Culture media could modulate sporozoite behaviour; for example, Hegge et al. showed that sporozoites transformed into exoerythrocytic forms in RPMI within 6 hours, while in DMEM in only 1 hour (223). Sporozoite neutralizing assays described previously have been performed using a variety of cell culture media, mainly DMEM and RPMI. To test a potential influence of cell media on infection rates, cells were cultured using different media for several days prior to and during the infection. As the human liver cell line HepG2 showed previously a higher infectivity than the mouse derived cell line BNL, it was used in this experiment together with other two human liver cell lines (Huh7 and HC04). DMEM was the media used initially in the experiments described so far, and the two other media tested were DMEM* (DMEM supplemented with non-essential amino acids and pyruvate) and RPMI. All media contained 10% FCS, p/s and L-glutamine. While addition of NEAA and pyruvate to DMEM increased the infectivity by 20% in Huh7 and HepG2, and in 40% for HC04, culturing cells with RPMI gave the highest levels of infectivity [Figure 1.4].
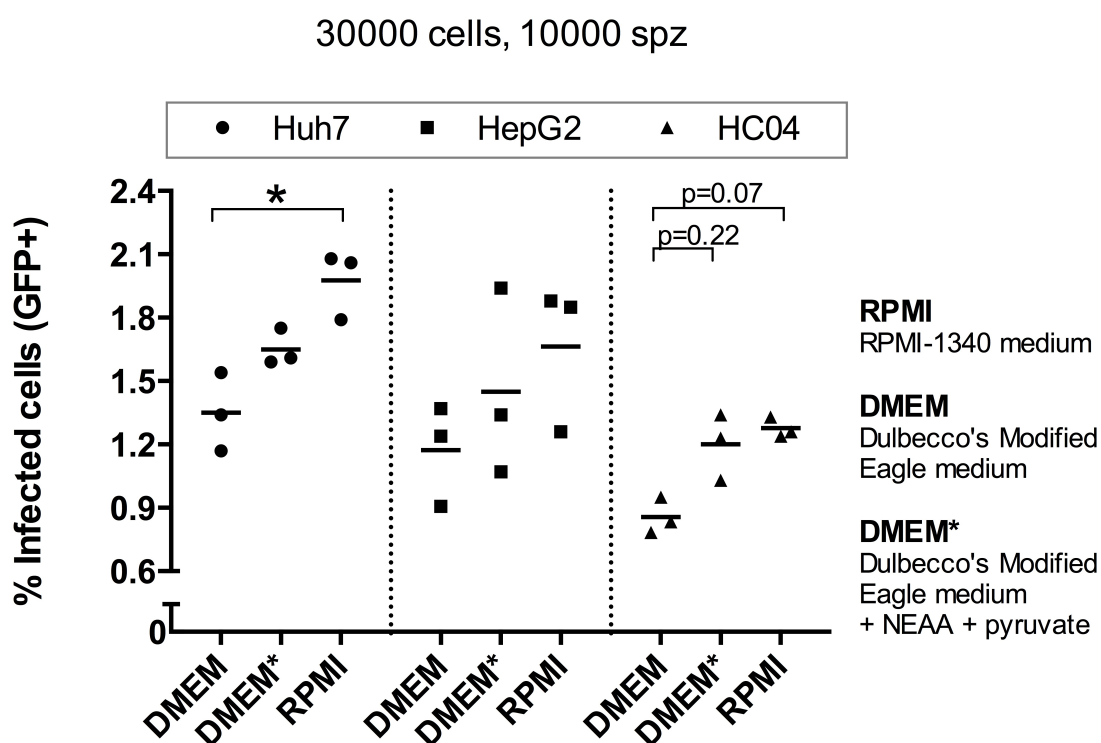

Figure 1.4. Effect of cell culture media on the percentage of infected cells. 30000 cells (Huh7, HepG2 or HC04) cultured in DMEM, DMEM* or RPMI, were seeded per well and infected with 10000 _P. berghei_ GFP sporozoites. Data was analysed with Kruskal-Wallis test, Dunn's multiple comparisons test (p-values below 0.3 are included in the graph).

When culturing with RPMI instead of DMEM an increase in the infectivity between 40 to 50% was observed for all cell lines. RPMI was therefore chosen as the media to use in future experiments. The effect of culture media on the percentage of infected cells may be related with an effect on the host-parasite interactions that take place on the cell surface or with a modulation of sporozoite or cell metabolism. An effect of the culture media on the cell line growth may also influence the percentage of infected cells measured.

With all different types of media, the highest level of infectivity was observed with Huh7 cells [Figure 1.4]. The following experiment was performed to directly compare the infectivity of these three cell lines with varying numbers of sporozoites [Figure 1.5]. Consistent with the previous experiment, Huh7 cells showed higher percentages of infected cells at all sporozoite numbers. In addition, Huh7 cells were easier to handle than HepG2 cells, as HepG2 cells have a greater tendency to clump together, which can cause problems when acquiring the samples on the flow cytometer. The differences found in infectivity among the three cell lines may be related with specific characteristics of their cell surface. Each cell line also have different speeds of division, which may influence in the percentage of infected cells measured. Carly Bliss (DPhil student) performed an experiment where she found that HC04 cell division rate was higher than the one of Huh7 cells. Non-infected cells may divide at a higher rate than infected cells and, as a result, the percentage of infected cells would become smaller in a faster dividing cell line at the time of readout.
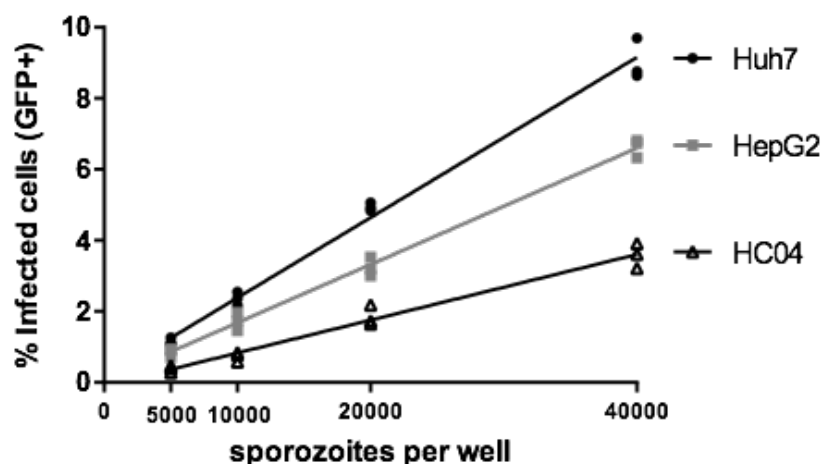
Figure 1.5. Infection of the liver cell lines Huh7, HepG2 and BNL with *P. berghei* GFP sporozoites. Percentage of infected cells when each cell line was infected with 5000 to 40000 sporozoites.

1.2.4. Pre-incubation and temperature during the first hour of incubation

To determine the best conditions to test the effect of post-vaccination serum samples, initial experiments were performed with a monoclonal antibody against *P. berghei* CS (mAb-*Pb*CS, 3D11 Fab fragment). Firstly, it was important to establish whether pre-incubation of sporozoites and antibodies was necessary to observe their inhibitory effect. Secondly, it was tested whether infectivity could be more efficient if the first hour of incubation was performed at 21ºC (room temperature) or at 37ºC (incubator). Sporozoites are very sensitive to temperature, when kept at room temperature for a long time they can become non-infectious, therefore sporozoites were kept on ice. Since infectivity decreases when sporozoites are maintained *in vitro* at 37ºC (224), it was important to determine whether maintaining cells at room temperature after addition of sporozoites could increase infectivity of the sporozoites.

Addition of 1 µg/mL of mAb-*Pb*CS antibody was shown to reduce the percentage of infected cells, however no difference was observed when pre-incubating the sporozoites for 15 or 30 minutes than when sporozoites and antibodies were simultaneously added to the cells [Figure 1.6.A]. This would suggest that antibody binding occurs rapidly, and waiting for longer does not increase the inhibitory effect. Consequently, pre-incubation of sporozoites with serum or monoclonal antibody was disregarded for future experiments.

Figure 1.6. The effect of temperature on different steps of the ISI assay.
A) Effect of the time of pre-incubation of 10000 *P. berghei* GFP sporozoites with 1 *µ*g/mL mAB-PbCS on ice prior to addition to 30000 Huh7 cells. B) Effect of the temperature during the first hour of incubation when infecting 30000 Huh7 cells with 20000 *P. berghei* GFP sporozoites.

Keeping plates at room temperature during the first hour of incubation did not increase the rate of infection [Figure 1.6.B] and while placing the plates at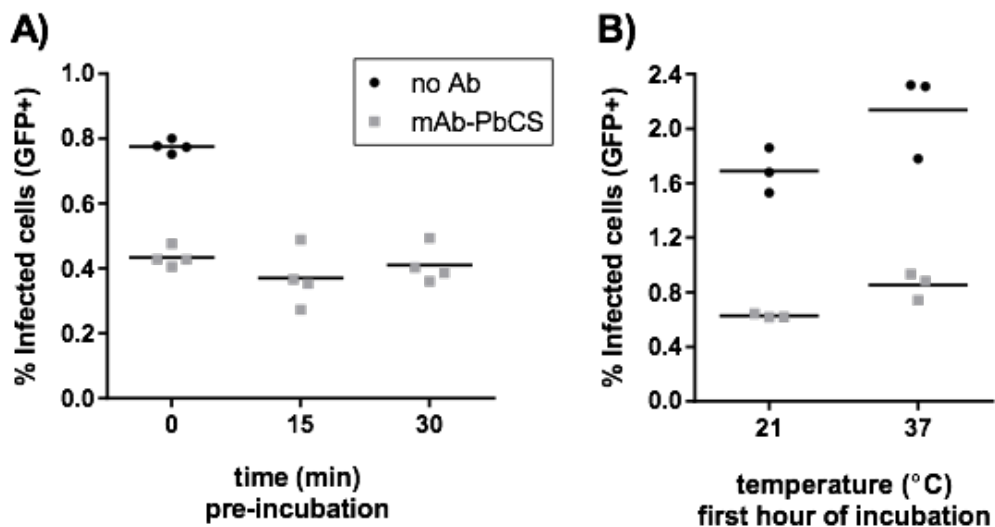 a temperature of 37ºC could have a detrimental effect, the data would suggest that sporozoites infect hepatocytes before losing infectivity capacity at 37ºC. Therefore, keeping the plates at room temperature for the first hour will not be necessary for future experiments.

1.2.5. Time of readout

The readout of the ISI assay by flow cytometry relies on the expression of GFP from the *P. berghei* parasite. Parasites used in this project expressed GFP only or the fused protein GFP-luciferase. In the experiments described above, parasites expressing GFP only were used and samples were run on the flow cytometer the following morning (16-18 hours incubation). When parasites expressing GFP-luciferase were initially used, almost no infected cells were detected when measuring at the same time post-infection. To find the best time to measure infectivity, two experiments were performed to establish the time course of GFP and GFP-luciferase expression [Figure 1.7]. Cells infected with *P. berghei* expressing GFP only could be detected by flow cytometry as early as 4 hours after infection [Figure 1.7.A]. However, it was not possible to detect cells infected with *P. berghei* expressing GFP-luciferase before 20 hours post-infection [Figure 1.7.B]. The presence of luciferase together with GFP could delay the expression of the protein, as the fused protein is a more complex product. The mean fluorescence intensity (MFI) of GFP+ cells (represented on the right Y axis) increased with the time of incubation, as the parasites developed within the cells and expressed a larger amount of protein. Although the MFI can not be compared between different experiments, the range of MFI recorded when using GFP is 1.5 log points while the range of MFI measured for GFP-luciferase is 0.6 log points [Figure 1.7]. The fact that the mean fluorescence intensity of the GFP is lower for GFP-luciferase infected cells, could be due to a fewer number of protein copies within the infected hepatocyte, or may indicate that fusion to luciferase could result in modifications of the GFP folding or quenching of the fluorescent emission.

An increase in the time of incubation seems to be associated with a decrease in the percentage of infected cells, probably due to cell division of uninfected cells (208). The profile of the curves in [Figure 1.7] indicates that an increase in the MFI had an effect on the percentage of infected cells detected. Both '% Infected cells' curves (GFP

and GFP: luciferase) follow similar kinetics with the main difference being that for GFP-luciferase, the GFP signal was not detected before 20 hours.

Based on these results, 24-28 hours after infection was chosen as the optimal time to measure cells infected with GFP-luciferase parasites. When cells are infected with GFP parasites, the rate of infectivity can be measured 3-4 hours after infection, but if it is measured after an overnight incubation (as it has been done in the experiments described so far) a large percentage of infected cells can still be detected.
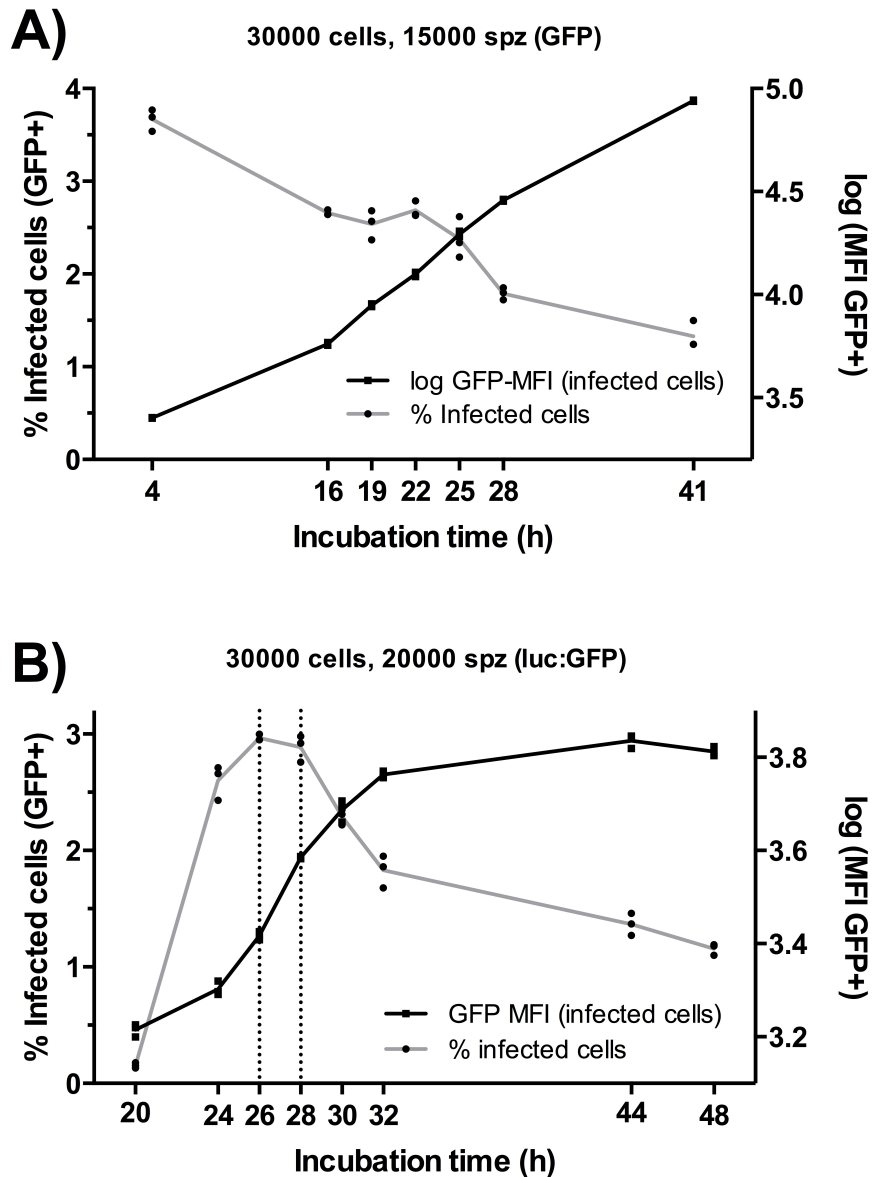
Figure 1.7. Effect of the incubation time on the percentage of infected cells detected. A) 30000 Huh7 cells per well were infected with 15000 *P. berghei* GFP sporozoites and the percentage of infected cells was measured between 4 and 41 hours after incubation. B) 30000 Huh7 cells per well were infected with 20000 *P. berghei* GFP-luciferase sporozoites and the percentage of infected cells was measured between 20 and 48 hours after incubation. In both graphs the log Mean Fluorescence Intensity (MFI) of the GFP+ events has been plotted on the Y right axis.

1.2.6 Blocking curve for mAb-*Pb*CS: concentration effect and sensitivity

mAb-*Pb*CS concentrations from 0.1 to 5 µg/mL mixed with either 15000 or 30000 sporozoites (GFP-luciferase) were added to each well (containing 30000 cells). mAb-*Pb*CS was found to inhibit the infection in a concentration-dependent manner, achieving a blocking of 50-60% with 1 µg/mL, 96% with 5 µg/mL and only 3% with 0.1 µg/mL [Figure 1.8.A]. While cells infected with 30000 sporozoites showed almost a doubling in the percentage of infected cells [Figure 1.8.A]. When transforming the data to show percentage of infection blocked, similar inhibition curves were obtained with addition of 15000 sporozoites and 30000 [Figure 1.8.B].
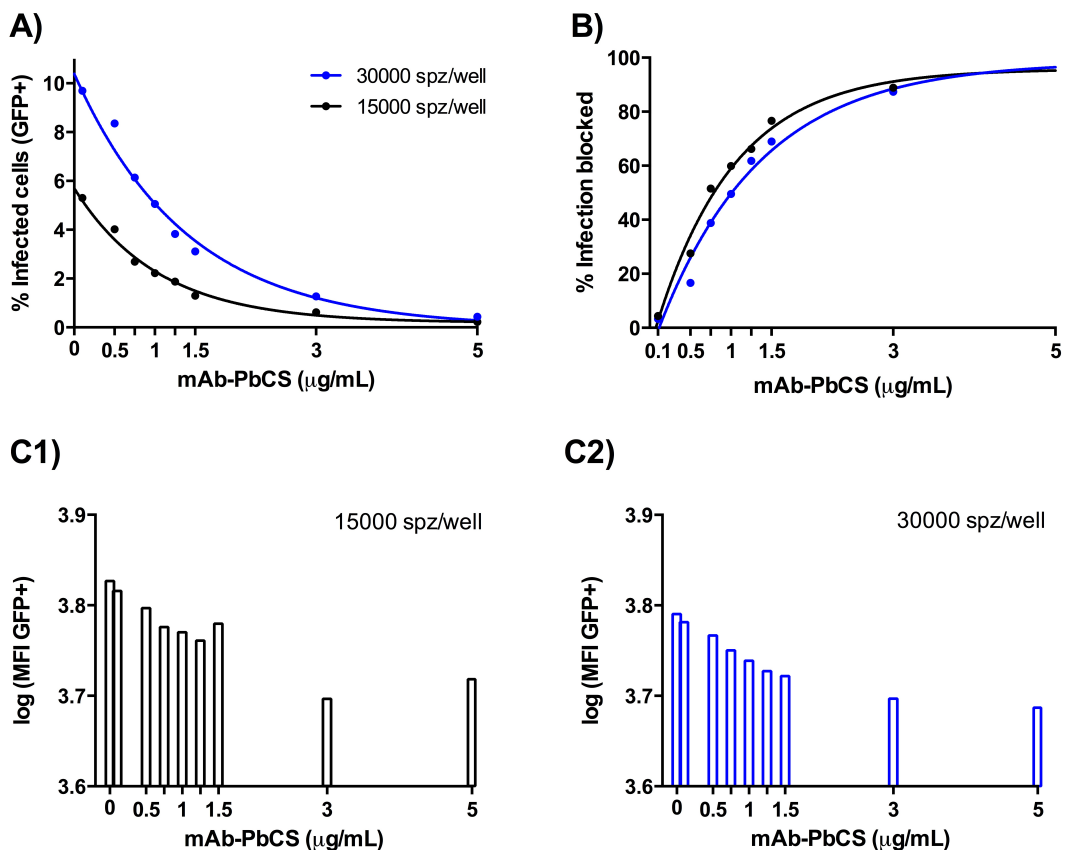
Figure 1.8. Effect of mAb-PbCS on sporozoite infection. 30000 or 15000 sporozoites (*P. berghei* GFP-luciferase) were added to 30000 Huh7 cells per well in the presence of a range of mAb-PbCS concentrations (0-5 *µg*/mL). A) Percentage of infected cells. B) Percentage of infection blocked. The log MFI found for the infected cells (GFP+) is represented when infecting with 15000 sporozoites (C1, black) and 30000 sporozoites (C2, blue).

The percentages of infection blocked were slightly higher when 15000 sporozoites were added, suggesting a limiting effect of antibody when more sporozoites were added into the well. Interestingly, doubling the amount of sporozoites did not effect the range of infection blocked by the monoclonal antibodies, which confirms that a smaller amount of sporozoites per well (15000) can be used without having a detrimental effect on assay sensitivity. This data would also suggest that fluctuations in the sporozoite infectivity or the error associated to the sporozoite count should not largely effect the ISI assay, as long as the data obtained is analysed with reference to infectivity in the control wells.

Interestingly, higher concentrations of antibody were associated with lower MFI values [Figure 1.8.C1, 1.8.C2]. Although the effect observed is very small (a decrease of the MFI from $10^{3.8}$ to $10^{3.7}$) it could be related to an effect of the antibodies on growth of the parasite within the infected cell.

1.2.7 Antibody effect on MFI

In the previous experiment, a trend towards slightly lower values of GFP MFI was observed when using higher antibody concentrations [Figure 1.8.C1,C2], suggesting that the presence of antibody could have an effect on the GFP MFI. Prudencio et al. (208) found a positive correlation between the intensity of the GFP signal and the number of parasite copies (measured by qRT-PCR), describing that flow cytometry could be used to assess parasite development within hepatocytes. The presence of antibody could impair sporozoite development (114,200,225), which might be detected as lower GFP MFI values.

An experiment was performed to find whether antibody could have a significant effect on GFP MFI when measuring at late time points (longer incubation times than 26h, which was the readout time in Figure 1.8). GFP-luciferase sporozoites were added in the presence of mAb-*Pb*CS at 0.25 and 0.5 μg/mL. These antibody concentrations blocked less than 50% of the infection, which enabled detection of an adequate number of infected cells where GFP MFI would be measured. No significant differences were observed when comparing GFP MFI from cells infected in the absence of antibody with those where monoclonal antibody was added together with the sporozoites (measuring at 26h and later time points after infection) [Figure 1.9].

In the experiment about the time of readout [Figure 1.7] it was described that GFP MFI increase with time of incubation was much more limited in GFP-luciferase

sporozoites (used in this experiment) than in GFP sporozoites. Antibody effects on parasite development might be measured using GFP sporozoites, or other kind of transgenic sporozoites, which count with a fluorochrome that can elicit a more intense fluorescent signal. In this work, the transgenic parasites of interest (*P. berghei* GFP-luciferase) did not generate a fluorescent signal strong enough to enable the measuring of variations on development caused by the presence of antibodies. For this reason, it was decided not to continue with the study of GFP MFI.
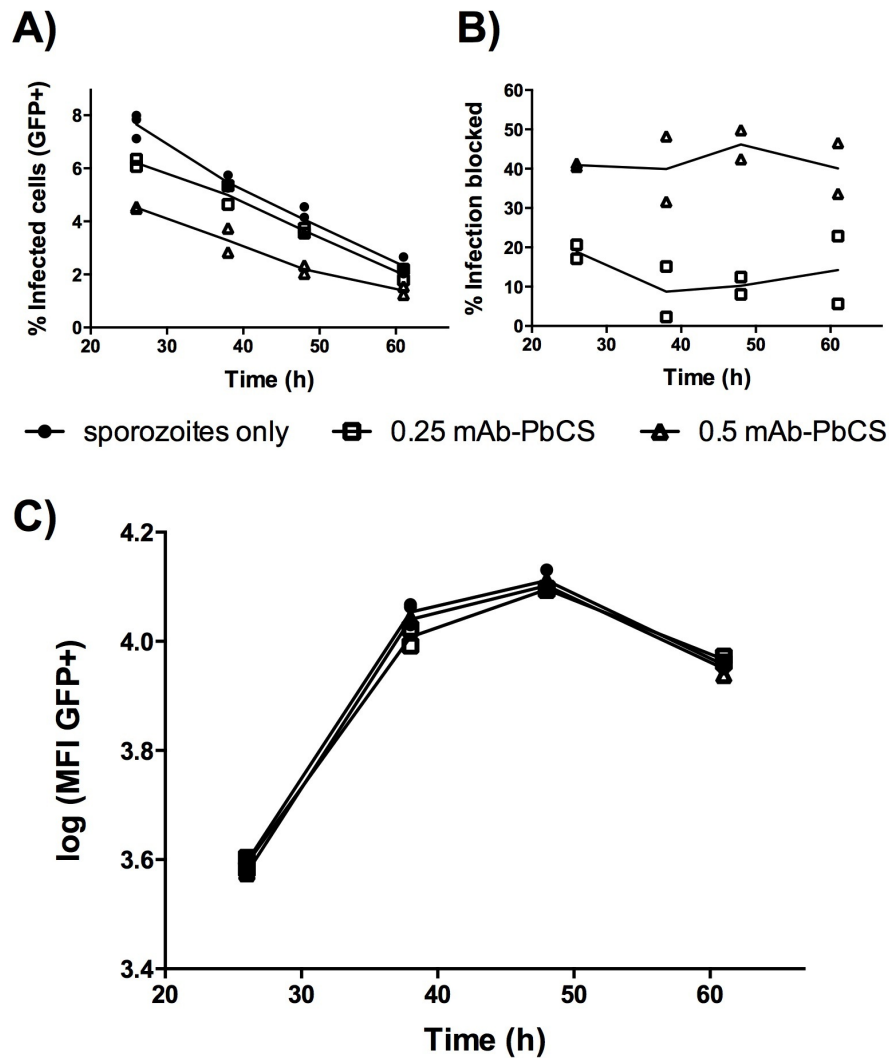
Figure 1.9. Effect of mAb-PbCS on GFP MFI. 15000 sporozoites (*P. berghei* GFP-luciferase) were added to 30000 Huh7 cells per well in the presence of mAb-PbCS (0.25 or 0.5 *μ*g/mL). A) Percentage of infected cells over time. B) Percentage of infection blocked over time. C) log MFI found for infected cells (GFP+). log (MFI GFP+) data was analysed with a Friedman test and Dunn's multiple comparisons test, confirming that means were not significantly different at any time point.

1.2.8. Non-specific effect of naive serum and heat inactivation

In future experiments, serum from naive mice will be used as a negative control. One of the described limitations of the ISI assay is the detection of blocking with non-specific serum (198,211,226). For this reason, an experiment was run to determine the effect of naive serum on sporozoite infectivity and to establish whether heat-inactivation could reduce the non-specific effect. Addition of non heat-inactivated (HI) serum up to 10% of the total final volume was shown to have a blocking effect of less than 20%, while addition of 20% non HI serum resulted in blocking more than 40% of the infection [Figure 1.10.A1].
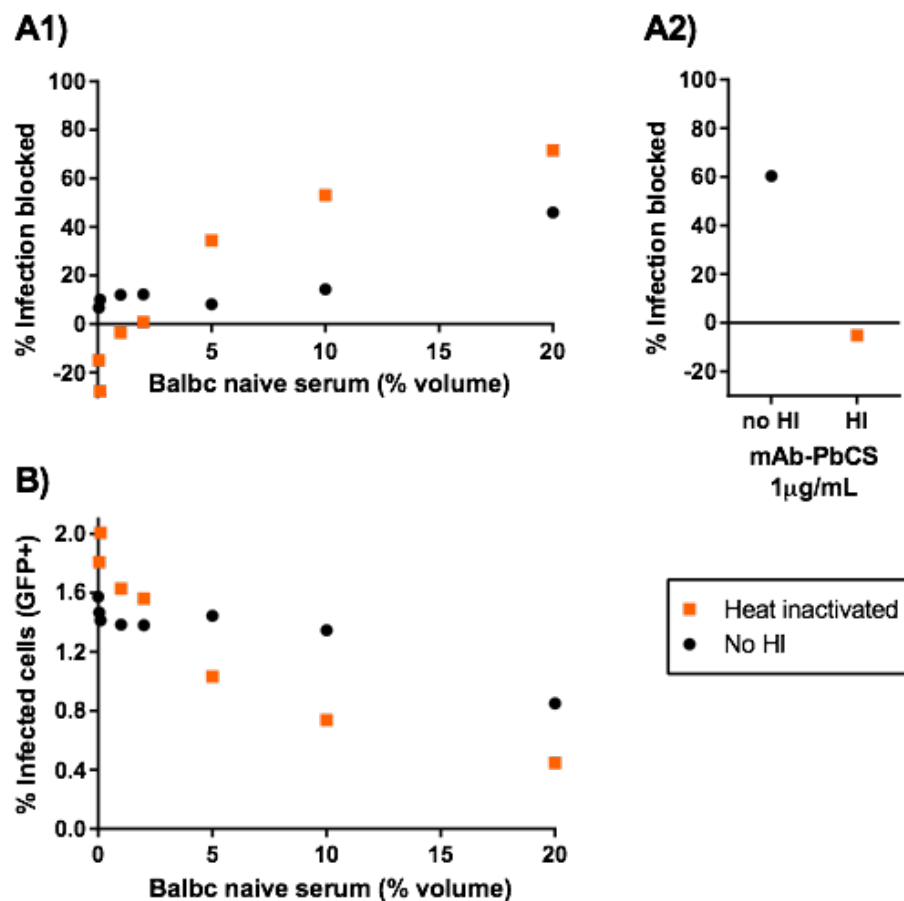
Figure 1.10. Effect of addition of naive serum on sporozoite infection of liver cells. 15000 sporozoites (*P. berghei* GFP-luciferase) were used to infect 30000 Huh7 cells per well in the presence of various concentrations (0-20%) of serum from naive BALB/c mice or 1 µg/mL mAB-PbCS. Heat inactivated (HI) serum and mAb-PbCS are shown in orange, and black is used for non HI samples. A) Percentage of infection blocked in the presence of HI/non HI BALB/c naive serum (A1) and HI/non HI mAb-PbCS (A2). B) Percentage of infected cells in the presence of HI/non HI BALB/c naive serum.

Low concentrations (≤ 2%) of HI serum did not have a blocking effect on the infection, while addition of 5%-20% HI serum had a greater effect on blocking the infection (more than 30% of blocking) than non HI serum [Figure 1.10.A1]. It is not clear why HI samples prevented the infection to a larger extent than non HI samples. In addition, heat inactivation of mAb-*Pb*CS inhibited its blocking capacity [Figure 1.10.A2], which discouraged the use of heat inactivation of the samples in future assays.

1.2.9. Monoclonal antibody against *P. falciparum* CSP (mAb-*Pf*CS)

Since an important number of serum samples to test in future experiments had been raised against *P. falciparum* CSP and there was an available monoclonal antibody (mAb-*Pf*CS, 2A10) against this protein, some experiments were performed to explore the blocking capacity of this antibody with the objective of establishing it as a positive control.
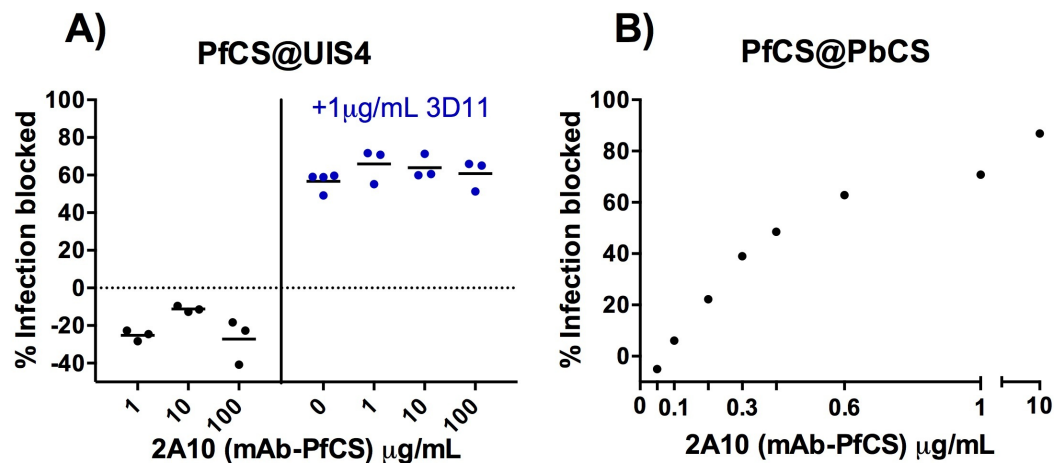
Figure 1.11. Effect of mAb-PfCS (2A10) on sporozoite infection. 15000 sporozoites (*P. berghei* GFP-luciferase) were added to 30000 Huh7 cells per well. A) PfCS addition sporozoites (PfCS@UIS4) with mAb-PfCS (2A10) at 1 to 100 µg/mL with (blue dots) or without (black dots) mAb-PbCS (3D11) at 1 µg/mL. B) PfCS replacement sporozoites (PfCS@PbCS) with mAb-PfCS at 0.05 to 10 µg/mL.

mAb-*Pf*CS used in concentrations up to 100 µg/mL did not block infectivity of *P. berghei* sporozoites expressing *P. falciparum* CSP and *P. berghei* CSP (*Pf*CS@UIS4; *Pf*CS addition sporozoites which express both *P. falciparum* and *P. berghei* CSP) [Figure 1.11.A]. It seems that the presence of *P. berghei* CSP maintains sporozoite functionality regardless that mAb-*Pf*CS may bind to *P. falciparum* CSP. When testing mAb-*Pf*CS at 1 to 100 µg/mL in combination with mAb-*Pb*CS (3D11) at 1 µg/mL, about 60% of the infection was blocked [Figure 1.11. A] which is the value observed for mAb-

*Pb*CS on its own at this concentration. However, when mAb-*Pf*CS (0.05-10 µg/mL) was used to prevent invasion by *P. berghei* sporozoites expressing *P. falciparum* CSP in which *P. berghei* CSP had been deleted (*Pf*CS@*Pb*CS; *Pf*CS replacement sporozoites), the infection was blocked in a concentration-dependent manner [Figure 1.11.B].

A)

PfCS@UIS4 GFP *P. berghei*

B)

GFP *P. berghei*
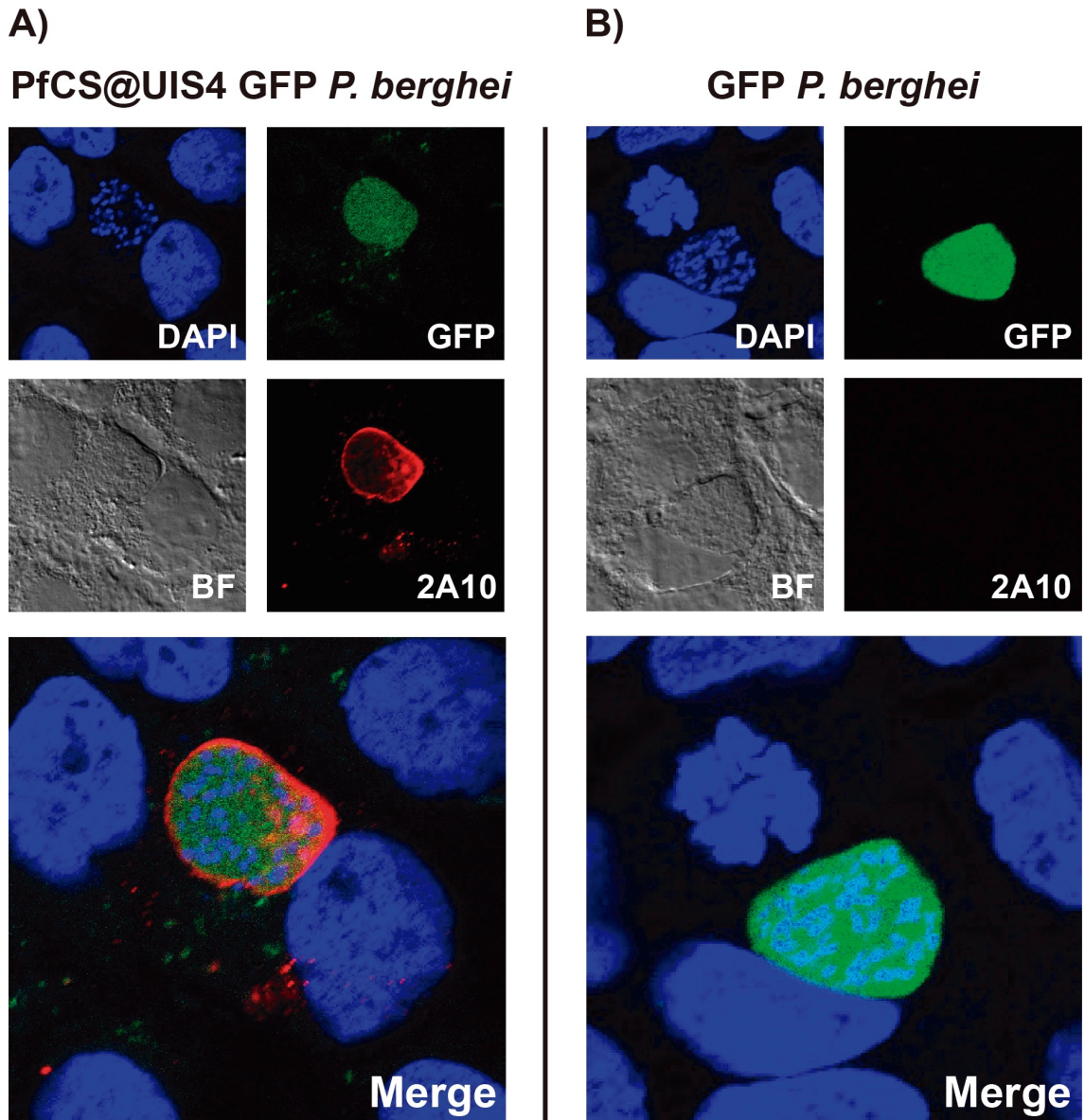
Figure 1. 12. mAb-PfCS (2A10) is detectable inside infected hepatocytes. 20000 sporozoites per well were added with mAb-PfCS at 3 µg/mL, to 60000 Huh7 cells seeded on a P24 plate and incubated during 45h. Sporozoites develop in a parasite vacuole inside hepatocytes, which is detectable through the green signal of GFP (expressed by chimeric parasites) and blue signal from DAPI staining parasite DNA. DAPI also stains hepatocyte nuclei. mAb-PfCS was detected with goat anti-mouse IgG conjugated to Alexa Fluor 594 (red). A) PfCS@UIS4 GFP *P. berghei* sporozoites B) GFP *P. berghei* sporozoites (negative control).

With the purpose of better understanding the interaction between mAb-*Pf*CS and *Pf*CSP@UIS4 sporozoites, confocal microscopy images were taken of cultures of Huh7 cells where addition sporozoites and mAb-*Pf*CS were added. Parasite vacuoles within hepatocytes where recognized for their GFP signal (green) and parasite DNA stained with DAPI (blue). DAPI also stained hepatocyte nuclei. An anti-mouse antibody conjugated to Alexa Fluor 594 (red) was used to detect mAb-*Pf*CS presence. Red signal (mAb-*Pf*CS) was found within the hepatocyte and surrounding the parasite vacuole [Figure 1.12.A], which suggests that sporozoites may have entered into hepatocytes with mAb-*Pf*CS attached to their surface and the antibody has been left on the surface of the parasite vacuole. As a negative control, when *P. berghei* sporozoites that did not express *P. falciparum* CSP, had mAb-*Pf*CS added, no red signal was detected [Figure 1.12.B].

1.3 Discussion

The aim of the work described in this chapter was the optimisation of a protocol to measure post-vaccination serum capacity to inhibit sporozoite invasion of liver cells. The following parameters were evaluated: the optimal number of sporozoites and cells to use per assay, the infectivity rates of several liver cell lines cultured in various media, the effect of sporozoite pre-incubation with antibodies, the temperature of the first hour of incubation, the best time of readout, the range of serum concentration to test and whether it was necessary to heat inactivate the serum.

The possibility of measuring changes in the number of infected cells (GFP+) caused by the presence of serum by flow cytometry was first tested by adding GFP expressing adenovirus to the liver cell lines HepG2 and BNL. Serum from mice that had been vaccinated with the same adenovirus was used in a range of concentrations to block virus entry into the cells [Figure 1.1]. As expected, the percentage of infected cells increased with the amount of virus added to the culture. In addition, the presence of serum blocked the virus infection in a concentration-dependent manner.

After this preliminary experiment with GFP adenovirus, HepG2 and BNL were both infected with various concentrations of GFP expressing sporozoites [Figure 1.2]. The number of infected cells was dependent on the number of sporozoites added, confirming flow cytometry as an adequate method to determine the number of infected cells. A percentage of infected cells between 1% and 5% was observed when adding 5000 to 40000 sporozoites to HepG2 cells. However, the percentage of infected cells for BNL was less than 0.5% in all cases tested. The discrepancy between infectivity rates found for each cell line could be due to the particular repertoire of surface receptors expressed on the hepatocytes.

A series of experiments were then performed with the aim of maximizing infectivity rates, since in the sporozoite neutralization assay the percentage of infection blocked is calculated using the number of infected cells in the absence and presence of post-vaccination serum. Then, performing the assay in the conditions were a higher number of infected cells could be achieved would increase the flow cytometry events upon which the percentage of infection blocked is calculated, increasing the reliability of the data.

Regarding the liver cell line to use in the assay, the BNL cell line was discarded due to the low infectivity rates observed, and HepG2 were compared with other human liver cell lines: Huh7 and HC04 [Figure 1.5]. In these experiments, the higher

percentages of infected cells were achieved when using Huh7 cells. One of the sources of variability when comparing infectivity rates is the growth rate of each cell line. Carly Bliss (DPhil student) performed an experiment where she found that HC04 cell division rate was higher than the one of Huh7 cells. If infected cells do not divide as rapidly as non-infected cells, the percentage of infected cells would be found to be smaller in a more rapidly growing cell line (such as HC04 compared to Huh7). Prudencio et al. found 1.5-4.5% infected cells in cultures of Huh7 infected with *P. berghei* (208), Kaushansky et al. measured 0.6-2.5% infected cells using HC04 cells (209) and *P. falciparum,* and Sinnis et al. described that when quantifying sporozoite invasion by flow cytometry at least 1% of total cells should be infected (217). Although direct comparison is not possible due to the different kind of cell lines and sporozoite strains used, sporozoite:cell ratio and variations in the protocols such as the time of readout; the percentage of infected cells found in this work is within the range previously described in the literature.

Sporozoite neutralizing assays described previously have been performed using a variety of cell culture media, mainly DMEM or RPMI. Huh7 cells and *P. berghei* sporozoites have been described to be cultured both in DMEM (217,227,228) and RPMI (208,229,230). Although successful infections can be achieved in both media, an experiment was performed to explore whether there could be differences on infectivity rates using DMEM, DMEM (supplemented with non-essential amino acids and pyruvate) and RPMI. Culture media could modulate sporozoite behaviour; for example, Hegge et al. showed that sporozoites transformed into exoerythrocytic forms in RPMI within 6 hours, while in DMEM in only 1 hour (223). RPMI has a higher concentration of glucose and phosphate, but lower of calcium than DMEM (231), which could have an effect on sporozoite metabolism. Host cell growth rates could also vary in different media, which could modify the infection rates. For Huh7 cells, superior infectivity rates were found using RPMI rather than DMEM [Figure 1.5]. As a result, RPMI was selected for future experiments.

The number of sporozoites was the limiting factor encountered when performing these assays. Sporozoites were obtained by homogenizing salivary glands dissected from infected mosquitoes and the parasites yields per mosquito were highly variable, usually between 4000 and 30000. To ensure efficient use of the sporozoites available, the assays were optimised to be performed with a minimal number of sporozoites. The cell:sporozoite ratio to plate was evaluated measuring the percentage of infected cells obtained when plating 30000 and 50000 cells per well and adding 10000, 20000 or 30000 sporozoites [Figure 1.3]. A higher percentage of infected cells was obtained

when platting fewer cells [Figure 1.3.A]. If a number of sporozoites would infect a certain number of hepatocytes, the number of infected cells would represent a larger percentage if the total number of cells was smaller. Nevertheless, the total number of positive events (100-200 total GFP+ cells) was in the same range regardless of the number of cells plated [Figure 1.3.B]. Therefore, 30000 cells and 15000 sporozoites were chosen as the standard number to plate per well (96-well plate) that would generate infectivity rates between 1-3%.

In order to test whether using a higher number of sporozoites would be possible to detect blocking effect of smaller antibody concentrations, a experiment was performed infecting hepatocytes with 15000 or 30000 sporozoites per well, in the presence of mAb-*Pb*CS concentrations from 0.1 to 5 µg/mL [Figure 1.8]. When plotting the percentage of infection blocked versus monoclonal antibody concentration [Figure 1.8.B], similar curves were obtained with both amounts of sporozoites used. Doubling the amount of sporozoites did not effect the range of infection blocked by monoclonal antibodies. It was concluded that 15000 sporozoites per well could be used without having a detrimental effect on assay sensitivity. It is interesting to note that when using 30000 sporozoites per well, the percentage of infected cells was twice as much than with 15000 sporozoites [Figure 1.8.A], but similar percentage of infection blocked was obtained in both cases. This result shows that in spite of obtaining differences in the percentage of infected cells, the values of percentage of infection blocked are consistent. This is especially relevant since the percentage of infected cells presented certain fluctuations in each experiment (probably due mainly to the quality of different batches of sporozoites and the error associated to the number of sporozoites added to each well).

Temperature has been described to play a role in sporozoite infectivity and in the process of transformation towards liver stage parasites. Sporozoites maintained *in vitro* at 37ºC rapidly lose their infectivity capacity (224), while infectivity could be preserved for longer periods of time when sporozoites were kept at lower temperatures (4-20ºC) (217). On the other side, Siau. et al showed that infectivity loss was delayed in the presence of human cells (232). A temperature elevation to 37ºC is required for efficient infection (232), causes early and faster sporozoite transformation into EEF (exoerythrocytic forms) (223) and is required to complete this transformation (233). It was considered, that the temperature of the first hour of infection might have an influence in the rate of infectivity achieved. As sporozoites lose capacity to invade cells at 37ºC, maintaining the sporozoites during the first hour of infection at room temperature instead of at 37ºC could increase the number of liver cells infected.

However, keeping the plates at room temperature did not have a positive effect increasing the infectivity rates measured [Figure 1.6.B]. Consequently, plates with sporozoites were placed in the incubator just after sporozoite addition in the subsequent experiments.

To determine whether pre-incubation of the parasites with antibodies had an influence on the blocking effect, sporozoites were pre-incubated on ice with monoclonal antibody against *P. berghei* CSP (mAb-*Pb*CS) prior to the addition of the mixture to the hepatocytes culture. The antibody blocking capacity after pre-incubation during 15 or 30 minutes, was the same as when there was no pre-incubation [Figure 1.6.A]. Therefore, sporozoites mixed with monoclonal antibody or serum were added to the cells without a pre-incubation in all the subsequent experiments. The interaction between antibodies and sporozoites appears to occur rapidly enough to disregard pre-incubation in the ISI assay.

Other factor explored when trying to maximize the number of infected cells measured, was the time of readout. In preliminary experiments with sporozoites expressing GFP, the percentage of infected cells was quantified by flow cytometry after 16-18h (overnight incubation). However, when measuring the number of cells infected with sporozoites expressing GFP-luciferase, the infectivity rates were much lower than expected after the usual time of incubation. For this reason, a time course was performed to determine how the percentage of infected cells measured changes over time. The percentage of infected cells (using GFP-luciferase sporozoites) reached a peak at 25-28 hours after infection, time established to measure the GFP signal; and later decreased with time, most likely due to cell division. When GFP is fused to luciferase, the expression of the protein could be delayed due to its overall larger size compared to GFP expressed on its own, as a result, the infected cell may need more time to accumulate enough reporter protein to be detected by flow cytometry. The presence of luciferase in the chimeric parasites allows performing *in vivo* imaging, constituting an important advantage for vaccination studies. For this reason GFP-luciferase was chosen as the reporter gene in chimeric sporozoites expressing *P. falciparum* antigens that would be used to test post-vaccination serum samples, and the assay needed to be optimised for these parasites. Traditionally, assays which measure antibody functionality preventing liver sporozoite infection have been divided in ISI, whose incubation time last from few hours up to a day, and ILSDA with incubations of one or several days (209,210). ISI measures neutralisation capacity of antibodies avoiding invasion of hepatocytes and ILSDA evaluates antibody capacity to prevent sporozoite development within the liver. The requirement of waiting at least 24h

when using GFP-luciferase sporozoites to allow enough time for the GFP signal to develop, situates the assay employed in this work in the limit between ISI and ILSDA (nevertheless, it is referred through out this text as ISI). If antibodies are able to act both avoiding invasion and preventing sporozoite development, measuring the percentage of infected cells at later time points may be a more realistic approach to evaluate the actual capacity of the antibody to avoid the progress of the liver stage.

Antibodies have been described to play a role on inhibition of parasite development (114,200,225). On other side, Prudencio et al. (208) found that the intensity of flow cytometry GFP signal of infected hepatocytes correlated with parasite load (measured by qRT-PCR), suggesting flow cytometry as a tool to evaluate parasite development within hepatocytes. GFP MFI (Mean Fluorescence Intensity) could be an indicator of parasite development within the hepatocyte, since a higher expression of GFP may indicate a larger number of parasite copies or a larger growth. The presence of antibodies could impair sporozoite development and therefore effect GFP MFI. A trend was detected towards slightly lower values of GFP MFI when increasing the concentration of mAb-*Pb*CS (3D11) [Figure 1.8. C1, C2]. For this reason it was decided to measure whether the presence of monoclonal antibody could decrease GFP MFI at later time points, when the antibody inhibition of parasite development might be more evident. However, no difference was detected on GFP MFI signal between hepatocytes infected in the absence or presence of mAb-*Pb*CS, even when measuring after 61 hours of incubation [Figure 1.9]. As it has been described in [Figure 1.7], the range of GFP MFI measured was larger using GFP only sporozoites rather than GFP-luciferase sporozoites. GFP-sporozoites were detected after 4h of incubation with a log(MFI GFP+) close to 3.5, which increased up to 5.0 after 41h of incubation. GFP-luciferase sporozoites presented a log(MFI GFP+) of 3.2, which only increased up to approximately 3.8 after 48h. GFP fused to luciferase produced a weaker signal at later time points. GFP-luciferase sporozoites may not generate a signal intense enough to allow evaluation of antibody effects on liver stage development by flow cytometry or the monoclonal antibodies employed may not have an effect on the development detectable by this technique. Consequently, it was decided not to continue with the study of GFP MFI in future experiments.

One of the limitations of the ISI assay is the non-specific blocking of sporozoite infection that may generate the presence of serum, evidenced in the finding of up to 30% of inhibition blocked using naive sera described in the literature (198,211,226). It was considered that heat inactivation of serum could be a way to reduce the non-specific effect observed. However, the percentage of non-specific infection blocked

observed after heat inactivation was higher than without it using serum dilutions of 5-20% [Figure 1.10.A1]. In addition, mAb-*Pb*CS lost its blocking effect after heat inactivation [Figure 1.10.A2]. Although heat inactivation is supposed to affect only the proteins of the complement, heat inactivation might elicit the loss of an active conformation of antibodies or cause antibody aggregation. As a result, heat inactivation of serum samples was disregarded.

In addition to mAb-*Pb*CS, which has been used as a positive control in some of the experiments to optimise the protocol and in the assays with serum samples (that will be described in chapter 2), a monoclonal antibody against *P. falciparum* CSP (mAb-*Pf*CS, 2A10) was also available. This monoclonal antibody could be an adequate positive control, when testing human, macaque and mouse anti-*Pf*CS sera. However, it was found that mAb-*Pf*CS was not able to block the sporozoite entry in hepatocytes when the parasites expressed *Pf*CS as an additional copy (*Pb*CS was also present in the sporozoites), even when using very high antibody concentrations [Figure 1.11.A]. mAb-*Pf*CS only acted efficiently blocking sporozoites expressing *Pf*CS as a replacement copy, which express *Pf*CS, but no *Pb*CS [Figure 1.11.B]. One possible explanation could be that, in the presence of functional *Pb*CS, even if *Pf*CS is blocked through antibody binding, the parasite can still enter into the cell. In fact, some confocal images were taken where mAb-*Pf*CS was detected inside the cell, surrounding the parasite vacuole [Figure 1.12.A]. This effect observed with mAb-*Pf*CS, seemed to also be happening with R21 serum (anti-*Pf*CS serum), which did not block sporozoites expressing *Pf*CS as an additional copy in the experiments that will be described in chapter 2. Consistent with these observations, Persson et al described that 2A10 (mAb-*Pf*CS) prevented *in vitro* and *in vivo* liver stage invasion of *P. berghei* sporozoites expressing *P. falciparum* CSP in the *P. berghei* CSP locus (equivalent to the 'replacement parasites' used here). The absence of blocking described when *P. berghei* CSP is also present, may highlight that antibodies are preventing the hepatocyte invasion because they block the function of a protein, rather than, for example, have a neutralization effect avoiding the contact between sporozoites and hepatocytes.

The experiments performed in this chapter established a protocol to test post-vaccination serum samples. The main assay limitations were sporozoite availability and the non-specific blocking effect of naive-serum. The percentage of infected cells presented certain variability, however, the measure of the serum blocking capacity was found to be very reproducible regardless the infectivity rate of a particular assay. The most important advantage that flow cytometry provides as a readout is the possibility of

using ISI assay as a high-throughput method to test antibody capacity to prevent sporozoite invasion.

Chapter 2:

Testing mouse, macaque and clinical samples with ISI assay

CHAPTER 2: Testing mouse, macaque and clinical samples with ISI assay

2.1 Introduction

This second chapter describes the results obtained when testing diverse serum samples with the Inhibition of Sporozoite Invasion assay, described in chapter 1.

Serum samples tested were obtained from pre-clinical and clinical studies performed by researchers at the Jenner Institute. The experiments described here were performed with the objective of better characterising the functionality of humoral immune responses generated through vaccines targeting pre-erythrocytic *P. falciparum* antigens.

In order to evaluate the capacity of the post-vaccination serum samples to reduce hepatocyte invasion, transgenic *P. berghei* parasites expressing the same *P. falciparum* antigen that was targeted through vaccination were used. These chimeric parasites, kindly provided by Ahmed Salman (DPhil student), were generated through GIMO ('Gene insertion/marker out') transfection (215). Chimeric parasites contain a construct, inserted in the genomic silent locus 230p, which includes a *P. falciparum* gene under *Pb* UIS4 promoter and a GFP-luciferase cassette under the control of *Pb* eef1α promoter.
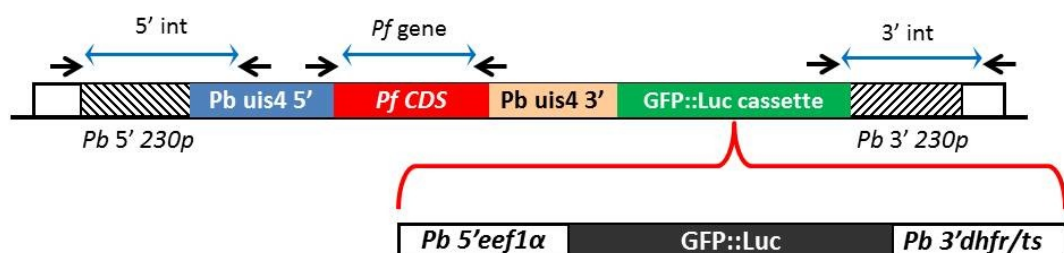
Figure 2.0. Schematic representation of the construct included in *P. berghei* chimeric parasites (additional copy). The graph represents the construct included in 230p (genomic silent locus) to generate *P. berghei* parasites which express a *P. falciparum* antigen (*Pf* CDS: *falciparum* coding sequence) under *Pb* uis4 promoter and GFP-luciferase under *Pb* eef1α promoter. Image provided by Ahmed Salman [DPhil Thesis, University of Oxford 2014].

UIS4 gene has been shown to be upregulated in salivary gland sporozoites, like other UIS (Upregulated in Infective Sporozoites) genes (234). Silvie et al. found high levels of UIS4 transcript in *P. berghei* sporozoites, but absence of UIS4 protein until one day after infection (235). However, in transgenic salivary gland sporozoites, they

observed mCherry expression under the control of UIS4 UTRs 5' (including UIS4 promoter) and UTRs 3' regions and consequently, they described a post-translational repression of UIS4 due to elements present in the UIS4 coding sequence (235). Therefore, UIS4 promoter was chosen to express pre-erythrocytic *P. falciparum* antigens in *P. berghei* sporozoites.

The *P. falciparum* antigens tested here were: CelTOS, LSA1, LSA3, LSAP1, LSAP2, UIS3, ETRAMP5, Falstatin, SPECT1, SPECT2, RP-L3, HT, CSP and TRAP. These antigens are pre-erythrocytic malaria vaccine candidates that are being currently evaluated at the Jenner Institute. *P. falciparum* CelTOS, UIS3, Falstatin, SPECT1, SPECT2, RP-L3, HT, CSP and TRAP are present both in *P. falciparum* and *P. berghei*, while the rest of the *P. falciparum* antigens tested do not have a *P. berghei* homolog. In this work, some experiments were performed with CSP replacement parasites in which *P. berghei* CSP has been deleted and *P. falciparum* CSP is expressed under the control of the *P. berghei* CSP promoter. This chimeric parasite was generated through the GIMO method, but replacing the *P. berghei* CSP by the *P. falciparum* CSP. Consistent with the additional transgenic parasites, *Pf* CSP replacement parasites express GFP-luciferase under *Pb* eef1α promoter in 230p. The rest of the chimeric parasites employed express the *P. falciparum* antigen under UIS4 promoter as an additional copy. This strategy allows the generation of transgenic parasites even when a *P. berghei* homolog is not present.

Both the genotype and phenotype analysis of the transgenic parasites were performed by Ahmed Salman [Ahmed Salman, DPhil thesis]. The correct integration of the construct in the chimeric parasites was demonstrated through southern analysis and PCR. The expression of the *P. falciparum* antigen was probed by IFAT (Immunofluorescence antibody test) using monoclonal antibodies whenever available or sera collected from vaccinated animals.

A short summary of information available in the literature for each of the *P. falciparum* antigens targeted through vaccination and expressed by the chimeric *P. berghei* parasites are included in the table [table 2.1]. Function and localization of these proteins are likely to have an effect on the role that antibodies targeting them may play inhibiting the invasion of sporozoites into hepatoma cell line.

Pre-erythrocityc antigens

	Name Localization	Function	Details	
CelTOS	**Cel**l-**T**raversal protein for **O**okinetes and **S**porozoites Located in micronemes (sporozoites)	CelTOS plays an important role in breaking through host cellular barriers. CelTOS is involved in cell-traversal capacity of ookinetes and sporozoites (at mosquito midgut and liver infection, respectively).	CelTOS-disrupted *P. berghei*: -infected HepG2 cells (hepatoma cell line) with a similar efficiency to WT. -showed damaged cell-passage activity *in vitro*. -showed reduced ookinete movement from the cytoplasm of the mosquito midgut epithelial cell to the basal lamina. -showed limited sporozoite movement through the liver sinusoidal cell layer (which includes Kupffer cells, hepatic macrophages), which was restored when using Kupffer cell-depleted rats. Immunisation with *P. falciparum* CelTOS has been shown to generate high immunogenicity and protection in mice (heterologous challenge with *P. berghei* sporozoites).	(236) (187)
LSA1	**L**iver-**S**tage **A**ntigen 1 Expressed only in liver stages. Located in the PV space.	LSA1 plays an important role in late liver-stage development.	LSA1-disrupted *P. falciparum*: -infected HCO4 cells (hepatoma cell line), but liver stage development was reduced at later stages. -did not achieve exo-erythrocytic merozoite formation in mice with humanised liver. Immune response to LSA1 have been found in individuals living in endemic areas.	(237) (188) (238) (122)
LSAP1	**L**iver-**S**tage-**A**ssociated **P**rotein-**1** Mainly expressed in liver stages. Located in the PV membrane.	Unknown	LSAP1 has been detected in primary human hepatocytes infected with *P. falciparum* and it might be expressed in small quantities in sporozoites. Anti-LSAP1 serum did not prevent sporozoite cell traversal (1% dilution), nor affected sporozoite invasion of primary hepatocytes (1 and 5% dilution).	(232)
LSAP2	**L**iver **S**tage-**A**ssociated **P**rotein-**2** Mainly expressed in liver stages. Located in the PV membrane.	Unknown	LSAP2 has been detected in primary human hepatocytes infected with *P. falciparum*, two days after the inoculation.	(232)

	Pre-erythrocityc antigens			
	Name **Localization**	**Function**	**Details**	
UIS3	**I**nfective **S**porozoites gene **3** Expressed in sporozoites and liver-stages. Located in the PV membrane. Belongs to ETRAMP family.	UIS3 is essential for early liver-stage development. UIS3 has been shown to interact with L-FABP (liver-fatty acid binding protein), so it could be involved with fatty acid acquisition by the liver stage parasite.	UIS3-disrupted *P. berghei*: -infected Huh7 cells (hepatoma cell line), but did not mature into liver schizonts. -were unable to establish blood-stage infection, *in vivo*. Mice immunised with *P. berghei* uis3(-) parasites were shown to be protected against WT sporozoites. P. yoelii UIS3 used in combination with other two antigens (P.y. falstatin and PY03661) elicited protection in CD1 mice.	(239) (240) (228) (241)
ETRAMP5	**E**arly **TRA**nscribed **M**embrane **P**rotein **5** Putative PV membrane protein.	ETRAMP5 binds human apolipoproteins (ApoA1, ApoE and ApoB)	Antibodies against ETRAMP5 have been found in sera of individuals from endemic areas.	(242) (243)
LSA3	**L**iver-**S**tage **a**ntigen **3** Expressed on sporozoites and in liver-stages.	Unknown	Immunisation with LSA3 peptides and lipopeptides elicited protection in chimpanzees challenged with *P. falciparum.* Humoral and cellular immune responses have been detected in individuals from endemic areas. Purified antibodies against LSA3 from volunteers living in endemic areas inhibited *P. yoelii* invasion of mouse hepatocytes and *in vivo* infection of BALB/c mice.	(244) (245) (246)
Falstatin	Expressed in sporozoites (secretory vesicles). Expressed in liver-stages.	Inhibition of cysteine proteases	Mouse antibodies (20% dilution) against *Pb* Falstatin blocked about 40% of *P. berghei* invasion of HepG2 cells. *P. yoelii* falstatin used in combination with other two antigens (P.y. UIS3 and PY03661) elicited protection in CD1 mice.	(247) (241)

	Pre-erythrocityc antigens			
	Name **Localization**	**Function**	**Details**	74
SPECT1	**S**porozoite microneme **P**rotein **E**ssential for **C**ell **T**raversal Located in micronemes (of salivary gland sporozoites)	SPECT1 is essential in cell-passage ability.	SPECT1-disrupted *P. berghei*: -infected HepG2 (hepatoma cell line) cells in a similar way to WT *P. berghei*. -lost cell-traversal ability *in vitro*. -showed a reduced liver infectivity in rats, compared to WT *P. berghei*. Disrupted and WT parasites had the same capacity of infection in Kupffer-cell depleted rats.	(248)
SPECT2	**S**porozoite microneme **P**rotein **E**ssential for **C**ell **T**raversal 2 Located in micronemes (of salivary gland sporozoites)	SPECT2 is essential for cell-traversal activity. SPECT2 has been suggested to mediate host cell membrane rupture through the pore forming activity of a MACPF (membrane attack complex/ perforin) related domain.	SPECT2-disrupted *P. berghei,* (like SPECT1): -infected HepG2 (hepatoma cell line) cells in a similar way to WT *P. berghei*. -lost cell-traversal ability *in vitro*. -showed a reduced liver infectivity in rats, compared to WT *P. berghei*. Disrupted and WT parasites had the same capacity of infection in Kupffer-cell depleted rats.	(249)
RP-L3	**R**ibosomal **P**rotein **L3** Expressed in liver stages	Unknown	In mice immunised with *P. yoelii* sporozoites (irradiated or in combination with chloroquine prophylaxis), CD8+ T cells targeted a peptide from RP-L3.	(250)
HT	**H**exose **T**ransporter Expressed in sporozoites and liver-stages (in the PVM).	Uptake of host hexoses such as fructose or glucose by the parasite.	-Liver development was inhibited using a D-glucose-derived specific inhibitor. -Sporozoites retained motility in the absence of D-glucose.	(251) (252)

Pre-erythrocityc antigens				
	Name **Localization**	**Function**	**Details**	
TRAP/ SSP2	**T**hrombospon din-**r**elated **A**nonymous **P**rotein/ **S**porozoite **S**urface **P**rotein **2** Located in micronemes and on the surface of sporozoites, and in liver stages.	Involved in invasion of salivary glands and hepatocytes. Essential for *in vitro* gliding motility. TRAP binds HPSGs on hepatocyte surface (through adhesion domains) and is connected to parasite cytoskeleton (through a cytoplasmic domain which interacts with actin binding proteins).	-Sera against TRAP reduce sporozoite invasion and development in primary human liver cells. -Immunisation with P. yoelii TRAP and CSP, elicited complete protection against P. yoelii. -Antibodies and T cell responses against TRAP have been detected in volunteers protected through immunisation with irradiated sporozoites. -TRAP has been included in a leading malaria vaccine: ChAd63 MVA ME-TRAP, which has been shown to generate high levels of T-cell responses, delay to the development of clinical disease and protection. TRAP-disrupted *P. berghei* sporozoites: -showed reduced infectivity of mosquito salivary glands. -generated blood infection in rats, but with a delay compared to WT. -lost *in vitro* gliding motility	(253) (225) (34) (254) (255) (256) (257) (258)
CSP	**C**ircum- **s**porozoite **p**rotein Located in micronemes and on the surface of sporozoites	CSP is involved in sporozoite formation within oocyst and mediates sporozoite attachment to hepatocytes and invasion.	In CS-disrupted *P. berghei* oocysts, sporozoite formation showed a drastically reduction compared to WT. Monoclonal antibodies against CSP prevented *P. berghei* infection in mice and *in vitro* invasion (WI38 cells). CSP is the target of the most advanced malaria vaccine (RTS,S / AS01), which has shown a protection of 30-50% and induces high antibody titers and moderate cellular responses. CS-disrupted *P. berghei* sporozoites: -showed a drastically reduced capacity to egress from oocyts. -decrease on the attachment to HepG2 surface*. -lost the capacity to infect rats*. (*using midgut sporozoites)	(259) (260) (261) (262) (192) (191) (263) (264)

Table 2.1. Pre-erythrocytic antigens tested with ISI assay in this chapter. Each row includes available information of each antigen about localization, function and other data related to immune response.

2.2 Results

2.2.1 Mouse samples

2.2.1.1 CS and TRAP

The first samples tested with the flow cytometry optimised ISI protocol were sera obtained from mice vaccinated with CS and TRAP expressing vaccines. Samples from three different studies were tested, where mice had been immunised with 10^7 or 10^8 iu AdCh63 followed at least 6 weeks later by 10^6 or 10^7 pfu of MVA. Serum samples were tested at a final concentration of 10, 5 or 2%.
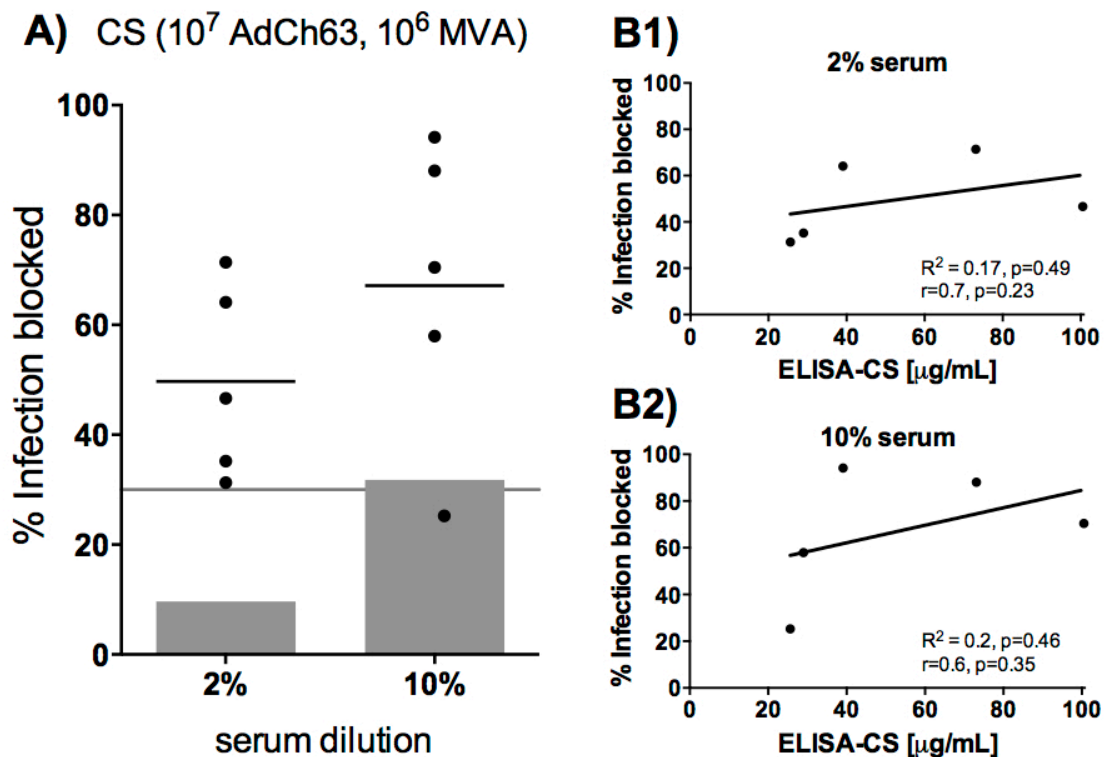
Figure 2.1. BALB/c mice were immunised with 10^7 AdCh63 CS and 10^6 MVA CS 8 weeks apart and serum collected 4 weeks after MVA vaccination. Serum samples were tested at 2 and 10% serum dilution and Huh7 cells infected with *P. falciparum* CSP *P. berghei* sporozoites in the presence of serum samples (A: black dots) or naive sera (A: grey bars). Results are expressed as the % Infection blocked, taking infectivity in the absence of serum as a reference. The line at 30% (A) indicates the threshold level for positive responses. Correlation between % Infection blocked and ELISA-CS is shown at 2% (B1) and 10% (B2) serum dilution. Data (B1, B2) was analysed with: nonparametric Spearman correlation test (r); linear regression (R2; p-value from F test: is the slope significantly different than zero?).

Chimeric *P. berghei* parasites expressing the *P. falciparum* protein (CSP or TRAP) were mixed with each serum sample and added to hepatoma cell line Huh7, which had been plated approximately 8 hours prior to infection. Tests were performed in duplicate or triplicate. Cultures were incubated at 37ºC for 24-28 hours prior to sample acquisition on the flow cytometer to determine the percentage of infected cells. Results are shown in the three figures: Figure 2.1 (BALB/c - CS), Figure 2.2 (C57BL/6 and CD1 -TRAP) and Figure 2.3 (BALB/c - CS and TRAP). The percentage of infection blocked (percentage of infection avoided in the presence of serum) has been plotted for each serum dilution used. CS samples showed a positive concentration-dependent blocking response [Figure 2.1.A, Figure 2.3.A]. BALB/c mice vaccinated against CS with a higher dose of ChAd63 and MVA [Figure 2.3.A] showed higher blocking responses (82% Infection blocked, with 2% serum dilution), than those that received the lower doses of vaccine [Figure 2.1.A] (50% Infection blocked, with 2% serum dilution).
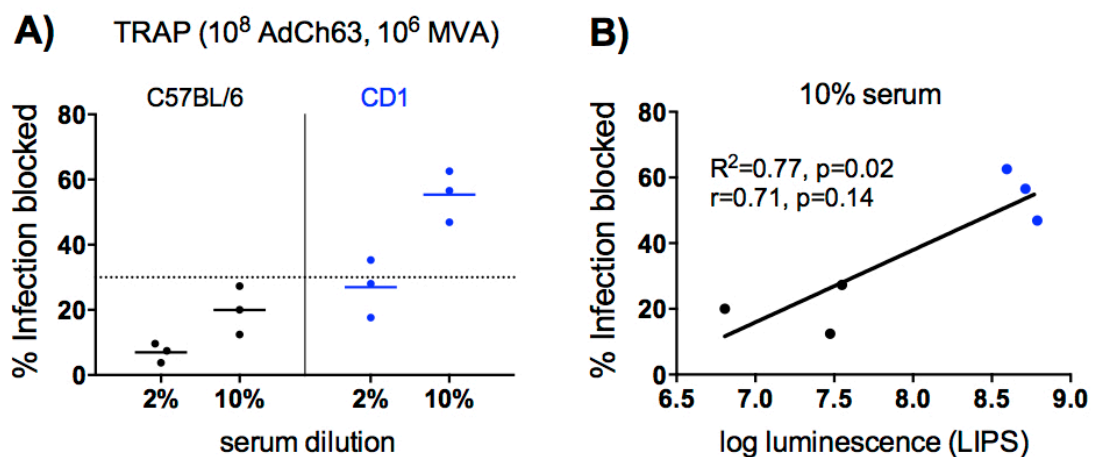
**Figure 2.2. C57BL/6 and CD1 mice, CS samples. Mice were immunised with 10^8 AdCh63 and 10^6 MVA, both vectors encoding ME-TRAP and administered 7 (C57BL/6) or 8 (CD1) weeks apart. Serum was collected one week after MVA vaccination. Each serum sample was tested in triplicates and at two concentrations, 2 and 10% (final serum volume). *P. falciparum* TRAP *P. berghei* sporozoites were employed to infect Huh7 cells. The % Infection blocked has been plotted in A), where each dot (black-C57BL/6, blue-CD1) represents an individual mouse. The line at 30% indicates the threshold above which blocking is considered positive. B) The graph shows the correlation between % Infection blocked and LIPS data, data was analysed with a nonparametric Spearman correlation test (r); linear regression (R2; p-value from F test: is the slope significantly different than zero?).**

C57BL/6 and CD1 mouse strains were tested for blocking capacity of antibodies after ME-TRAP vaccination. It was possible to detect positive responses at 10% dilution for CD1 mice, but not for C57BL/6 [Figure 2.2.A].

Figure 2.3 shows the blocking obtained with serum from BALB/c mice vaccinated against CS [Figure 2.3.A] or TRAP [Figure 2.3.B]. Both antigens elicited strong blocking responses, higher in the case of CS (at 2% serum dilution: CS generated 82% of blocking and TRAP, 69%).
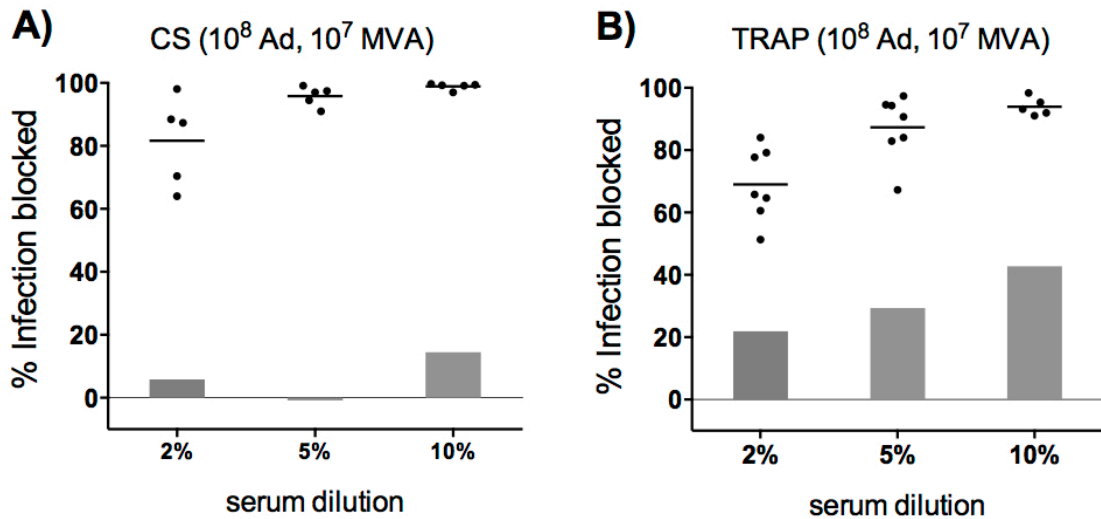
Figure 2.3. BALB/c mice were immunised with 10^8 iu AdCh63 followed 8 weeks later by 10^7 pfu MVA, expressing either CS (A) or TRAP (B). Serum samples were taken 8 to 14 days after the last vaccination and each sample tested in duplicates and at three dilutions: 2, 5 and 10% serum. Transgenic *P. berghei* sporozoites (*Pf*CS *Pb* in A and *Pf*TRAP *Pb* in B) and serum were added to Huh7 cells. In both graphs, each dot represents an individual mouse and the blocking effect (non-specific background effect) of naive serum is shown as bars.

To determine whether the level of infection blocked is due to the amount of antigen specific antibodies, the level of % Infection blocked was compared to LIPS (Luminescence ImmunoPrecipitation System) or ELISA values. While ISI assay is a measurement of antibody functionality, LIPS and ELISA give information about antibody concentration. Comparing ISI assays with other studies may give an idea of the relative effect of antibody concentration on the blocking of sporozoite infection and allow appreciation of the different information obtained by each assay. In Figure 2.1, %Infection blocked at 2% (B1) and 10% (B2) serum dilution was plotted against ELISA values. Although samples with lower ELISA titres tend to have a smaller blocking effect, there was not a strong correlation between the two values, most likely due to the small number of samples tested. In Figure 2.2, the regression line for %Infection blocked against LIPS values had a slope significantly different than zero (linear regression), but the correlation between the two parameters was not statistically

significant (Spearman test). Unfortunately, LIPS / ELISA data was not available for samples used in Figure 2.3 to enable a correlation analysis on a larger data set.

The experiments described confirmed that, using the optimised flow cytometry based ISI assay, it is possible to detect a reduction on the rate of infection with the addition of post-vaccination serum samples. These first results emphasise the importance of the serum dilution employed in the ISI assay, the effect of the vaccination dose on the antibody responses, the influence of using a particular mouse strain on the antibody responses, and support (with the trends found between LIPS and ELISA assays and % Infection blocked) the search for possible correlations between ISI and other antibody assays in future studies.

2.2.1.2 Comparing candidates: CelTOS, LSA1, LSA3, LSAP1, LSAP2, UIS3, ETRAMP5 and Falstatin.

Eight pre-erythrocytic *P. falciparum* antigens (CeLTOS, LSA1, LSA3, LSAP1, LSAP2, ETRAMP5 and Falstatin) were studied as potential candidates to be included in a malaria vaccine. Immunogenicity and protective efficacy for each candidate were evaluated in BALB/c mice after vaccination with ChAd63 and MVA vaccines expressing each antigen (190). The humoral immune responses were analyzed with LIPS assay and used in this study to determine the capacity to inhibit sporozoite invasion of liver cells.

Mice were immunised against a *P. falciparum candidate* with 10^8 iu ChAd63, followed by 10^6 or 10^7 pfu MVA eight weeks later, with serum collected two weeks after the last vaccination. Independent experiments testing serum samples from mice vaccinated against each candidate in combination with the correspondent transgenic parasite were performed in the standard experimental set-up. Monoclonal antibody against *P. berghei* CS (3D11) was utilised as a positive control in each experiment (data not shown in this chapter, but chapter 1 includes Figure 1.8 showing the %Infection blocked with different concentrations of this monoclonal antibody). BALB/c naive mouse serum was used as a negative control, to determine the effect of non-specific blocking. Due to the high non-specific blocking observed with naive sera, a threshold was established where only blocking effect was considered positive when at least 30% of the sporozoite infectivity was blocked.

Serum samples from two different MVA vaccination doses (10^6 or 10^7 MVA) were tested and each sample was analysed at 2% and 10% serum dilution. Results are shown in Figure 2.4. Almost no blocking effect was detected at 2% serum dilution [Figure 2.4. A1, A2], therefore the serum concentration was increased to 10% [Figure 2.4. B1, B2] to detect positive blocking effects and find differences between antigens. The MVA dose, had an effect on the antibody responses detected by ISI, which were higher when mice were vaccinated with 10^7 MVA instead of 10^6 MVA [Figure 2.4. B1, B2]. The candidate antigen which showed the highest blocking effect was CelTOS, with an inhibition of almost 70% [Figure 2.4. B2] in mice vaccinated with 10^7 MVA and at 10% serum dilution. In the same conditions, LSAP1 reached a percentage of infection blocked of 50%, but the rest of the candidates showed mean blocking values below the 30% threshold [Figure 2.4. B2]. CelTOS showed a slightly higher blocking response than the other candidates at 2% serum dilution and 10^7 MVA vaccination dose [Figure 2.4. A2] and at 10% for 10^6 MVA [Figure 2.4. B1], supporting the idea that antibodies

generated against this antigen seem to have a higher capacity to block the sporozoite infection compared to the other antigens.
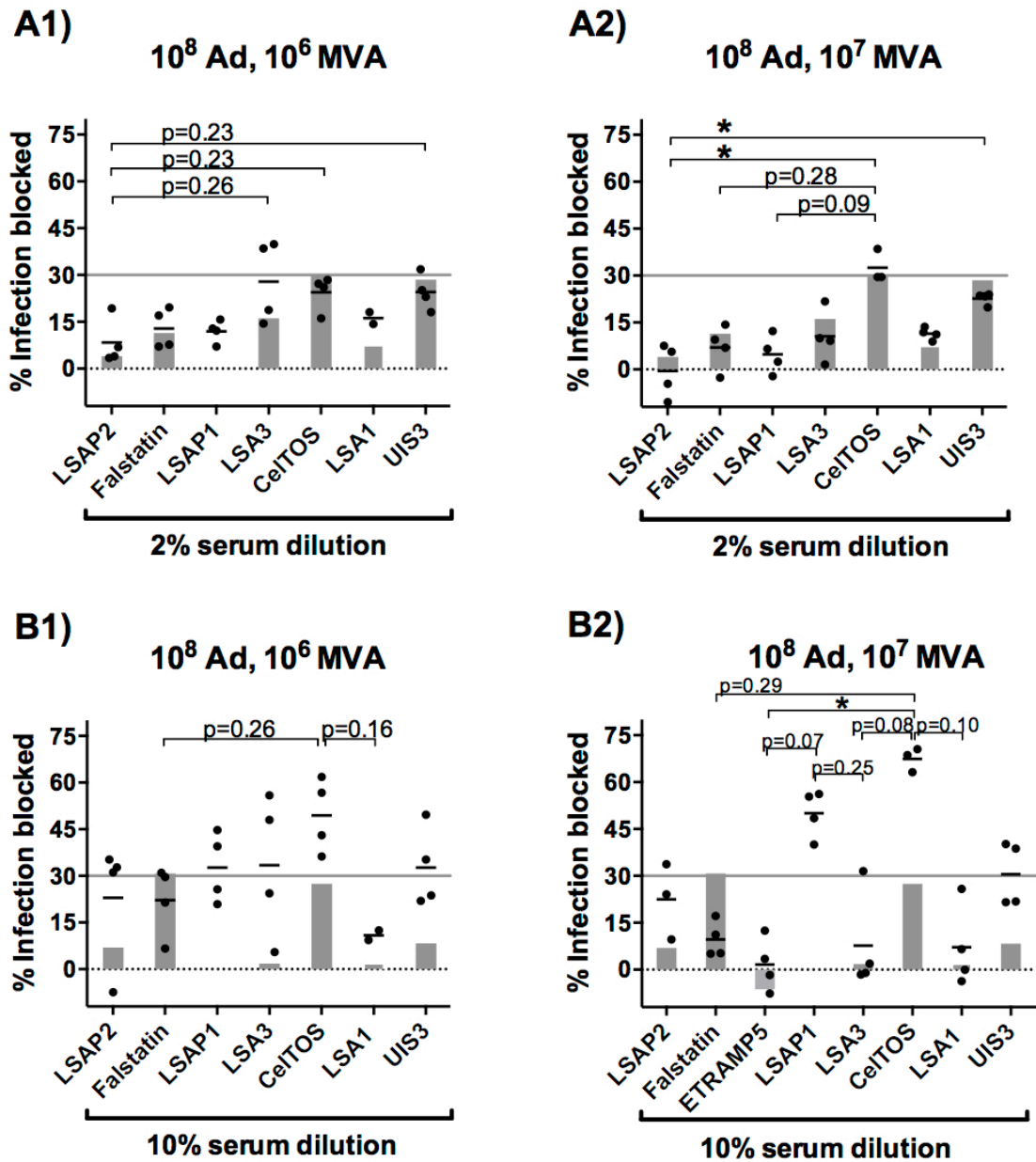
Figure 2.4. Percentage of infection blocked by serum samples against different *P. falciparum* **antigens. Each antigen (LSAP2, Falstatin, LSAP1, LSA2, CelTOS, LSA1 and UIS3) was tested independently using** *P. berghei* **sporozoites expressing the** *P. falciparum* **antigen of interest. BALB/c mice were vaccinated with prime-boost viral vector regimes, 10^8 iu ChAd63 followed by 10^6 pfu MVA (A1, B1) or 107 pfu MVA (A2, B2). Serum samples were taken two weeks after MVA vaccination and added at 2% (A1, A2) or 10% (B1, B2) serum dilution, with the appropriate transgenic parasite, to Huh7 cells. The results from the ISI assay are presented as percentage of infection blocked (referred to the infectivity in the absence of serum), each dot is the average value of duplicates or triplicates and represents an individual mouse, horizontal lines show the mean value response of each group and grey bars represent naive serum mean response. Data was analysed with Kruskal-Wallis test, Dunn's multiple comparisons test (p-values below 0.3 are included in the graph).**

In Figure 2.4, each antigen was assessed in an independent experiments (one per antigen) with data combined for presentation and statistical analysis (Kruskal-Wallis test, Dunn's multiple comparisons test) to compare mean blocking values between different antigens. It is essential to bear in mind that the values obtained in each column have been obtained using different *P. berghei* chimeric parasites, which raises concerns about the ability to compare antigens, so it is important to be cautious considering the p-values obtained. The data showed for each candidate was obtained using the serum samples of mice vaccinated against a *P. falciparum* antigen but tested against a *P. berghei* chimeric parasite expressing both the *P. berghei* and *P. falciparum* protein (where a homolog exists in both species). The percentage of infection blocked by each sample is referred to the infectivity achieved in the absence of serum. The presence of a *P. falciparum* protein on the *P. berghei* parasite could cause differences in parasite infectivity. Nevertheless, each transgenic *P. berghei* should conserve all of its functional capabilities, as no protein has been removed from the parasite. The additional *P. falciparum* protein could support the function of an ortholog *P. berghei* protein or add a new function if no equivalent protein was present in *P. berghei*. The percentage of infected Huh7 cells (in the absence of serum), measured in the different experiments, varied between 1.5 to 4.5% (data not shown). However, this variability is probably more related to the number of sporozoites added, to the time of readout or to the health and viability of the sporozoites at the moment of infection, rather than to the kind of transgenic parasite used. In all experiments, 1 µg/mL of monoclonal antibody against *P. berghei* CSP (3D11) was used as a positive control and showed a consistent percentage of infection blocked (about 60%) and would therefore suggest all parasites had similar rates of infectivity and ability to be blocked were all behaving similarly and there is a true difference between the blocking effect on different *P. falciparum* antigens.

IFAT (Immunofluorescence antibody test)

To gain a better understanding of the antibody responses in terms of the ability of serum Ab to bind to sporozoites surface proteins, IFAT (Immunofluorescence antibody test) was performed for each antigen with the different vaccination doses and dilutions employed in ISI assay. IFAT is an assay based on the same principle as an ELISA, with the advantages of presenting the antigens in their native conformation and not needing to purify the antigen; but with some disadvantages, like the difficulty in quantifying the antigen-antibody interaction. *P. berghei* sporozoites expressing the relevant *P. falciparum* antigen were stained with the corresponding post-vaccination

serum (2% or 10% dilution) and bound antibody detected using a secondary antibody (goat anti-mouse Ig-G Alexa Fluor 488, green). Sporozoite nuclear DNA was stained with Hoechst-33342. In an attempt to compare the levels of antibody binding, green signal was recorded for 7 seconds and blue signal for 3-4 seconds with adjusting brightness and contrast occurring in the same proportion, to allow comparison between different images. Maximum signal was also always modified in the same proportion, but the minimum signal (background signal) was modified differently in a few images. If it was not possible to detect sporozoites in the image, the minimum signal was slightly reduced (removing less background than in the rest of the images), which has been stated in the figures as *bl [Figure.2.6]. If the image had a very high minimum signal (very high background), the minimum signal was increased to achieve a better contrast between the sporozoite signal and the background (stated as *br [Figure 2.5, Figure 2.6]). Blue images were modified to obtain the best image possible for each case, as the purpose of the nuclear stain was primarily to indicate location of sporozoites on the slide. Merged images (which combine signals from green and blue channels) and bright field images are shown in Figure 2.5 (sera from vaccination dose: 10^8 ChAd63, 10^7 MVA) and Figure 2.6 (sera from vaccination dose: 10^8 ChAd63, 10^6 MVA).

With this technique it was possible to collect interesting qualitative information, but it is essential to take into account its limitations and be cautious when comparing antigens. Sporozoites were obtained from the mosquito salivary glands, and even when careful dissections were performed, it was extremely hard to avoid contamination with other small particles from the mosquito. In addition, the sporozoites were fixed on a slide on the day of dissection and were kept on PBS (at 4ºC), in some cases for months. It is understandable that contamination and possible deterioration, could have affected each slide differently and had an effect on the image acquired (possibly affecting the amount of background signal or sporozoite quality). However, in spite of these drawbacks, it is still possible to obtain valuable information with IFAT.
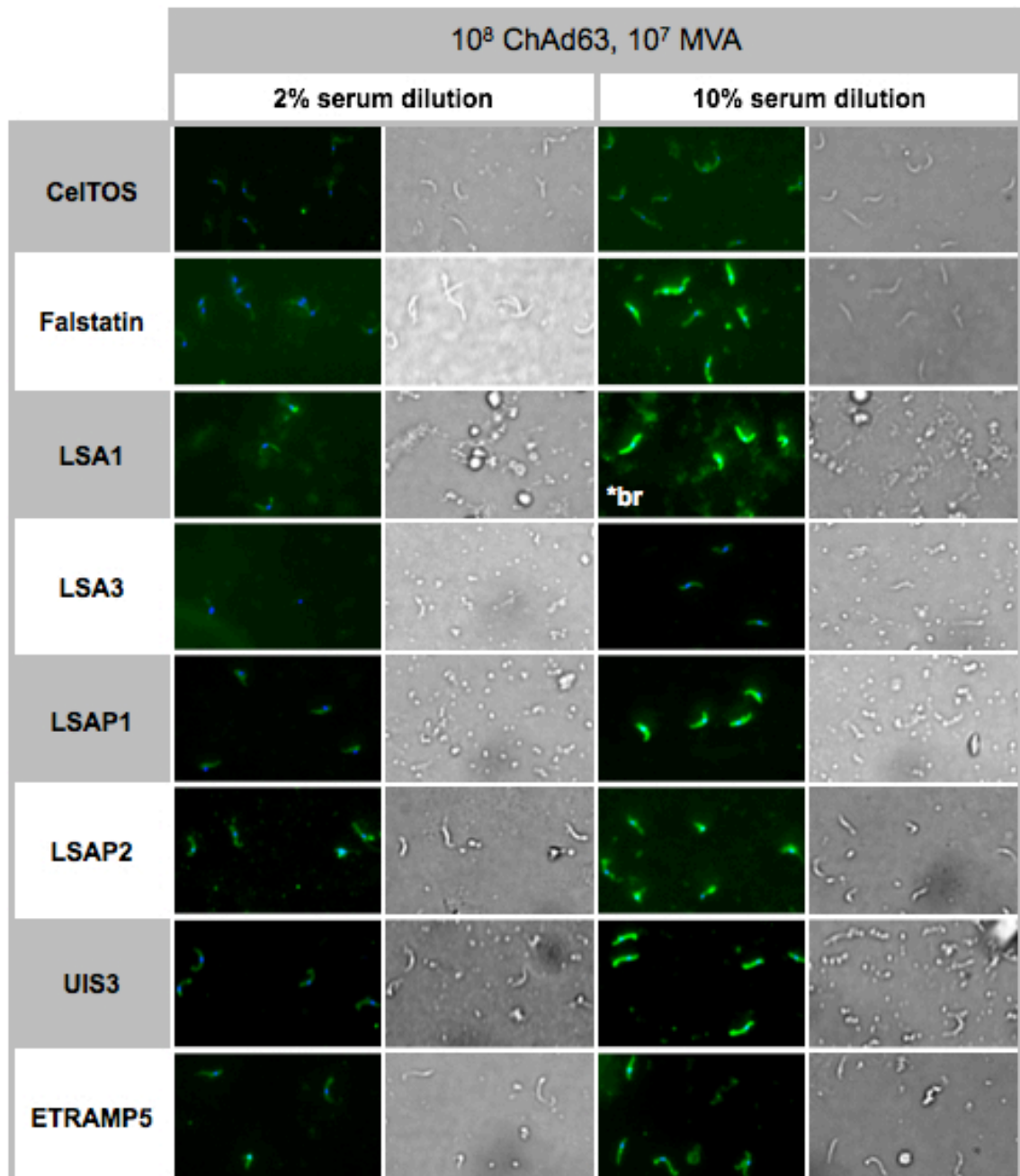
Figure 2.5. IFAT: post-vaccination serum 10^8 ChAd63, 10^7 MVA with corresponding (*Pf* antigen) *Pb*. Sporozoites fixed on slides were prepared for each strain and stained with 2% (2nd column) and 10% (4th column) serum dilution. Bright field images (3rd and 5th columns) and composite images (merged of green and blue colour, 2nd and third columns) are presented. Green signal was collected during 7 seconds, and all images were adjusted for brightness and contrast in the same proportion. Maximum signal was always modified in the same proportion, but when it is stated (*br) minimum signal was decreased because the image presented a high green background. *br: higher amount of background removed than in the rest of the images.
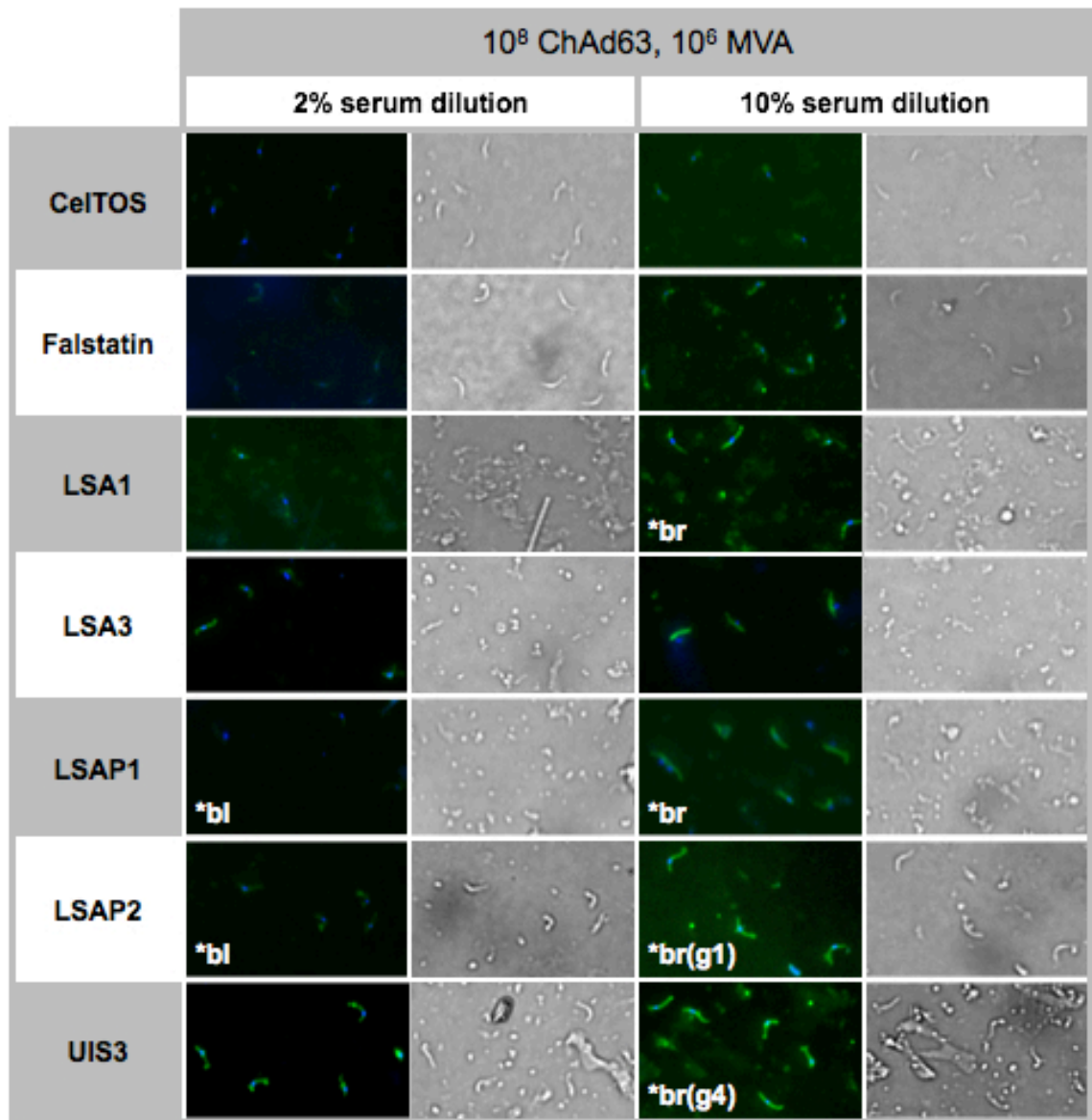
Figure 2.6. IFAT: post-vaccination serum 10^8 ChAd63, 10^6 MVA with corresponding (*Pf* antigen) *Pb*. Sporozoites fixed on slides were prepared for each candidate and stained with 2% (2nd column) and 10% (4th column) serum dilution. Bright field images (3rd and 5th columns) and composite images (merged of green and blue colour, 2nd and third columns) are presented. Green signal was collected during 7 seconds and blue signal for 2-3 seconds. All green images were adjusted for brightness and contrast in the same proportion. Maximum signal was always modified in the same proportion except, whenever is stated the background signal was either increased (if it was not possible to detect stained sporozoites) or decreased (if the image presented a high green background). *br: higher amount of background removed than in the rest of the images, *bl: less background removed than in other images. Green exposition was reduced in LSAP2 and UIS3 (4th column) because the background signal was so high, that the image obtained after 7 seconds was saturated. g1: green signal collected during 1 second, g4: green signal recorded during 4 seconds.

In general terms, the staining produced by post-vaccination serum with the smaller MVA dose [Figure 2.6] was less intense than observed with the higher MVA dose [Figure 2.5]. This supports what has been also observed with ISI assay: a higher MVA dose generates a higher antibody response. Interestingly, *Pf* CelTOS *Pb* sporozoites were weakly stained by post-vaccination serum, even when serum from the highest vaccination dose was used at 10% dilution [Figure 2.5. First row, 3rd column], while serum against CelTOS showed the larger inhibition capacity in ISI assay. In contrast, UIS3, showed one of the best staining (high signal recorded and sporozoites uniformly stained) for the two sera and both concentrations tested [Figure 2.5 and 2.6, 7th row]. LSA1, LSAP1 and Falstatin also showed a high signal at 10% serum dilution and higher MVA dose [Figure 2.5]. LSA1 was not expected to present IFAT staining since it is only expressed in liver stages. The signal detected here [Figure 2.5] could be due to cross-reaction with other sporozoite proteins, or to a different pattern of LSA1 expression in the transgenic parasite where LSA1 is expressed under *Pb* UIS4 promoter.

The additional *P. falciparum* protein may present different levels in the *P. berghei* chimeric parasite (all the falciparum antigens are expressed under the same promoter, but the complexity of the protein could influence the levels of expression and protein stability would also have an effect on the frequency of degradation), and also, a higher presence of the protein could generate more opportunities for interaction with antibodies. Nevertheless, this could also have an effect on the inhibition of the sporozoite invasion, because there would be a higher number of interactions antigen-antibody preventing the normal function of the *falciparum* protein. One alternative explanation is that not all the *P. falciparum* proteins may be equally accessible to specific antibodies in the serum, but as the sporozoites are fixed, internal proteins become available to Ab binding.

ISI assay has proved to be concentration dependent. Therefore, we could expect that CelTOS (the antigen that elicited the highest blocking response) would show an intense IFAT signal, indicating high antibody titer. However, according to that IFAT, there was a higher antigen-antibody interaction with other candidates that did not shown a blocking effect. There could be two possible explanations for this finding. On one side, proteins that are accessible to antibodies on fixed sporozoites (which present holes on their membranes), may not be accessible in live sporozoites. On the other side, it is possible that only antibodies against a protein which has a role on hepatocyte infectivity may play a role on the inhibition of sporozoite invasion. All the candidates tested are pre-erythrocytic antigens expressed in sporozoite and/or liver stages.

Information about their function is only available for some of the candidates. As it has been described in the introduction, UIS3 and LSA1 function has been related with development within the hepatocyte, while CelTOS is a protein involved in the sporozoite motility. Diverse protein functions could explain differences seen in the ISI assay. This assay evaluates antibody capacity to avoid a particular step of the malaria infection: sporozoite invasion. Functionality of antibodies targeting proteins whose function is related to other malaria stage, would require an alternative assays. Nevertheless, antigens that have not been associated with a function in the ISI assay may act as negative controls and help to understand how the assay works. It is important to bear in mind that no definitive conclusions can be stated about protein functionality using *P. berghei* chimeric sporozoites expressing *P. falciparum* antigens, since *P. falciparum* proteins are not being expressed under their normal promoter to their usual levels or in their normal context. *P. falciparum* sporozoites would be the appropriate model to use in order to gain a better understanding on how protein functionality may be interfered by antibodies.

LIPS (Luminescence Immunoprecipitation System)

In the absence of purified protein, it is possible to measure antigen specific antibodies by the LIPS assay (265) where antibodies are measured based on the ability to bind antigen fused to a reporter gene (Ruc, renilla luciferase). Cell lysates from HEK 293 transfected cells (human embryonic kidney cells) are used in the assay, avoiding the need to purify soluble antigen. Figure 2.7 has been plotted from data provided by R. Longley. It is not possible to compare between antigens because there is no way to normalise for the difference between constructs, and, for this reason a statistical analysis comparing between groups has not been included. Nevertheless, it is interesting to have a rough idea of the response elicited by the different antigens. UIS3 generated one of the highest LIPS signals [Figure 2.7. A, B] which corresponds with the IFAT response found. LSA3 and CelTOS which did not show a good IFAT signal, have LIPS responses at the lower end of the spectrum [Figure 2.7. A,B]. Falstatin, LSAP2, and LSA1 with high and medium LIPS values [Figure 2.7. A,B], gave better than average IFAT signals [Figure 2.5 and 2.6]. Possibly the highest discrepancy between IFAT and LIPS is for LSAP1 which showed low LIPS signal [Figure 2.7. A,B] although it showed one of the best IFAT signal [Figure 2.5 and 2.6]. Different antibody assays emphasise diverse aspects of the antibody response, like concentration, avidity or functionality. The varied information obtained with ISI, IFAT and LIPS supports the

importance of not limiting the analysis of antibody responses to only one type of experiment.
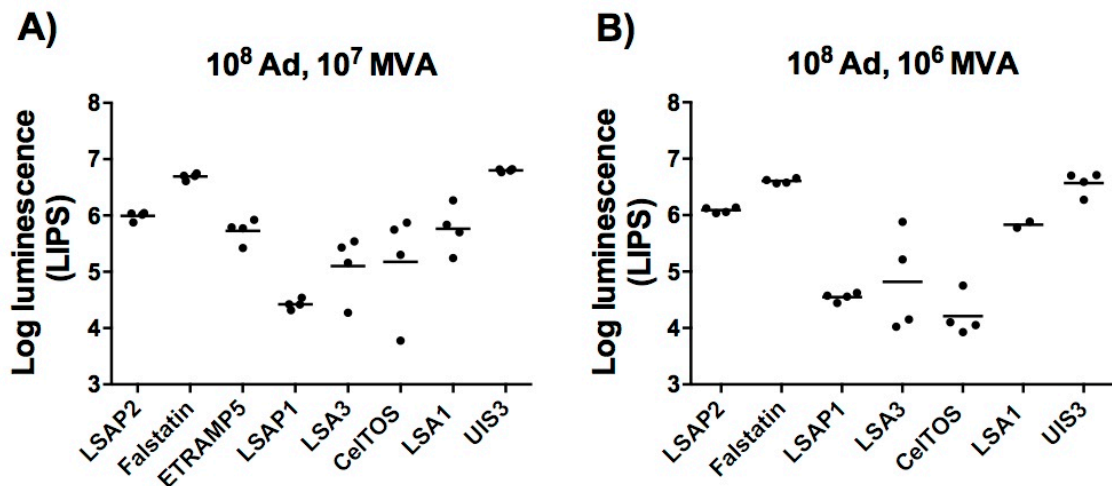
Figure 2.7. LIPS assay. These graphs have been plotted with LIPS data generated by Rhea Longley [Rhea Londgley, DPhil thesis]. LIPS (Luminescence immunoprecipitation system) allows the detection of antigen-specific antibodies without the need to purify the antigen. A cellular lysate is obtained from cultures of mammalian cells expressing the antigen of interest fused to a reporter gene (Ruc, Renilla luciferase). This lysate is mixed with the serum samples and added to a plate coated with A/G beads. Fused protein bound to antibody is retained on the beads, while non-bound antigen is washed away. Adding luciferase substrate, a light signal proportional to the amount of antigen bound to antibody can be recorded. Results are expressed as the log of luminescence (light units). Data for sera obtained with 10^8 ChAd63, 10^6 MVA is presented in A and with 10^8 ChAd63, 10^7 MVA in B.

P. berghei effect

To determine the effect of cross-reactive *Plasmodium berghei* proteins present on the sporozoites the ISI assay was performed with *P. berghei* sporozoites expressing only GFP-luciferase and each post-vaccination sample from mice vaccinated with 10^8 ChAd63, 10^7 MVA (high MVA dose) and 10% dilution. The results, shown in Figure 2.8, indicate a blocking effect of 54% of CelTOS and 67% of LSAP1. There is a high degree of homology between *P. berghei* and *P. falciparum* CelTOS ortholog (187), so it is not surprising that antibodies against *falciparum* CelTOS may elicit a blocking effect against *P. berghei*. On the contrary, a LSAP1 homolog has not been identified in *P. berghei*. One possible explanation for this result could be the presence of factors in the serum responsible of an antibody-independent blocking response. Alternatively, antibodies elicited through vaccination against *P. falciparum* LSAP1 may cross-react with other *P. berghei* proteins with similar epitopes.

In Figure 2.8, the spread of the data seems to be higher than in the equivalent figure where each serum was tested with the corresponding chimeric parasite [Figure 2.4. B2]. A possible way to justify this is that serum against vaccination is polyclonal and generated differently in each mouse. The serum from a particular mouse targets a particular set of epitopes, generating different cross-reactivity with diverse *P. berghei* proteins that may be especially noticeable in the absence of the targeted *falciparum* protein.
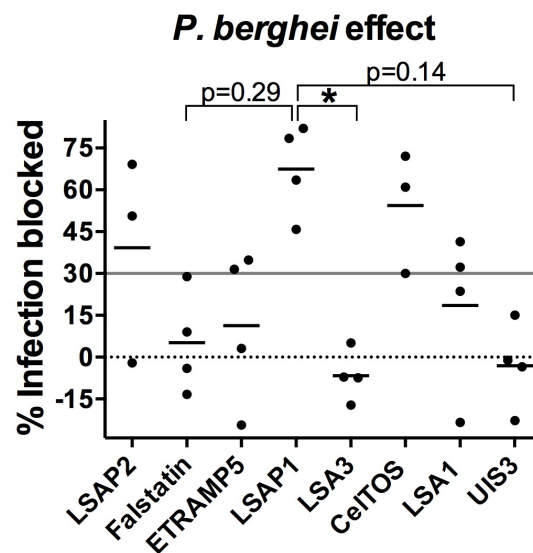
Figure 2.8. *P. berghei* effect. ISI assay was performed employing *P. berghei* parasites that do not express a *P. falciparum* protein. The % Infection blocked measured for each mouse sample is represented with dots and horizontal lines indicate the mean value of the group. The horizontal line at 30% indicates a positive cut-off with responses under this level considered a non-specific background effect. All serum samples were tested at 10% serum dilution and are from mice vaccinated with 10^8 ChAd63, 10^7 MVA. Data was analysed with Kruskal-Wallis test, Dunn's multiple comparisons test.

IFAT (Immunofluorescence antibody test)

IFAT staining was also performed with *P. berghei* sporozoites (that did not express *P. falciparum* proteins), for each post-vaccination serum. Sporozoites stained with anti-LSAP1 serum showed high green signal [Figure 2.9], supporting the mentioned suggestion that post-vaccination serum against LSAP1 (which does not have a *berghei* ortholog) could cross-react with other *P. berghei* proteins. anti-Falstatin and anti-LSA1 serum stained sporozoites also showed strong IFAT signal [Figure 2.9] (but these sera did not had a significant blocking response [Figure 2.8]). *P. berghei* has a Falstatin ortholog, which justifies the observed strong IFAT signal, but no ortholog has been identified for LSA1. Anti-UIS3 serum presented a high IFAT signal for *Pf*UIS3

Pb sporozoites [Figure 2.5, Figure 2.6] and a medium-high IFAT signal with *Pb* sporozoites [Figure 2.9], consistently with the fact that UIS3 has a *P. berghei* ortholog. Low green signals were recorded for sporozoites stained with serum from mice vaccinated LSA3, LSAP2 and ETRAMP5 [Figure 2.9], but none of them have *P. berghei* homologs. CelTOS, in spite of having a *P. berghei* ortholog, also showed weak staining [Figure 2.9], as happened when *Pf* CelTOS *Pb* was employed [Figure 2.5, 2.6] (probably due to a low antibody titer or poor protein stability).
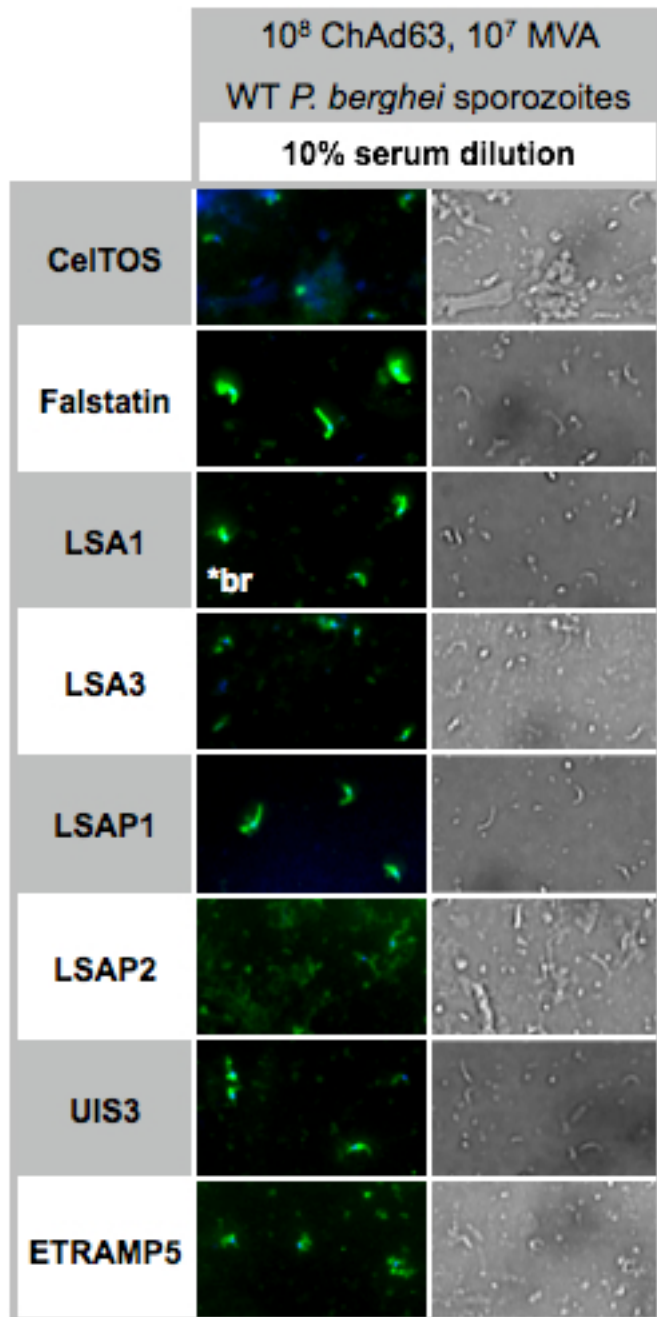
Figure 2.9. IFAT: post-vaccination serum 10^8 ChAd63, 10^7 MVA and *P. berghei*. Sporozoites were stained with 10% serum dilution. Bright field images (3rd column) and composite images (merged of green and blue colour, 2nd column) are presented. Green signal was recorded during 7 seconds and blue signal for 2-3 seconds. All green images were adjusted for brightness and contrast in the same proportion. Maximum signal was always modified in the same proportion, but whenever is stated (*br) the background signal was decreased (because the image presented a high green background). *br: higher amount of background removed than in the rest of the images.

2.2.1.3 Comparing candidates: SPECT1, SPECT2, RP-L3 and HT

A second group of pre-erythrocytic *P. falciparum* candidate malaria antigens and corresponding chimeric *P. berghei* parasites were generated and evaluated by Ahmed Salman. BALB/c and CD1 mice were vaccinated with 10^8 ChAd63 and 10^7 MVA (with an interval of 8 weeks), both vectors expressing one of the pre-erythrocytic candidates: SPECT1, SPECT2, RP-L3 or HT. Serum samples were collected between 8 to 14 days after MVA boost and tested in the ISI assay at 2 and 10% serum dilution.

The ISI assay was the first experiment performed to evaluate antibody responses generated by these candidate vaccines and it took place on the same day of challenge. Sporozoite availability is the limiting factor for the ISI assay. Each mouse is typically challenged with 1000 sporozoites, while to analyse each single serum sample at least 30000 sporozoites (duplicates, 15000x2) are required per sample. Performing the assay on the same day of the challenged proved to be a good way to optimise the insectary resources and enabled the use of left-over sporozoites. This particular screening study of four candidates was the only occasion when ISI assay took place on the same day of challenge (as the rest of the serum samples tested in this thesis belonged to studies that were carried out months before the ISI assay was optimised). Nevertheless, it proved to be an efficient way of performing both experiments in the future, provided that two researchers are available on the day.
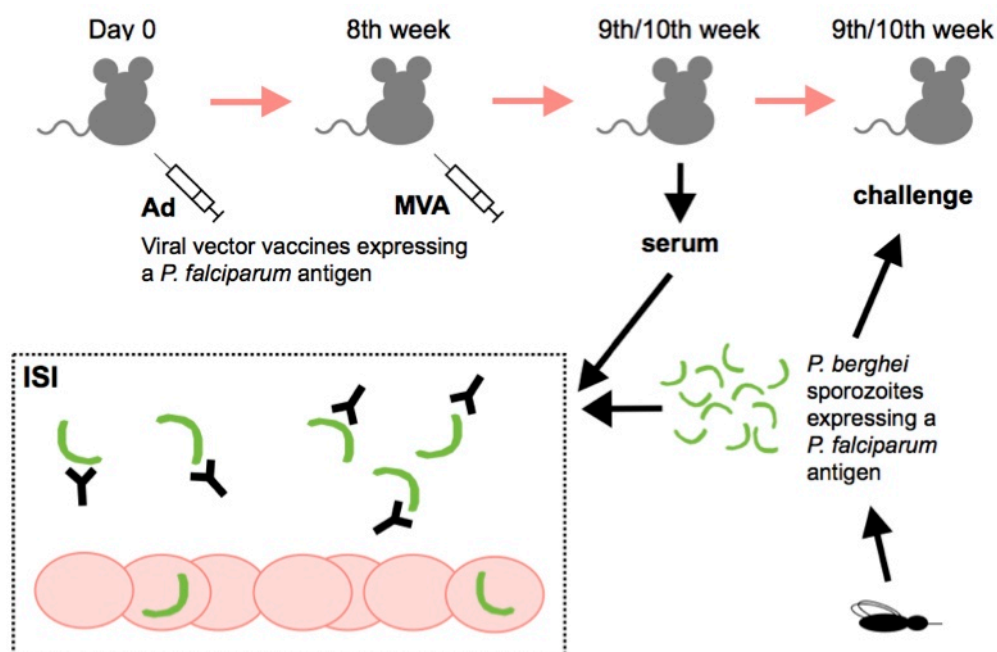
Figure 2.10. Vaccination, challenge and ISI assay. Mice were vaccinated with a prime-boost viral vector regimen. Serum was collected and frozen one or two weeks after the last vaccination dose. Sporozoites were obtained and used to both challenge vaccinated mice and for the ISI assay.

The percentages of infection blocked by serum against each of the four antigens from the screening study and CS are presented in Figure.2.11. CS was included to compare its blocking effect with the other candidates, since it was obtained by the same researcher in the same period of time as the other samples. In the previous set of *P. falciparum* candidates tested (2.2.2) very little blocking was detected with 2% dilution [Figure 2.4.A2], but here it was necessary to use this dilution to observe differences between antigens [Figure 2.11.A] (at 10% serum dilution, all samples inhibited almost 100% of the infection [Figure 2.11.B]). The fact that with this second group of antigens a much higher blocking response was observed, may be due to antibody levels or to the particular function of the antigens tested. However, the large difference between both groups could be more likely related with the fact that the serum samples employed for the first study of *P. falciparum* antigens (2.2.2) had been stored for about two years and frozen and thawed a number of times, which could decrease the blocking capacity (due to degradation or lost of functional conformation of the antibodies).
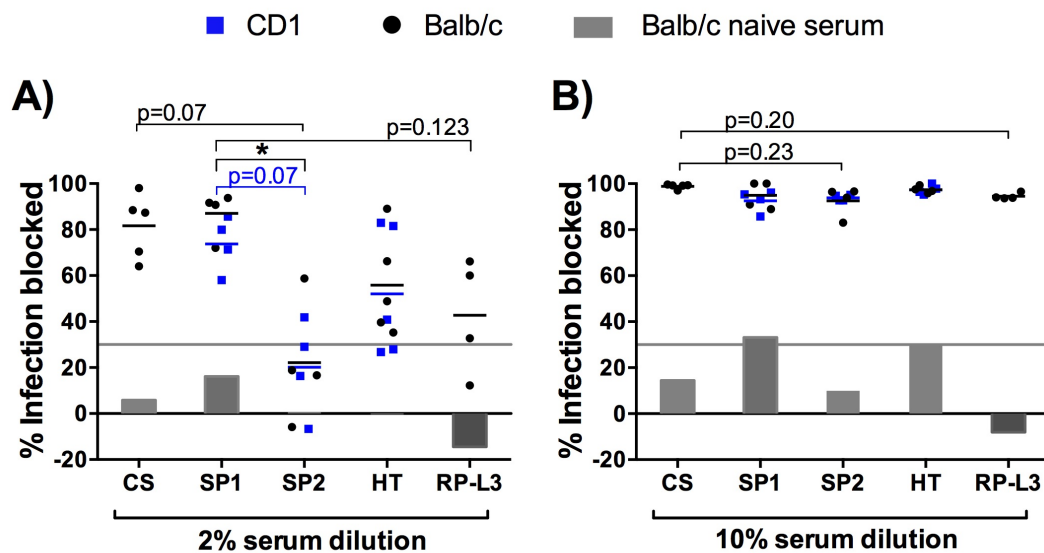
 Figure 2.11. Percentage of infection blocked by post-vaccination serum samples against different *P. falciparum* antigens. Each serum was tested independently using *P. berghei* parasite expressing the corresponding *P. falciparum* protein. BALB/c (black in the graph) and CD1 (blue) mice were vaccinated with 10^8 ChAd63 followed by 10^7 MVA. Serum samples were collected 8-14 days after MVA vaccination and were added to Huh7 cells at 2% (A) or 10% (B) serum dilution, with the appropriate transgenic parasite. The results from the ISI assay are presented as percentage of infection blocked (referred to the infectivity in the absence of serum): each point (average of duplicate tests) represents an individual mouse, horizontal lines show the mean per group and grey bars represent naive serum mean response. Horizontal line at 30% Infection blocked indicate the threshold to consider a positive blocking effect. Data was analysed (BALB/c and CD1, separately) with a Kruskal-Wallis test, Dunn's multiple comparisons test (p-values below 0.3 are included in the graph).

At 10% serum dilution all candidates blocked almost 100% of the sporozoite invasion [Figure 2.11.B]. At 2% serum dilution, blocking ranged from approximately 20% to 100% [Figure 2.11.A]. The highest responses observed were with serum from SPECT1 (SP1, BALB/c mean: 87%) or CS (BALB/c mean: 82%) vaccinated mice, followed by moderately positive response from HT (BALB/c mean: 56%) and RP-L3 (BALB/c mean: 43%) vaccinated mice. SPECT2 did not show a positive response at 2% dilution [Figure 2.11.A].

SPECT1 and SPECT2 are expressed in sporozoites and have been associated with cell-traversal ability (248,249). Interestingly, serum from SPECT1 vaccinated mice displayed inhibition of a similar magnitude to CS serum samples. HT, expressed on sporozoites and during the liver-stage, is involved in the uptake of D-glucose, and sera against this protein produced a positive blocking response (251,252). RP-L3 is mainly expressed in liver-stages (250) and its function is unknown. RP-L3 blocking response is not as good as CS or SPECT1, but having a detectable antibody response at 2% serum dilution could be an indicator of good antibody function.

Samples from CD1 mice were tested for SPECT1, SPECT2 and HT antigens. CD1 mean blocking responses were nearly the same level as obtained with serum from BALB/c against each antigen [Figure 2.11.A]. CD1 are outbred mice, what could effect the overall magnitude of immune responses or the variability between mice, however vaccination elicited antibodies with similar functionalities in CD1 and BALB/c mice.

IFAT (Immunofluorescence antibody test)

As in the previous study (2.2.2), IFAT test was performed to gain more information about antibody responses. At 2% [Figure 2.12. 2nd column] serum dilution, SPECT2 showed the highest signal, RP-L3 and SPECT1 had moderate binding and HT binding was not detected.

SPECT2 seems to have a higher antibody titre than SPECT1 as determined by IFAT, but only SPECT1 showed a positive blocking capacity at 2% serum dilution. Antibodies generated against SPECT1 might be more functionally efficient, even at a lower antibody concentration, than those against SPECT2. It is possible that although both proteins have been associated with the same function (cell-traversal), they do not have the same relative importance in the process and antibodies bound to these antigens limit sporozoite motility to differing extents.
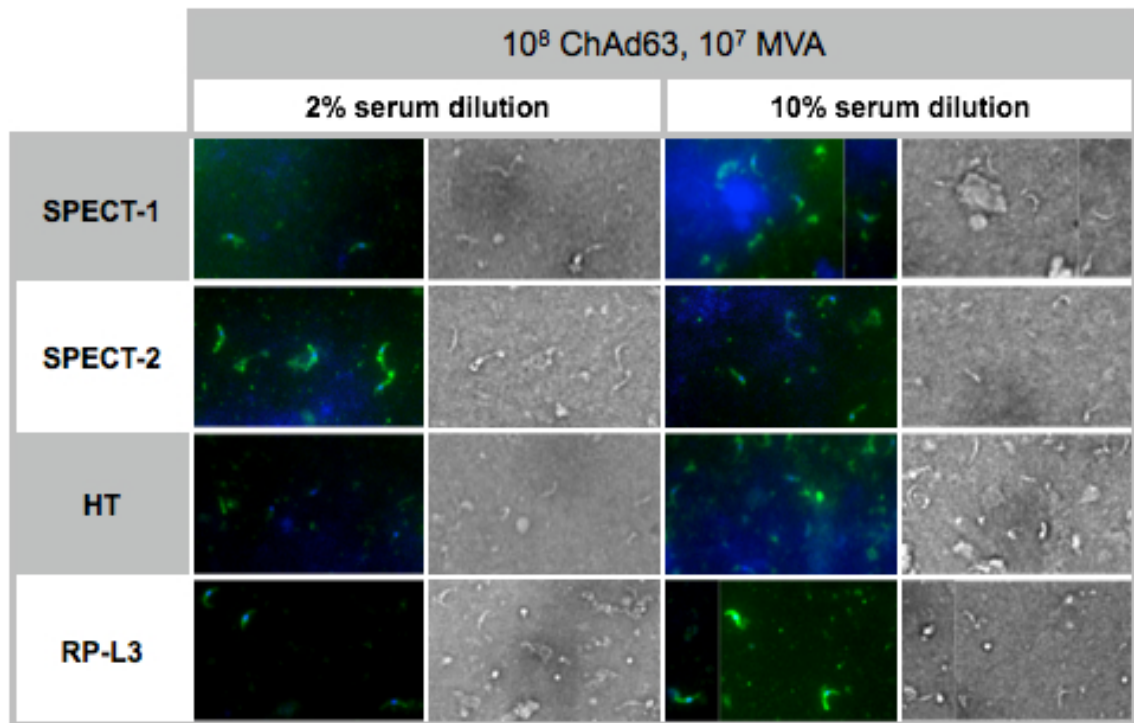
Figure 2.12. IFAT: post-vaccination serum 10^8 ChAd63, 10^7 MVA with corresponding (*Pf* antigen) *Pb*. Sporozoites were stained with 2% (2nd column) and 10% (4th column) serum dilution. Bright field images (3rd and 5th columns) and composite images (merged of green and blue color, 2nd and third columns) are presented. Green signal was collected during 7 seconds and blue signal for 1 to 4 seconds. All green images were adjusted for brightness and contrast in the same proportion.

P. berghei effect

ISI assay was also performed with each serum (2% dilution) with *P. berghei* sporozoites that did not express any *P. falciparum* protein. A positive effect (69% Infection blocked) was only detected for SPECT1 [Figure 2.13]. *P. berghei* and *P. falciparum* orthologs of SPECT1 share 45.6% of sequence identity (248). This data would suggest that antibodies generated against *P. falciparum* SPECT1 were able to bind *P. berghei* SPECT1 and reduce sporozoite infectivity. When using *Pf* SPECT1 *Pb* it was possible to detect an increase in blocking by about 20% [Figure 2.11], suggesting that although there is some cross-reactivity there is still an effect of anti-*Pf* SPECT1 vaccinated serum.

SPECT2, HT and RP-L3 *P. falciparum* proteins all have *P. berghei* homologs. HT and RP-L3 had a blocking effect on sporozoite invasion with *P. berghei* parasite expressing the corresponding *P. falciparum* proteins [Figure 2.11. A], but not with control *P. berghei* parasites, indicating the blocking effect is through interaction with the transgenic *P. falciparum* proteins.
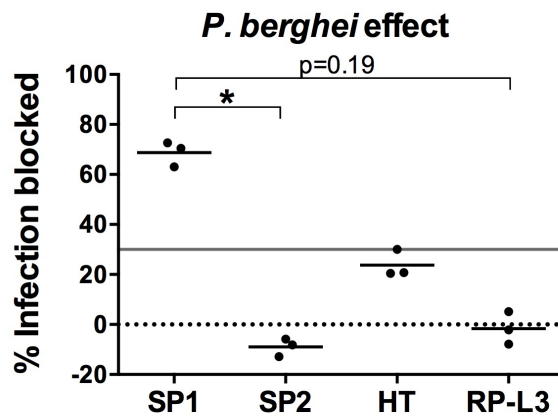
Figure 2.13. *P. berghei* effect. *P. berghei* parasites expressing GFP-luciferase were treated with different ChAd63-MVA *P. falciparum* post-vaccination serum (2% dilution). Serum samples were taken from mice immunised with 10^8 ChAd63, 10^7 MVA. Due to the limited number of sporozoites available, serum samples from 4-6 mice vaccinated against the same *P. falciparum* antigen were mixed together and tested in triplicates (each dot represents one of this single triplicates and horizontal lines the mean value). Horizontal line at 30% indicates the positive threshold level. Data was analysed with a Kruskal-Wallis test, Dunn's multiple comparisons test.
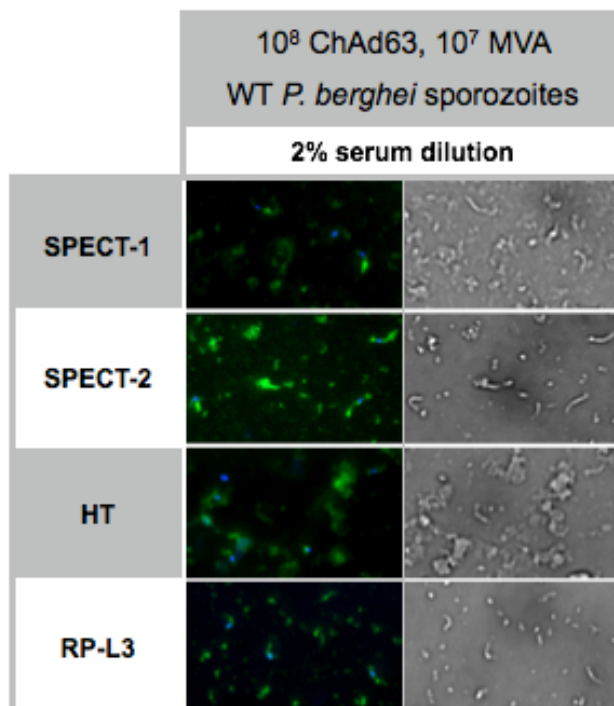
Figure 2.14. IFAT: post-vaccination serum 10^8 ChAd63, 10^7 MVA and *P. berghei*. Sporozoites were stained with 2% serum dilution. Bright field images (3rd column) and composite images (merged of green and blue colour, 2nd column) are presented. The green signal was recorded for 7 seconds and blue signal for 2-3 seconds. All green images were adjusted for brightness and contrast to the same proportion.

To further analyse the effect of the cross-reactivity with *P. berghei* proteins, *P. berghei* sporozoites not expressing any P. *falciparum* protein were stained with 2% dilution of serum from each of the four *P. falciparum* antigen vaccinated mice. The staining observed was not strong for any antigen [Figure 2.14], although all of them have *P. berghei* orthologs. SPECT2 appeared to give a higher signal, supporting the idea that there is cross-reactivity between orthologs which could be influencing the ISI assay. Antibodies against *P. falciparum* SPECT1 may cross-react with *P. berghei* SPECT1 and impair sporozoite infectivity to a greater extent than a higher titre of SPECT2.

2.2.1.4 R21 study

RTS,S, which is expected to be licensed in 2015 (currently in a phase III trial involving 16000 children), is the most advanced malaria candidate vaccine and has shown efficacy of 30-50% (266), (267). RTS,S targets CSP (circumsporozoite protein, an abundant protein on the sporozoite surface) using a VLP (Virus Like Particle) comprised by two proteins (RTS and S). RTS,S , which is produced in *S. cerevisiae,* contains 189 amino acids from the CSP protein (including B and T cell epitopes) fused to HBsAg (Hepatitis B surface antigen) and HBsAg on its own (268). R21 has been designed to improve RTS,S immunogenicity and efficacy through increasing the ratio CSP:HBsAg present in the vaccine. R21 contains the same CSP regions as RTS (minus 14 aa which do not play a role in RTS) fused to HBsAg. R21 is produced in *Picha pastoris* without HBsAg co-expression, therefore the VLP is formed from only the CSP-HBsAg fusion protein and is composed of larger percentage of CSP compared to RTS,S . R21 was developed by Katharine Collins as part of her DPhil research project [Katharine Collins, DPhil thesis, University of Oxford, 2014] and provided serum samples for assessment with the ISI assay.

Three experiments were performed using R21 post-vaccination serum samples. First, an experiment measuring the inhibition of sporozoite invasion detected using the *P. falciparum* CSP addition chimeric parasite (*P. berghei* parasite expressing *P. berghei* CSP and *P. falciparum* CSP) compared to the CSP replacement transgenic parasite (*P. berghei* parasite expressing *P. falciparum* CSP under the control of the *P. berghei* CSP promoter). In the second experiment, the effect on inhibition of sporozoite invasion of administering R21 with different adjuvants was determined and the third experiment compared the effect of vaccination with RTS,S and R21 formulated with AS01B .

Experiments were carried out, as described previously, at final serum concentrations of 0.2, 2 and 10%. 0.2% serum dilution was selected, because these samples had shown antibody levels much higher (log ELISA endpoint titer: 4-7) than those found in prime-boost viral vector vaccinated mice (ELISA µg/mL: 0-100). Plates were centrifuged (6min, 1600rpm) to improve sporozoite infection and incubated at 37º for 24-28 hours. Samples were acquired on a flow cytometer, detecting infected cells with the GFP-luciferase expressed by the transgenic parasites. The data is presented as the percentage of infection blocked, the reduction in % of infected cells compared to when no serum was added. In %Infection blocked graphs, each dot represents an individual mouse and is the average of duplicate wells, while bars indicate the infection

blocked by BALB/c naive serum. Pre-vaccination data is not available for each mouse, but as they are genetically identical, naive serum has been used to have an idea of the non-specific background effect. Although in the three experiments, the percentage of infection blocked by the naive serum is very low or even negative (when the sporozoite infectivity was found to be higher in the presence of naive serum than in absence of serum), due to the variability of this effect and the fact that higher background values have been found in previous experiments, only values over 30% were considered positive.

a) CS addition vs CS replacement

In a pilot experiments with R21 samples and CS addition parasites, the percentage of infection blocked was lower than observed with ChAd-MVA vaccinated serum, in spite that R21 serum is known to have higher Ab titres. As this finding was in total contrast to the expected results, we wondered whether the presence of very high Ab titers cause the parasite to change its behaviour. With the aim of finding whether using the CS replacement parasite with R21 samples could overcome this different behaviour, serum dilutions of 0.2%, 2% and 10% were added with CS addition or replacement parasite to Huh7 cells. Sera samples were obtained from BALB/c mice immunised with 0.5 µg R21 given with Abisco adjuvant (12 µg). Mice received 2 vaccinations with an interval of 8 weeks and serum was collected 3 weeks after the final vaccination.

A higher percentage of infection blocked was achieved at every serum dilution when using the CS replacement parasite instead of CS addition [Figure 2.15]. A concentration dependent blocking of sporozoite invasion was observed using the replacement parasite [Figure 2.15.A1]. The blocking effect found with the addition parasite was lower, supposedly due to the presence of *P. berghei* CSP [Figure 2.15.A2] which could still carry out the CSP function.

For the CS replacement parasite, there was a trend to increased blocking with increased NANP IgG ELISA titer, with 2% and 0.2% serum dilution [Figure 2.15.B1]. With a serum dilution of 10% the ISI assay seems to be saturated and no differences in blocking capacities were observed between the different samples [Figure 2.15.B1]. For the CS addition parasite, no correlation was found with ELISA data [Figure 2.15.B2].
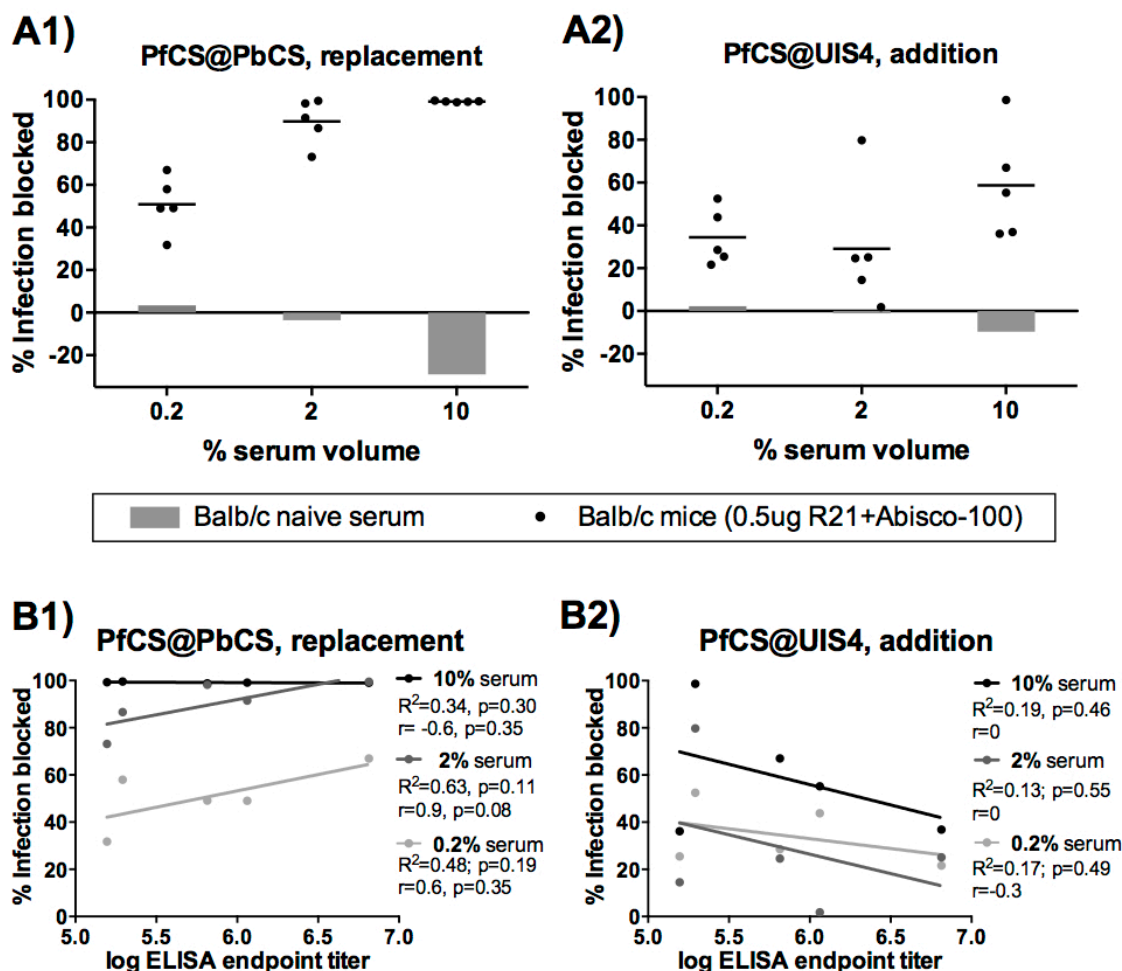
Figure 2.15. R21 samples: addition vs replacement CS transgenic. Serum samples from BALB/c mice vaccinated with two doses of 0.5 µg R21 formulated with Abisco adjuvant administered 8 weeks apart with serum collected 3 weeks after the last dose. Huh7 cells were infected with *Pf* CS replacement *P. berghei* (A1) or *Pf* CS addition *P. berghei* (A2) in the presence of 0.2, 2 or 10% post-vaccination R21 serum (dots) or 0.2, 2 or 10% BALB/c naive serum (bars). The percentage of infection blocked by each serum is represented in A1 and A2. B1 and B2 show the correlation between the percentage of infection blocked and the ELISA (IgG NANP) endpoint titer for each sample. Data was analysed with a nonparametric Spearman correlation test (r); linear regression (R^2; p-value from F test: is the slope significantly different than zero?).

It is not clear why R21 samples failed to show a blocking effect as high as samples from prime-boost viral vector regimens when used in combination with the CS addition parasite. CS viral vector vaccines (CS ChAd63 and CS MVA), RTS,S and R21 target *P. falciparum* CS protein. All these vaccines contain NANP repeats (tandem repeats of 4 aa located in the CSP central region), which constitute an immunodominant epitope for B cells. However, certain differences in the specific CSP regions included in the protein (viral vectors include the whole CSP protein except for some of the NANP repeats, while RTS,S and R21 contain 189 aa from the C terminal$_T$ region) could elicit antibodies against different CSP epitopes that might behave differently on blocking sporozoite invasion. The absence of a high blocking effect with

addition parasites could also be related to a high antibody concentration or avidity which could cause the release of CSP or an increase of the host-pathogen interactions.

R21 vaccinated mice were challenged with CS addition parasites. Inhibition of sporozoite invasion *in vivo* should be easier to achieve than *in vitro*, as the serum is not diluted and the sporozoite need to travel through different tissues and cellular barriers before reaching the hepatocytes. Nevertheless, the same low blocking capacity observed *in vitro* with the addition parasite, could be happening *in vivo*. If this was the case, higher efficacy might be observed in mice challenged with the replacement parasite.

b) R21 administered with different adjuvants

To evaluate whether the adjuvant used in combination with R21 could have an effect on the sporozoite blocking capacity, post-vaccination serum from BALB/c mice vaccinated with 0.5 µg R21 without adjuvant or with one of the following adjuvants: Alhydrogel (85 µg), Abisco (12 µg), AddaVax (50 µL) or AddaVax and Abisco (50 µL and 12 µg, respectively) was assessed by ISI. Mice received 3 immunisations with a 3 week interval and serum was collected 3 weeks after the last vaccination.

R21 with Alhydrogel sera showed the lowest blocking activity: significantly less than Abisco at 0.2% and 2% serum volume and significantly less than AddaVax and AddaVax and Abisco at 10% [Figure 2.16. A1, A2, A3]. Abisco and AddaVax (together or on its own), or R21 in the absence of adjuvant were found to have similar capacity to block the sporozoite invasion [Figure 2.16. A1, A2, A3]. A significant correlation (Spearman test) was observed between the percentage of infection blocked values and the ELISA endpoint titer at all the serum dilutions tested [Figure 2.16. B1, B2, B3].

When these mice were challenged, mice vaccinated with R21 AddaVax showed a delay in time to 1% parasitaemia (compared to naive mice), while mice which received R21 Abisco were sterilely protected [Katharine Collins, DPhil Thesis]. Both groups of mice had similar antibody titres measured with ELISA. One possible explanation for the differences observed in protection between these two groups, could be that vaccination with Abisco elicited antibodies with a greater capacity to block the sporozoite invasion of hepatocytes (more functional antibodies). However, no significant differences were detected between Abisco and AddaVax with the ISI assay. It was also found that R21 + Abisco vaccinated mice have a higher T cell response by ELISPOT (R21 Abisco generated a median group response of 211 SFC/10^6 splenocytes, while R21 AddaVax, only the half (96.5 SFC/10^6 splenocytes) [Katharine Collins, DPhil Thesis].
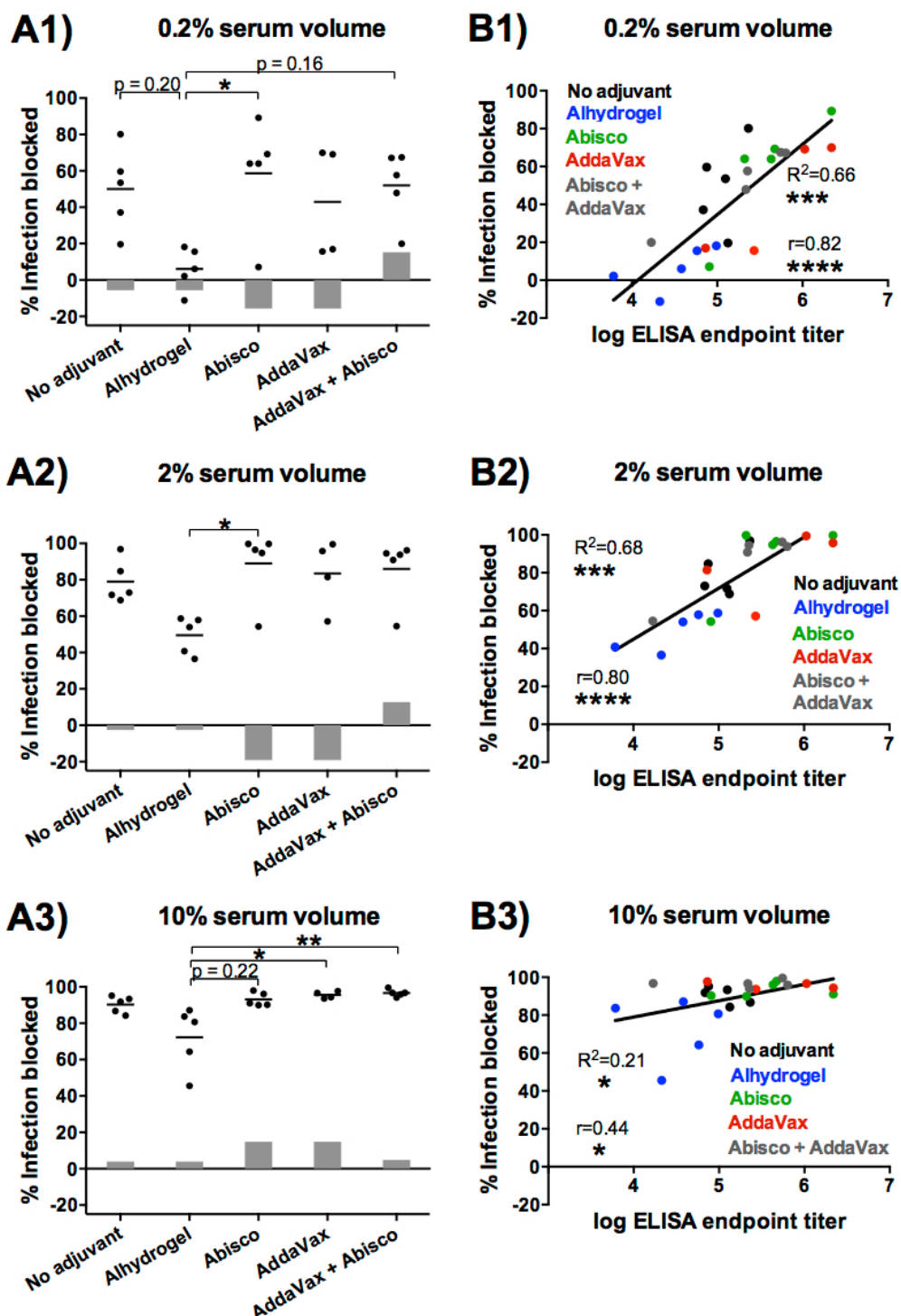
Figure 2.16. R21 with different adjuvants. BALB/c mice were vaccinated with 3 doses (3 weeks apart) of 0.5 µg R21 and serum was taken three weeks after the last dose. 5 groups of mice were tested according to the adjuvant given with R21: no adjuvant, Alhydrogel, Abisco, AddaVax or AddaVax and Abisco. Huh7 cells were infected with *Pf* CS replacement *P. berghei* in a final serum concentration of 0.2, 2 or 10%. The results from the ISI assay are presented in A1, A2 and A3 (each dot represents a different mouse, and the bars represent naive serum) and its correlation with ELISA (IgG NANP) data on B1, B2 and B3. Serum samples and ELISA endpoint titres were provided by Katharine Collins, DPhil student. A1, A2 and A3 were analysed with Kruskal-Wallis test and Dunn's multiple comparisons test. B1, B2 and B3 were analysed with nonparametric Spearman correlation test (r) and linear regression (R^2; p-value from F test: is the slope significantly different than zero?).

The fact that no functional differences were found with the ISI assay, suggests that the differences in protection between these two adjuvants may be due to the different T cell responses. This example supports the importance of ISI assay for gaining a better understanding of the immune mechanisms working on a pre-erythrocytic malaria vaccine.

c) RTS,S vs R21

To compare the ability of antibodies generated by R21 to inhibit sporozoite invasion with the blocking ability of antibodies generated by the most advanced malaria vaccine in development, RTS,S, BALB/c mice were vaccinated with 0.5, 1.6 or 5 µg of either RTS,S or R21 and serum tested in the ISI assay. Both vaccines were administered in combination with AS01B (50 µL) with two doses given with an interval of 8 weeks and serum taken 11 days after the last vaccination.

There was no significant difference in the percentage of infection blocked achieved with the various groups of vaccinated mice, suggesting that R21 is as good as RTS,S at preventing infection of liver cells in this model. Antibodies from mice immunised with either vaccine (RTS,S or R21) were able to inhibit almost 100% of sporozoite invasion at very low serum concentrations (serum dilution of 2%) [Figure 2.17.A2]. This is the highest level of blocking seen at this serum dilution compared to the other vaccines assessed in this model. It is possible that by diluting the serum below 0.2%, larger differences between doses or vaccines might be detected. However, it is probably not needed to dilute the serum more than 0.2%, as with this concentration was already possible to register a range of percentage infection blocked from 40 to 90% [Figure 2.17.A1]. Diluting the serum further would also increase the spread of the data. Probably, the most interesting information would be to know which antibodies continue to be more functional over time, collecting sera at later time points and testing them with the ISI assay.
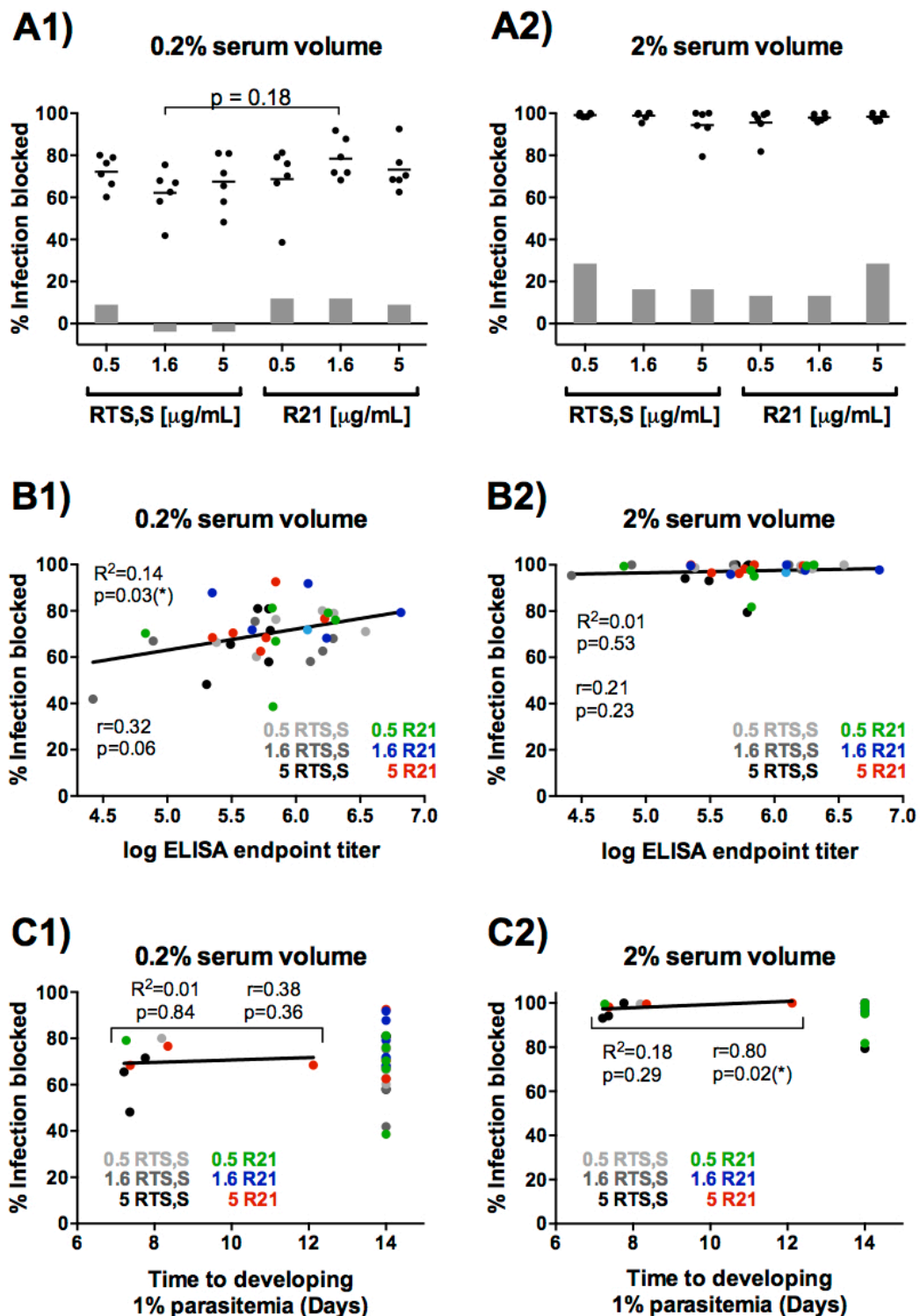
Figure 2.17. R21 vs RTS,S. BALB/c mice were immunised with 0.5, 1.6 or 5 µg of either RTS,S or R21, with AS01B (50µL). Two doses were given with an interval of 8 weeks and serum was collected 11 days after the last vaccination. Huh7 cells were infected with *Pf* CS replacement *P. berghei* in the presence of 0.2 or 2 % serum. A1 and A2 show ISI assay results for each vaccination group (dots represent each mouse sample and bars represent naive serum). B1 and B2 show the correlation of these data with ELISA (IgG NANP) values. C1 and C2 represent the correlation between the percentage of infection blocked and the time to develop 1% parasitaemia for each mouse. Serum samples, ELISA endpoint titer and time to develop 1% parasitaemia were provided by Katharine Collins (DPhil student). Data was analysed with Kruskal-Wallis test, Dunn's multiple comparisons test (A1, A2); and nonparametric Spearman correlation test (r), linear regression (R^2; p-value from F test: is the slope significantly different than zero?) (B1, B2, C1, C2).

There was a trend in the correlation with ELISA data, although not statistically significant (p=0.06, Spearman test) with 0.2% serum [Figure 2.17.B1]. The correlation was weaker in this experiment compared to the previous one (R21 with different adjuvants). One possible explanation may be that in the other experiment, the values of ELISA endpoint titres were more evenly spread along a range of two logs, while in the present experiment a high number of the samples had ELISA values spread within less than one log. The ELISA data only included the isotype IgG. Possibly, a stronger correlation between the ISI assay and antibody titres could be found if the ELISA antibody titres included all antibody isotypes.

Percentage of infection blocked data were also plotted against the time that took to each mouse to develop 1% parasitaemia. Mice that did not develop parasitaemia by day 14 were considered to be protected, they appear in the graph at day 14, although no parasite infection was detected in these mice. The percentage of infection blocked correlated with the time to develop 1% parasitaemia excluding protected mice. A significant correlation was found for 2% samples [Figure 2.17.C2]. Nevertheless, this correlation must be considered with caution as the percentages of infection blocked measured are very close to each other and ISI assay is unlikely to be able to discriminate blocking effect differences of less than 10%. In these graphs including the time to 1% parasitaemia [Figure 2.17.C1, C2], it is interesting to note that some mice whose antibodies have a high capacity to inhibit sporozoite invasion developed parasitaemia, while mice with lower inhibition activity did not develop parasitaemia. These data would support that antibody-mediated inhibition of sporozoite invasion is not the only mechanism operating to provide protection in RTS,S or R21 vaccines.

2.2.2 Macaque samples

2.2.2.1 TRAP

Rhesus macaques were immunised with $5\bullet10^7$ iu/animal AdCh63 ME-TRAP followed 8 weeks later by $8\bullet10^7$ pfu/animal MVA ME-TRAP, and serum samples were collected three week after the MVA vaccination. ME is a construct fused to TRAP that includes CD4+ and CD8+ epitopes that belong to six pre-erythrocytic antigens: LSA1, CSP, STARP, LSA3, Exp1 and TRAP. These samples, kindly provided by Dr. Alexandra Spencer, were tested with the ISI assay to determine antibody-mediated inhibition of malaria infection. First, a group of 5 samples was tested at 2% and 10% serum dilution, both with and without prior heat inactivation (56ºC, 30min). The best results were observed for non-heat inactivated samples, at 10% dilution, therefore, an additional group of samples were tested in this condition. Figure 2.18 shows the results obtained for these experiments, including the percentage of infection blocked measured for pre-vaccination and post-vaccination serum from each subject.

At 10% serum dilution, post-vaccination sera achieved a statistically significant higher prevention of sporozoite entry in hepatocytes (Infection blocked mean: 53%) than pre-vaccination sera (Infection blocked mean: 37%) [Figure 2.18.A2]. No significant increase of the blocking capacity of antibodies was observed at 2% serum dilution [Figure 2.18. A1] or whenever the serum samples were heat inactivated [Figure 2.18.B1,B2].

Macaque humoral responses were expected to show more complexity and present higher background responses than genetically identical mice like BALB/c. In fact, with 10% serum dilution, pre-vaccination sera avoided about 40% of the sporozoite invasion [Figure 2.18.A2].

In this context, heat inactivation of the serum was considered again as a possible solution to obtain values with lower background signal. Nevertheless, it has been shown already that heat inactivation [Figure 1.10.A2] was found to prevent the blocking effect of a monoclonal antibody. Similarly, the significant increase of blocking effect after vaccination found at 10% serum dilution was not detected when the samples had been heat inactivated previously.
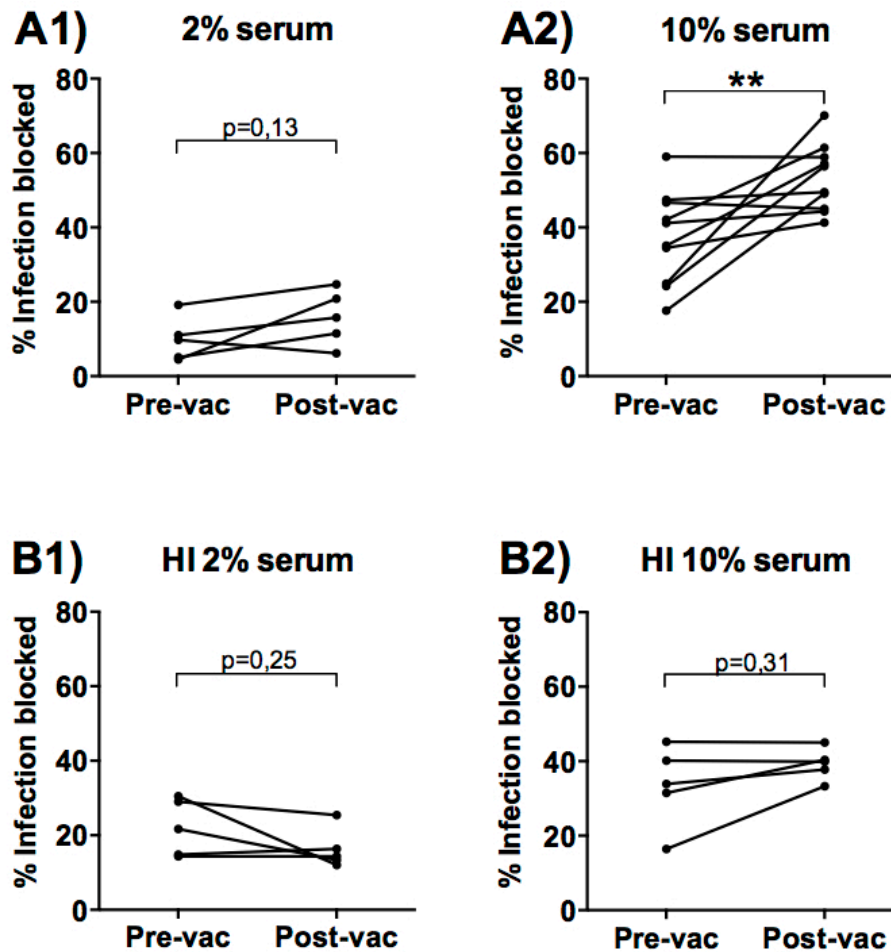
Figure 2.18. Rhesus macaques were vaccinated with ME-TRAP. Each animal received 5·10⁷ iu AdCh63 ME-TRAP, 8·10⁷ pfu/animal MVA ME-TRAP (8 weeks apart) and serum taken three weeks after MVA vaccination. A1 and A2 show %Infection blocked at ISI assay by serum samples at 2% and 10% dilution, respectively. The same experiment was performed with heat inactivated samples (56°C, 30min) and results obtained are included in B1 (2%) and B2 (10%). Data was analysed with Wilcoxon matched-pairs signed rank test (two-tailed).

%Infection blocked data obtained with ISI assay were plotted against LIPS and ELISA data obtained by Dr. Alexandra Spencer. Figure 2.19 (A1 and B1) contains the LIPS (A1) and ELISA values (B1) available for both pre- and post-vaccination serum samples. A significant correlation was found between LIPS signal and %Infection blocked [Figure 2.19.A1] and between ELISA endpoint titre and %Infection blocked [Figure 2.19.B1]. The analysis was also performed representing ISI data as Blocking increase (% Infection blocked with post-vaccination serum - % Infection blocked with pre-vaccination serum) against the fold increase found after vaccination with ELISA and LIPS assays [Figure 2.19.A2,B2]. Although the data analysed is the same, significant correlations were not found with this last approach. This observation is probably due to the reduction of the number of samples to the half (instead of considering each sample on its own, each post-vaccination figure is related to its pre-

vaccination value) and to the accumulation of variability associated with each experiment when values from post-vaccination samples are related to values from pre-vaccination ones. In the next section of this chapter, experiments performed with human samples from clinical trials are described. Correlations with ISI results were presented with Blocking increase (%) for normalisation purposes, in an attempt to remove the non-specific blocking effect from each volunteer. For macaque serum samples, both analysis approaches have been included [Figure 2.19] to stress the effect of the number of samples included in the correlation and the increase on variability that may be associated to a result obtained from two different serum samples in the ISI assay.
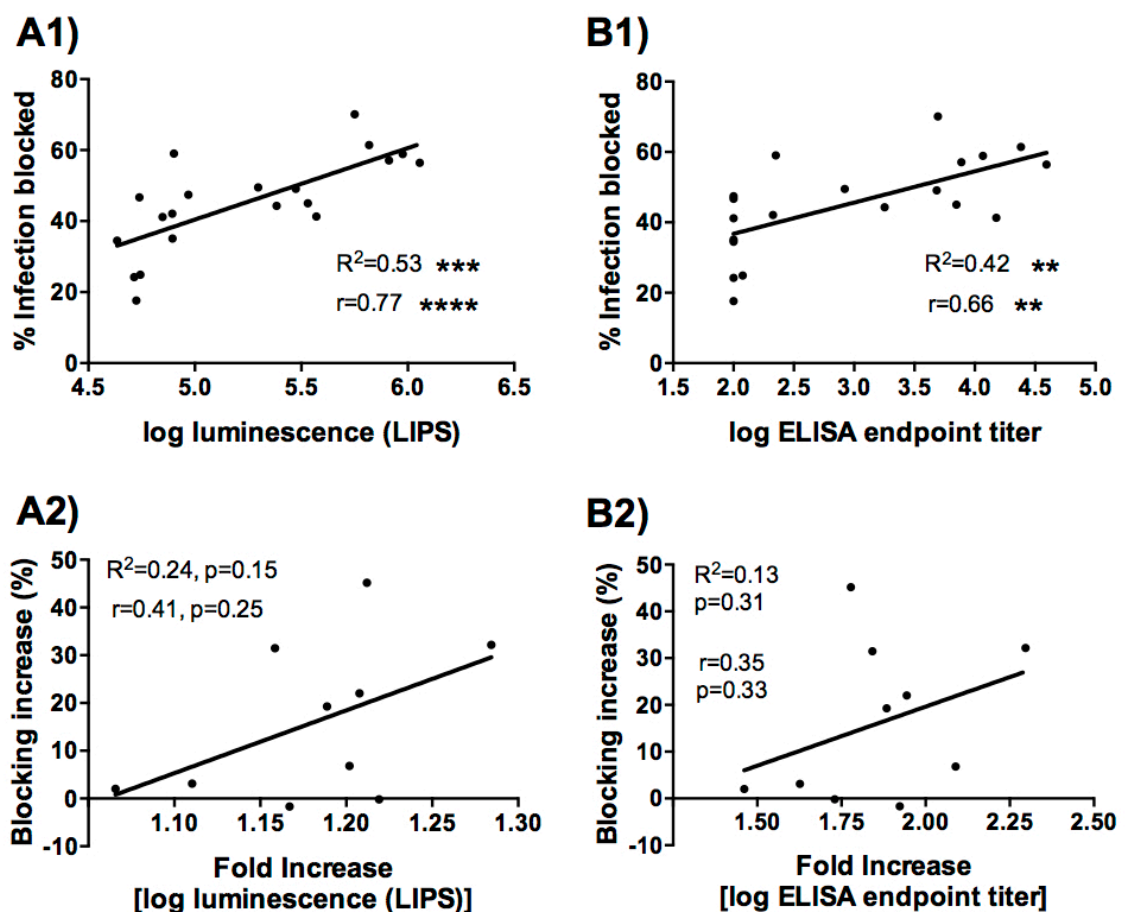
Figure 2.19. ISI assay data vs LIPS/ELISA data. Rhesus macaques were immunised with ME-TRAP AdCh63 and MVA (8 weeks apart) and serum collected three week after MVA. %Infection blocked obtained for each pre- and post-vaccination sample at 10% serum dilution has been plotted against LIPS values (A1) or ELISA values(B1) obtained for each sample. Blocking increase (which is obtained as %Infection blocked (post-vaccination serum) minus % Infection blocked (pre-vaccination serum) has been represented against fold increase of LIPS values (A2) or ELISA values (B2). Data was analysed with nonparametric Spearman correlation test (r); linear regression (R2; p-value from F test: is the slope significantly different than zero?).

2.2.3 Clinical trial samples

2.2.3.1 CS clinical trial

UK adults were immunised with $5 \cdot 10^{10}$ vp ChAd63-CS followed 8 weeks later by $2 \cdot 10^8$ pfu MVA-CS and serum samples were taken 2 weeks after the last vaccination. On the same date, volunteers were challenged with *P. falciparum* sporozoites administered through mosquito bite. Serum samples were tested using *P. berghei* chimeric sporozoites expressing *P. falciparum* CSP either as an additional copy (*Pf* CS *P. berghei* addition), or replacing *P. berghei* CSP (*Pf* CS *P. berghei* replacement). The use of both kinds of parasites was due to the results obtained for R21 samples [Figure 2.15], where serum samples showed a greater capacity to inhibit the sporozoite invasion of *Pf* CS *P. berghei* replacement than of *Pf* CS *P. berghei* addition.

First, six serum samples were tested with *Pf* CS *P. berghei* addition sporozoites and using 2% and 10% serum dilutions [Figure 2.20]. Secondly, the ISI assay was performed for five samples (some of them were already tested in the first experiment, some of them were not) with *Pf* CS *P. berghei* replacement sporozoites [Figure 2.21]. A higher difference between pre- and post-vaccination blocking response was detected at 10% serum dilution, rather than at 2% [Figure 2.20. A1, A2]. In an attempt to increase the difference observed at 10% serum dilution, in the second experiment serum samples were tested at 10% and 20% serum dilution. ISI assay results from both experiments have been presented with other graphs were ISI values are plotted against ELISA data available for these samples (this data and the serum samples were provided by Georgina Bowyer and Dr. Katie Ewer) [Figure 2.20, Figure 2.21]. A statistically significant (p<0.05, Wilcoxon test) increase of the blocking capacity of post-vaccination sera compared to pre-vaccination was not found [Figure 2.20. A1, A2; Figure 2.21 A1,A2]. Nevertheless, at 10% serum dilution the p-values obtained were low: p=0.09 with addition parasites [Figure 2.20.A2] and p=0.13 with replacement parasites [Figure 2.21.A1], indicating that the detected increase of the blocking effect after vaccination could be due to a real effect with a probability of at least 87%. When using the replacement parasites the mean %Infection blocked for post-vaccination sera was 56% [Figure 2.21.A1], while with addition parasites was 26% [Figure 2.20.A2]. Pre-vaccination blocking effect was also higher with replacement parasites, with a %Infection blocked mean of 44% [Figure 2.21.A1], while the mean response with addition was 5% [Figure 2.20.A2].

Figure 2.20. UK adults were immunised intramuscularly with $5 \cdot 10^{10}$ vp ChAd63-CS, followed 8 weeks later by $2 \cdot 10^8$ pfu MVA-CS and serum samples were collected 2 weeks after MVA vaccination. In addition, 2 weeks after MVA vaccination, volunteers were challenged with *P. falciparum* sporozoites. Results from ISI assay using *Pf* CS *P. berghei* addition sporozoites are shown for pre- and post-vaccination samples, at 2% serum dilution (A1) and 10% serum dilution (A2). Blocking increase (%Infection blocked by post-vaccination serum - %Infection blocked by pre-vaccination serum) for each sample at 10% dilution was plotted against ELISA units (obtained for post-vaccination samples) (B), isotype OD (C), day of diagnosis (D) and parasitaemia at day 7.5 (E). Serum samples and values for ELISA, isotype OD, day of diagnosis and parasitaemia were provided by Georgina Bowyer and Dr. Katie Ewer. Data was analysed with Wilcoxon matched-pairs signed rank test (two-tailed) (A1,A2); nonparametric Spearman correlation test (r) (B,C,D,E) and linear regression (R^2; p-value from F test: is the slope significantly different than zero?) (B,D,E).

The higher blocking values observed with chimeric replacement sporozoites could be related to a lower amount of CS protein on the surface of the sporozoite, which would become more vulnerable in the presence of anti-CS antibodies from the serum sample. At 20% serum dilution with replacement parasites, rather than achieving a better differentiation of the pre- and post-vaccination blocking effect, the blocking responses were more similar, probably due to an elevated non-specific effect in the presence of high protein concentration. Replacement parasites did not provide a better system than addition parasites to evaluate the anti-CS clinical sera, differently to what occurred in the R21 study. Already in previous experiments, addition parasites also allowed the detection of high %Infection blocked values for serum samples from mice vaccinated with viral vector prime-boost strategies [Figure 2.1.A; Figure 2.3.A].

Interestingly, a statistically significant correlation (Spearman test) was found between the increase of %Infection blocked (obtained with *Pf* CS *P. berghei* addition, at 10% serum dilution) with ELISA units, day of diagnosis and level of parasitaemia 7.5 days after challenge [Figure 2.20. B,D,E]. Although significance was not achieved for these correlations when the sporozoites employed were *Pf* CS *P. berghei* replacement, low p-values (Spearman test) reflected the same effect [Figure 2.21. B,D,E]. Blocking increase% values were obtained as: (%Infection blocked by post-vaccination sample) - (%Infection blocked by pre-vaccination sample), with the objective of normalising background responses among volunteers. Presumably, the blocking increase should be a measure of the functionality of antibodies to avoid sporozoite invasion gained through vaccination (due to the generation of specific antibodies against the antigen included in the vaccine). ELISA units included in the correlation were the values obtained for each post-vaccination sample. Pre-vaccination ELISA data was not obtained for all the clinical data and when it was available, no differences were detected among pre-vaccination sera. For these reasons, it was decided to analyse the correlation using post-vaccination ELISA values in all clinical trials. A significant correlation between Blocking increase (10% dilution) and ELISA Units [Figure 2.20.B], indicates the determinant influence of antibody titre in the prevention of the sporozoite invasion. A significant correlation between Blocking increase (at 10% serum dilution) and the level of a particular antibody isotype (IgA, IgM, IgG1, IgG2 or IgG3) was not detected, but a trend was found for IgM (p=0.06) with addition sporozoites, and IgA (p=0.08) with replacement [Figure 2.20.C, Figure 2.21.C]. In the replacement experiment, it is interesting to observe that the two samples with higher ELISA units and higher Blocking increase (10% dilution) were probably achieving these effects through the production of either IgM or IgA.
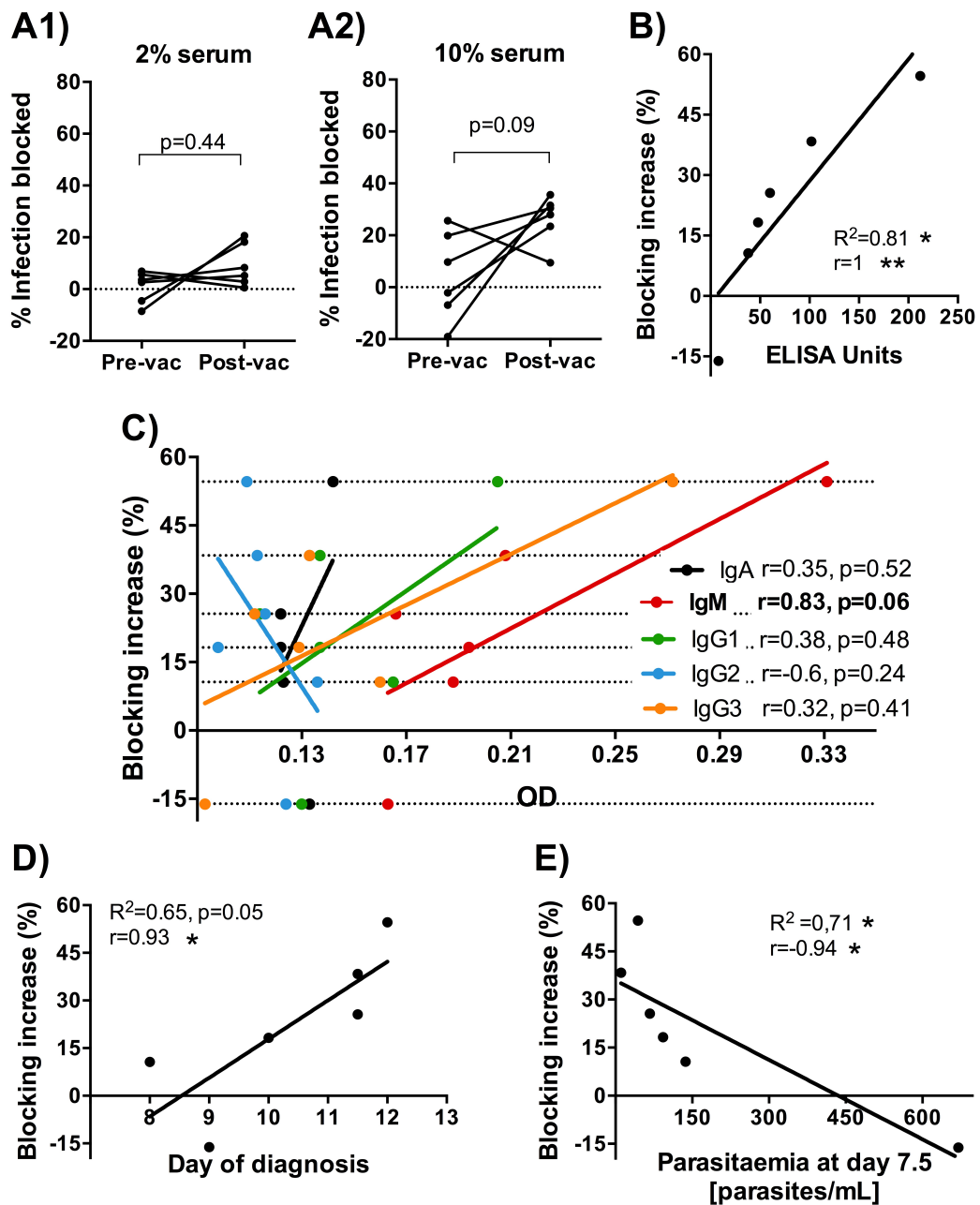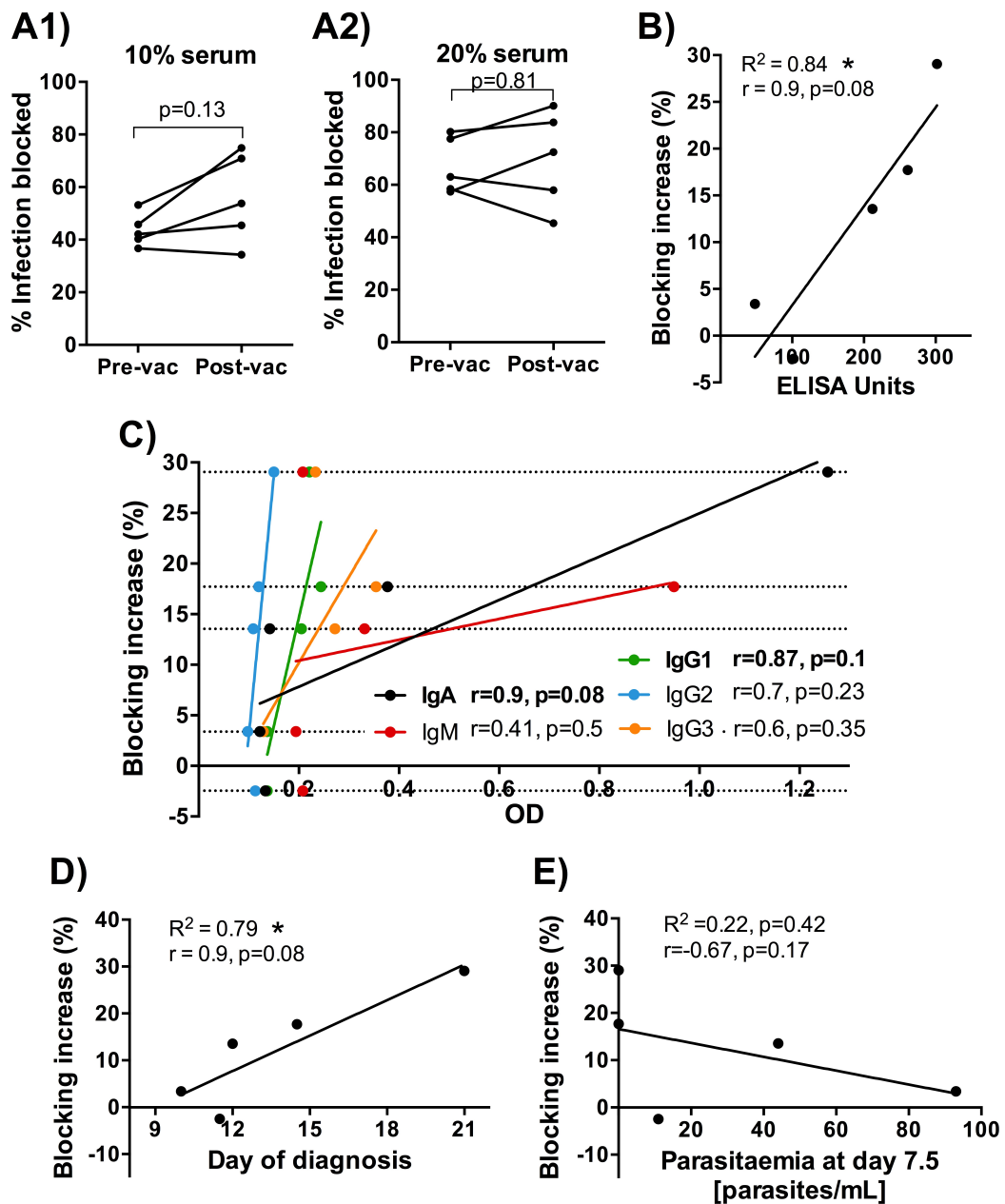
Figure 2.21. UK adults were immunised intramuscularly with $5 \cdot 10^{10}$ vp ChAd63-CS followed 8 weeks later by $2 \cdot 10^8$ pfu MVA-CS and serum samples were collected 2 weeks after MVA vaccination. In addition, 2 weeks after MVA vaccination, volunteers were challenged with *P. falciparum* sporozoites. Results from ISI assay using _Pf_ CS _P. berghei_ replacement sporozoites are shown for pre- and post-vaccination samples, at 10% serum dilution (A1) and 20% serum dilution (A2). Blocking increase (%Infection blocked by post-vaccination serum - %Infection blocked by pre-vaccination serum) for each sample at 10% serum dilution was plotted against ELISA units (obtained for post-vaccination samples) (B), isotype OD (C), day of diagnosis (D) and parasitaemia at day 7.5 (E). Serum samples and values for ELISA, isotype OD, day of diagnosis and parasitaemia were provided by Georgina Bowyer and Dr. Katie Ewer. Data was analysed with Wilcoxon matched-pairs signed rank test (two-tailed) (A1,A2); nonparametric Spearman correlation test (r) (B,C,D,E); linear regression (R2; p-value from F test: is the slope significantly different than zero?) (B,D,E).

Since the individuals were challenged with *P. falciparum*, data about the day of diagnosis and the levels of parasites on blood at day 7.5 was available. Higher levels of Blocking increase at 10% serum dilution were associated with a delay of the clinical day of diagnosis [Figure 2.20.D]. A greater functional capacity of serum to block the sporozoite invasion would play an essential role delaying the establishment of the malaria infection and consequently: the greater the capacity of the sera to prevent sporozoite invasion was, the smaller the quantity of parasites/mL detected in blood at day 7.5 [Figure 2.20.E]. The correlations between ISI and day of diagnosis and parasitaemia were statistically significant in the experiment performed with addition sporozoites.

2.2.3.2 TRAP clinical trial

Serum samples from volunteers vaccinated with ME-TRAP were tested in the ISI assay and results were contrasted with other data obtained previously. These serum samples and data were provided by Georgina Bowyer and Dr. Katie Ewer. UK adults were vaccinated with $5 \cdot 10^{10}$ vp ChAd63-ME-TRAP and $2 \cdot 10^8$ pfu MVA-ME-TRAP, 8 weeks apart, and serum samples were collected 2-3 weeks after MVA. Volunteers were challenged with *P. falciparum* 2-3 weeks after MVA vaccination. Two experiments were performed, each one with samples from a different clinical trial: one had been conducted on July 2012 [Figure 2.22] and the other took place on June 2009 [Figure 2.23].

ISI assay was performed, as previously described, at 10% serum dilution (20%, also in the first experiment) and *Pf* TRAP *P. berghei* addition parasite (no replacement parasite was available for *Pf* TRAP). Contrary to what was expected, post-vaccination serum samples presented an inferior capacity to avoid sporozoite invasion than pre-vaccination serum samples. This decrease after vaccination was statistically significant in the second experiment [Figure 2.23.A], where pre-vaccination samples showed a mean blocking response of 25% and post-vaccination samples, only 12%; and a trend to lower post-vaccination %Infection blocked values was observed in the first experiment at 10% (p=0.08) and 20%(p=0.08) serum dilution [Figure 2.22. A1, A2]. Using 20% serum dilution in the first experiment did not help to measure a higher difference between pre-vaccination and post-vaccination values compared to 10% serum dilution and increased the background responses: pre-vaccination %Infection blocked mean at 20% dilution was 62% [Figure 2.22. A2], while with 10% dilution was 46% [Figure 2.22. A1].

In previous experiments (with macaque serum samples) heat inactivation (30min, 56ºC) did not help to get rid of background responses and decreased the differences between blocking values measured. To confirm that it was not advisable to heat inactivate samples prior to ISI assay, a small group of samples was tested in the ISI at 20% dilution after being heat inactivated. Once again, the difference in blocking responses between groups at the same serum dilution [Figure 2.22. A2] disappeared upon heat inactivation treatment [Figure 2.22.A3].

Significant correlations were not found between Blocking increase% at 10% serum dilution and ELISA units, Isotype OD, IgG avidity, Day of diagnosis or Parasitaemia at day 7.5. The strongest correlation was found in the first experiment

between Blocking increase% and IgG2 OD (p=0.07) and IgM OD (p=0.12) [Figure 2.22.C].

In the first experiment, the largest blocking decrease value was obtained for the sample with the highest value of ELISA units [Figure 2.22.B]. Similarly, in the second experiment, the samples which lost more blocking capacity after vaccination had better than average ELISA units [Figure 2.23.B]. The described trends between Blocking increase% and IgM or IgG2 [Figure 2.22.C] also suggest that higher levels of the isotype decreased the blocking capacity. These results contradict what was found for macaque samples, where vaccination with ME-TRAP increased the blocking capacity of the serum [Figure 2.18.A2]. One possible explanation for the surprising effect observed in humans vaccinated with ME-TRAP, could be that higher levels of antibodies (generated after vaccination) may promote sporozoite invasion of hepatocytes, possibly by increasing pathogen-host contacts. The ISI assay is performed with a human liver cell line, and human antibodies bound to TRAP on the parasite surface could be recognised by Fc receptors on the surface of the hepatocyte. If the antibody did not prevent sporozoite invasion through its binding to TRAP, it could have just helped the anchoring of the parasite to the hepatocyte surface. Also, the *P. berghei* parasite used here is expressing *P. falciparum* TRAP as an additional protein, so *P. berghei* TRAP is also present on the surface. This could have as a consequence that, even if the antibody could achieve a good 'blocking' through binding to *P. falciparum* TRAP, the parasite may still conserve the functionality of *P. berghei* TRAP in the presence of specific antibodies against *falciparum* TRAP.

Finally, samples from ME-TRAP clinical trial (July 2012) used in the first experiment were the only samples with available IgG avidity data. Like antibody concentration has been regarded as an important factor contributing to the level of infection blocked, the avidity of antibodies may be playing also an important effect on the interaction between a specific antibody and its target antigen. Here, no correlation or trend was found between Blocking increase% and IgG avidity [Figure 2.22 D]. Nevertheless, this result can not be generalised, specially as serum samples had an effect on sporozoite invasion opposite to what had been found in previous experiments.
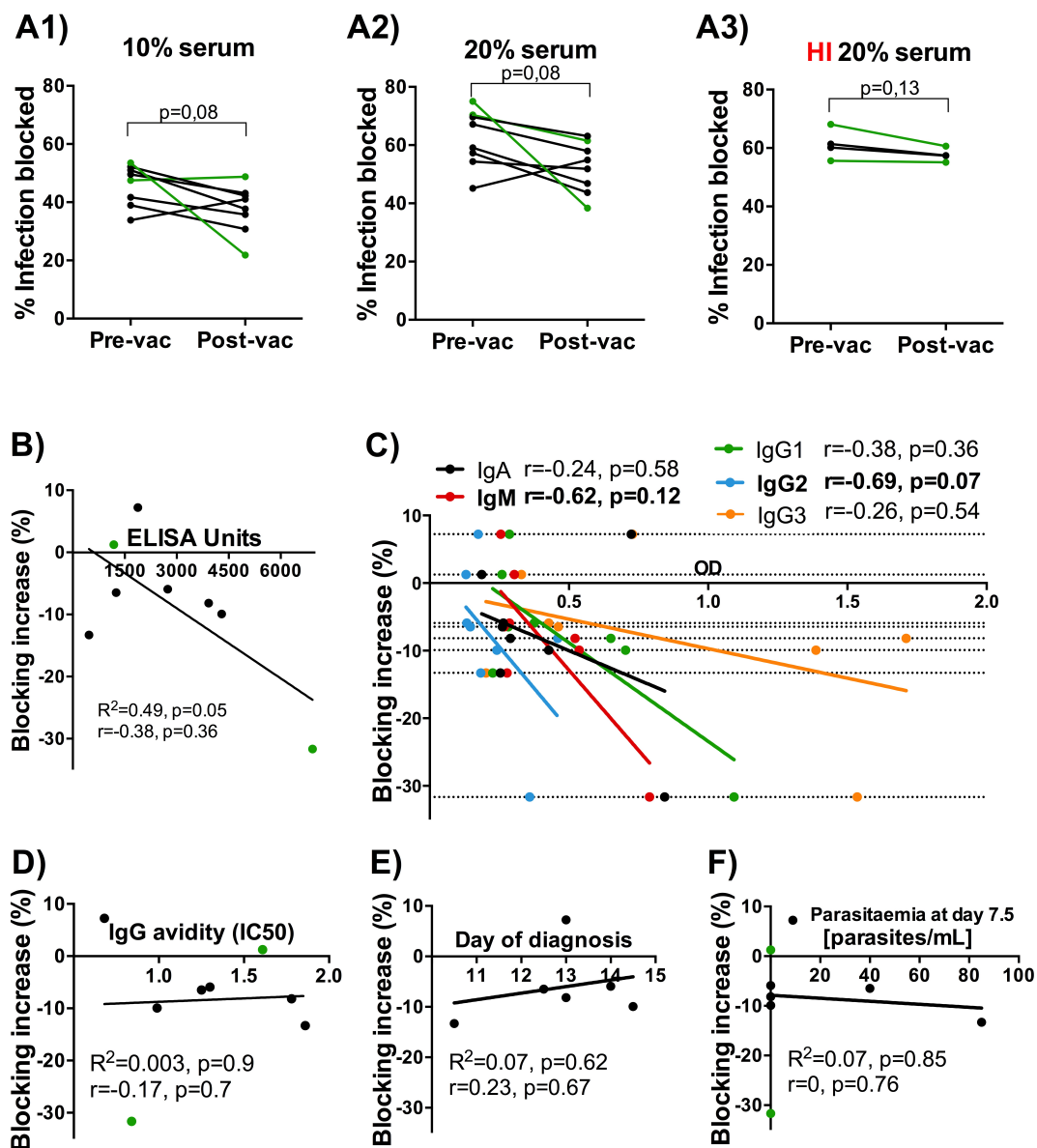
Figure 2.22. UK adults received intramuscularly 5•10^{10} vp ChAd63-ME-TRAP and 2•10^8 pfu MVA-ME-TRAP, 8 weeks apart. Serum samples were taken 2 weeks after the last vaccination, the following day volunteers were challenged with *P. falciparum*. ISI assay was performed using *Pf* TRAP *P. berghei* with 8 samples at serum dilution of 10% (A1), 20%(A2), and four samples were tested also at 20% serum dilution after being heat inactivated (30min, 56ºC) (A3). Blocking increase (%Infection blocked by post-vaccination serum - %Infection blocked by pre-vaccination serum) for each sample (at 10% dilution) was plotted against ELISA units (obtained for post-vaccination samples) (B), isotype OD (C), IgG avidity (D), day of diagnosis (E) and parasitaemia at day 7.5 (F). Serum samples and values for ELISA, isotype OD, IgG avidity, day of diagnosis and parasitaemia were provided by Georgina Bowyer and Dr. Katie Ewer. Data was analysed with Wilcoxon matched-pairs signed rank test (two-tailed) (A1,A2,A3); nonparametric Spearman correlation test (r) (B,C,D,E,F); linear regression (R^2; p-value from F test: is the slope significantly different than zero?) (B,D,E,F). Green colour in A1, A2, A3, B, D and F indicates that the samples belong to volunteers who were protected upon challenge.
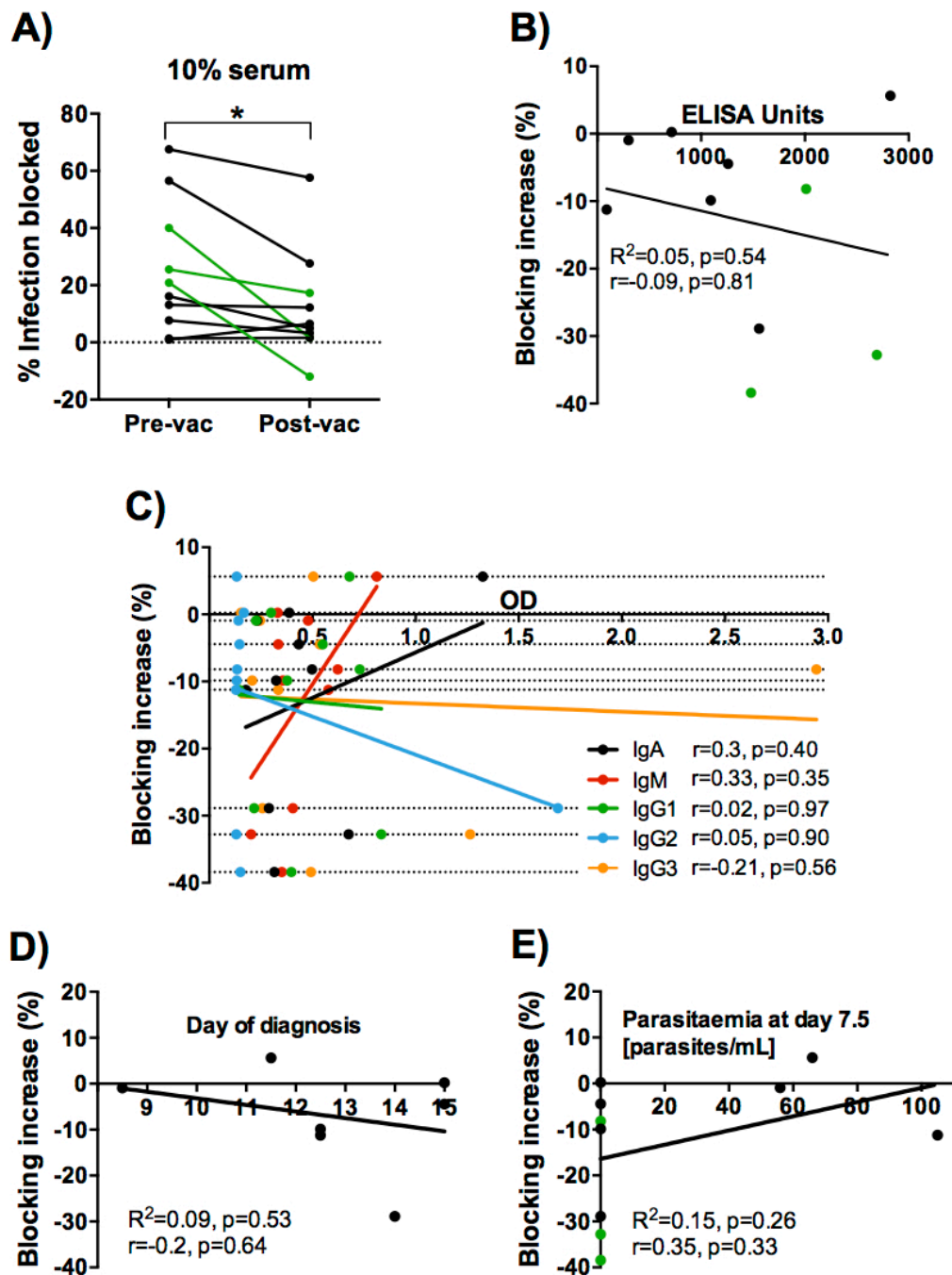
Figure 2.23. UK adults received intramuscularly 5•10[10] vp ChAd63-ME-TRAP and 2•10[8] pfu MVA-ME-TRAP, 8 weeks apart. Serum samples were taken 2 or 3 weeks after the last vaccination, volunteers were challenged with *P. falciparum* the following day. ISI assay was performed using *Pf* TRAP *P. berghei* with 10 samples at 10% serum dilution (A). Blocking increase (%Infection blocked by post-vaccination serum - %Infection blocked by pre-vaccination serum) for each sample (at 10% dilution) was plotted against ELISA units (obtained for post-vaccination samples) (B), isotype OD (C), day of diagnosis (D) and parasitaemia at day 7.5 (E). Serum samples and values for ELISA, isotype OD, IgG avidity, day of diagnosis and parasitaemia were provided by Georgina Bowyer and Dr. Katie Ewer. Data was analysed with Wilcoxon matched-pairs signed rank test (two-tailed) (A); nonparametric Spearman correlation test (r) (B,C,D,E); linear regression (R^2; p-value from F test: is the slope significantly different than zero?) (B,D,E). Green colour in A, B and E indicates that the samples belong to volunteers who were protected upon challenge.

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2.3 Discussion

Once the flow cytometry based ISI assay was optimised (process described in chapter 1), the developed protocol was used to test a number of serum samples from mouse, macaque and human clinical trials. In studies with mouse samples, the assay proved to be useful comparing the functional capacity of antibodies generated against different antigens and with different vaccination strategies, such as the adjuvant or vaccination dose. A positive increase of antibody functionality after vaccination was shown for some macaque and volunteer samples, and interesting correlations were found for ISI assay data and other antibody assays and clinical data. In all cases, samples tested were sera obtained after vaccination against a pre-erythrocytic *P. falciparum* antigen, and the sera was tested for its capacity to avoid the invasion of Huh7 cells by a *P. berghei* sporozoite expressing the same *P. falciparum* antigen targeted through vaccination.

There are limitations with the ISI assay, the most important is the difficulty to obtain live sporozoites, since mosquito yields can be variable and the number of sporozoite needed for the assay may require several hours of salivary gland dissection. The assay is also affected by significant levels of inhibition observed with non-specific sera, as it has been reported in the literature (198,211,226) and could be observed in the experiments described. For this reason, a threshold level of 30% was set above which blocking was considered positive. In addition, the high variability in the assay can result in differences between samples within 10-15% blocking not considered relevant. Some sources of this variability could be error in the amount of cells, sporozoite and serum plated in each well or variations on the viability of the sporozoites. Variability could be compensated by including a higher number of replicates and a greater number of samples per group, but it would require also a higher number of sporozoites. Despite these limitations, the flow cytometry based protocol optimised in this work proved to be a reliable and efficient approach to test antibody capacity to avoid sporozoite invasion. It is particularly important to highlight the high throughput capacity achieved with this method, which enabled to test a large number of samples. The flow cytometry readout makes it possible to routinely use the ISI assay with clinical and pre-clinical studies and overcomes the subjectivity of the traditional microscopy readout.

CSP and TRAP

The first experiments with mouse samples tested the capacity of CS and TRAP post-vaccination serum to inhibit sporozoite invasion. A range of positive blocking responses were observed for CS and TRAP, which are consistent with previous studies, helped to validate the assay and confirmed that 2% and 10% serum dilutions were appropriated concentrations to test samples. Some serum samples were tested at 20% serum dilution but resulted in a high non-specific effect and when using 2% serum dilution, sometimes no positive blocking responses were detected. For other serum samples (anti-CS serum from mice vaccinated with R21) positive blocking was detected even at 0.2% serum dilution. Following these results in mice, serum samples from humans vaccinated against CS were tested, obtaining a trend towards an increase in blocking responses after vaccination. With regard to TRAP, a statistically significant increase on the blocking response after vaccination was detected at 10% serum dilution with serum samples from macaques but, surprisingly, the human anti-TRAP serum samples behaved differently: a trend towards a decrease in the blocking response was observed after vaccination which was statistically significant in one of the clinical studies. There is not a clear explanation for an increase of sporozoite invasion in the presence of post-vaccination serum. It could be suggested that human anti-TRAP antibodies may be facilitating host-cell contact through the interaction between their Fc region and the Fc receptors on the human hepatocyte surface.

Antibodies against CSP have been largely described to have the capacity to inhibit sporozoite invasion. Serum and monoclonal antibodies, both from mouse and human origin, targeting CSP have been shown to block sporozoite invasion *in vitro* and *in vivo*. Mouse monoclonal antibodies against *P. berghei* CSP injected into mice protected them from challenge with *P. berghei* (192,269) and also blocked the sporozoite entry into WI38 cells and HepG2 cells (191,199,270). Sera from mice or rabbits immunised with peptides or recombinant protein containing a *P. falciparum* CSP epitope (tandem repeats) inhibited *P. falciparum* entry in HepG2 cells (271,272) and in primary human hepatocytes (200). The same effect was observed with *P. berghei* sporozoites expressing *P. falciparum* CSP treated with mouse post-vaccination sera (against CSP repeats) and added to HepG2 cells (273). Monoclonal antibodies from a volunteer vaccinated with RTS,S protected mice with humanised liver against *P. falciparum* challenge and avoided sporozoite entry in HepG2 and Huh7 cells (264). Post-vaccination sera from some volunteers vaccinated with recombinant protein R32tet (containing a repeating region from CSP) blocked *P. falciparum* entry in HepG2 cells (274,275). With regard to TRAP, post-vaccination sera from mice immunised with

recombinant proteins of *P. falciparum* TRAP fragments inhibited *P. falciparum* sporozoites invasion in cultures of primary human hepatocytes (225) and in HepG2 cells (276). Charoenvit et al. found that sera from mice immunised with peptides from TRAP partially inhibited *P. falciparum* invasion of HepG2 cells (277). In other study, immunisation or passive transfer of monoclonal or polyclonal antibodies against certain TRAP epitopes did not protected mice from *P. yoelii* infection (278).

CSP and TRAP are the antigens which have been more extensively studied as pre-erythrocytic malaria antigens. Therefore, is not surprising that previous studies on the capacity of antibodies to prevent hepatocyte invasion focus mainly on CSP and TRAP. It is extremely complicated to compare inhibition results obtained by different studies. Apart from the variability that could be associated to the researcher performing the experiment, the assay used in different laboratories present many variations: cell type, strain of sporozoites, times of incubation, use of purified antibodies or whole sera or method of readout. In addition, serum against a particular antigen is obtained in each study following different vaccination strategies (delivery method, epitopes targeted, adjuvants, dose…), which is likely to affect the concentration of specific antibodies in sera and their functionality. Therefore, it has not been attempted to make any quantitative comparison between results described here and those reported in other studies.

Mouse samples: screening studies

In this work, serum samples from mice vaccinated against a number of candidate pre-erythrocytic antigens were tested following the same ISI protocol, which provided some suggestions of which antigens may had a greater capacity of generating antibodies with better functionality to inhibit sporozoite invasion. Two sets of mouse samples obtained in two screening studies were assessed, one included eight antigens: CeLTOS, LSA1, LSA3, LSAP1, LSAP2, ETRAMP5 and Falstatin; and the other, four candidates: SPECT1, SPECT2, RP-L3 and HT. In the first group of antigens, CelTOS elicited the highest blocking response, inhibiting almost 70% of sporozoite invasion. anti-LSAP1 serum blocked about 50% of the infection, but the rest of the samples did not show inhibition over the threshold of 30%. Consistent with these results, sera from mice immunised with recombinant CelTOS has previously been described to block *P. falciparum* sporozoite entry in HepG2 cells (187). In contrast to these current results, Siau et al. found that anti-LSAP1 mice serum did not block *P. falciparum* invasion of primary hepatocytes (232), but this effect was described using a

lower serum dilution (1% and 5%) and in any case, the inhibition observed here for this antigen was not strong. It has also been described that mouse sera against *Pb* ICP (*P. berghei* Falstatin homolog, which share 40% amino acid sequence identity) blocked about 40% of *P. berghei* invasion of HepG2 cells at 20% dilution (247).

In the second screening study, almost 100% blocking was obtained for all the serum samples at 10% serum dilution, potentially because these samples have only been thawed once and were stored for less than one month at -20°C before performing the ISI assay. In contrast the samples used in the first screening study had been frozen and thawed a number of times and stored for about two years at -20°C (which could have affected protein degradation or favoured the lost of functional antibody conformation). Therefore, the best concentration to use in the assay may be dependent on the particular characteristics and quality of the serum samples, in fact, for the samples from the first screening positive blocking responses where only observed at 10% serum dilution, while for the second study at the same concentration the blocking responses were close to saturation and it was not possible to compare between antigens. The most significant finding of this experiment, was the high blocking response obtained by anti-SPECT1 serum at 2% serum dilution (87% mean infection blocked), which was equivalent to the blocking response observed for anti-CS serum (82% mean infection blocked). CS is the target of the most advanced malaria vaccine in clinical trials: RTS,S, which induces an antibody response associated with protection (140,264,279). Pre-erythrocytic antigens, such as SPECT1, that could elicit strong humoral responses could be included with CS in a vaccine to achieve higher levels of sterile efficacy.

Using chimeric sporozoites in the ISI assay enabled the testing of *P. falciparum* antigens in a *P. berghei* model, regardless whether the protein was or was not originally present in *P. berghei* sporozoites (all the antigens tested in this work have a *P. berghei* homolog except: LSA1, LSA3, LSAP1, LSAP2 and ETRAMP5). To evaluate the role of the *P. berghei* protein on the observed inhibition, serum samples were tested with *P. berghei* sporozoites that did not express any *P. falciparum* antigen. In this system, anti-CelTOS serum samples also elicited high blocking responses, which is not surprising since there is a high degree of homology between *P. falciparum* CelTOS and *P. berghei* CelTOS (187). However, it was not expected to find a high blocking response to LSAP1 that does not have a *P. berghei* homolog (232). It is possible that the serum contained factors that caused a non-specific inhibition response or the anti-LSAP1 antibodies cross-reacted with other *P. berghei* protein. In the second screening study, where all the four antigens tested had *P. berghei*

homologs, a higher blocking capacity was detected with chimeric sporozoites expressing *P. falciparum* antigens than with WT *P. berghei*, likely due to a better interaction between the specific antibodies and the actual proteins they are targeting.

IFAT staining was performed to gain a better understanding of the ability of antibodies to bind to sporozoites. Surprisingly, the best IFAT signals were not obtained with the candidates that presented the best blocking effect within each group: CelTOS and SPECT1. UIS3 showed one of the best IFAT staining in the first study, together with high signals from LSA1, LSAP1 and Falstatin. In a similar way, SPECT2 showed better staining than SPECT1. Therefore, the lack or low concentration of antibodies is not the only cause of absent or low blocking responses found for some antigens. These discrepancies between IFAT and ISI, may be due to protein accessibility and/or protein function. *P. falciparum* proteins not exposed on the surface of live sporozoites may not be accessible to specific antibodies in ISI assay, while in IFAT staining, these internal proteins become accessible since the sporozoites are fixed to the slide. Serum samples from mice vaccinated against antigens whose function has been mainly related with parasite development within the hepatocyte such as LSA1, LSAP1, LSAP2, UIS3, RP-L3 and HT showed reduced or absent blocking response. Apart from antibody accessibility to antigen, a low blocking response could be due to a non-sufficient antibody level or to a weak interaction, but also, to a lack of function of the target protein in the process of hepatocyte invasion. It could be hypothesised that antibodies that bind proteins which play a role on hepatocyte invasion may be more likely to have an effect on the blocking of sporozoite entry in liver cells. Nevertheless, it is important to bear in mind that the ISI has been performed using chimeric *P. berghei* sporozoites expressing *P. falciparum* antigens and no definitive statements regarding functionality of proteins can be made, using *P. falciparum* sporozoites would be a more appropriate model.

It is interesting to point out that outstanding blocking responses compared with other antigens were observed for serum samples obtained from mice vaccinated against TRAP, CS, CelTOS and SPECT1. As it has been mentioned in the introduction to this chapter, these antigens can be found on the sporozoite surface and micronemes (secretory organelles that contain proteins released upon host cell contact to the sporozoite surface). Microneme proteins, move on the sporozoite surface from the apical end of the parasite to the posterior end supporting sporozoite motility, therefore, these proteins would be exposed to specific antibodies present in the serum samples tested. TRAP, CS, CelTOS and SPECT1 have been associated with functions such as sporozoite motility, cell traversal, hepatocyte attachment and invasion, which could

presumably be interfered by bound antibodies, impairing the process of sporozoite entry into the hepatocyte. For example, CelTOS and SPECT1 are involved in breaking through host barriers, but they do not seem to play a role on hepatocyte invasion since *P. berghei* parasites which had these proteins disrupted infected HepG2 cells (236,248). However, even though the protein targeted by specific antibodies do not play a role at the moment of invasion, the binding of specific antibodies could immobilise the sporozoite, preventing further steps even in an *in vitro* assay where the sporozoites are added directly on the hepatocytes. In fact Bergmann-Leitner et al. showed that CelTOS antibodies interfered with sporozoite motility (187), Stewart et al. reported that anti-CSP antibodies immobilised sporozoites (270) and Okitsu et al. described a positive correlation between migration inhibition and invasion inhibition (280).

Mouse samples: R21 vaccine

Following the screening studies experiments with the ISI assay, a set of experiments were performed with serum samples from mice that had been vaccinated with R21, a virus like particle vaccine which targets *P. falciparum* CSP. Two experiments were conducted: one to know whether the adjuvant employed in R21 vaccine delivery had an effect on antibody functionality to prevent infection of hepatocytes and the other, to compare the functionality between antibodies elicited by R21 vaccine and RTS,S vaccine. In the adjuvants experiment, serum samples from mice vaccinated with R21 administered with Alhydrogel showed a lower blocking response than other adjuvants at 0.2% serum dilution, this difference was statistically significant between Alhydrogel and Abisco. Vaccination with R21 in two other adjuvants, AddaVax and Abisco, demonstrated similar ELISA antibody titres, but Abisco vaccinated mice were sterilely protected while AddaVax only elicited a delay to parasitaemia [Katharine Collins, DPhil Thesis]. ISI assay did not detect differences in antibody responses after vaccination with AddaVax or Abisco, which is consistent with the ELISA results. Abisco vaccinated mice showed higher T cell response by ELISPOT than AddaVax mice, and as the humoral responses were similar in terms of titre and functionality evaluated with ISI assay, it seems that the protection achieved by R21+Abisco may be enhanced with a cellular immune response. This example shows how ISI assay can help to gain a better understanding of the immune mechanisms working on a pre-erythrocytic malaria vaccine.

In the second experiment, there was no significant difference between the capacity to inhibit sporozoite invasion by the serum samples from mice vaccinated with

RTS,S and those vaccinated with R21, showing that R21 elicited an antibody response with an invasion blocking capacity as good as RTS,S, measured by ISI. It would be interesting to measure with ISI serum samples from later time points, to determine whether either vaccine is capable of maintaining antibody functionality and preventing hepatocyte invasion.

Interestingly, high levels of inhibition were only achieved with these R21 samples when the chimeric *P. berghei* sporozoites used in the assay expressed only *P. falciparum* CSP (replacement parasite), not as an additional copy to *P. berghei* CSP (addition parasite). This observation was surprising since serum samples obtained from mice vaccinated with viral vectors targeting *Pf* CSP showed a high blocking effect with chimeric addition CS parasites, and clinical trial samples did not show a better blocking when using replacement CS parasites. It seemed that the use of replacement sporozoites with ISI could provide an advantage only for certain anti-CSP serum samples. According to ELISA, R21 serum samples had a high CS antibody titer, and, in fact, positive blocking responses were observed even at 0.2% serum dilution. The cause of the differential behaviour observed with replacement and addition chimeric parasites is not clear, but could be related to the high specific antibody concentration in R21 samples or the avidity of these antibodies. A very strong antibody binding to *Pf*CSP could cause shedding of the protein (known to occur naturally on sporozoite invasion), while the chimeric addition sporozoite would still conserve CSP functionality from the *P. berghei* protein.

Addition and replacement sporozoites

CSP was the only antigen with a chimeric replacement parasite available, so for all the other antigens tested, chimeric addition parasites were always employed. The presence of the *P. berghei* protein could reduce the blocking effect, if antibodies could bind the *P. falciparum* antigen but leaving free the *P. berghei* homolog to perform its normal function. The decrease of the blocking effect observed with human post-vaccination anti-TRAP serum samples could be related to the use of the chimeric TRAP addition parasite. It would be interesting to evaluate these samples again with ISI assay using a TRAP replacement chimeric sporozoite, when it becomes available.

Replacement sporozoites would constitute a cleaner model to study antibody function of sporozoite invasion, since antibodies are targeting a single sporozoite protein. When both the *P. falciparum* and *P. berghei* versions of the protein are available, the specific antibodies may be targeting both proteins or only one of then, and the amount of expression of the protein of interest may be overrepresented.

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ISI compared with other antibody assays

To evaluate the effect of antigen specific antibody concentration on the inhibition of sporozoite invasion, the values obtained with the ISI assay were compared with ELISA or LIPS data, both of which provide a measure antibody concentration. ELISA is an assay commonly used to obtain the titre of antigen specific antibodies present in a serum sample and LIPS, which also evaluates antibody concentration, was used when purified protein was not available. For some of the experiments, particularly those with large numbers of samples, a statistically significant correlation was found between ISI and ELISA or LIPS values, such as the study of anti-CS serum from R21 vaccinated mice, anti-TRAP macaque pre- and post-vaccination serum samples or one of the experiments testing CS clinical samples. Although not significant, a trend towards a larger blocking effect was observed with serum samples with higher ELISA values.

Previous studies have usually sought a correlation between ISI and ELISA, rather than ISI and LIPS, which is an assay that has been introduced later and it has been less used. While no definitive answer can be given about ELISA correlation with ISI assay from this current study, this is consistent with previously published data where both significant correlation (280–282) and the absence of correlation (206,274) have been described.

Taking all the data together in this study, ISI assay has been shown to be concentration dependent, with higher blocking of sporozoite invasion observed when using a higher concentration of monoclonal antibody or a higher percentage of serum dilution. Therefore, it is not surprising to find a correlation for ISI and ELISA values. Nevertheless, several factors could cause discrepancies between an assay focused on evaluating antibody concentration, measuring antigen-antibody interaction, and an assay like ISI. For example, there could be differences in the conformation of the antigen expressed in the sporozoite compared to purified antigen used in ELISA, or antigen obtained from transfected cell lysates used in LIPS. In the ISI assay, antigens targeted by specific antibodies may not be exposed on the sporozoites or may not play a role on sporozoite invasion, and therefore, even if antigen specific antibodies are present in the serum sample, they will not be detected. It is likely that correlation of ISI with ELISA or LIPS will be dependent on the characteristics of the antigen (expression, localisation and function) targeted by the antibody evaluated, the vaccination strategy which may enhance a certain kind of immune response, the range of serum dilution employed in the ISI assay and other factors dependent on the specific antibody like its fine specificity and isotype.

With regard to the antibody isotype, the relationship between the blocking response and the isotype was studied for CS and TRAP clinical trials samples. No statistically significant correlation was found for any isotype, probably due in part to the low number of samples (5-10 per experiment). In the literature, positive correlations have been described between sporozoite inhibition capacity and IgG levels (100,199), and both IgG and IgA (283).

It could be considered that ISI assay may offer a better model to evaluate humoral responses than ELISA or LIPS, due to its higher degree of similarity with an *in vivo* system. On the other side, ISI assay is focused only on one step of the malaria cycle, and measures antibody functionality to block this specific stage, but not the effect that antibodies could have on a different site of the cycle.

ISI correlation with protection

In the first screening study on mice whose samples were analysed with ISI, the highest protection for BALB/c mice had been observed when mice were vaccinated against LSA1 or LSAP2 (in both cases of 87.5% mice were protected) and was shown to be mediated primarily through CD8$^+$ T cells (190). CelTOS vaccination did not induce sterile protection or even a delay in parasitaemia, even though it was the antigen that generated a higher blocking response in the ISI assay. Antibodies against CelTOS may had not reached a minimum level to generate *in vivo* protection with this vaccination regimen.

In the second screening study, vaccination with SPECT1 vaccines conferred both sterile protection (37.5% BALB/c and 70% CD1) and a significant delay to 1% parasitaemia, no protection or delay was described for HT, nor RP-L3, and unfortunately, protection data for SPECT2 was not available [Ahmed Salman, DPhil Thesis]. The protection achieved by SPECT1 was even higher than conferred by CSP vaccination (31.25 BALB/c and 33.3% CD1), and both candidates showed high blocking responses in the ISI assay.

Serum samples from mice vaccinated with either R21 or RTS,S (both vaccines targeting CSP), were tested in the ISI assay where some protected mice showed lower inhibition levels than unprotected animals. The percentage of infection blocked significantly correlated with a delay in developing parasitaemia at 2% serum dilution, but not at 0.2% where the differences between samples were better distinguished (at 2% serum dilution the assay was probably working on the saturation limit).

The most interesting finding regarding the relation between ISI assay and protection was a statistically significant correlation between the increase of the blocking response in UK adults vaccinated against CS with the day of diagnosis and with the parasitaemia at day 7.5 [Figure 2.20]. Samples from the same study were evaluated using addition and replacement *Pf* CSP chimeric parasites and statistically significance was only observed with addition sporozoites, although the same trend was also detected with replacement parasites, probably due to the low number of samples. Although the immune response elicited after vaccination was not sufficient to confer protection, it seemed that a higher level of functional antibodies reduced the liver burden and resulted in a pre-patent blood-stage period and later day of diagnosis. However, no correlation was found with serum samples from TRAP clinical trials with protection, day of diagnosis or parasitaemia.

Previous studies that have tested human or animal serum samples with a functional antibody assays have in some cases supported a correlation with protection (100,187,198,275,284,285), but in others no correlation in terms of resistance to malaria or delay has been found (226,268,274,286,287). The small number of samples studied has limited the statistical significance of the results found. However, the cellular immune response not evaluated in the ISI assay and may be playing a key role in protection. In addition, the humoral response may carry out its functions in a way that is not evaluated by this kind of assays, such as opsonising activity (110) or it may even take place before reaching the liver, for example retaining the sporozoites in the skin (288). On other hand, targeting a particular antigen, or the particularities of vaccine delivery such as dose or adjuvant, can also have an effect on the immune response generated, making it difficult to compare between different studies.

SUMMARY AND CONCLUSIONS

Chapter 1 described the process to optimise a protocol for a flow cytometry based Inhibition of Sporozoite Invasion assay. The main criteria observed was maximising the infectivity rates, in order to increase the number of positive events acquired. The "percentage of infection blocked" was calculated using the percentage of infected cells, which if it was based in a higher number of events, would increase the reliability and reproducibility of the data. When comparing several cell lines and culture media, HuH7 cell line cultured in RPMI were selected since higher infectivity rates were observed with this combination.

Other parameters were evaluated, such as pre-incubation of sporozoites with antibodies which appeared to be unnecessary; and the temperature of the first hour of incubation (room temperature or 37º) where no differences were observed and it was decided to place the plate containing the co-culture in the incubator after infection and centrifugation. With the objective of testing as many samples as possible with the number of sporozoite available, several cell:sporozoite ratios were tested, finally deciding to plate 30000 cells and 15000 sporozoites per well in 96-well plates. These numbers did not have a detrimental effect on sensitivity, generated an acceptable number of positive events and allowed the use of replicates (duplicates or triplicates).

A time course was performed to determine the best time of sample acquisition on the flow cytometer. Hepatocytes infected with transgenic parasites expressing GFP were detectable by flow cytometry much earlier (4 hours and less after infection) than those whose reporter was GFP fused to luciferase which were better detected between 24 and 28 hours post-infection. It is essential to perform a time course every time a different reporter protein is being used in a transgenic parasite, because infected cells may not been detected at a certain time point due to low levels of reporter protein expression.

Since non-specific sera inhibition contributed to a significant background effect, it was set a threshold level of 30% above which blocking was considered positive. This value was chosen after observing the behaviour of naive serum samples at 2% and 10% serum concentration. For human and macaque samples, due to the important effect of background immune responses in each subject, pre and post-vaccination sera was tested for each individual. In addition, due to the high variability in the assay (caused by error in the amount of cells, sporozoites and serum plated and by variations in sporozoite viability and infectivity), differences between blocking values within 10-

15% were not considered relevant. With regard to normalisation of variations due to changes in infectivity rates in each particular experiment, the percentage of infected cells achieved in wells without serum (calculated as the average of three or more replicates) was used to calculate the "percentage of infection blocked", which was considerably consistent between experiments.

In Chapter 2, the optimised protocol proved to be useful to test mouse, macaque and human serum samples using *P. berghei* transgenic parasites expressing *P. falciparum* antigens. It is specially relevant the high throughput capacity of the assay: performing the assay for all the samples in a vaccination group would take usually one day (depending on the size of the group of samples, usually 6-8) and the time to acquire samples on the flow cytometer the following day.

The transgenic parasites expressing *P. falciparum* antigens and a reporter protein (GFP fused to luciferase) were first developed in order to establish a challenge system to test protection elicited after vaccination with viral vector vaccines including the *P. falciparum* antigens. Without the need of purified protein to perform ELISA, this same transgenic parasites already available in the laboratory, provide also a tool to evaluate humoral responses.

In studies with mouse samples, the assay proved to be useful comparing the functional capacity of antibodies generated against different antigens and with different vaccination strategies, such as the adjuvant or vaccination dose. A positive increase of antibody functionality after vaccination was shown for some macaque and volunteer samples, and interesting correlations were found for ISI assay data and other antibody assays and clinical data.

With regard to the results obtained with mouse samples, high blocking responses were detected for the antigens: CeLTOS, CSP, TRAP, SP1, SP2, RP-L3 and HT. The inhibition of sporozoite invasion was particularly higher in CSP and SP1, with more than 80% of sporozoite infection blocked only at 2% serum concentration. The finding that SP1 could generate antibody blocking responses similar to CSP, which is the antigen included in RTS,S, together with the protection elicited upon challenge, supports the further progress of a vaccine including SP1.

In other study, higher blocking responses were elicited when R21 (virus like particle vaccine which targets *P. falciparum* CSP) was administered with the adjuvant Abisco compared to Alhydrogel (a result consistent with previous ELISA data). R21 vaccine induced an antibody response with an invasion blocking capacity as good as RTS,S. An important concern raised when evaluating R21 serum samples, was that

high blocking responses were only achieved when the transgenic *P. berghei* sporozoites used in the assay expressed *P. falciparum* CSP instead of *P. berghei* CSP (replacement parasites), and not with those expressing *P. falciparum* CSP as an additional copy (addition parasites). Although the cause of these differences is not clear, it is relevant to point that the design of transgenic parasites can have important consequences when evaluating antibody functionality with ISI assay.

Significant correlation was found between ISI and ELISA values with: R21 samples, serum samples from macaques vaccinated against TRAP and with human anti-CSP serum samples (blocking responses were significantly increased after vaccination in all these studies). Although this association was not always found, and no definitive statement can be made, it is not surprising that correlations can be established with data obtained by ELISA since ISI assay is also concentration dependent.

ISI assay data for UK adults vaccinated against CSP significantly correlated with the day of diagnosis and parasitaemia at day 7.5. In the same way, SP1 vaccinated mice, which were sterile protected or presented a delay to 1% parasitaemia, showed high blocking capacity measured by ISI. However, for instance, in RTS,S and R21 study some protected mice showed lower inhibition levels than unprotected animals. Similarly, from the potential candidates evaluated in mice studies, the highest protection in BALB/c mice was shown for LSA1 or LSAP2, which did not present outstanding blocking data. Discrepancies between ISI assay and other immunological and clinical data can be related to the presence of various immune mechanisms involved in protection, for example, LSA1 and LSAP2 vaccines primarily induced protection through CD8^{+} T cells. In fact, ISI assay would only be an important indicator of *in vitro* protection in vaccines which elicit high antibody responses that prevent sporozoite invasion. Nevertheless, other vaccines, even those mainly targeting cellular responses, also induce humoral responses whose role in host immunity to malaria is important to characterise.

Future directions:

The most important limitation for the ISI assay is to obtain live sporozoites, since if mosquito yields (which vary between mosquito batches) are low, to collect the required amount of sporozoites can be time-consuming or not enough sporozoites may be available to perform the assay. For this reason, any technical improvement facilitating mosquito salivary gland dissection (which needs specialised training and

practice) and research towards achieving higher sporozoite yields in mosquito salivary glands would greatly benefit the assay. Testing a larger number of samples would provide more relevant statistic information on potential correlations between ISI assay and other antibody assays and clinical data. In addition, establishing a standardised protocol for the assay, would allow data comparison between studies and research centres.

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