The role of the spleen in mediating pathology in Plasmodium Yoelii (P.yoelii) infection

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**THE ROLE OF THE SPLEEN IN MEDIATING
PATHOLOGY IN *PLASMODIUM YOELII*
(*P. yoelii*) INFECTION**

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Abstract

Differences in the ability of the spleen to deal with the infected red blood cells (iRBCs) are linked to differences in virulence. Using virulent and avirulent *Plasmodium yoelii* strains, we investigated how parasite virulence modulates overall spleen function. Following parasite invasion, a difference in parasite virulence and the corresponding disease outcome was observed to associate with different spleen morphology, immune response and iRBC rigidity, all of which contributing to enhanced parasite clearance. The iRBC rigidity as modulated by the spleen was demonstrated to regulate disease outcome. Moreover, the early activation of pro-inflammatory responses in the spleen appears to help to control the parasite development, confirming that early spleen responses are a key factor in directing the clinical outcome of an infection. This work highlights the biological responses to control malaria disease development, and also provides a potential tool for fast and easy diagnosis and prognosis of malaria patients.

ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
ANOVA	ANalysis Of Variance
APS	Ammonium persulfate
BSA	Bovine serum albumin
CaNa ₂ EDTA	Calcium disodium versenate
CCL	C-C motif chemokine
CR1	Complement receptor 1
cRPMI	Complete RPMI
CSF1R	Colony Stimulating Factor 1 Receptor
CXCL	C-X-C motif chemokine
DC	Dendritic cell
DMSO	Dimethyl Sulfoxide
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Experimental cerebral malaria
ef1	Elongation factor 1
ELISA	Enzyme-Linked Immunosorbent Assay
FACs	Fluorescence-activated cell sorting
gDNA	Genomic DNA
GDP	Gross domestic product
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
hr	Hour
HSD	Honest Significant Differences
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular adhesion molecule 1
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP	Intraperitoneal injection
iRBC	Infected red blood cell
iRPMI	Incomplete RPMI
IRS	Indoor residual spraying
ITNs	Insecticide-treated mosquito nets
IV	Intravenous injection
KCl	Potassium chloride
KH ₂ PO ₄	Monopotassium phosphate

MARCO	Macrophage receptor with collagenous structure
M-CSFR	Macrophage Colony-Stimulating Factor Receptor
min	Mins
MLE	Maximum Likelihood Estimation
MSP1	Merozoite surface protein-1
Na ₂ HPO ₄	Disodium phosphate
NaCl	Sodium chloride
NACLAR	National Advisory Committee for Laboratory Animal Research
NaHCO ₃	Sodium bicarbonate
NK cell	Natural killer cell
<i>P. berghei</i>	<i>Plasmodium berghei</i>
<i>P. chabaudi</i>	<i>Plasmodium chabaudi</i>
<i>P. cynomolgi</i>	<i>Plasmodium cynomolgi</i>
<i>P. inui</i>	<i>Plasmodium inui</i>
<i>P. knowlesi</i>	<i>Plasmodium knowlesi</i>
<i>P. malariae</i>	<i>Plasmodium malariae</i>
<i>P. ovale</i>	<i>Plasmodium ovale</i>
<i>P. schwetzi</i>	<i>Plasmodium schwetzi</i>
<i>P. simian</i>	<i>Plasmodium simian</i>
<i>P. vinckei</i>	<i>Plasmodium vinckei</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
<i>P. yoelii</i>	<i>Plasmodium yoelii</i>
<i>P.falciparum</i>	<i>Plasmodium falciparum</i>
PALS	Periarteriolar lymphoid sheath
PbA	<i>Plasmodium berghei</i> ANKA
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PF4	Platelet factor 4
PfEMP1	P falciparum Erythrocyte Membrane Protein 1
PHZ	Phenylhydrazine-HCl
RA	Retinoic acid
RBC	Red blood cell
RT-PCR	Reverse transcription polymerase chain reaction
SI	Selectivity Index
SIGLEC-1	Sialic-acid-binding immunoglobulin-like lectin 1
SIGNR1	Specific intercellular adhesion molecule-3-Grabbing non-integrin R1
SPF	Specific pathogen free

<i>ssurra</i>	Small subunit of ribosomal RNA
STEVOR	Subtelomeric variable open reading frame
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
Tris-HCl	TRIS hydrochloride
uRBC	Uninfected red blood cell
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter 1

Introduction

1.1 Overview of malaria

1.1.1 Malaria: a top global health concern

Malaria is a mosquito-borne disease that is caused by parasitic protozoan of the genus *Plasmodium*. It is transmitted through female Anopheles mosquitoes. It has been affecting human for over 50,000 years [1], yet it remains a public health threat with about half of the world's population—3.3 billion people still being at risk of malaria [2]. According to the most recent World Health Organization (WHO) record, there were 96 countries and territories still having ongoing malaria transmission [3]. Despite the achievements and progress to treat and prevent malaria in the past decade, there are still about 214 million cases with an estimated 438,000 death reported in 2015 [3]. Among these cases, 88% of all malaria cases and 90% of all malaria deaths occurred in sub-Saharan region, mostly among the children below five years old [3]. In fact, malaria claims 20% of the total childhood deaths in Africa, whereas pregnancy malaria results in 10-50% of maternal deaths and roughly 200,000 infant deaths every year [2]. Malaria is usually associated with poverty, with people living in the poorest countries being the most vulnerable to malaria. But correlatively it is also a cause of poverty and major hindrance for economic development with an estimated cut of 1.3% in gross domestic product (GDP) and nearly 40% cost of public health expenditures [2].

There are approximately 70 out of more than 500 species of *Anopheles* that have the capacity to transmit human malaria parasites, with 41 of these being considered to be the dominant vector species [4]. About 200 species of *Plasmodium* have been described to infect various hosts ranging from birds and reptiles to mammals [5, 6]. Among these, there are six known species that can infect humans naturally, namely *Plasmodium falciparum* (*P. falciparum*), *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*

and the very recently reported *P. cynomolgi* [7-9]. Among these species, *P. falciparum* and *P. vivax* pose the greatest threat. *P. falciparum* is the most deadly species that has the highest rates of complications and mortality, accounting for more than 95% of malaria deaths [10]. However, in *vivax*-endemic areas, *P. vivax* is responsible for the major cause of morbidity. A large number of severe or fatal cases caused by *P. vivax* have been reported from different areas such as Indonesia and Papua New Guinea [11, 12]. Moreover, though there is currently no evidence of human-to-human transmission for *P. knowlesi* and *P. cynomolgi*, they constitute a potential threat to human health. They are non-human primate parasites and their natural hosts are often in close contact with humans particularly in the forest. Other non-human primate parasites including *P. inui*, *P. simian* and *P. schwetzi* have also been recorded to possess the ability for human infection [13-15]. Indeed, *P. knowlesi* has been shown to intimidate human in multiple countries [8, 16, 17].

1.1.2 Malaria symptoms, treatments and preventions

Malaria is an acute febrile illness that initially causes relatively mild symptoms like fever, headache, chills and vomiting in a non-immune individual. The onset of malaria usually takes place within several days (usually 10-15 days) following an infective mosquito bite, but longer incubation period may occur [18]. Moreover, the disease condition may rapidly progress to severe illness if no treatment is received [18]. Malaria can be broadly categorized as either uncomplicated or severe (i.e. complicated). Patients with the severe forms of the disease develop one or more of the symptoms including hemoglobinuria with renal failure, pulmonary oedema, hypoglycaemia, anemia, cerebral malaria and metabolic acidosis that often result in death [19-22]. Therefore, more attention and rapid referral to an intensive care unit (ICU) is required in cases of severe malaria [23]. Consequently, early diagnosis and treatment of malaria is absolutely needed to reduce disease and

promote survival.

The current best available first-line treatment recommended by WHO, in particular for *P. falciparum*, is artemisinin-based combination therapies (ACTs). Artemisinin, also known as *Qinghaosu* in Chinese, is a traditional Chinese medicine used in treatment of various illnesses for thousands of years [24]. Artemisinin and its derivatives target the parasitic asexual blood stage to reduce parasite loads as well as disease symptoms. It is currently highly effective. However, artemisinin resistant parasites have been detected in recent years in 5 countries of the Greater Mekong subregion [3]. In addition, despite efficient and rapid acting antimalaria drugs, cerebral malaria and respiratory distress in relation to metabolic acidosis still have a mortality of 15-20% [25]. An alternative antimalaria treatment is needed before full artemisinin resistance develops and spreads to larger geographical areas, and also to reverse the complications of malaria infections.

Malaria transmission depends on factors related to the parasite, the human host, the vector and the environment. Malaria transmission is more intense in places where the mosquito lifespan is long and the climatic conditions are suitable for mosquito survival [18]. On the other hand, it has also been suggested that parasite virulence, defined as the damage to the host caused by infection, is positively related to the transmission rate [26]. The differences in parasite virulence can be attributed to a number of factors including the parasite genetic factor, which is often closely linked to malaria severity [27]. In fact, *Plasmodium* parasites display significant variation in their virulence. For example, *P. ovale* and *P. malariae* are avirulent whereas *P. falciparum* and *P. knowlesi* can trigger severe disease conditions that can kill the host [28-30]. Moreover, there are significant differences in replication rate of *Plasmodium* parasites, which contribute to virulence. For example, rodent malaria parasite lines typically replicate every 20-24 hours (hr) while human malaria parasite lines have a life-cycle of 48 hr for *P. falciparum*, *P. vivax*, *P. ovale*

and *P. cynomolgi* and 72 hr and 24 hr for *P. malariae* and *P. knowlesi* respectively [18, 31]. The differences in malaria pathology associated with different parasite strains and species complicate disease management, and it is further complicated by the fact that in endemic areas more than 1 species of *Plasmodium* can infect an individual. On top of all these are variation in drug resistance patterns of different parasite strains across different geographic regions [32, 33]. These together with the case of parasite mixed strain infections, create particular challenges in disease management. While patients with uncomplicated diseases can be effectively treated by oral antimalarials, patients with severe conditions are recommended to be hospitalized and treated with parenteral antimalaria therapy [18]. Diagnostic tools that can predict the risk of developing severe disease could significantly reduce the cost of disease management and allow the application of the best treatment options for each case.

Besides treatment, antimalaria medicines are also employed to prevent malaria. It is recommended for travellers to prevent malaria through drug chemoprophylaxis, and for pregnant women and infants in high transmission areas through intermittent preventive treatment with sulfadoxine-pyrimethamine [18]. On the other hand, malaria vector control is the main way to reduce malaria transmission. There are two forms of vector control being broadly implemented, namely insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) with insecticides. These intervention strategies together with the application of antimalaria drugs have proven very effective especially in eliminating malaria in low-transmission areas. Since 2000, there has been a significant increase in the number of countries moving towards malaria elimination. Among the 106 countries with ongoing malaria transmission in 2000, 57 countries have achieved reductions in new malaria cases of at least 75% by 2015 [3]. In addition, malaria mortality rates have fallen by more than 25% globally since 2000 [18]. To further support this, WHO has also introduced Seasonal Malaria Chemoprevention (SMC)

during high transmission seasons in March 2012, which involves monthly courses of amodiaquine plus sulfadoxine-pyrimethamine to all children below 5 years old. This acts as an additional prevention strategy for areas of the Sahel sub-Region of sub-Saharan Africa [18].

1.1.3 Malaria biology and rodent malaria model

Human malaria parasites have a complex life cycle that involves two hosts—the female *Anopheles* mosquito and a human. While the mosquito vector does not suffer from the presence of malaria parasites, the human host develops the symptoms as described in Section 1.1.2. The life cycle can be subdivided into three different stages as shown in Fig1.1. The male and female gametocytes taken up by the mosquito through a blood meal develop and multiply in the mosquito mid-gut, which is known as sporogonic stage. The sporogonic stage takes approximately 10-18 days and it has been shown to be temperature dependent [34]. The malaria-infected mosquito inoculates malaria sporozoites to a human host during another blood meal. Subsequently it goes into the exoerythrocytic stage where the sporozoites infect the liver cells and mature into schizonts within the hepatocyte which then will rupture and release merozoites. Once the merozoite released into bloodstream, it starts the erythrocytic stage with the invasion of suitable erythrocyte. Within the erythrocyte, the parasite undergoes distinct morphological changes from ring to schizont, where merozoites will form in the mature schizont stage that are released to invade another erythrocyte following schizont rupture. The asexual erythrocytic cycle is repeated with multiplication, release and re-invasion. It is responsible for the clinical manifestations. Some merozoites after invasion can differentiate into the sexual forms known as gametocytes, which can be ingested by mosquito as mentioned above, such that the life cycle continues.

better understanding of the parasite biology, malaria immunology, pathogenesis, as well as basic discovery, drug testing and vaccine development. The role of the animal model in providing biological insight continues today, being the main source of biological materials in some instances such as the research on ookinetes and zygotes, and central in the studies on invasion of hepatocytes by sporozoites [37]. Indeed, animal models have successfully complemented the research on human malaria parasites. Many of the experimental procedures used now were initially developed and established for animal malaria models [38]. Recently, there has been a debate on the relevance of the experimental animal models on human diseases such as cerebral malaria, highlighting some of the limitations of these models [39-41]. However, there are also quite a number of advantages on using animal models. It allows the study on the progressive development of an infection, and permits the investigation on specific organs to which the parasite accumulates. Animal models also provide the possibility to examine the pathophysiological processes more detailedly [42, 43]. It is true that no single species of non-human *Plasmodium* can be a perfect model of human malaria parasites; however, different species of parasites exhibit different phenotypes in different hosts that can mimic different aspects of the human diseases [44]. It is thus important to select the appropriate combination of parasite strain and host to carefully set up the experimental model. It is also essential to closely link the experimental studies to the human aspects, and extrapolate the data from animal models to human disease with caution.

The rodent *P. yoelii* model has been employed in malaria research for decades. Genomic data has shown that there is a high degree of conservation of synteny between *P. falciparum* and *P. yoelii* particularly in the regions of housekeeping genes with the exception of the telomeric and subtelomeric regions [38]. Different clones of *P. yoelii* result in very different clinical outcomes in the BALB/c mouse that mimic the human malaria diseases. For example, clones like YM and 17XL

cause a lethal infection, while YA and 17X1.1 result in a self-limiting infection that is eventually cleared by the host [45, 46]. Interestingly, these parasite lines have been characterized for their potential host cell preference by a statistic measurement termed “Selectivity Index” (SI) [47], with virulent parasites such as YM associated with a low SI indicating that the invasion is non-selective to all ages/types of red blood cells (RBCs), while avirulent parasites such as 17X1.1 with a significantly higher SI implying a strong preference to invade reticulocytes [48]. In addition, genome sequences are available for both the avirulent strain 17X1.1 and the virulent strain YM, while genetic crosses of *P. yoelii* are relatively easily to produce [49]. Gene targeting techniques are also well established in the *P. yoelii* model [50] to facilitate the study on specific gene and parasite virulence. Furthermore, the availability of many antibodies against invasion-related molecules in *P. yoelii* and their ability to grow in irradiated mice [51] make it as a good model to study the pathology and immune response for malaria.

1.2 Malaria and the spleen

1.2.1 Spleen physiology

1.2.1.1 Spleen structure

The spleen is the largest lymphoid organ that has been extensively studied in particular in regulating the immune system. Physiologically, it is an elongated dark-red organ located along the curving lateral border of the stomach in the left abdomen. It is protected by a capsule that consists of mostly collagenous and elastic connective tissue interspersed with a fair amount of smooth muscle. It is attached to the retro-peritoneum by fatty ligaments. It has a smooth serosal surface and it is roughly triangular in cross section. The gross appearance and size of the spleen vary among the species and depend on the degree of distension, with a normal healthy adult human spleen being approximately 11cm in length and 150g in weight [52]. The relative ratio of the splenic weight to body weight stays rather constant regardless of the age in one species, while changes in the splenic weight usually associate with splenic events such as immune activation. [53]. Through the observations from scanning microscopy and corrosion cast studies as well as the histological researches, the spleen architecture has been described clearly and it is comprised of two functionally and morphologically distinct compartments namely 1) the red pulp and 2) the white pulp, which are separated by 3) the marginal zone (Fig1.2).

Fig1.2. Structure of the human spleen. (a) The anterior view of the spleen with the splenic artery and vein indicated. (b) Histology of the fibrous capsule protected spleen, showing different compartments and blood vessels involved in splenic flow. (Adopted from Laboratory investigations in anatomy & physiology)

1. Red pulp

The red pulp is the major part of the spleen in human that is responsible for 70-80% of the volume of the whole spleen [54]. It is comprised of a three dimensional meshwork of connective tissue known as splenic cords and many splenic venous sinuses. The splenic cords, also called cords of Billroth, are open spaces that lack an endothelial lining. They consist of highly active macrophages along with reticular fibers and reticular cells that are of fibroblast origin, and the reticular fibers are ensheathed by the reticular cells [55]. The reticular fibers and reticular cells make up the red pulp meshwork, which is also named reticular meshwork. The spaces between the cords are filled with blood particles and cells including hematopoietic cells, erythrocytes, granulocytes, monocytes and activated lymphocytes as well as plasma cells and plasmablasts after antigen specific differentiation [56]. These cells within the spaces and the actively phagocytic red pulp macrophages facilitate the immune surveillance of blood-born materials as well as old and damaged erythrocytes. There is an addition region named

perifollicular zone in the human spleens, which is absent in mice. It forms part of the red pulp, but phenotypically it is distinct from the remainder of the red pulp. There are certain antigens including thrombomodulin intensely expressed in the perifollicular sinus endothelia [57]. Since cellular components of the blood are concentrated in the spleen, the reticular meshwork is filled with blood of very high hematocrit. The red pulp is intimately associated with the venous system draining the spleen. The blood passes from the cords into the venous sinuses, which is then collected into the trabecular vein. The venous sinuses, also known as sinusoids, are small vessels lined by endothelium with an unusual discontinuous structure. There are stress fibers running parallel to the cellular long axis of the endothelial cells and they extend underneath the basal plasma membrane, connecting the endothelium to the extracellular matrix [58]. These stress fibers form the interendothelial slits that facilitate the blood filtration, and the contractility of the stress fibers may help in the retention of the erythrocytes in the spleen [59], therefore contributing to the splenic blood storage.

2. White pulp

Unlike the red pulp that contains large quantities of RBCs, the white pulp of the spleen holds the majority of immune effector cells including lymphocytes, macrophages and dendritic cells. It predominates the mouse spleen and it can be divided into periarteriolar lymphoid sheaths (PALS) and follicles, which are also known as T-cell zones and B-cell zones respectively. PALS consist of two layers—the inner layer PALS are a T-cell dependent region that is more prominent in T cell quantity than the outer PALS. The T-cells of inner PALS are largely CD4⁺ T cells with a small number of CD8⁺ T cells and interdigitating dendritic cells (DCs) as well as migrating B cells may be present [60]. On the other hand, the outer PALS contain small and medium lymphocytes including both T-cells and B-cells, and they are important sites for transiting B cells to develop an adaptive

immune response [61]. The B-cell zone organized in follicles is continuous with the PALS and is composed primarily of B-cells with few follicular dendritic cells and CD4⁺ T cells but no CD8⁺ T cells. Follicles may contain germinal centers where upon antigenic stimulation, mature B cells undergo proliferation and differentiation to produce antibodies as well as antibody isotype switching [62]. It has been shown that the organization and maintenance of the proper white pulp compartments is controlled by specific chemokines that attract T and B cells respectively, thereby forming the specific zones. The C-X-C motif chemokine 13 (CXCL13), also known as B lymphocyte chemoattractant, is required for B cell migration to the B-cell follicles as indicated by its name [63]. Nevertheless, it has been shown that C-C motif chemokines 19 (CCL19) and CCL21 are involved in the T-cell zone formation by attracting the T cells and dendritic cells [64].

3. Marginal zone

The red pulp and the white pulp are separated by the marginal zone, which is sometimes considered as an isolated compartment rather than part of the white pulp. The marginal zone is an important transit area for the cells that are leaving the bloodstream and entering the white pulp, which was shown to be an active process involving G-protein-coupled receptors signalling [65]. It is a rim like structure that consists of marginal sinus, reticular fibroblasts, dendritic cells, marginal zone B cells as well as some specific subsets of macrophages. Being at the interface, these specific subset of macrophages have specific locations, with the marginal zone macrophages positioned at the outer boundary of the marginal zone adjacent to the red pulp while the marginal metallophilic macrophages are present at the inner boundary of the marginal zone near the white pulp border; while other cells like B cells and dendritic cells are in between [66]. They are important in facilitating the marginal zone to screen and remove the circulating pathogens [56], with depletion of marginal zone macrophages and marginal metallophilic

macrophages resulting in an absence of trapping of particulate antigen in C57BL/6 mice infected with *Listeria monocytogenes* [67]. These two specific subsets of macrophages differ by the expression of some molecules and receptors. The marginal zone macrophages are characterized by the type I scavenger receptor MARCO (macrophage receptor with collagenous structure) and the C-type lectin SIGNR1 (specific intercellular adhesion molecule-3-Grabbing non-integrin R1, also known as CD209b), while marginal metallophilic macrophages are featuring in the adhesion molecule SIGLEC1 (sialic-acid-binding immunoglobulin-like lectin 1) [68-70]. However, in contrast, it has been indicated that there are no marginal metallophilic macrophages in the human spleens, and the macrophages present in the human marginal zone do not exhibit any special phenotype distinct from that of the red pulp macrophages [57]. Also, the marginal sinus is absent in the human spleens, resulting in a direct contact of the marginal zone and the follicles [71]. The differences in the follicular periphery potentially lead to the variation of marginal zone B cell phenotypes. The marginal zone B cells in human represent recirculating cells [57]. On the other hand, the marginal zone B cells in mice form a unique subset of noncirculating B cells with a phenotype of IgM⁺/IgD⁻, which is different from the white pulp follicular IgM⁺/IgD⁺ B cells [60], and they are involved in T-cell-independent responses [72]. The marginal zone also contains a variety of trafficking populations including dendritic cells and T cells. In fact, the marginal zone directs their traffic to the white pulp [73], and T cell trafficking has been shown to go through the fibroblast channels without crossing an endothelium [74] .

1.2.1.2 Splenic microcirculation

The spleen is a highly vascular organ that has very complex and highly branching vessels, as illustrated in Fig1.3. The splenic vascular arrangements among species are of great variation. The basis of species variations is classified by the

differences in the structure and morphology of the venous sinuses to group spleens into sinusal and nonsinusal, where the human spleen belongs to the sinusal group while the mouse spleen falls into the nonsinusal group [75, 76]. Comparatively, the venous sinuses of sinusal spleens are larger, more abundant and have a characteristic wall structure that are encircled by rings of reticular fibers, while the venous sinuses of nonsinusal spleen are very different in structure and some researchers name it “pulp venule” due to the existence of the typically large enough venular mural apertures [76]. In general, the spleen possesses a very complex blood microcirculation regardless of the features of the venous sinuses. There are two types of microcirculation that have been identified – the slow open circulation and the fast closed circulation [77]. The blood enters the spleen through splenic arteries which branch to enter the hilum of the spleen. The splenic arteries branch further to penetrate the organ from the capsule and pass along the trabeculae. The trabeculae act as a fibroelastic coat of the spleen and constitute the splenic frame-work. The white pulps are closely associated with the arterial tree. The trabecular arteries continue to divide into arterioles once reaching the white pulp PALS, which are then termed central arteries or central arterioles. Branches of the central arterioles may end in either the white pulp or the marginal zone. In rodents, some of these arteriole branches terminate in the marginal sinus, the space between the white pulp and the surrounding marginal zone; whereas in humans without the marginal sinus, blood can directly drains into capillaries of the perifollicular zone [74]. The terminal portion of these arterioles lose their lymphatic tissue sheath and eventually subdivide into penicillar arteries that direct the blood to the red pulp [78]. These penicillar arteries then give rise to arterial capillaries. In the fast closed pathway, the blood flow through the arterial capillaries radially enters the adjacent venous sinuses, which are open-ended tubes continuous to the marginal zone, such that the blood bypasses the red pulp reticular meshwork. In the other pathway, the blood passes through the reticular meshwork where filtration of abnormal RBCs normally takes place, and is collected into

venous sinuses which then enters the trabeculae and is finally drained by the trabecular veins. In both sinusal spleen and nonsinusal spleen, this circulatory pathway involves longer distance through the labyrinthine reticular meshwork which lacks the endothelial continuity, thus it is called the slow open microcirculation [79]. The slow open microcirculation accounts for around 10% of the splenic blood flow in human [80], which also differs in species [78, 81], the remaining of the circulating blood is through the fast closed circulation. It has been implicated in some species that switching of the blood flow between the alternate pathways can occur when some changes are triggered, such as elevated blood pressure by administration of norepinephrine [82] or dilation of the spleen by perfusion against increased venous outflow pressure [83]. However, though the co-existence of both circulation pathways in human spleen is largely accepted, there is still some debate on it [80, 84].

Fig1.3. Splenic microcirculation. The main compartments of the human spleen and major blood vessels as well as the two identified blood circulation modes are marked. (Adapted from Junqueira's Basic Histology: Text and Atlas, 12th Edition)

1.2.1.3 Spleen functions

The spleen is a very busy organ, and one of its important functions is to filter blood. The red pulp is the main site for this. The red pulp reticular meshwork containing the reticular cells and reticular fibers creates a tortuous path for the erythrocytes. And the presence of a large amount of highly phagocytic red pulp macrophages in the meshwork aid to survey the blood-born particles and also the conditions of the erythrocytes. Through recognizing the expression of CD47 on the RBC surface, which will have conformational changes upon erythrocyte aging, red pulp macrophages get activated and phagocytosis will occur [85, 86]. In addition, the blood passing through the red pulp sinuses has to squeeze into the narrow endothelial slits which generate an additional challenge that some of the cells will get trapped or damaged. There are some factors that contribute to the blood filtration aspect, including the erythrocyte size, shape and membrane components, which will be elaborated more later on. By filtering blood, the spleen helps to remove aged, damaged or infected RBCs (iRBCs) as well as the intraerythrocytic inclusions in a process known as pitting. Pitting retains the integrity of the RBC but removes the inclusion-containing non-deformable portion of the RBCs, which is subsequently phagocytised by splenic macrophages. Importantly, it has been suggested that the basic filtration beds – the red pulp meshwork, provide a sufficient filtration with modest filtration capacity under normal physiological condition, but not when the spleen is stressed. In fact, when the spleen is stressed such as during an infection, in some species, there are stromal cells termed barrier cells being activated and augmented to the red pulp meshwork to facilitate the blood clearance, such that the splenic clearance capacity expands [87, 88] .

Concurrently, the spleen assists in recycling iron while removing the abnormal erythrocytes, which is facilitated by the red pulp macrophages [89]. In the phagolysosome of the macrophages, the erythrocyte is hydrolysed and the hemoglobin is digested and proteolytically degraded to release heme, which is further catabolised into biliverdin, carbon monoxide and ferrous iron. The iron is

then either released from the cells or stored as ferritin in the splenic macrophages [90]. Nevertheless, continuing damage of the plasma membrane in physiological or pathological conditions results in intravascular haemolysis, such that haemoglobin released which is then rapidly captured by acute phase protein haptoglobin. The haptoglobin-haemoglobin complexes are recognized by CD163 that expressed on the surface of the macrophages, leading to receptor-mediated endocytosis and therefore scavenging the haemoglobin from the circulation in the spleen [91]. The release of iron stored in the splenic macrophages is regulated according to the requirement of the bone marrow [57], but the underlying mechanism is still poorly understood.

The spleen in some species is a site for hematopoiesis, such as mice, particularly in fetal and neonatal stages. The iron recycled from erythrocytes through spleen filtration contributes to hematopoiesis. While in humans, the splenic erythropoietic functions cease after birth, but the spleen retains the ability to produce lymphocytes [92]. The splenic hematopoiesis has long been suggested to play an important role for emergency or backup in cell development during stress or disease, such as anaemia, inflammation or neoplasia [93].

The spleen is also a storage site of blood. In some species, such as cat and horse with contractile spleens, the spleen can store up to 50% of the total blood and expel out into the general circulation when it is needed. While in human whose spleen is noncontractile, the capacity of storing blood is greatly reduced [94]. However, human spleen is a major site for storing platelet with around 1/3 of the total platelets being stored in the spleen, and upon stimulation such as epinephrine the platelets can be released [95]. Moreover, the spleen also contains a large amount of monocytes, plasmablasts and plasma cells mainly residing in the red pulp. In response to specific antigens, these cells can migrate, differentiate and proliferate to fight against the pathogens [96].

Being the largest secondary lymphoid organ, the spleen contains a well-developed network of lymphatic vessels. It is a key regulator in the circulatory system that plays an important role in body fluid homeostasis, keeping the daily fluid levels in balance. It also assists in regulation of blood volume through fluid extravasation from the splenic circulation into the lymphatic reservoirs by direct modulation of splenic capillary resistance [97, 98]. The lymphatic network also contributes to circulating lymphocytes [99], facilitating the rapid responses of the immune system. The splenic macrophages are highly phagocytic and together with other immune cells residing in the spleen, help to efficiently defend the body against infection. The large number of platelets present in the spleen have been implicated in immunological processes such as direct pathogen-killing [100]. The perfect location of the macrophages and dendritic cells in the marginal zone enables them to facilitate antigen capture and presentation, which will then prime the T-cell responses. Moreover, there are distinct T-cell and B-cell zones within the splenic white pulp that can generate different immune responses against different pathogens. The role of the spleen in immune system against pathogens in particular *Plasmodium* parasites will be elaborated in the next section.

1.2.2 The role of the spleen in malaria infection

1.2.2.1 Overview of spleen functions in malaria

Since the spleen acts as a blood filter and plays an important role in surveying pathogens in circulating blood, it has a central role in controlling and clearing the malaria parasites. Its importance has been confirmed through the studies in infected splenectomized humans and rodents [101-105]. The spleen is a major site

for parasitized erythrocyte removal from the circulation, *via* its special architecture as mentioned in Section 1.2.1.3. It has also been suggested that through filtering blood and selectively destroying the infected erythrocytes, the spleen helps in modulating the antigens expressed on the surface of the RBCs [106]. Nevertheless, the spleen is an important site for erythropoiesis and haematopoiesis during the infection [107, 108], thereby replenish the erythrocytes to prevent anaemia that is associated with the destruction of iRBCs, and also to rapidly generate large amount of immune cells to fight against the parasites. Moreover, the spleen is also the bridge between innate and adapted immunity. The special arrangement of the spleen allows the antigen presenting cells easily migrate to the distinct areas for T cell and B cell residence within the splenic white pulp upon destroying the parasitized erythrocyte. Thereby pathogen-specific T-cell and B-cell responses will be activated [109, 110].

1.2.2.2 Spleen remodelling in malaria

In response to malaria parasite infection, the spleen undergoes various types of remodelling. One of the most obvious observations is the splenic enlargement as a pathological association known as splenomegaly. Splenomegaly in part is due to the increased influx of lymphocytes, as well as the enlarged erythrocyte volume. In acute cases of imported malaria to non-endemic countries, splenomegaly is palpable in approximately 20% of febrile patients [111]. In fact, splenomegaly has been shown to be commonly associated with malaria infection in various hosts, and in general the enlargement of the spleen enables the expansion of its filtration capacity such that help to accelerate the clearance of abnormal erythrocytes as compared to patients with spleens of normal sizes. However, it has also been demonstrated that after antimalarial treatment, patients with spleens of normal sizes cleared the abnormal erythrocytes faster than patients with splenomegaly [112, 113]. The enlargement of the spleen leads to increased splenic vasculature as well as the width of splenic blood vessels and thus increased blood retention in the

spleen. It has been proposed that with splenomegaly, there is elevated retention of infected erythrocytes in the spleen facilitating the splenic parasite clearance and also reducing the risk of cerebral malaria. Meanwhile there is also increased splenic retention of uninfected erythrocytes that may contribute to severe anaemia [80]. There is an extreme form of splenomegaly known as hyperreactive malaria splenomegaly, which usually arises in responses to recurrent infections and is associated with high mortality. It is implicated to involve disruption of T cell regulation of effective humoral immunity [114, 115].

Besides the changes in splenic appearance, the spleen in response to malaria parasite infection also exhibits some structural disorganization and remodelling, such as expansion of the red pulp, hypercellularity with extensive hematopoiesis in the red pulp, transient loss of the marginal zone as well as indistinct separation of the T- and B-cell zones [109, 116, 117]. It has also been shown in the mouse malaria model that upon infection with non-lethal parasite strains, the small amount of barrier cells originally present in the spleen became activated immediately after infection and started multiplication in both BALB/c mice infected with *P. yoelii* 17XNL and C57BL/6 mice infected with *P. chabaudi*. Together with the red pulp reticular fibers, these increased number of barrier cells fused to form syncytial sheets that ultimately form physical barriers to temporarily change the splenic open circulation to closed circulation, resulting in the closure of the splenic filtration bed and thus resembling an “asplenic” condition [88, 118]. By closing the filtration bed, these barriers protect the spleen hematopoietic and immunologic capacities from the parasites, as a result, when the filtration bed re-opens the parasites can be rapidly cleared and the anaemia conditions would be ameliorated. However, these syncytial sheets have also been demonstrated to contain receptors for cytoadherence of parasitized erythrocytes, which may contribute to the parasite escape from clearance by macrophages as well as the establishment of chronic infections [119]. In contrast, though the barrier cells in

BALB/c mice infected with the lethal *P. yoelii* 17XL parasites have also been shown to be activated and have infected erythrocytes attached on their surface, there are a large number of macrophages associated on their surface as well. The presence of these macrophages is potentially linked to the observation of the degeneration and death of the barrier cells. Therefore, these barrier cells failed to develop into competent blood-spleen barriers [88], suggesting a differential spleen remodelling in response to different *Plasmodium* parasites and differentiating the lethal and nonlethal infections.

1.2.2.3 Effect of splenectomy

Since splenomegaly is commonly associated with severe malaria infection, it is classical to have splenectomy to release the pain and discomfort, and more importantly to prevent splenic rupture. The spleen has actually been shown to be a nonvital organ that sometimes it is neglected. Despite its importance, a full splenectomy does not generally involve severe complications. However, it has been shown that splenectomized patients are more prone to infections and exhibit more severe symptoms upon infections as well as higher mortality can result in general, especially for young children [120, 121]. It has also been shown that in mouse model, splenectomized BALB/c or C57BL/6 mice failed to resolve the infection with *P. yoelii* 17X avirulent parasites and eventually died [101, 103]. While in human disease, splenectomized patients invariably showed an increase in parasitemia with *P. falciparum* infection regardless of the anti-malaria agents used [122]. In addition, splenectomized humans infected with *P. falciparum* again required markedly prolonged period for parasite clearance after artemisinin treatment [123], implying the importance of the spleen in rapid post-therapeutic parasite clearance. Nevertheless, the late stages parasites which is normally sequestered to avoid clearance, have also been found in the peripheral blood of *P. falciparum* infected patients with spleen removal, [124], suggesting that the spleen is involved in modulating parasite phenotypes. Studies have also demonstrated that

architecturally intact spleen is required for fighting against the malaria infection but not solely the splenic immune effector cells [125]. It has been shown that the removal of the spleen has effects on other organs particularly the liver [126]. As a result, a partial splenectomy is usually more recommended and favoured if clinically appropriate. It has been demonstrated that in mice model with half splenectomy, there is maintenance of spleen functions against malaria infection in terms of controlling parasitemia which resembles the conditions in spleen intact mice [127]. However, it has also been reported that in some species, splenectomy results in a normally chronic infection to be eliminated in the host [128], suggesting a pathological role of the spleen in malaria parasite infection that sometimes the splenic environment may favour to the growth of some parasites but not to the host. Indeed, it has also been demonstrated in rhesus monkeys that spleen has both protective and suppressive role in host defence against *P. inui*, which usually causes persisting infection for years. In this model, rhesus monkeys with splenectomy before the infection resulted in high mortality in a few days while splenectomy that occurred 2-3 months after the infection led to an abrupt rise in parasitemia following with parasite clearance within 1 year [129].

1.2.3 Splenic filtration of erythrocytes

1.2.3.1 Malaria and red blood cell: preference and modification

The spleen is a site for removal of abnormal erythrocytes. Though grossly damaged RBCs are primarily taken up by the liver since it has a comparatively higher mass and blood flow than the spleen at physiological condition, the spleen has been shown to obtain the ability to selectively remove erythrocytes that are even slightly damaged and would escape retention elsewhere [130]. Naturally, the

RBCs can undergo remarkable deformation. They can transit through the skeletal muscle, capillaries with diameters between 3-7 μm , and also the spleen interendothelial slits [131, 132]. The RBC deformability and durability is attributed to their membrane skeleton. Upon malaria parasite infection, starting from the merozoite attachment to successful invasion into the erythrocyte, and the subsequent intracellular development into mature schizont stage, the parasite undergoes serial developmental processes that are associated with changes in host cell sizes, shapes as well as the deformability [133]. During these processes, the iRBC cytoplasm hemoglobin content decreases along with the synthesis of parasite proteins, cellular volume increases, the cell membrane is stretched and modified. The membrane cytoskeleton interacts with exported parasite proteins forming extra cross-linking in the spectrin network. As a result, the iRBC becomes more rigid and less deformable [134, 135]. This reduced deformability in RBC upon parasite infection has been demonstrated through different methods including ektacytometry [136], optical tweezers [137], laminar flow system [138], micropipette aspiration [139] and microfluidic devices [140].

In addition, it has also been shown that RBCs upon malaria parasite infection become more adhesive to a number of cell types including vascular endothelial cells, platelets and also other RBCs regardless whether they are normal or infected [141-143]. The adhesive phenotype of the parasitized erythrocytes enables the parasite to sequester and avoid clearance, and it is important for parasite survival and pathogenicity. From various studies, it has been shown that this adhesive phenotype is mediated by the interaction between host cell receptors and parasite proteins expressed on the surface of the infected erythrocytes, and these parasite surface proteins involved in *P. falciparum*-mediated malaria include the variant PfEMP1 (*P. falciparum* Erythrocyte Membrane Protein 1), RIFIN and STEVOR (subtelomeric variable open reading frame) [144-146]. In fact, spleen filtration seems to play a role in modifying the expression of the surface ligands. It has been

shown that with spleen removal, the normally sequestered mature stage infected erythrocytes are found in the peripheral blood. Moreover, these infected erythrocytes failed to bind to the host cell receptors, and the detection of the expression of some known surface proteins like PfEMP1 STEVOR by RT-PCR (reverse transcription polymerase chain reaction) could not be achieved. However, these functions were shown to be restored after *in vitro* culture over a period of time [106, 124]. On the other hand, changes in the expression of parasite proteins on the merozoite or infected erythrocyte surface have also been implied to alter the parasite preference to the host cells [147, 148]. Indeed, different strains of parasites naturally have different preference on RBC types, with some species/strains more reticulocyte-prone like *P. vivax* and *P. yoelii* 17X1.1, while some others like *P. falciparum* and *P. yoelii* YM have a broader preference of host cells and usually can invade erythrocytes of all ages [149, 150]. Moreover, it has also been shown that patients with certain red cell disorders are generally protected from severe malaria infection and have reduced morbidity and mortality [151], suggesting that malaria parasites have driven selection of these RBC variants.

Differences in red cell preference of *Plasmodium* parasites for invasion frequently correlate with their virulence, and it has been demonstrated that reduced parasite virulence is in association with the RBC repertoire switch from normocytes to reticulocytes [148, 152]. Comparatively, the young erythrocytes, namely reticulocytes, have irregular shapes and are bigger than the biconcave mature erythrocytes (also known as normocytes) which have a diameter of 7.5-8.7 μm [132, 153]. Upon expulsion from the bone marrow, reticulocytes mature into normocytes within 24-36 hr. During this maturation process, the cells undergo dramatic changes in structure, morphology as well as mechanical properties. The ribosomes will disappear and the membrane-bound organelles, membrane lipids, surface molecules and hemoglobin etc. will decrease in number [154-157]. Besides that, the membrane skeleton will be rearranged significantly [158] and the

surface-to-volume ratio of the cell will decrease [159]. Therefore, the reticulocytes are expected to have different mechanical properties as compared to the normocytes. Moreover, the maturation process has been demonstrated to be associated with a general streamlining of cellular metabolism, such that reticulocytes have a more complex, enriched metabolic profile and also contain a richer repertoire of carbon sources and other essential nutrients than the normocytes, which may be preferred or required for invasion by some *Plasmodium* parasites [160]. In addition, with the presence of more specific surface molecules than normocytes, invasion to reticulocytes by certain strains of malaria parasites are likely enhanced [149, 161]. The surface proteins and membrane lipids that are diminished during the reticulocyte maturation process have been demonstrated to be secreted into vesicles, which are also known as exosomes, and then released from reticulocytes into peripheral circulation and get cleared eventually [162]. Recent studies have characterized the RBC proteome including both the cytoplasmic and membrane proteins, for both normocytes and reticulocytes [163-165]. The information on RBC proteome facilitates the studies on intracellular trafficking as well as understanding the mechanism of reticulocyte maturation and the role of reticulocytes in disease. Interestingly, though the exosome proteome has not yet been fully characterized, *Plasmodium* proteins were found in the exosomes secreted from infected reticulocytes in *P. yoelii* malaria model, and these exosomes have been speculated to have an immunomodulation role [166].

Besides the alternations on infected erythrocytes, malaria parasites have also been shown to have some impacts on the uninfected RBCs (uRBCs) both *in vivo* and *in vitro*. The uRBCs in the infected hosts have been found to have a significant increase in the linoleic acid level and also changes in fatty acid composition of neutral lipids. In addition, these uRBCs also show progressive decreases in the net surface negative charge, as well as increased apoptosis and shortened lifespan

[167-170]. Taken together, these changes in uRBCs in the infected hosts indicate that the parasites secrete some substances that are able to change the biological properties of the circulating RBCs.

1.2.3.2 Mechanisms of splenic filtration

From the observation of various studies and clinical cases, the spleen undoubtedly plays a central role in malaria parasite clearance, and one of the major mechanisms involved is *via* the splenic filtration of infected erythrocytes. There are a few elements involved in splenic filtration. Through the study on the effect of different alternations in RBCs, it has been demonstrated that increased splenic filtration is largely associated with changes in erythrocytes including increased sphericity, inhibition of membrane sulfhydryl groups, loss of surface charge as well as reduction on deformability [171]. These changes in some situations can occur concurrently, and can be classified into two main aspects: the deformability and the surface properties.

Red cell deformability is commonly associated with the splenic mechanical trapping machinery. There are many factors that can contribute to the altered cell deformability, including the size, shape, viscoelasticity of the cell membrane, the state of the cytoplasm, and the fluidity of the hemoglobin [172]. The presence of the rigid parasite intracellularly also greatly affects the deformability of the iRBCs. As mentioned above, parasitized erythrocytes gradually lose their deformability along the parasite developmental stages. These less deformable RBCs are easily trapped for pitting or phagocytosis when passing through the red pulp and particularly the narrow interendothelial slits. This mechanical trapping of the less deformable cells, resulting from malaria parasite infection, heat or chemical treatment, has been demonstrated through different studies by *ex vivo* spleen perfusion system [80, 173] .

However, the interendothelial slits do not exist in nonsinusal spleens, and these the splenic filtration relies more on the red pulp meshwork. The red pulp reticular meshwork and the presence of the highly phagocytic macrophages form the foundation the filtration system, but the interstices of the reticular meshwork are actually slightly larger than the normal RBCs. Thus at physiological condition the red pulp filtration bed does not constitute a test for deformability like the interendothelial slits do. Instead it has been proposed that the abnormal as well as the immature RBCs can adhere to the fine structures of the reticular meshwork [174, 175], which would then be targeted by the splenic immune cells. Moreover, it has been demonstrated that the high hematocrit in the red pulp meshwork, which is as much as twice that of the arterial hematocrit, is a result of the RBC surface interactions whereby the RBCs have a decreased flow rate in contrast to that of the plasma [176]. Indeed, it has been shown that red pulp meshwork itself is the major site for retention of the immature and abnormal erythrocytes, while the interendothelial slits in sinusal spleens offers a second mean of filtration [75]. Therefore, RBC mechanical retention in the spleen is believed to be one key mechanism facilitating the removal of infected erythrocytes and parasite clearance. Upon infection, the reticular meshwork in the red pulp mechanically challenges the erythrocytes and parasitized erythrocytes with compromised deformability are more likely to be retained in the red pulp before destruction [177]. The expansion of red pulp during splenomegaly in response to *Plasmodium* infection is therefore thought to provide a larger filtration capacity and accelerate erythrocyte clearance. Conversely, transformations in the red pulp and splenic vasculature may modulate the mechanical retention threshold of erythrocyte and regulate the microcirculatory trapping of blood cells in spleen. Increased splenic retention of infected erythrocytes assists the clearance of malaria parasites, lowering the risk of severe malaria. However, excessive blood retention could lead to malaria anemia and is undesirable [80].

1.2.3.3 RBC deformability and pathogenesis in malaria

RBC deformability in relation to malaria pathogenesis has been discussed extensively [178-181]. Upon infection, the presence of the intracellular *Plasmodium* parasite bring about the cytoskeleton remodelling of the host cell, significantly impairing the host erythrocyte ability to deform [182], such that iRBCs have decreased deformability. Though the mechanisms underlying the changes in cell deformability during *Plasmodium* infection are not yet fully understood, it is clear that reduced RBC deformability would potentially contribute to impaired blood circulation in particular the capillary flow [183] as well as organ dysfunction [184]. Moreover, it has been observed that besides iRBCs, deformability of uRBCs are greatly reduced in patients with *Plasmodium* infection when comparing to the healthy subjects [136, 180], thereby potentially triggering the uRBC retention in the spleen and contributing to malaria anemia. In addition, data from *in vitro* studies as well as field reports have suggested the association between RBC deformability and malaria severity [136, 138, 185, 186], though so far there is still no direct evidence showing the correlation among spleen remodelling, RBC deformability and infection severity of *Plasmodium* parasites. However, a lot of evidence has indicated that, besides ligand-receptor interactions, reduced RBC deformability during malaria parasite infection is greatly linked to the increased spleen retention thereby significantly influencing the spleen clearance [80, 138, 173]; while spleen retention of parasitized RBCs starts very early during the infection, increased retention of iRBCs may help to activate the antigen-specific responses in the spleen [181].

1.2.4 Splenic immune responses

1.2.4.1 Adaptive immunity

Compared to the innate immunity, host adaptive immunity in particular the humoral immunity, against malaria parasites provides more specific and long-lasting protection. Specific antibodies against parasite surface antigens, including MSP1 (merozoite surface protein-1) and glutamate-rich protein, have been shown to facilitate phagocytosis as well as antibody-dependent cell inhibition through Fcγ receptors [187, 188]. In addition, specific antibodies have also been shown to inhibit merozoite invasion and parasite growth in both *in vivo* and *in vitro* systems [189, 190]. A recent report also showed that immunization with the exosomes containing parasite proteins of avirulent strain can provide protection for virulent strain infection in *P. yoelii* model [166], highlighting the role of exosomes in modulating host adaptive immunity. However, the natural generation of specific antibodies usually requires recurrent or long-lasting infections. Moreover, the parasites have high rate of antigen variation that assist in immune evasion and this may be the reason why all the malaria vaccines identified and developed so far have turned out to be only marginally effective. Even the most advanced vaccine against *P. falciparum*, known as RTS, S/AS01, showed only 18% and 26% reduction in clinical malaria cases in infants and children respectively with a 3-dose primary vaccination. The extra booster dose administered 18 months after the primary schedule only increased the protection to 26% and 36% respectively in infants and children [191].

1.2.4.2 Innate immunity

Innate immune responses act as the first line defence against invading pathogen. Depletion of macrophages has been demonstrated to result in increased parasitemia and exacerbated anemia in both lethal and nonlethal infections in *P. yoelii* model

[192], suggesting an essential role of macrophages in controlling parasite development. In malaria parasite infection, the red pulp macrophages in the spleen have been considered as one of the major players in removing infected cells [193, 194]. There are a few mechanisms that have been proposed for macrophages to fight against the blood stage malaria parasites. Red pulp macrophages have been suggested as an early sentinels for *Plasmodium* infection that produce a large amount of type I Interferons (T1IFNs) against malaria in the *P. chabaudi* system [195]. A decrease in the highly phagocytic marginal zone macrophages have been shown to correlate with around a 20% decrease of splenic trapping of iRBCs, and this loss in marginal zone macrophages has been suggested to be associated with the Tumor necrosis factor (TNF)-alpha production during malaria infection [196]. In addition, macrophages express the scavenger receptor CD36 on the surface that can directly bind to infected erythrocytes and mediate the non-opsonic phagocytosis [197]. This is an important part of innate immunity protection against malaria parasite infection, in particular for the immunologically naïve individuals that lack *Plasmodium*-specific antibodies. In addition, parasitized erythrocytes have been shown to be able to promote macrophage activation or alternatively lead to complement activation leaving the complement fragment C3b deposition on infected red cell surface. This complement fragment is then recognized by macrophages through complement receptor 1 (CR1), resulting in opsonic complement-mediated phagocytosis [198, 199]. In malaria-immune individuals, opsonic antibody-mediated phagocytosis also plays an important role in parasite clearance [200].

Nevertheless, macrophages together with DCs are important antigen presenting cells that are able to uptake and express the parasite antigens on their surface and prime the CD4⁺ T cells to activate the adaptive immune system [201, 202]. In particular, DCs are the professional antigen presenting cells and splenic DCs are specialized in antigen processing and presentation. It has been shown that the

splenic DCs as well as macrophages in mice model infected with *P. yoelii* are able to process and present antigens to T cells [203]. Moreover, administration of retinoic acid (RA), a downstream derivative of Vitamin A, has been shown to be able to regulate the development of a certain subset of splenic DCs for MHC class II-restricted antigen presentation [204]. Though there are some reports on parasitized erythrocytes impairing the DC maturation [205, 206], it has also been shown that blood stage parasites can activate DCs and macrophages through signalling of toll-like receptors (TLRs) [207, 208]. TLRs are a family of pathogen recognition receptors that are involved in the initial detection of pathogens. It has been shown that TLR4, TLR9, MyD88 and NF κ B signalling are required for DC activation in malaria infection [209]. In fact, the members of the TLR family are involved in pathogen recognition and many of the TLRs have been implicated in human malaria infection. For example, TLR 11 on DCs is activated by a protozoan profilin-like protein in *P. falciparum* [210]. In addition, through TLR signalling, cytokine production from a wide range of innate immune cells in response to the interaction with parasite-associated components is promoted [211], and macrophages non-opsonic phagocytosis of iRBCs mediated by scavenger receptor CD36 is enhanced [212].

Besides the direct phagocytosis and stimulation of adaptive immune responses, activated macrophages and DCs secrete cytokines to stimulate further immune cell activation for downstream signalling as well as further cytokine production to enhance the clearance mechanisms. Cytokine responses in malaria have been extensively studied, demonstrating that the timing and magnitude of the cytokine production are crucial yet no conclusive cytokine profile that leads to protection rather than pathology has been identified. However, it has been suggested that the absolute levels and ratios of the proinflammatory and anti-inflammatory cytokines have great impact on the infection susceptibility and disease pathologies [213]. It is generally accepted that proinflammatory cytokines Interferon (IFN)-gamma and

Interleukin (IL)-12 in the early infection stage play a crucial role in the parasite clearance, where IL-12 has been found to be essential for the clearance of nonlethal strain parasites *via* IFN-gamma production in rodent malaria models [214-216]. Conversely, IFN-gamma is also involved in the acute symptoms of infection with overproduction of IFN-gamma and TNF-alpha linked to severe pathology [217, 218]. On the other hand, anti-inflammatory cytokine IL-10 plays a complicated role in malaria infection, as illustrated from patients of malaria-endemic areas and experimental malaria models. IL-10 is known to inhibit the protective immunity against malaria infection, and high levels of IL-10 has been shown to be associated with high levels of parasitemia and severe anaemia [219-221]. Moreover, IL-10-deficient mice showed lower parasitemia than the wildtype mice during infection, yet they developed severe diseases such as hepatic pathology and cerebral pathology [222-224], indicating a suppressive role of IL-10 in the severe malaria pathology. Many other cytokines have been suggested in malaria pathology as well. For example, proinflammatory cytokines like TNF, IFN-gamma, IL-6 and IL-1alpha have been shown to induce the expression of adhesion molecules, such as E-selectin, ICAM-1, V-CAM1 and CD36 on the surface of endothelial cells, facilitating and strengthening the binding of infected erythrocytes for sequestration [225, 226]. Inflammatory responses to lethal strains of malaria parasites appear to contribute to structural and functional changes in the spleen, as demonstrated in the studies in *P. vinckei* and *P. chabaudi*, where suppression of erythropoiesis is shown to be mediated by TNF production [227, 228]. IL-2 has also been shown to be involved in the expansion of the regulatory T cell population during *P. chabaudi* infection, which may be essential for the effector T cell modulation towards the recognition of parasite-specific peptides [229].

There are some other important cells or proteins involved in the innate immunity or in bridging the innate and adaptive immunity during malaria infection as well.

Natural killer (NK) cells which are mainly found in peripheral blood, spleen and bone marrow have been suggested to be one of the ideally placed immune cells for parasite clearance. It has been found that NK-cell-mediated cytotoxicity and IFN-gamma production are induced during malaria infection. Production of IFN-gamma by NK cells is found to be essential for developing protective immunity, where NK cell depletion results in decreased IFN-gamma levels and increased virulence of non-lethal *P. yoelii* and *P. chabaudi* infections [230-232]. In addition, it has been demonstrated that there is cross-talk between NK cells and DCs. This cross-talk is bi-directional that IL-12 production by DCs is critical for NK cells activation to produce IFN-gamma during *P. chabaudi* infection [233, 234], while *P. chabaudi*-activated NK cells can also stimulate splenic DCs to mature and produce cytokines [234].

Besides NK cells, it has also been proposed that platelet may provide an influential protective role in malaria infection recently [235]. When platelets bind to infected erythrocytes and become activated, platelet factor 4 (PF4/CXCL4) are released, which then can bind to the infected cell and get internalized and relocate to the parasite digestive vacuole resulting in organelle lysis and parasite death [236]. Since there are a larger number of platelets present in the human spleen and the number and mass of platelet exceed that of all leukocytes in the circulation [237], the powerful splenic clearance against malaria parasite infection may in part rely on the platelet functions.

1.3 Aims and Objectives

The spleen plays an important role in malaria parasite infection. It exhibits distinct but extensive remodelling in response to *Plasmodium* parasites. The types of the spleen response determine the pathological development as well as the disease outcome in malaria. However, the detailed characterization of distinct spleen responses that can be directly linked to the clinical conclusion has not yet been established. Due to the ethical and technical constraints, most of the information known so far on the human spleen in malaria has been obtained indirectly from *ex vivo* studies. However, spleen remodelling is a dynamic and complicated response, *ex vivo* studies alone do not provide sufficient information to understand disease development and predict disease outcome. On the other hand, rodent malaria model have the advantage to follow the progressive disease development and allows the *in vivo* investigation on the complex interactions in the organ in details.

With the importance of the spleen in malaria, the principle aims of this project are to investigate the spleen remodelling in response to *Plasmodium* infection *in vivo*, and identify the factors that are involved in parasite clearance by the spleen. Though there are anatomical differences between human and mouse spleen as mentioned in Section 1.2.1, many morphological features and functions of the spleen including the blood filtration are actually conserved [238]. We hypothesize that the difference in spleen remodelling upon malaria parasite infection directs the disease development and it is the key factor to determine disease outcome. To achieve our specific aims, we utilize different strains of parasites of *P. yoelii* with different disease outcomes in the BALB/c mouse model to address the following objectives:

1. Characterization of detailed changes in overall spleen morphology in response to infections by the virulent and avirulent parasite lines.

2. Identification of early molecular signals in the spleen in response to infection with the virulent and avirulent parasite lines.
3. Investigation on parasite host cell selection, modifications and their association to spleen clearance as well as disease outcome.

1.4 Scope of thesis work

To address the objectives stated above, we followed the infection period of mice with either virulent or avirulent parasite infections and examined and compared the infected spleens in details of the size, internal structure including the cellular composition as well as the blood circulation. We also studied the mechanical properties of the infected cells and their association with spleen remodelling. The early immunological response in the spleen was also investigated and compared between lethal and non-lethal infection. This work highlights a way to control malaria disease development through avirulent parasite infection and its characteristic “non-lethal” spleen response. In addition, this study also provides a potential tool for fast and easy diagnosis and prognosis of malaria patients using the cell deformability information.

Chapter 2

Methods and Materials

Ethics statement

This study was carried out in strict accordance with the recommendations of the NACLAR (National Advisory Committee for Laboratory Animal Research) guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore. The protocol was approved by the by the Institutional Animal Care and Use Committee (IACUC) of the Nanyang Technological University of Singapore (Approval number: ARF SBS/NIE-A0234). All efforts were made to minimize the suffering.

2.1 Materials

2.1.1 Animals

Wildtype Male BALB/c mice of 4-8 weeks old were obtained from Sembawang Laboratory Animal Center, National University of Singapore or Biological Resource Center, A*Star or InVivos Singapore. Mice were subsequently bred under specific pathogen free (SPF) condition at Nanyang Technological University Animal Holding Unit.

BALB/c-CD11b-DTR mice were gifts from Dr. Ruedl (Nanyang Technological University, Singapore). They were subsequently mated and bred under SPF condition at Nanyang Technological University Animal Holding Unit, with the same breeding conditions as the wildtype BALB/c mice. These transgenic mice use CD11b promoter to control the diphtheria toxin receptor (DTR), followed with a reporter protein dTomato fused to the C-terminal. Thereby they are able to conditionally ablate most of the innate immune cells that are CD11b positive upon administration of diphtheria toxin (DT).

2.1.2 *Plasmodium yoelii* parasites

P. yoelii parasites were either obtained directly from MR4 (The Malaria Research and Reference Reagent Resource Center) or generated in our laboratory. Parasite lines were grown and expanded in normal BALB/c mice and infected blood was cryopreserved in liquid nitrogen for future use.

2.1.3 Microfluidic device

Microfluidic devices used to examine the cell deformability were designed and made by Dr. Huang Sha (Massachusetts Institute of Technology, USA and Singapore-MIT Alliance for Research and Technology, Singapore) and the same device has been used in some published studies [140, 239]. It contains repeated

bottleneck structures with dimension of 3 x 4.2 μm^2 (Width x Height), which is similar to the critical mesh size of mouse spleen [140].

2.1.4 Chemical, reagents and kits

Table1. Chemicals and reagents used for mouse *in vivo* studies

Name	Active constituent	Brand	Dosage	Usage
Valabarb euthanasia solution	Pentobarbitone sodium	Jurox	40-60 mg/kg (in saline)	Euthanasia
Ketamine	Ketamine	Ceva	100 mg/kg (in saline)	Anesthesia
Xylazil-20	Xylazine	Ilium	20 mg/kg (in saline)	Anesthesia
Isoflurane, USP	Isoflurane	HSB Veterinary Supply Inc.	Inhalation	Inhalting anesthesia
Septin (Co-Trimexazole Suspension)	96% Sulphamethoxazole +108.0% Trimethoprim	Beacons Pharmaceutical	50-60 mg/kg (in drinking water)	Antibiotic
Meloxicam	Meloxicam	Ilium	0.2 mg/kg (in PBS)	Pain killer
D-Luciferin	D-Luciferin, firefly, potassium salt	Caliper Life Sciences	150 mg/kg (in PBS)	<i>In vivo</i> luciferase assay
Pyrimethamine	Pyrimethamine	Sigma-Aldrich/MP biomedical	10 mg/kg (in DMSO, for ip.); 70 $\mu\text{g}/\text{ml}$ (in drinking water)	Selectively kill parasites without DHFR expression
Phenylhydrazine -HCl (PHZ)	Phenylhydrazine	Sigma-Aldrich	50 mg/kg (in distilled water)	Stimulate reticulocytosis
Heparine	Heparine	Sigma-Aldrich	0.2 mg per mouse (in PBS)	Anticoagulant
Diphtheria toxin (DT)	Diphtheria toxin	Sigma-Aldrich	20 ng/kg (in PBS with 1% mouse serum)	Selectively kill the DTR positive cells

Table2. Other chemicals and reagents used for experiments.

Name	Brand
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Histodenz TM nonionic density gradient medium	Sigma-Aldrich
RPMI Media 1640	Thermo Fisher Scientific
Fetal Bovine Serum	Thermo Fisher Scientific
Goat serum	Thermo Fisher Scientific
Gentamicin	Thermo Fisher Scientific
Ficoll-Paque PLUS	GE Healthcare
Geimsa Stain	Sigma-Aldrich
Reticulocyte Stain	Sigma-Aldrich
Xylene	Sigma-Aldrich
Paraffin Wax	VWR
Hematoxylin	Sigma-Aldrich
Eosin Y disodium salt	Sigma-Aldrich
1X RBC Lysis Buffer	Affymetrix eBioscience
RIPA buffer	Sigma-Aldrich
1 kb DNA Ladder	New England Biolabs
Formalin solution, neutral buffered, 10%	Sigma-Aldrich
Agarose	1 st Base
Laemmli buffer	Sigma-Aldrich
Western blot blocking buffer	Odyssey
PageRuler TM Plus Prestained Protein Ladder, 10 to 250 kDa	Thermo Fisher Scientific
Protease and Phosphatase Inhibitor cocktail	Thermo Fisher Scientific
10% SDS solution	Bio-Rad
Acrylamide/Bis-acrylamide (37.5% w/v)	Bio-Rad
TEMED	Bio-Rad
Ammonium persulfate (APS)	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Sodium bicarbonate (NaHCO ₃)	Sigma-Aldrich
TRIS hydrochloride (Tris-HCl)	Sigma-Aldrich
Calcium disodium versenate (CaNa ₂ EDTA)	Sigma-Aldrich
Sodium chloride (NaCl)	Merck Millipore
Potassium chloride (KCl)	Merck Millipore
Disodium phosphate (Na ₂ HPO ₄)	Sigma-Aldrich
Monopotassium phosphate (KH ₂ PO ₄)	Sigma-Aldrich
Hydrochloric acid (HCl)	Merck Millipore
Ethanol (CH ₃ CH ₂ OH)	Merck Millipore
Methanol (CH ₃ OH)	Merck Millipore
Sodium hydroxide (NaOH)	Merck Millipore
Sodium citrate (C ₆ H ₅ Na ₃ O ₇)	Sigma-Aldrich
Triton-X 100	USB® biochemicals
TWEEN® 20	Amresco Inc

Table3. Kits used for experiments.

Name	Brand	Usage
Mercox II Embedding Kit	Ladd Research Industries	Plastination
Basic Parasite nucleofector solution kit II	Lonza	Parasite transfection
Pierce TM BCA Protein Assay Kit	Thermo Fisher Scientific	Protein quantification
prepGEM blood kit	ZyGEM	DNA extraction
KOD Hot Start DNA Polymerase	Merck Millipore	PCR reaction
NucleoBond [®] Xtra Midi kit	MACHEREY-NAGEL	Plasmid purification
Ready-Set-Go! [®] ELISA kits for IL-1beta, IL-2, IL-6, IL-10, IL-12p70, TNF-alpha, IFN-gamma	Affymetrix eBioscience	ELISA
Mouse MCP-5 Instant ELISA kit	Affymetrix eBioscience	ELISA
Mouse VEGF-A Platinum ELISA kit	Affymetrix eBioscience	ELISA
Restriction enzyme and its corresponding reaction buffer for ApaI, BamHI, NcoI and SacII	New England Biolabs	DNA digestion
Novocastra BOND reagents	Leica Biosystems	Histology examination
Mouse Cytokine Antibody Array, Panel A	R&D Systems	Cytokine detection and quantification

2.1.5 Home-made media, buffers and solutions

Table4. Media/buffers/solutions used for experiments.

Name	Composition
incomplete RPMI (iRPMI) (for parasite culture)	Dissolve 16.2 g RPMI 1640 powder and 2.3 g sodium bicarbonate in 1L distilled water, filter through 0.2 µm membrane
complete RPMI (cRPMI) (for parasite culture)	iRPMI with 20% FBS and 30 µg/ml Gentamicin
Histodenz buffer (for histodenz solution resconstitution)	pH 7.5, containing 5 mmol/L Tris-HCl, 3mmol/L KCl and 0.3mmol/L Ca Na ₂ EDTA.
Histodenz solution (for separation of different stages parasites)	Dissolve 27.6 g Histodenz powder into 100ml Histodenz buffer, autoclave for 20 min at 120°C and store at 4°C.

1X phosphate buffered saline (PBS)	pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ and 2 mM KH ₂ PO ₄
FACs buffer	1XPBS with 1% BSA
Saline	Dissolve 0.9g NaCl in 100 ml distilled water, and filter through 0.2 µm membrane
ELISA wash buffer (PBST)	1XPBS with 0.05% Tween-20
Sodium citrate solution (anticoagulant for white blood cell isolation)	Stock solution prepared in 20% (w/v) in distilled water

2.1.6 Commercial antibodies and dyes

The commercial antibodies used for experiments in this study are listed in Table5. Among these, the antibodies with conjugation were mainly used for FACs or fluorescent western blotting, while those without conjugation were used for immunohistochemistry (IHC) examination.

Table5. Commercial antibodies used for experiments.

Anti-mouse antibody	Label	Clone and Isotype	Company
CD11b	Alexa Fluor® 647	M1/70, Rat IgG2b, K	BioLegend
CD11b	Alexa Fluor® 660	M1/70, Rat IgG2b, K	Affymetrix eBioscience
F4/80	Alexa Fluor® 488	BM8, Rat IgG2a, K	Affymetrix eBioscience
Ly6G	PE	1A8, Rat IgG2a, K	BioLegend
Ly6C	Alexa Fluor® 488	HK1.4, Rat IgG2c, K	BioLegend
Rat IgG2b K Isotype Control	PE	eB149/10H5	Affymetrix eBioscience
Rat IgG2b K Isotype Control	APC	eB149/10H5	Affymetrix eBioscience
Rat IgG2b K Isotype Control	FITC	eB149/10H5	Affymetrix eBioscience
Rat IgG2c K Isotype Control	Alexa Fluor® 488	RTK4174	BioLegend
Lamp-1 (CD107a)	Alexa Fluor® 488	1D4B, Rat IgG2a, K	Affymetrix eBioscience
Lamp-3 (CD63)	PE-Cyanine7	NVG-2, Rat IgG2a, K	Affymetrix eBioscience
P-selectin (CD62P)	NA	Rabbit polyclonal	LifeSpan Biosciences, Inc.

CD33	NA	EPR4423, Rabbit monoclonal	LifeSpan Biosciences, Inc.
CSF1R (CD115)	NA	Rabbit polyclonal	LifeSpan Biosciences, Inc.
CD3	NA	SP7, Rabbit monoclonal IgG	Abcam
CD19	NA	Rat monoclonal IgG2a	Abcam

Table6. Stains and dyes used for Fluorescence-activated cell sorting.

Dye	Content	Company
7-AAD Viability Staining Solution (for cell survival)	7-amino-actinomycin D	Affymetrix eBioscience
BD Retic-Count™ Reticulocyte Reagent System (for reticulocyte detection)	Thiazole Orange solution	BD Biosciences
Hoechst 33342 nucleic acid stain (for nucleus staining)	Hoechst 33342	Thermo Fisher Scientific

2.1.7 Computer software

Table7. Computer software used for experiment acquisition and data analysis.

Name	Developer
FlowJo V10	TreeStar Inc, Ashland, OR
Graphpad Prism 5.0	GraphPad Software, La Jolla, CA, USA
ImageJ	Wayne Rasband (retired from NIH)
Inveon	Siemens Medical Solutions USA, Inc., USA
Matlab	The MathWorks, Inc., Massachusetts, USA
Living Image® V4.3.1	Caliper Life Sciences, Inc., USA
SPSS	SPSS Inc., Chicago, USA
OriginPro 8	OriginLab Corporation, USA
Image-Pro Plus V6	Media Cybernetics, Inc., USA

ImageQuant TL software	Amersham, GE Healthcare
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2.2 Methods

2.2.1 Parasite infection in rodent host

Mice of 6-8weeks old were infected with cryopreserved stocks of different *P. yoelii* parasite lines individually by intraperitoneal injection (i.p.). These parasite lines include wildtype YM, YM-GFP, YM-mCherry, YM-luc, wildtype 17X1.1 (referred as 17X), 17X-GFP and 17X-luc as well as SERA2-KO lines. Parasitemia was monitored by thin blood smears fixed in methanol and stained with Giemsa solution or by Fluorescence-activated cell sorting (FACs) LSRII or LSR Fortessa X-20 (BD) system.

2.2.2 Mice splenectomy

Mice of 4-5weeks old were used to for mice splenectomy. In detail, mouse was first anaesthetized by ketamine and xylazine mixture by i.p., and then pain-killer Meloxicam was given by i.p.. The mouse was laid on its right side, with the left side facing upward, and its left side abdomen skin near back was wiped with 70% ethanol after which the loose hairs were removed. A small transverse incision in the skin and then the body wall were made using the dissection scissors. The spleen was gently pulled out using blunt forceps, and both ends of the spleen were tied tightly using the absorbable string of the surgical suture, following which the entire spleen was then removed using dissection scissors. The body wall and then the skin were sewed up by surgical suture one after another. Mouse was then supplied with 1ml of 1X PBS by i.p., and then kept in a clean and warm cage. A combination of antibiotics (Septtrin) was provided in drinking water for continuous 3 days after the surgery. Mice were allowed to keep in individual cages for 10-15 days for recovery before any parasite infection.

2.2.3 Parasite transfection

Mice infected with the wildtype parasite lines, either YM or 17X, were used as parasite donors. When the parasitemia in donor mice reached 15%-60%, mice were terminated using Valbarb by i.p. and infected blood was collected by cardiac puncture with syringe containing heparin solution. Parasitized blood was centrifuged at 2100 rpm with break 0 for 5 min at room temperature to remove serum and buffy coat and then washed once with iRPMI. Different stage parasites (rings, trophozoites and schizonts) were separated and harvested using a density gradient with 50%-80% Histodenz diluted in iRPMI. Schizont layer was separated and washed once with iRPMI, and then were cultured till maturity in cRPMI with gentle shaking at 37°C. Mature schizonts were transfected with the nucleofector solution from the Basic Parasite nucleofector solution kit II and the linearized construct containing the gene of interest, fluorescent signal (GFP or mCherry) and a drug selectable marker TgDHFR-TS. The transfection procedure was done using the Amaxa electroporator (Lonza) and the published protocols [240, 241]. Transfected parasites were then introduced into new BALB/c mice by intravenous injection (i.v.) and tranfectants were selected with pyrimethamine by either i.p. or in drinking water. Transfected parasites were subject to several rounds of pyrimethamine drug selection and then diluted to single parasite and injected back to new BALB/c mice. Obtained parasite lines were then checked and confirmed for the successful integration by microscope and FACs.

2.2.4 Parasite growth curve

To assess the parasite virulence, 5-10 mice as a group were each injected intravenously with 1000 mature schizonts of parasite. Same infections were done in splenectomized mice to compare the parasite phenotype. Similar procedure was done for the mixed infection and co-infection models, except that the mice received two different lines of parasites with 24 hr interval in the case of mixed infection or on the same day in the case of co-infection. Parasitemia was recorded

daily from day 3 pi by FACs.

2.2.5 Reticulocyte impact

To access whether reticulocyte has any impact on parasite clearance by the spleen, a reticulocyte- enriched model was used for parasite infection. Briefly, mice were intraperitoneally injected with PHZ with 50 mg/kg body weight per treatment with total three treatments to stimulate reticulocytosis on day-2, day-1 and day 0. Enrichment of recitulocytes was confirmed by microscope with Giemsa staining and Reticulocyte staining or by FACs system coupled with Retic-count staining. Mice were then infected with YM-GFP and when the parasitemia reached 40-60%, mice were terminated. Parasites in trophozoite and schizont stages were separated by Histodenz density gradient. Normal culture without reticulocyte enrichment was prepared similarly and used for comparison. Splenectomized mice were included to examine the spleen function. Mouse with pre-infection of 1000 mature schizonts of 17X-GFP 24 hr prior were injected with 5×10^8 normal YM-GFP or reticulocyte-enriched YM-GFP (r-YM-GFP). And GFP signal corresponding to parasite was detected by FACs system 1, 4, 24, 48 and 72 hr pi.

In some experiments the reticulocytosis in mice was stimulated by repeated bleeding. Briefly, mice were anaesthetized and bled through retro-orbital bleeding for 300-400 μ l of blood every alternate day. Reticulocyte count was monitored using Retic-count staining coupled with FACs system. When the reticulocyte count was above 10%, mice were ready to use.

2.2.6 Parasite bioluminescent assay

To investigate the parasite load in the spleen, parasite lines expressing GFP-luciferase throughout the parasite life cycle were generated and used for infection. Similarly, mice were infected with a standard inoculation of 1000 mature

infected cells and on different post-infection days mice were subjected to bioluminescent assay. Briefly, mice were injected with luciferin by i.p. 7 min before the *in vivo* peripheral luciferase signal detected by an IVIS imaging system (PerkinElmer). Mice were then anesthetized and subjected for whole body perfusion by 1X PBS. The spleen was removed and put on a clean Petri dish. The spleen luciferase signal was again detected by the IVIS imaging system. Ratios of the luciferase signal of the spleen to peripheral blood for each mouse were calculated and compared.

2.2.7 Genotyping of CD11b-DTR mice

Mouse genomic DNA (gDNA) was extracted using prepGEM blood kit from mouse tail blood according to the manufacturer's protocol. Briefly, 2-3 µl mouse tail blood was mixed with 10 µl of prepGEM buffer and 1 µl of extraction enzyme, and topped up with distilled water to a final volume of 100 µl per reaction. The extraction reaction was done in a PCR machine using an incubation setup with 75°C for 2 min followed by 95°C for 5 min. The gDNA containing supernatant was then obtained through centrifugation with 13000rpm for 5 min. 1 µl of each gDNA sample was then used for PCR reaction using KOD DNA polymerase to amplify the dTomato fragment (~700bp). The reaction was set up using an annealing temperature of 61 °C for 10second and an extension temperature of 72 °C for 45second in the first 6 cycles, and subsequently changed to 59 °C for 10second and 72 °C for 35second for another 24 cycles. The specific primer pair used is—

F: 5'—TCCGAGGACAACAACATGGC—3'

R: 5'—TACAGCTCGTCCATGCCGTA—3'

PCR products were run on 1% agarose gel to check for the positive amplification.

2.2.8 DT treatment and parasite infection

Prior to the infection, mice were weighed and received two consecutive daily doses of DT treatment by i.p. before parasite infection. As illustrated in Fig2.1, mice received another three DT treatments every three to four days after parasite infection.

Fig2.1. Experimental design for DT/DTR system-mediated inducible cell ablation approach and malaria parasite infection.

2.2.9 Spleen plastination

To study the spleen vascular changes upon parasite infection, five mice infected with 1000 mature schizonts of either YM-GFP or 17X-GFP as a group were used for spleen plastination every day from day 3 until day 7 pi, and two more points—day 17 and day24 pi for mice infected with 17X-GFP. Five normal mice as the control were included in the experiment. In detail, mice were first injected with heparin to prevent blood clotting and then mice were terminated with Valbarb euthanasia solution. Mice were cut open to expose the whole thoracic cavity and abdomen including the spleen. A 22GA catheter (BD) was surgically inserted into the heart and the descending aorta. Following the instruction protocol, the spleen was first perfused with 1X PBS followed with complete Mercor II solution through injection from the catheter. Mice were left overnight for Mercor polymerization, after which mice were immersed into 10% NaOH solution for tissue digestion. NaOH solution was changed every other day for a period of 10-14 days for complete sample digestion. Spleen samples were washed with distilled water, and spleen casts were air-dried and then collected for analysis.

2.2.10 Spleen cast analysis

The spleen casts were scanned using an Inveon CT® (Siemens) scanner by IMCB at Biopolis *ASTAR, with a configuration of an 80 W tungsten anode, 30-80kVp variable focus X-ray source and a 165 mm detector. The scan was acquired with 2 by 2 binning at an exposure time of 7800 ms per projection. A total of 1200 projection for high resolution and high magnification image scan was performed at 40 kVp anode voltage and anode current 110 μ A, giving a good dynamic range of histogram, at an effective pixel size of 19.5 microns. The acquired images were assembled and saved in .dicom files using Inveon software. Images were processed, reconstructed and visualized using Matlab. 3D-splenic vein of each specimen was “grown” by manually planting a seed at the region of interest after appropriate thresholding and masking of the entire image. The files were then saved in .tiff format and vessels complexities were analyzed using FIJI [242].

2.2.11 Serum Vascular endothelial growth factor (VEGF) determination

To examine the vascular changes upon parasite infection, mouse serum VEGF levels were measured and compared using sandwich Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer protocol. For this, mouse serum was first prepared. Briefly, mouse blood samples were collected and allowed to clot at 4°C. The samples were then centrifuged at 10000rpm for 10 min at 4°C to separate the serum, which was then stored at -20°C for future use. The ELISA kit comes with microwells precoated with polyclonal antibody to mouse VEGF-A. The microwells were first washed twice with the Wash Buffer provided by the kit and then Assay Diluent was added into the wells, followed by which the Standard or the sample diluted with Sample Diluent was added into the well with 1:1 ratio of the Assay Diluent. The microwells were incubated at room temperature for 2 hr before washing with the Wash Buffer for twice. Biotin-Conjugate was then added to the wells for 1 hr incubation at room temperature. After two washing with the Wash Buffer, Streptavidin-HRP was added into the well for 1 hr incubation at

room temperature. After another two washing, the TMB Substrate Solution was finally added in, followed with an incubation of 30 min at room temperature under dark. Stop Solution was added to quickly stop the enzyme reaction. The absorbance was determined in a micro-plate reader (Bio-Rad) using 450nm as the primary wavelength and 620nm as the reference wavelength. Data was analyzed using excel, and statistical significance was determined with Origin software using the ANOVA coupled with Tukey test.

2.2.12 Histology examination on spleens

To access the broad spleen morphology changes as well as cellular changes in response to parasite infection, five mice infected with 1000 mature schizonts of either YM-GFP or 17X-GFP as a group were used to prepare histology samples every day from day3 to day7 pi, and a few more points for mice infected with 17X parasites. Matched control mice with 3 each were included in the first and last points, with additional three intermediate points (day10, day17 and day24 pi) for 17X infection group. The body weight of mice on the day of infection (day 0) and the experimental day were recorded and compared. Mice were terminated and cut open to expose the abdomen as well as the thoracic cavity. Mouse blood was collected. Serum samples were prepared as described in section 2.2.11 and stored at -20°C for future use. Mouse liver, spleen and kidneys were cut out carefully and measured by weight before immersing into a container with 10% buffered formalin. The organ samples were then processed in a histology lab in IMCB at Biopolis *ASTAR for paraffin embedding and sectioning as well as staining. Each section was cut at 5 µm thickness using a Leica RM2255 microtome, and then was placed onto a properly labeled microscope slide.

2.2.12.1 Hematoxylin and Eosin (H&E) staining

When the microscope slide with the tissue section was dried, H&E staining was performed using the Leica Automated Slide Stainer XL with the following protocol

(Table 8).

When the program finished, the slides were then coverslipped using Cytoseal 60 on the Leica CV5030 Automated Coverslipper. Images were captured using an Olympus BX 61 microscope.

Table8. Program setup for spleen section H&E staining.

Step	Station	Reagent	Time	Exact
1	1	Xylene	5 min	No
2	2	Xylene	5 min	No
3	3	100% Ethanol	2 min	No
4	4	100% Ethanol	2 min	No
5	5	100% Ethanol	2 min	No
6	6	95% Ethanol	2 min	No
7	7	70% Ethanol	2 min	No
8	Wash 1	Gentle running water	2 min	No
9	8	Haematoxylin 2	4 min	Yes
10	Wash 5	Gentle running water	2 min	No
11	9	Clarifier 2	2 min	Yes
12	Wash 4	Gentle running water	2 min	No
13	10	Bluing Solution	2 min	Yes
14	Wash 3	Gentle running water	2 min	No
15	11	70% Ethanol	2 min	No
16	12	Eosin Y/Phloxine Solution	2 min	Yes
17	13	70% Ethanol	2 min	Yes
18	14	95% Ethanol	2 min	Yes
19	15	100% Ethanol	2 min	No
20	16	100% Ethanol	2 min	No
21	17	100% Ethanol	2 min	No
22	18	Xylene	2 min	No
23	Exit	Xylene	N/A	No
24	-	Exit for coverslipping	-	-

2.2.12.2 Immunohistochemistry (IHC) staining

IHC staining was done using Leica BOND-MAX automated system. Briefly, the tissue section was first deparaffinized in Bond™ Dewax Solution and rehydrated through 100% ethanol to 1X Bond™ Wash Solution. The sample was then incubated with Bond™ Epitope Retrieval Solution 2 for 40 min at 100°C for antigen epitope exposure. The slide was then cooled to room temperature and

washed 4 times with 1X BondTM Wash Solution. Endogenous peroxidase was blocked for 15 or 45 min in 3-4% (v/v) H₂O₂ and then the slide was rinsed three times with 1X BondTM Wash Solution before blocking using 10% goat serum for 30 or 60 min. Primary antibody was diluted in BondTM Antibody diluents to a concentration stated in Table9 and added to the sample for 15 min incubation. Again three times rinse was performed using 1X BondTM Wash Solution. After that, Polymer was added to the sample for 5 min, and the slide was rinsed again 4 times with 1X BondTM Wash Solution followed by once with deionized water. BondTM DAB Refine was applied to the sample for 7 min and then stopped through washing with deionized water. The slide was incubated with hematoxylin for 5 min for nuclei counterstaining, and then rinsed once with 1X BondTM Wash Solution and once with deionized water. The slide was then dehydrated and mounted in synthetic mounting media. Images were captured using an Olympus BX 61 microscope and analyzed with Image-Pro Plus.

Table9. Antibody dilution used for IHC staining of spleen sections.

Antibody	Dilution
P-selectin (CD62P)	1:200
CD33	1:100
CSF1R (CD115)	1:100
CD3	1:100
CD19	1:100

2.2.13 Cytokine and Chemokine study

To study the early immune responses of the spleen, mouse parasite infection was done similarly as the above mentioned procedure with i.v. injection of 1000 mature schizonts of either YM-mCherry or 17X-GFP as single infection model, or both with the infection of 17X-GFP first followed by YM-mCherry infection 24 hr later. The spleens were collected from mice on day4, day5 and day6 pi.

2.2.13.1 Cytokine and Chemokine screening

Mouse cytokine array (R&D systems) was applied with infected mice spleen to determine the different responses upon parasite single infection with either 17X-GFP or YM-mcherry line on day4 and day5 pi. Following the manufacturer protocol, infected mouse spleen was excised and smashed in 1X PBS containing protease and phosphatase cocktail. Triton X-100 was added in to the solution with a final concentration of 1%. After a freeze-thaw cycle, cellular debris was centrifuged down and spleen lysate was collected and stored at -80°C for future use. All the kit reagents including the membranes were warmed up to room temperature prior use. Membranes were blocked using the provided blocking buffer for 1 hr at room temperature on a rocking platform shaker. Spleen lysates were thawed and two samples with the same parasite line infection and post-infection day were mixed together which then mix with the reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail to incubate at room temperature for 1 hr. The sample-Ab mixtures after incubation were applied to the blocked membrane and incubated overnight at 4°C. After washing with the Washing Buffer three times, Streptavidin-HRP was added to the membranes and incubated for 30 min at room temperature with shaking. The membranes were washed three times again and prepared Chemi Reagent Mix was added. Signals on the membranes were captured by LAS-4000 (Fujifilm) and the acquired images were analysis using ImageJ. Each signal of the 40 cytokines and chemokines was quantified within the membrane and the comparison across membranes was done through the normalization by the reference spots.

2.2.13.2 Cytokine /Chemokine Enzyme Linked Immunosorbent Assay (ELISA)

8 targets among the 40 cytokines and chemokines detected by the Mouse cytokine array were selected to study the responses upon infection in detail using conventional ELISA. Five mice as a group were used for each point, and the mice were either with single parasite line infection or mixed strain infection. Spleen

protein lysate was prepared in RIPA buffer supplemented with proteases and phosphatases cocktail. Briefly, spleen was excised and frozen immediately using dry ice and stored at -80°C for future use. When doing the experiment, the spleen was thawed on ice and washed once with 1X PBS supplemented with proteases and phosphatases cocktail. Thawed spleen was then diced and washed once with RIPA buffer supplemented with proteases and phosphatases cocktail. Diced spleen samples were then added into the tubes containing the beads that have been pre-washed using 1X PBS for three times and RIPA buffer for one time. Spleen homogenization was processed using the Bullet Blender Homogenizer (Next Advance Inc.) with three rounds of procedures under Program 8 for 3 mins. Spleen tissue debris was pelleted down and supernatant was collected and stored at -80°C in 11 aliquots. Spleen protein content was determined by BCA assay following the manufacturer protocol. Briefly, samples and protein standards were 1:1 mixed with the Working Reagent in a 96-well plate and incubated for 2 hr at 37°C . After the plate has been cooled down to room temperature, absorbance was detected using the wavelength of 562nm. Protein concentration was then determined according to the absorbance of the protein standards. ELISA was performed using a standard protein concentration of 30 ng/ml and the procedures were done following the manufacturer protocol. Absorbance was detected by a micro-plate reader (Bio-Rad) using the primary wavelength of 420nm and the reference wavelength of either 570nm or 620nm according to each manufacturer protocol. Data was analyzed using excel, and statistical significance was determined with SPSS software using the Bonferroni test.

2.2.14 Red blood cell deformability measurement using a microfluidic device

To investigate the mechanical properties of the RBCs in different infection models, a microfluidic deformability device as described in Section 2.1.3 was used. Prior to each experiment, the device was first mounted to a microscope (Olympus), flushed with cRPMI and then incubated at room temperature for approximately 20 min.

Meanwhile, freshly collected mice blood was diluted with cRPMI such that each sample had a final concentration between 0.1 – 1% hematocrit. The optimal concentration range was highly desired to minimize potential clogging event due to blood cell overloading or limited RBC capturing due to over-dilution. Finally, approximately 3 μl of blood sample was loaded to the device reservoir and a constant pressure gradient of 0.36 Pa/ μm was applied as described previously. The signals were detected under a 60x objective attached to a confocal microscope and 2000 to 3000 frames were captured using 4X4 binning setting by a CCD camera. RBC velocity is defined as the horizontal displacement as RBCs traverse across the device divided by the time in terms of the number of frames taken.

To differentiate the normal RBCs and reticulocytes in the circulating blood, blood sample was stained with Retic-count solution for 5 min followed by a wash with cRPMI before going through the same experimental procedure as above.

2.2.15 Maximum Likelihood Estimation (MLE)

Let Θ denote the two possible infection outcomes (i.e. lethal or non-lethal infection), and x denote the observed RBC deformability. The likelihood function L is then represented simply as the probability density of the observed deformability x fall at a specific infection state (i.e. disease outcome). Mathematically,

$$\Theta = \{\text{Lethal}, \text{Non-lethal}\} \quad (1)$$

$$L(\theta; x) = f(x|\theta) \quad (2)$$

Then the MLE function of $\hat{\theta}$ is simply

$$\hat{\theta} = \arg \max_{\theta \in \Theta} L(\theta; x) \quad (3)$$

2.2.16 Quantification of monocytes/macrophages in circulation and the spleen

Mice were infected with 1000 mature parasites of either YM-mCherry or 17X-GFP as previously described. DTR mice received the DT treatment prior and during the infection as mentioned above. Normal BALB/c mice were included as control. Mouse blood and spleen sample were collected for analysis.

2.2.16.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Mice were either anesthetized with isoflurane or euthanized by Valbarb and blood was collected via retro-orbital route or cardiac puncture respectively. Blood was collected into tube containing 3% Sodium citrate solution to prevent coagulation. This mixture was subsequently layered above Ficoll-Paque PLUS, a solution that is commonly used to separate PBMCs from erythrocytes. The gradient was centrifuged at 900 g for 30 min with no brake at room temperature. After centrifugation, a layer of cells consisting of PBMCs would be formed and this layer of cells was transferred to clean tube containing 5 ml 1X PBS with 2% BSA for washing.

2.2.16.2 Spleen collection, processing and isolation of cells

Mice were euthanized with Valbarb and the spleen was excised and washed once with iRPMI. Excised spleen was then smashed and passed through a 70 µm cell strainer to obtain single cell suspension. The cell suspension was spun down and the pellet was resuspended with 1X RBC lysis buffer and incubated for 10 min at 4°C for RBC lysis. The cell suspension was centrifuged and the pellet was washed once with 1X PBS containing 2% BSA.

2.2.16.3 Cells labeling for flow cytometry

The PBMCs and the splenocytes were prepared as above. For staining of cell surface antigen, fluorochrome-labelled antibodies were incubated with the cells at 4 °C for 40 min to 1 hr. After incubation, the cells were washed once and

resuspended in 1X PBS containing 2% BSA for analyses. For detection of cell death and apoptosis, single cell suspension was stained with 7-AAD Viability Staining Solution for 30 min at 4°C. Samples were acquired on a LSR II or LSR Fortessa X-20 (BD) system. Data analysis was done using Flowjo.

2.2.17 Stimulation of anti-inflammatory response by Retinoic Acid (RA)

To investigate the impact of early activation of anti-inflammatory response on parasite growth, RA was used to stimulate mice response before the virulent YM parasite infection. RA was reconstituted in DMSO and diluted with DMSO. Mice were either received single dose of 500 µg RA on day-1 or two doses of RA with 300 µg each on day-1 and day+1. Control mice received DMSO. Treated mice were infected with 1000 YM-GFP parasites by i.v. on day0, and parasitemia was monitored daily from day4 pi by FACs.

2.2.18 Exosome isolation, characterization and immunization

Mouse reticulocytosis was stimulated through repeated bleeding and when reticulocyte count was >20%, mice were infected with avirulent 17X-GFP parasites. When the parasitemia was >20%, mouse blood was collected and exosomes were isolated as previously described [166]. Briefly, plasma was obtained from mouse blood through sequential centrifugations at 500 g for 30 min, 15,000 g for 45 min and 100,000 g for 2 hr at 4°C. After that, the pellet was resuspended in 1X PBS and filtered through a 0.22 µm membrane, followed by a final centrifugation at 100,000 g for 2 hr at 4°C. Exosome pellet was resuspended in 1X PBS and stored at -20°C for future use.

To verify the exosome isolation, purified exosomes were lysed with laemmli buffer and heated at 100°C for 5 min. Exosome samples were separated on a 10% SDS-PAGE gel. The blot was incubated with blocking buffer at 4°C overnight

followed by incubation at room temperature with rat anti-lamp1 (1: 800 in blocking buffer) and rat anti-lamp3 (1:600 in blocking buffer). After washing with 0.1% PBST 4 times with 5 min each, the blot was incubated with Alexa fluor-647 conjugated goat anti-rat IgG(H+L) (Thermo Fish Scientific, 1:1000 in blocking buffer) at room temperature for 1 hr. The blot was washed again with 0.1% PBST 4 times and then once with PBS, 5 min for each wash, before it was dried completely and scanned with the Typhoon Scanner (GE Healthcare). Scanned images were analyzed using ImageQuant TL software.

2.2.19 Statistics analysis

Statistical significance was determined with either SPSS or Origin software. Differences among different experimental groups were analyzed for statistical significance by means of one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honest Significant Differences) Test unless otherwise specified. A p -value <0.05 was considered statistically significant.

Chapter 3

**The spleen controls parasite growth and
determines host survival in *P. yoelii*
nonlethal strain infection**

3.1 Single strain infection model

The rodent malaria model *P. yoelii* has long been employed in malaria research to complement the research on human malaria. There are different parasite clones with different red cell preference, growth behavior and virulence that result in very different clinical outcomes in mouse. Strains like YM and 17XL invade RBC of all ages and cause a lethal infection, while strains like YA and 17X1.1 preferentially invade reticulocytes and result in a self-limiting infection that is eventually cleared by the host [243, 244]. In this study, BALB/c mice were used as the host for infection with either the lethal strain YM or the non-lethal strain 17X parasites.

3.1.1 Phenotypic assessment of genetically modified parasites

Here in this study, genetically modified parasite lines that express fluorescent signal throughout the life cycle were utilized to facilitate better visualization and easier comparison.

3.1.1.1 Generation of the gfp- and mCherry - positive parasites

Previous studies have shown that integration of the pL0016 plasmid into the parasite genome at the small subunit ribosomal RNA site will not affect the normal parasitic development in *P. berghei* [245].

Fig3.1. Plasmid construction for mCherry-positive parasite. Antibiotic resistance and drug-selectable markers as well as the fluorescent signal fragments are indicated in brown. A- The original published pL0016 plasmid; B- pL0016 derivate with mCherry as the fluorescent signal in replacement of the *gfpM3*; C- Restriction digestion profile of purified plasmids of selected clones to screen for correct orientation of the mCherry insert.

Here in this study, we used the published pL0016 plasmid (Fig3.1A) containing the *tgdhfr* drug selectable marker and a fluorescent reporter *gfpM3* driven by the constitutive promoter *pbef1* (elongation factor 1) as the backbone to generate pL0016-mCherry construct for producing the mCherry-positive parasites (Fig3.1B). A DNA fragment of mCherry was amplified and purified to replace the *gfpM3* fragment by using the restriction enzyme *BamHI*. Orientation of the insertion was determined by the restriction digestion profile of the purified plasmids of selected clones through double digestion by *NcoI* and *SacII*, and separation of the digested plasmid DNA was on a 1% gel (Fig3.1C). The band size of 1.3 kb indicated the

correct orientation while the 1kb band represented the wrong orientation. As a result, plasmid 1 was selected for transfection to generate the mCherry-positive *P. yoelii* parasites. And the original pL0016 was used for the generation of gfp-positive *P. yoelii* line. Both plasmids were linearized through the unique SacII and ApaI sites at the d-ssu-rna region targeting the small subunit ribosomal RNA in the parasite genome. Total amount of 5-10 µg of each cut plasmid in a volume of 5-10 µl was used for each transfection reaction.

3.1.1.2 Growth behaviour of the parasites in spleen-intact and splenectomized mice

Previous studies have demonstrated that infection with the *P. yoelii* YM line is lethal while the *P. yoelii* 17X line is non-lethal [244]. Here we examined the growth behaviour of the generated genetically modified parasites. Similar to the wildtype parasite line, when the mice were infected with the genetically modified YM lines (Fig3.2A and B), regardless of the presence of the host spleen, the parasites multiplied rapidly and reached peak parasitemia (>50%) on day7 post-infection (pi) followed by host death. In contrast, with the presence of the intact spleen in mice (Fig3.2C), the genetically modified 17X parasites multiplied slowly and showed a much lower peak parasitemia (~17%) on a much later post-infection day (day14). They showed a significant different replication profile from day5 pi onwards, as compared to YM (one-way ANOVA, p -value <0.05). The avirulent parasites were subsequently cleared from the mice blood circulation by day17 pi. On the other hand in the absence of the intact spleen, the avirulent 17X parasites showed significant difference in growth rate from day4 pi onwards (p -value <0.05), as compared to that in the host with the intact spleen. As shown in Fig3.2D, the majority of the splenectomized mice (~80%) died from the infection with high parasitemia (>30%) even when infected with the avirulent 17X parasites, and the remainders suffered from a long-lasting infection that lasted beyond 23 days after infection.

Fig3.2. *Plasmodium yoelii* growth behavior in BALB/c mice. A- Growth curve for YM-GFP line in spleen-intact and splenectomized mice; B-Growth curve for YM-mCherry line in spleen-intact and splenectomized mice; C-Growth curve for 17X-GFP line in spleen intact and splenectomized mice; D- Survival curve for splenectomized mice infected with 17X-GFP line. Data are mean $\pm$ SEM results from three or more individual experiments with total 10-15 mice. † denotes death of mice.

3.1.2 Spleen remodelling upon parasite infection

The data from the parasite growth and host survival clearly indicates that the spleen plays an important role in regulating differential parasite growth profiles and host survival, what distinguishing the virulent and avirulent parasite infections. Therefore, the spleen responses upon parasite infection were next studied to investigate potential mechanisms accounting for the different disease outcomes.

3.1.2.1 Overall morphological changes of the spleen upon infection

Upon infection, an increase in spleen size was observed for both virulent YM and avirulent 17X parasite lines (Fig3.3A and B), though there was no significant difference between YM and 17X until day7 pi (p -value <0.05). In particular, we noted the enormous splenomegaly observed in 17X-infected mice, such that their spleen lengths increased by close to three-fold on day14 pi when peak parasitemia was achieved (Fig3.3B). Subsequently, the spleens slowly reduced back to normal size after parasite clearance.

Besides the palpable changes in spleen size, the color of the spleens gradually changed from bright red to dark brown during the course of the infection. The dark brown color indicates RBC disruption and is associated with increased parasite load. For example the spleens for YM-infected mice were darker brown on day7 pi as compared to day5 pi when the parasitemia was much lower (Fig3.3A). Moreover, other organs such as liver and kidney from the same mouse were excised, examined and compared, confirming that the increase in spleen is stimulated by the parasite infection and is independent to the normal growth of the mouse. In addition, when comparing the ratios of spleen/liver weight and spleen/kidney weight between the two parasite infections, the avirulent 17X infection showed significantly higher ratios comparing to those of the virulent YM infection on day4 pi (Fig3.3C and D, p -value <0.05), indicating a stronger spleen response stimulated by the 17X infection in the early infection stage.

Fig3.3. Spleen morphological changes upon parasite infection. A-Selected images of spleens from control or infected mice on different post-infection days; B-Measurement of spleen length on different post-infection days; C-Comparison of spleen and liver weight on different post-infection days; D-Comparison of spleen and kidney weight on different post-infection days. Data are mean $\pm$ SEM results from 5 mice per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates p -value <0.05 .

3.1.2.2 Architecture changes of the spleen upon infection

Besides the changes in spleen appearance, malaria-induced morphological changes in spleen architecture were also examined through brightfield microscopy coupled with hematoxylin and eosin (H&E) stain.

Fig3.4. H&E staining of spleen sections for different post-infection days. Panel I- 20x magnification; Panel II- 100x magnification, with red arrow indicating the infected RBCs. Data presented as one representative picture of five spleen samples per group.

For mice infected with the virulent YM line, the splenic red pulps showed minimal to mild expansion associating with congestion starting from day4 pi. There were minimal increased in lymphocytes from day5 pi onwards, and minimal increased extramedullary hematopoiesis on day7 pi. The splenic white pulps showed gradual disruption from day5 pi with the disappearance of the marginal zone (Fig3.4I). Free and intracellular golden brown parasite pigments were observed from day4 pi

(Fig3.4II). For the mice infected with the avirulent 17X line, the expansion of the red pulps associating with congestion was also starting from day4 pi. Nevertheless, there was minimal to moderate increased lymphocytes and minimal to marked increases extramedullary hematopoiesis starting from day4 pi (Fig3.4I and Appendix I). Similar to the YM-infected mice, the splenic white pulps of the 17X-infected mice were also gradually disrupted from day5 pi, which slowly reformed after parasite clearance on day17 pi, followed by the marginal zone reappearance (Appendix I). In addition, there was minimal to mild single cell necrosis of lymphocytes in white and red pulps observed from day7 pi till day24pi in 17X-infected mice. Free and intracellular parasite pigments were observed one day earlier in mice infected with the non-lethal 17X line (day3) as indicated by the red arrows in Fig3.4II, indicating a preferential trapping of the 17X-infected RBCs compared to YM-infected RBCs.

3.1.2.3 Structural changes of the spleen upon infection

Upon malaria parasite infection, the spleen exhibits great responses. Besides spleen appearance and architecture, the changes in splenic microcirculatory network upon parasite infection were studied here in details.

3.1.2.3.1 Observation on splenic vasculature

In collaboration with Ms Lai Chun from Prof Matsudaira's lab (National University of Singapore/Singapore-MIT Alliance for Research and Technology), mouse spleen plastination casts were prepared on different post-infection days to gain information on the changes in the circulation in the spleen upon parasite infection. The plastic spleen casts were analyzed by a CT scanner and the re-constructed images were analyzed using Matlab (Fig3.5). As shown in Fig3.5A, the splenic veins and arteries were identified (indicated by the yellow arrows). In addition, the internal vessels as well as the red pulp meshwork can be recognized. In general, both the virulent YM infection (Fig3.5B) and the avirulent 17X

infection (Fig3.5C) stimulated increased complexity of the splenic vasculature.

A. Control

B. YM-GFP

C. 17X1.1-GFP

Fig3.5. Reconstructed spleen cast images. A- Spleen casts of control mice, with splenic vein and artery are indicated by the yellow arrows; B- Spleen casts of YM-GFP infected mice; C- Spleen casts of 17X-GFP infected mice. Data presented as one representative picture of three spleen casts per group.

3.1.2.3.2 Quantification of splenic vascular changes

To further investigate the complexity of splenic vasculature, the splenic internal vessels in terms of the number of venous branches and the relative size of vein to artery were examined and both appeared to vary with parasite infection and parasitemia changes. Fig3.6A plots the relative diameters of splenic veins to arteries during the course of disease development. The vein-to-artery diameter ratios increased gradually in both infection models, with significant difference from the control from day4 pi onwards (p -value <0.05). Moreover the ratios in the YM infection model increased more significantly on day7 pi as compared to 17X infection (p -value < 0.05). We noted that the diameters of splenic arteries remained relatively constant in both infection models throughout the post-infection days and the significant increases in the ratio were largely due to dilation of the splenic veins upon infection (Appendix II). The vein-to-artery ratio returned to normal after the parasite clearance in mice infected with the avirulent 17X line (Appendix II). In addition to the splenic vessel size, we also quantified the number of venous branches on different post-infection days. Fig3.6B indicates that more veins formed upon avirulent 17X infection compared to virulent YM infection, and the number of venous branches returned to normal after parasite clearance. Systemic vascular endothelial growth factor (VEGF) level in the serum was also measured (Fig3.6C), confirming that there were new vessels synthesized in response to parasite infection. We observed a significant increase in VEGF concentration upon parasite infection (p -value <0.05). However, though comparatively the VEGF levels were higher in mice infected with the avirulent 17X line, they were not significantly different between the hosts infected with YM and 17X on the time points tested. It is therefore not yet clear what molecular mechanism is driving the difference in venous branch development observed between the virulent and avirulent parasite infections.

Fig3.6. Quantification of splenic vascular changes. A- Measurement of vein to artery diameter ratio. Data are represented as mean $\pm$ SEM for results from the three main pairs of veins and arteries of one representative spleen cast of three samples per group; B- Quantification of venous branches. Data are from one representative spleen cast of three samples per group; C- Expression of VEGF in mouse serum. Data are means $\pm$ SEM results from a total of five serum samples per group, collected in two replicate experiments.

3.1.2.4 Comparison of parasite trapping in spleen

The more prominent changes in the splenic vessels and the resulting microcirculation after the avirulent 17X infection, suggest that there is a significant increase in the overall blood volume that is filtered in the spleen and subsequently collected by the increased venous outflow in 17X infections, while the observed earlier deposition of the parasite pigments in the 17X-infected spleens indicates a more preferential splenic trapping towards the avirulent 17X-infected RBCs. A quantification of the parasite trapped in the spleen was then performed with the help of the luciferase-expressing parasite lines and bioluminescent assay.

Fig3.7. Plasmid used for producing luciferase-expressing parasites.

The luciferase-expressing parasite lines again were generated on both virulent YM and avirulent 17X lines using the pL0027 vector (Fig3.7). Similar to pL0016, pL0027 was also linearized at the d-ssu-rrna region through the unique SacII and ApaI sites for the parasite transfection. Similar to the parasites transfected with the

pL0016 plasmid, successful transfected parasites of either virulent YM (YM-luc) or avirulent 17X (17X-luc) with the pL0027 vector would contain the *tgdhfr* drug selectable marker and express GFP-luciferase under control of the constitutive *Pbefl* promoter. Transfected parasites were first selected by pyrimethamine treatment and a second selection procedure was performed by FACs sorting GFP-expressing parasites. Sorted parasites were then diluted and used to infect new mice with each receiving 10 sorted parasites of either 17X-luc or YM-luc. The parasites were again subjected to three-day consecutive pyrimethamine treatment before the parasite stocks were prepared.

A previously study has shown that in luciferase-expressing *P. berghei*, there is a strong positive linear correlation between luminescence intensity and parasite density [246]. Here in this study, with these generated luciferase-expressing *P. yoelii* parasite lines, parasite load reflected through luciferase signal in peripheral blood as well as the spleen was accessed and compared (Fig3.8). On the same post-infection day, mice infected with the virulent YM line showed significantly higher luciferase signal in peripheral blood compared to those infected with the avirulent 17X line on both day4 and day5 pi (Fig3.8A), consistent with the higher peripheral parasitemia observed in the gfp- or mCherry- expressing parasite lines (Fig3.2). In contrast, the luciferase signal in the spleens from YM-infected mice after perfusion showed much lower signal than the 17X-infected mice spleens (Fig3.8B), indicating a higher number of parasite retained in the avirulent 17X-infected spleen. Since there are great differences in the circulating blood parasitemia between the virulent and avirulent infections even on the same post-infection day, instead of direct evaluation, relative luciferase signal of the spleen to the peripheral blood was assessed for better quantification and comparison. 17X-infected mice showed significantly higher relative luciferase signal when comparing to that in YM-infected mice on both day4 pi and day5 pi (Fig3.8C), confirming that 17X-infected RBCs are more

preferentially trapped in the spleen, and this is consistent with the previous result that earlier and higher parasite deposition observed in the avirulent 17X-infected spleen.

Fig3.8. Parasite load reflecting through luciferase signal. A- Parasite load in peripheral blood; B- Parasite trapped in the spleen; C- Comparison of parasite retention in the spleen between virulent and avirulent parasite lines. Data are mean $\pm$ SEM results pooled from two individual experiments each with 3 mice per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates p -value < 0.05.

3.2 Mixed infection model

Our data clearly show that the spleen plays a central role in controlling the parasite growth and determining disease outcome in avirulent parasite infection. Given the diverse spleen remodelling and retention between virulent and avirulent parasite infections, we speculated that avirulent parasite infection through its characteristic non-lethal spleen-dependent response could stimulate a non-lethal disease outcome against a subsequent infection with virulent parasites.

3.2.1 Parasite growth behaviour in mixed infection model

To verify this hypothesis, a mixed infection model was set up, where the mice were pre-infected with the avirulent 17X line and 24 hr later challenged with the virulent YM line. Splenectomized hosts were also included to reveal the spleen influence.

As shown in Fig3.9A, the total parasitemia in the mixed infection increased very slowly in mice with intact spleen and the peak parasitemia was around 10% on day11 pi followed by parasite clearance by day17 pi. Again, the protective effect only applies for mice with intact spleen; in hosts with the spleen removed, the majority (Fig3.9B) developed severe disease and mice started to die from day 9 pi onwards with a typical parasitemia close to 30%. When looking at the parasite growth on the same post-infection day relative to YM infection, we also noted that compared to the single parasite infection model, the parasitemia of YM-infected cells in the mixed infection model was significantly lower in both spleen-intact and splenectomized hosts (Fig3.9C). For example, in the mixed infection model, the peak YM parasitemia was suppressed to around 7% and 15% with spleen-intact

and splenectomized mice respectively. On the other hand, unlike the virulent strain YM, the avirulent 17X-infected cells (Fig3.9D) showed similar growth patterns in the spleen-intact and splenectomized mice respectively, regardless the infection types of either single or mixed.

3.2.2 Parasite growth in modified mixed infection

In order to further investigate the mixed parasite infection, three modified mixed infection models were set up. To examine the possible interference on parasite growth by the co-appearance of both GFP and mCherry fluorescent proteins, an infection model (Model 1) with co-infecting 1000 mature schizonts of YM-GFP and 1000 mature schizonts of YM-mCherry were carried out (Fig3.10A). Similar to the single infection model, the parasitemia in Model 1 increased rapidly and the mice started to die from day7 pi and by day8 pi all mice were dead, indicating that the co-appearance of the two fluorescent report proteins have no effect on parasite growth.

Fig3.9. Mixed infection model of *Plasmodium yoelii*. A-Parasite growth curve of total parasites in mixed infection in spleen-intact mice and splenectomized mice; B-Survival curve of splenectomized mice in mixed infection; C-Parasite growth curve of YM in mixed infection as well as single infection in spleen-intact mice and splenectomized mice; D-Parasite growth curve of 17X in mixed infection as well as single infection in spleen-intact mice and splenectomized mice. Data are mean $\pm$ SEM results pooled from three or more individual experiments with total 10-15 mice. † denotes death of mice.

With the assumption that parasite number increased 10 times in each multiplication cycle, a modified mixed infection model was performed with pre-infection of 100 mature schizonts of YM-mCherry and then 24 hr later challenged with 10^4 mature schizonts of 17X-GFP (Model 2). As shown in Fig3.10B, in this model, the parasitemia of both virulent YM and avirulent 17X showed to be very low at the early stage (day3-day6 pi), followed by rapid increase of YM-mCherry parasites with a peak parasitemia of 45.9% appeared on day9 pi; the avirulent 17X line remained to be very low. In Model 2, though the growth of the virulent YM parasites was not successfully suppressed, there was a delay of host death compared to that in the single infection model, where the mice started to die only from day8 pi and all the mice were dead by day11 pi. On the other hand, when the mice were co-infected with 1000 mature schizonts of YM-mCherry and 10^4 mature schizonts of 17X-GFP (Model 3), the parasitemia was suppressed compared to that in the single infection models, particularly for the virulent YM parasites. The peak parasitemia appeared on day12 pi, with the avirulent 17X line being close to 8%, while the virulent YM line was around 10% (Fig3.10C). The majority of the hosts in Model 3 survived from the infection, suggesting that both the timing and the amount of avirulent parasites present are important to stimulate the spleen response against the virulent parasite infection.

Fig3.10. Parasite growth in different mixed infection models. A- Model 1, with co-infection of 1000 YM-mCherry and 1000 YM-GFP; B- Model 2, with pre-infection of 100 YM-mCherry and then 24hr later challenged with 10^4 17X-GFP; C- Model 3, with co-infection of 1000 YM-mCherry and 10^4 17X-GFP. Data are mean $\pm$ SEM results from 5 mice.

To further investigate the protective effect of the pre-infection of avirulent parasites in the mixed infection model, another modified mixed infection model was set up using SERA2-knockout (SERA2-KO) line in replacement of the avirulent 17X line (Fig3.11).

Fig3.11. Parasite growth behaviour in modified mixed infection model. A- Parasite growth curves of individual parasite line. Data are mean $\pm$ SEM results from 10 mice; B- Survival curve of the mice infected with SERA2-KO and challenged with YM-mCherry.

A previous study has shown that SERA2-KO parasites have greatly reduced virulence and increased selectivity index (SI) compared to the parental virulent YM line [152]. When the mice were pre-infected with 1000 mature schizonts of SERA2-KO and then 24hr later challenged with 1000 mature schizonts of

YM-mCherry, the SERA2-KO parasitemia as assessed by the GFP signal remained very low all the time until the host had recovered. On the other hand, the mCherry signal indicating the parasitemia of virulent YM line increased gradually and reached a first peak (~26%) on day10 pi, followed by two days of parasite clearance. However, this trend was reverted and the virulent YM parasite levels increased again until ~35% on day17 before the parasites were cleared again (Fig3.11A). The majority of the mice (~67%) survived from the infection (Fig3.11B) and all parasites were cleared by day27 pi without any subsequent re-occurrence.

3.2.3 Assessment of parasite trapping in the spleen

The data from the mixed infection model indicates that the virulent YM parasites are suppressed by the pre- or simultaneous infection of avirulent parasites. To investigate the parasite retention in the spleen in the mixed infection, luciferase-expressing YM line was used in replacement of the YM-mCherry in the mixed infection. Parasite load was determined by the luciferase signal (radiance).

As shown in Fig3.12, the luciferase-expressing virulent YM line had much low radiance in the mixed infection comparing to that in the single infection (Fig3.8A), with an average radiance of 4.98×10^5 and 4.3×10^6 in the mixed infection while 9.6×10^6 and 2.1×10^7 in single infection on day4 and day5 pi respectively. And the parasite signals in the spleen after perfusion also showed to be lower in the mixed infection (Fig3.12B) with the average radiance being 1.21×10^6 and 9.81×10^6 on day4 and day5 pi respectively, comparing to that of 1.3×10^7 and 2.7×10^7 respectively in the YM single infection model (Fig3.8B).

Again, since the circulating parasitemia was very much different between the YM single infection and the mixed infection even on the same post-infection day, the relative luciferase signal of the spleen to the peripheral blood was assessed instead of the absolute quantification. Similar to 17X single infection, the relative luciferase signal of the spleen to the peripheral blood was significantly higher in the mixed infection model (2.53 and 2.28 respectively) compared to that in the YM single infection model (1.57 and 1.53 respectively) on both day4 and day5 pi, indicating a higher spleen trapping of YM-infected RBCs in the mixed infection.

Fig3.12. YM parasite load in mixed infection. A- Parasite load in peripheral blood; B- Parasite trapped in the spleen; C- Comparison of YM retention in the spleen in single infection and mixed infection. Data are mean $\pm$ SEM results pooled from two individual experiments each with 3 mice per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates p-value <0.05 .

3.3 Discussion

Though the virulent YM and the avirulent 17X lines have a highly similar genetic background, they have very much different phenotypes such as parasite growth rate, host RBC preference, surface protein expression as well as stimulation of immune response [148, 243, 247]. In this study, we take a new perspective and attempt to understand malaria through spleen remodelling and retention. The essential role of the spleen in malaria pathogenesis was verified by examining the effect of splenectomy on both lethal and non-lethal infected hosts. Removal of the spleen resulted in severe disease outcomes even for avirulent 17X parasite infection, in line with the spleen controlling parasite growth and determining disease outcome in non-lethal parasite infection.

We next investigated malaria-induced splenic remodelling that may promote parasite retention. The spleen is a highly vascular organ that has very complex and highly branching vessel. And the spleen size is usually important in its pathological evaluation [53], for example, enlargement of the spleen, commonly referred as splenomegaly, has been used as a clinical marker to estimate malaria transmission [248]. Our data showed rapid and distinct splenic responses in both morphology and architecture during infection of different malaria parasite strains. While in both YM and 17X infections the spleen undergoes remodelling, it is clear from our data that in 17X there is a significant increase in the venous pore size and the splenic vein- to-artery diameter ratio. The rapid enlargement of the spleen and its associated prominent increase in splenic vasculature in mice infected with the avirulent 17X parasites implies that there is a significant increase in the overall blood volume that is filtered in the spleen and subsequently collected by the increased venous outflow in 17X infections, such that contributing to the splenic ability on controlling parasite load. Together with the observation that earlier parasite deposition as well as higher preferential iRBC trapping in the spleen in

mice infected with the avirulent 17X line than the virulent YM line, suggest a mechanism to distinguish lethal and nonlethal infection through the splenic response and its resulting splenic clearance of parasites. This was verified by our mixed infection model. The observed significantly higher spleen retention rate of the YM-infection RBCs in the mixed infection compared to that in the single infection, together with its non-lethal disease outcome in the spleen-intact hosts, indicating that pre-infection with avirulent 17X induced an “avirulent” like spleen response and protected the mice from the virulent parasite infection. This “avirulent” like spleen response is associated with increased splenic filtration capacity and an early protective immunological reaction may potentially be involved which still needs to be further investigated. Overall, our data supports the prospect that the strain-specific spleen remodelling is likely to modulate pathological outcome of malaria, while the increased retention of iRBC is a key mechanism by which the spleen functions to control avirulent parasite infections.

Interestingly, the protective effect from the avirulent strain parasites in parasite mixed strain infection seems to be correlated with the infection timing as well as the parasite amount. A previous study has demonstrated that the time between the avirulent and virulent parasite infections is important for disease outcome, where mice can be protected against the virulent parasite infection when the challenge was made 3 days after the avirulent parasite infection, but not completely protected if the challenge was made 1 day after the avirulent parasite infection [249]. However, in our model, 24 hr interval between the avirulent and virulent parasite infection was enough for the host to respond and get protected from the virulent parasite infection, and this protection is associated with the host spleen responses which is absent in the splenectomized hosts and resulting in lethal disease outcome. In addition, our data also indicates that besides the timing, the sequence of the infection of the mixed parasite strains as well as the ratio of the virulent and avirulent parasites are important to stimulate the protective responses. This was

confirmed by our modified mixed infection models, where changes in the infection sequence of the virulent and avirulent parasites or their ratio resulted in different disease outcomes. Comparatively, our modified mixed infection models imply that the presence of a larger amount of the avirulent parasites when infecting the virulent parasites could potentially overcome the time interval factor and stimulate the host responses towards the avirulent type.

It is commonly thought that the virulent strain YM parasites have a growth advantage compared to avirulent strains, explaining strain specific intrinsic virulence. However this common presumption clearly is not supported in our mixed infection model study, where the avirulent 17X parasite infection actually suppressed the growth of the virulent YM parasites. It is also counter intuitive to expect the combined peak parasitemia in the mixed infection model to be lower than that in single strain infections. Moreover, though the spleen shows rapid and dramatic changes in malaria infection, our data showed that the suppression of the lethal strain parasite in the mixed infection is spleen-independent as demonstrated by its existence in the splenectomized mice model. It raises a question on how this phenomenon could be achieved considering the small amount of the starting parasites (1000 schizonts) in such a short time (24 hr interval). There must be a powerful system to facilitate this, and the parasite molecules that could stimulate such rapid response remain to be elucidated. On the other hand, the information obtained from the mixed-infection model as well as the modified mixed infections also highlights the possibility in controlling the lethal disease by co-infection with the avirulent strain parasites, changing the spleen retention of the iRBCs so as to modulate the parasite clearance by the spleen.

Chapter 4

**Deformability of the red blood cell acts as
a biomarker for parasite virulence
prognosis**

4.1 Cell deformability profile

The data from the luciferase bioluminescence assay indicates there is preferential trapping of the avirulent 17X parasites compared to the virulent YM parasites, resulting in differential clearance of iRBCs by the spleen. There are many factors influence the spleen clearance of malaria parasites. In fact, a lot of evidences suggest that, besides ligand-receptor interactions, reduced red cell deformability is greatly associated with splenic clearance [80, 138, 173]. Therefore, here in this study we attempt to understand malaria pathology through spleen remodelling and retention, from the point of view that spleen trapping is a mechanical process. This part of work was done in collaboration with Dr Huang Sha from Prof Han's lab (Massachusetts Institute of Technology/ Singapore-MIT Alliance for Research and Technology).

4.1.1 Cell deformability profile in mice with parasite single strain infection

Changes in RBC deformability have been shown to be one way the spleen uses to recognize and subsequently destroys iRBC. To investigate whether the observed changes in spleen morphology are related to differences in the biomechanical properties of the iRBC, we set out to explore this in the different parasite infection models used here.

The microfluidic device used in this study has repeated bottleneck structures with dimension of 3 x 4.2 μm^2 (Width x Height,), which is similar to the critical mesh size of mouse spleen [140]. These repeated bottleneck structures would continuously challenge the RBCs mechanically when they pass through the device. With a given distance of the bottleneck structures, the less deformable cells would migrate slower as compared to the more deformable ones. As a result,

the deformability profile of infected and uninfected RBC as interpreted by the transit velocity of individual cells was established over the course of different *P. yoelii* infections (Fig4.1). Low RBC velocity corresponds to impaired cell deformability and vice versa. In agreement with prior works, in both YM and 17X infection models, iRBCs consistently exhibited lowered cell deformability (i.e. velocity) throughout (Fig4.1). Whereas both infected (red) and uninfected (black) RBC deformability remained fairly stable across the post-infection days (day4-7 pi) for the YM-infected mice, RBC deformability varied significantly over the course of infection in 17X-infected mice (p -value <0.05). In the peripheral blood of 17X-infected mice, the mean velocities of the uninfected cells (black) were similar to that of YM-infected mice, and remained relatively constant during early infection days until a significant dip was observed on day12-14 pi. On the other hand, the 17X-infected cells (blue) exhibited significantly lower deformability as compared to YM-infected cells (red) during early infection when the parasitemia was less than 1% (day 5 pi) (p -value <0.05); and the deformability of 17X-infected RBCs decreased further by over 40% from day9 pi onwards, until all detectable parasites were cleared.

The deformability of blood extracted from mice spleen (i.e. splenic blood) was also assessed during the course of the infection (Fig4.1). In YM-infected mice, the mean velocities of both the infected and uninfected red blood cells in the splenic blood were significantly lower than that in the peripheral blood (p -value <0.05). Similarly, in 17X-infected mice, the splenic minced uRBCs were less deformable as compared to that of peripheral blood, but no significant change in velocity for the infected cells between the splenic blood and peripheral blood was observed in the time points tested. However, no further stiffening was observed when we compared splenic infected cells against peripheral infected cells on day10-14 pi. Interestingly, there were no iRBCs detected in the splenic blood on day7 pi. This could be due to the relatively low parasitemia observed in

the peripheral blood on that day, or special changes in the spleen that adhere and separate the infected cells, as proposed previously [250].

To further investigate the interaction of splenic retention and cell deformability, the deformability of peripheral blood RBCs from splenectomized mice was also measured. In the absence of the spleen, similar deformability was observed for YM- and 17X-infected RBCs (Fig4.1). There was no significant difference in deformability of the infected cells between YM and 17X except day5 pi (p -value =0.036). The post-infection day dependent decrease observed in the 17X-infected RBC deformability profile was no longer observed either; instead, their deformability remained fairly stable across day5-9 pi, with no significant changes in velocity between adjacent time points tested in both infection models.

Fig4.1. Deformability/velocity profiles of the RBCs in mice with parasite infection. Black- uninfected RBCs; Red- YM-GFP-infected RBCs; Blue- 17X-GFP-infected RBCs. The lines connect the mean value of each RBC population. Data are pooled from at least three individual experiments each with three mice per group. Data are pooled from at least three individual experiments each with three mice per group. Statistic analysis was done using one-way ANOVA with Tukey test.

4.1.2 Cell deformability profile in mice with parasite mixed infection

The finding that removal of the spleen impacts the deformability of iRBC was surprising as it implicates that the spleen directly regulates the biomechanical properties of the iRBC. It suggested to us that the complex interplay between the parasite and the host spleen could trigger signals that impact iRBC deformability and thereby the clinical outcome that may be independent of the parasite strain. To explore this, we utilized the mixed infection model and assessed the RBC deformability.

Fig4.2. Deformability/velocity profile of RBCs in mixed infection. Black- uninfected RBCs; Red- YM-GFP-infected RBCs; Blue- 17X-GFP-infected RBCs. The lines indicate the mean value of each population of RBCs. Data are pooled from two individual experiments each with three mice per group.

Consistent to prior results, regardless of the presence of the spleen, the uRBCs were more deformable as compared to iRBCs (p -value <0.05). In particular, iRBCs from the spleen-intact mice were close to 2-fold less deformable as compared to those of the splenectomized hosts; even the YM-infected RBCs were very stiff with a mean velocity close to 1.0 (Fig4.2). Though there were

some differences between YM- and 17X-infected RBCs in spleen-intact hosts on day9 pi, the p -value of 0.04066 indicating only a marginal trend towards the statistical significance. Both YM- and 17X-infected RBCs from splenectomized hosts exhibited significantly higher deformability (p -value <0.05) as compared to those from the spleen-intact mice, implying a less efficient parasite clearance by the capillary circulation can be achieved [251].

4.2 Application of cell deformability in malaria prognosis

The substantial changes in cell deformability along with the changes in spleen microcirculatory network, allows a more efficient clearance of the avirulent parasites, which probably account for the less severe disease outcome. The high correlation between RBC deformability and the severity of the disease led us to explore the possibility of using RBC deformability as a biomarker for fast malaria prognosis.

With a similar parasite load of around 5% parasitemia, we first constructed Gaussian fitting curves for the deformability profiles of the respective infection models (Fig4.3A). The mean and standard deviation of iRBC deformability from YM-, 17X-, and mixed-infection models were 2.08 ± 0.75 , 0.5 ± 0.41 and 0.77 ± 0.79 respectively. Pairwise separation resolution (SR) describing how well two given populations are apart from each other was also calculated in order to compare the deformability difference between lethal (i.e. YM) and non-lethal infections (i.e. 17X or mixed infection model). From equation 4, the deformability SR between YM- and 17X-infected RBCs, and between YM- and mixed model iRBCs was 0.67 and 0.42 (Fig4.3A).

$$SR_{i,j} = (\mu_i - \mu_j)/2(\sigma_i + \sigma_j) \quad (4)$$

where μ_i and μ_j are the mean deformability values of RBCs infected with parasite infection model i and j respectively. And σ_1 and σ_2 denote the standard deviations of deformability values in respective infection models.

Since in our animal models, YM infections always induced a lethal disease outcome (i.e. the death of the host) whereas 17X always lead to a non-lethal infection, we then estimated the parasite infection severity using the maximum likelihood estimation (MLE) based on the deformability of a given single iRBC alone. Briefly, we randomly subsampled 15 out of 34 RBCs each from the YM and 17X single infection model (Fig4.3A), and termed them as the reference group. The remaining 19 YM-infected, 19 17X-infected as well as 29 mixed model iRBCs (including 15 YM-infected RBCs and 14 17X-infected RBCs) were used as test group. Gaussian fittings as described earlier (Fig4.3A) were then constructed using the reference group, such that the deformability distributions of lethal and non-lethal infections were defined by the 15 YM- and 17X-infected RBCs respectively. We next calculated the probability densities of the remaining RBCs in the test group against the reference group and applied MLE to assign individual RBCs to the disease outcome in which they are more likely to fall into (refer to the Methods and Materials for more details for the calculation).

The MLE result after one subsampling is illustrated as Fig4.3B. Close to 90% of the YM-infected RBCs, 100% of the 17X-infected RBCs, and 76% of the mixed model iRBCs, all from the test group, correctly identified their corresponding disease states. Note that in this evaluation, we considered the disease outcome of mixed infection as “non-lethal”. Similarly, with 20 reiterations of the random subsampling, our deformability data could identify a lethal infection at over 90% of true positive (TP) and true negative (TN) rate in single infection models

(Fig4.3C). Even in the mixed infection model where the deformability distribution is more complex, the MLE could still produce a TN rate of 72%.

Fig4.3. Prognosis of malaria. A-Gaussian fitting curves for the deformability profiles of respective infection models. Red- YM single infection; Blue- 17X single infection; Green- mixed infection; B-Illustration of MLE result after one sub-sampling; C-Evaluation scores of the different infection models.

Our data show clear differences in the iRBC deformability between virulent and avirulent parasites lines, and their deformability profile can be potentially used as a robust predictor of clinical outcome. To investigate this further, we assessed the cell deformability information on other *Plasmodium* infection models. *P. berghei* ANKA (PbA) causes an acute infection in C57BL/6 (B6) mouse that is lethal in about a week post-infection, which is also commonly used as experimental cerebral malaria (ECM) model [252]; while for BALB/c mouse, a mouse strain

that is usually resistant to ECM, PbA infection will not be lethal within the ECM window [253]. A preliminary result showed that when the mice were infected with 1000 mature schizonts of PbA and reached a similar parasitemia about 2% on day8 pi, the uRBCs of both BALB/c and B6 mice peripheral blood showed no significant difference and had a similar deformability/velocity level with a mean value around 3.2 (Fig4.4), which is consistently higher than the iRBCs and comparable to that in the *P. yoelii* infection. For the iRBCs, PbA-infected B6 mice, corresponding to a lethal infection, showed a significantly higher mean value (close to 2-fold) in deformability than the PbA-infected BALB/c mice (p -value <0.05).

Fig4.4. Deformability/velocity of RBCs in *Plasmodium berghei* ANKA infection on day8 pi. Black- uninfected RBCs; Blue- infected RBCs in BALB/c mice; Red- infected RBCs in B6 mice. The lines indicate the mean value of each population of RBCs. Data are pooled from three mice per group.

4.3 Discussion

RBC deformability in relation to malaria pathogenesis has been discussed extensively [178-181]. Though the mechanisms underlying the changes in cell deformability during *Plasmodium* infection are poorly understood, the presence of the intracellular *Plasmodium* parasite brings about the cytoskeleton remodelling of the host cell, significantly impairing red cell ability to deform [182], and RBCs with reduced deformability are more likely to be retained within the reticular meshwork in the red pulp and eventually destroyed [177]. Indeed, RBC mechanical retention in the spleen is believed to be one key mechanism facilitating the removal of infected erythrocytes during malaria parasite infection. Our finding that the avirulent parasite infection resulted in significantly lower iRBC deformability compared to the virulent infection, implying that increased retention of iRBC is a key mechanism by which the spleen functions to control avirulent parasite infections. This is verified in both *P. yoelii* and *P. berghei* infection models. The strong correlation between the iRBC deformability and the disease outcomes suggests that the deformability of iRBCs may be adopted as a potential diagnostic marker for malaria prognosis and aid the early decisions undertaken by medical staff for effective treatment of malaria.

Our data also indicates that there is a direct interplay between iRBC deformability and spleen responses. This was confirmed by our mixed infection model in which pre-infection with avirulent 17X induced an “avirulent” like spleen response and protected the mice from the virulent parasite infection. Notably, in the mixed infection model, the deformability of RBCs infected with virulent parasite strain was decreased considerably to a level that is significantly different from those in the single infection model. In fact, the circulating RBCs in mixed infection model exhibit similar deformability as that in the 17X-infection alone. In addition, removal of the spleen leads to increased iRBC deformability in both the avirulent

17X infection as well as the mixed infection, highlighting the role of the spleen in modulating the biomechanical properties of the RBCs. Though the mechanism behind this modulation on biomechanical properties is largely unknown, previous studies have suggested that the spleen is involved in modifying the expression of iRBC surface antigens [106, 124], which resulting in different RBC membrane properties with and without the presence of the spleen. Moreover, the observed increased iRBC deformability in splenectomized mice was in association with the changes in disease outcomes from nonlethal to lethal. The observation suggests the potential of using RBC deformability as a novel, important marker to predict the disease severity of malaria.

When a patient is infected with malaria parasite, it is difficult to make an early-stage prognosis on whether the infection will be lethal or non-lethal. According to the WHO guideline, the patient would first be given standard oral treatment, unless further complications develop. The prospect of performing early-stage infection severity prediction and applying different anti-malarial treatment accordingly would be of great medical and economic value. Here in this study, we demonstrate that iRBC deformability can be used as a robust predictor of clinical outcome. Based on just single iRBC, we could predict infection severity with an overall accuracy over 90% for single infection model. In fact, we would argue that all iRBCs follow approximately identical independent distribution (IID), and with our high-throughput microfluidic platform with the capability of measuring hundreds of RBCs within minutes, we may have a good chance of predicting infection lethality with further improved accuracy and making decisions through comparing more similar blood samples at very early post infection days with even lower parasitemia. In our study, the lowest parasitemia used for the deformability detection was 0.4%. As a detection tool, this is not sufficient and far below the gold standard of thick blood smears microscopy [254]. But as a diagnostic tool for treatment decision, this is believed to be a very good estimate.

However, in order to apply this microfluidic platform in the field, some modifications of the whole system will have to be done, such as pre-preparation of the PDMS devices, control of the internal flow and also minimization of the microscopy detection system.

To conclude, we investigated the changes in RBC deformability upon parasite infection, and during the course of disease development. Removal of the spleen resulted in increased deformability of RBCs in circulating blood upon infection with the avirulent 17X parasites, highlighting the role of the spleen in modulating cell mechanical properties. Reduced RBC deformability correlates with more efficient parasite clearance. Parasitized RBC deformability was therefore, for the first time, proposed to be an important marker for early-stage prognosis of the virulence of parasite infection. As a simple demonstration, our MLE model could achieve over 90% prediction accuracy when the host suffers single-strain parasite infection. This provides a potential tool for fast and easy diagnosis and prognosis in malaria, helping to make a rapid response in choosing the correct treatment.

Chapter 5

**Innate immune response varies between
virulent and avirulent parasite infections**

5.1 Splenic innate immune responses upon parasite infection

The data that pre-infection of avirulent parasites protects from a subsequent virulent infection in a host with an intact spleen, suggests that a fast splenic response is a key factor in controlling the parasite growth as well as the disease outcome. Since the spleen is the largest secondary lymphoid organ in the body and it is involved in activation of the immune system to fight circulating pathogens, we hypothesize that the innate immune response plays an important role against the parasite infection.

5.1.1 Splenic cytokine/chemokine responses

Innate immunity is a nonspecific response that provides an immediate defence against infection. The elements that make up the innate immunity include anatomical barriers, secretory molecules and cellular components [255]. To study the splenic innate immune responses against parasite infection, we first looked at the secretory molecules, i.e. cytokines and chemokines production that happens in response to a parasite infection.

It has been suggested that relative cytokine amounts are crucial for determining whether a response will be protective or pathologic [256]. To distinguish differences in the cytokine and chemokine responses in virulent and avirulent malaria parasite infection, a broad screening on 40 major cytokines and chemokines was performed using the Mouse Cytokine Antibody Array (refer to Methods and Materials for more details). For this, the spleen lysate was obtained from mice 4 and 5 days after infection with 1000 mature schizonts of either the avirulent 17X or the virulent YM parasites. The relative signal of YM-infected

spleen to 17X-infected spleen for respective cytokine or chemokine was calculated and compared between day4 and 5 pi. There were a number of targets shown to be differentially expressed between the two different parasite line infections and also between the two different post-infection days, including IL-1 β , IL-3 and IL-27 (Fig5.1). Since IL-27 belongs to the same Interleukin family as IL-12 [257], while IL-3 has been shown to lack homology between human and mice [258], they were excluded in the further analysis. Based on our result and published data a total 8 cytokines/chemokines (IL-12, IL-2, IL-1beta, TNF-alpha, IFN-gamma, IL-6, IL-10 and MCP-5, indicated by the arrows in Fig5.1) were chosen to be analyzed using conventional ELISA so as to have better sensitivity and more accurate quantification.

Fig5.1. Screening of cytokines and chemokines in spleen lysate. Data are presented as the ratio between the signals obtained from mice infected with YM-GFP and that of the mice infected with 17X-GFP. Arrows indicate the selection for further analysis.

Fig5.2. Comparison of cytokine and chemokines responses during malaria infection among different infection models. Data are mean $\pm$ SEM results from 5 mice per group. Statistic analysis was done using Bonferroni test. * indicates statistically significant with p -value <0.05 as compared to the control.

As shown in Fig5.2, the cytokine/chemokine responses in mice with the mixed infection were broadly similar to those of the mice infected with the avirulent 17X alone but very much different to those of the virulent YM-infected mice. Taking into consideration the disease outcomes, where 17X infection and mixed infection result in a nonlethal disease development while YM infection leads to a lethal disease outcome (Fig5.3), the pro-inflammatory cytokines IL-12, IL-2 and IL-1beta showed a general and similar pattern in the nonlethal infection, with a significant higher expression (p -value <0.05 , Bonferroni test) than the control mice as well as the YM-infected mice early on in the infection (day4 pi), followed by decrease towards normal level by day6 pi. Other pro-inflammatory cytokines including IFN-gamma and TNF-alpha showed peak expression on day5 pi in avirulent 17X-infected mice but an earlier peak on day4 pi in mice with mixed infection. IL-6, having both pro- and anti-inflammatory roles in the immune system, also showed a similar expression pattern in the nonlethal infections, with peak expression on day4 pi that were significantly higher than those of the control as well as the YM-infected mice (p -value <0.05). The expression of anti-inflammatory cytokine IL-10 showed a similar pattern in the nonlethal infections as well but peaked on a slightly later day (day5 pi). On the other hand, in the lethal infection where mice were infected with YM parasite, these pro- and anti-inflammatory cytokines peaked later except for IL-12p70, which showed limited changes with no significant difference among the three post-infected days tested in this study. Moreover, the pro-inflammatory cytokines including IL-1beta, TNF-alpha and IFN-gamma showed continuous increase from day4 to day6 pi, with the increase in IFN-gamma being very dramatic. The anti-inflammatory cytokine IL-10 in mice with the virulent YM infection also showed a gradual increase from a level below the control to 2-fold of the control. Chemokine MCP5 that is responsible for recruitment of monocytes and macrophages, also peaked on day5 pi in the nonlethal infection, with a significantly higher expression compared to the control and the lethal infection (p -value <0.05 , Bonferroni test). This was

followed by a rapid decrease towards normal level during the nonlethal infection, while the expression of MCP5 in the lethal infection increased gradually from day4 to day6 pi. This result showed very different cytokine/chemokine expression profiles between the lethal and nonlethal infections, where a general late expression of cytokine/chemokine tested here (peaked on day6 pi) was found in the lethal infection. Our finding demonstrated that the rapid recruitment of monocytes and macrophages as well as the early pro-inflammatory cytokine responses the spleen in particular the IFN-gamma and TNF-alpha responses may be important for the disease outcome.

Fig5.3. Parasite growth behavior in spleen-intact BALB/c mice. Blue- Growth curve for 17X-GFP in single infection; Red- Growth curve for YM-GFP in single infection; Green- Growth curve for mixed infection. Data are mean $\pm$ SEM results from three or more individual experiments with total 10-15 mice. † denotes death of mice.

5.1.2 Quantification of splenic immune cell activation

As a lymphoid organ, the spleen plays a vital role in malaria parasite infection through its immunological reaction. Besides the cytokine/chemokine responses, we also investigate the activation of immune cells in the spleen.

5.1.2.1 Platelet/endothelial cell activation upon parasite infection

Platelets are well known as the cellular mediator of thrombosis. However, it has been shown that platelets are linked to the pathogenesis of many inflammatory diseases such as atherosclerosis and rheumatoid arthritis [259]. There is now growing evidence supporting the important immune and inflammatory roles of platelets in both health and disease. Recent findings have demonstrated that the platelets are able to kill intracellular parasites directly [235, 236]. In addition, it has been shown that platelet-derived microparticles as well as other microparticles derived from several other types of cells including erythrocytes exert a strong pro-inflammatory activity [199]. Moreover, there are a large amount of platelets present in the circulating blood with more than 200 million platelets per 1 ml of blood in a healthy human [260]. Taken altogether, it implicates platelets as a powerful weapon in fighting malaria. On the other hand, endothelial cell activation also plays an important role in the course of malaria parasite infection and it has been shown to be associated with parasite sequestration [261]. Furthermore, interactions between platelets and endothelial cells have also been implicated in malaria pathogenesis [262]. Therefore, we examined the activation of platelets/endothelial cells in the spleen upon parasite infection.

Fig5.4. Expression of P-selectin on splenic cells. A- Selected images of the spleen sections; B- Quantification of the positive P-selectin cells. Data are mean $\pm$ SEM from 8-12 images of one representative spleen out of 5 samples per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates statistically significant with p -value <0.05 .

P-selectin is a marker for activated endothelial cells as well as activated platelets, and it has been implicated to promote platelet aggregation and interaction between platelet and neutrophils and monocytes [263, 264]. As shown in Fig5.4, it was elevated in mice with parasite infections compared to the control significantly from day3 pi (p -value <0.05), in line with the previous finding that

platelets play a substantial role in the antiparasitic immune defence. Despite the parasite multiplication (Fig5.3), the P-selectin levels in YM-infected mice showed relatively constant with no significant difference among day3 to day7 pi except for day4 pi. While in mice infected with the avirulent 17X line, the P-selectin signal in the spleen started to drop significantly from day7 pi onwards (Fig5.4B). Though it seemed to revert back on day10 pi, there was no significant difference among the levels for the control, day7 and day10 pi. On the other hand, while the P-selectin signal in 17X infection decreased towards normal level on day7 pi, it remained high in YM infection on the same post-infection day. Whether this is associating with the spleen remodelling in response to different parasite infection or the antiparasitic action is largely unknown and further studies will be required. In addition, it is not yet clear whether the change in the P-selectin level on day4 pi in YM infection (Fig5.4B) is meaningful or not, and this also requires further investigation. However, there was no significant difference between the virulent YM and avirulent 17X infections on the same post-infection day from day3 to day6 pi, except for day4 pi, suggesting a limit impact of platelets/endothelial cells in regulating the disease development.

5.1.2.2 Monocyte/macrophage responses upon parasite infection

Since our data on cytokine/chemokine screening suggests the recruitment of monocytes and macrophages plays an important role in fighting malaria parasites, we quantified and compared the activation of immune cells in particular monocyte and macrophages in the spleen over the course of the infection.

Fig5.5. Expression of CD33 on splenic cells. A- Selected images of the spleen sections; B- Quantification of the CD33-positive cells. Data are mean $\pm$ SEM from 8-12 images of one representative spleen out of 5 samples per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates statistically significant with p -value <0.05 .

CD33 is a myeloid progenitor marker that is weakly expressed on granulocytes and has a high expression on monocytes [265]. As shown in Fig5.5A, upon infection, the signal of CD33 increased in the spleens as compared to the control ones, regardless the of the parasite strain used. However, the detailed

quantification of the CD33 expression in the spleen sections over the infection period (Fig5.5B) showed significantly higher expression in YM-infected spleens as compared to the control spleens on day3 pi (p -value <0.05), while in 17X-infected spleen it showed a significantly lower expression than the control (p -value <0.05). But this was completely changed on day4 pi, where 17X-infected spleens showed a rapid increase of CD33 signal. Indeed, there were significantly higher expression of CD33 in 17X-infected spleens as compared to YM-infected spleens during the early infection stage (day4-day6 pi, p -value <0.05), indicating an earlier and faster recruitment of myeloid cells including monocytes to the spleen in the avirulent 17X infection. In addition, the number of CD33-positive cells remained high in 17X-infected mice, even after the peak parasitemia (day14 pi), suggesting an important role of the myeloid cells in controlling the disease development.

Fig5.6 shows the staining and quantification of the Colony Stimulating Factor 1 Receptor (CSF1R) which is also known as Macrophage Colony-Stimulating Factor Receptor (M-CSFR). CSF1R is a cytokine that controls the production, differentiation and function of macrophages. In the spleen, expression of CSF1R is mainly on the residential red pulp macrophages [266]. Comparing the infected spleens to the control ones, there was no significant change in CSF1R level in YM-infected spleens across the infection period (day3-day7 pi). On the other hand, the quantification of CSF1R showed significantly higher amount in 17X-infected spleens on day3 pi (close to 2-fold), indicating a higher activation of red pulp macrophages in the 17X infection. This was followed by gradual decrease to a level that was close to 4-fold less than the normal condition on day12 pi (Fig5.6B), implying a parasite-induced death of red pulp macrophages and suggesting an active interaction between the red pulp macrophages and the avirulent 17X parasites. Taking into consideration the disease outcomes, our data suggest that the early activation of red pulp macrophages is important in

controlling parasite development. The CSF1R amount was in a trend back to the normal level after parasite clearance on day17 pi in 17X infection (Fig5.6B), suggesting a recovery in the spleen.

Fig5.6. Expression of CSF1R on splenic cells. A- Selected images of the spleen sections; B- Quantification of the positive CSF1R cells. Data are mean $\pm$ SEM from 8-12 images of one representative spleen out of 5 samples per group. Statistic analysis was done using one way ANOVA with Tukey test. * indicates statistically significant with p-value <0.05.

5.1.2.3 Lymphoid cell responses upon parasite infection

In addition to the myeloid immune cells, markers for lymphoid cells were also studied. As shown in Fig5.7A, the quantification of T cell marker CD3 indicated that infection with virulent YM parasites stimulated significantly higher T cell responses in the spleen compared to the avirulent 17X infection from day4 to day7 pi (p -value <0.05). Moreover, the CD3 signal showed gradual decrease from the normal level upon 17X infection, and it was significantly lower than the control level from day5 pi onwards (p -value <0.05). The data suggests that the T cell response is less important in controlling the parasite development, and early stimulation of T cell responses may favour the parasite development and result in lethal disease outcome.

On the other hand, examination of B cell marker CD19 showed no significant changes on splenic B cell amount upon virulent YM parasite infection as compared to the control, except for day7 pi. And in the 17X-infected mice spleens, the expression of CD19 also showed no significant difference when comparing to the control spleen (Fig5.7B). It is worth to note that our quantification of B cells was somehow far below the normal case of 40-55% of cells in the spleen [267]. However our result was consistent among all the samples examined. On the other hand, it is not surprising to see limited changes in B cell percentage in the spleen after parasite infection, as the infection period for YM was only 7 days. However, taking into consideration of the increase in spleen sizes along with the parasite growth, in particular in 17X infection (Fig3.3), the relative constant B cell percentage potentially is associating with an increase in the total B cell count along with the disease development. Using the ratio of the spleen/kidney weight as a reference for the increase in spleen size (Fig3.3D), the changes in B cell count were estimated (Table5.1). For example, when compared the mice with 17X infection to the control, there were 1.61, 4.42 and 12.60 fold changes in the spleen/kidney weight in 17X-infected mice on day4, day7 and day14 pi respectively. Correspondingly, the estimated CD19⁺ cell

percentages were 1.64%, 5.26% and 16.43%, representing 1.11-, 3.55- and 11.10- fold increases in the B cell amount respectively.

Fig5.7. Expression of CD3 on splenic cells. A- Quantification of the positive CD3 cells; B- Quantification of the positive CD19 cells. Data are mean $\pm$ SEM from 8-12 images of one representative spleen out of 5 samples per group. Statistic analysis was done using one way ANOVA with Tukey test.

Table10. Estimation of CD19-positive cells in the spleen relative to the control.

	Control	YM				
Post-infection day		3	4	5	6	7
spleen/kidney weight (normalized to control)	1.00	1.08	1.16	2.30	3.62	5.23
Estimation of CD19 ⁺ %	1.48%	1.79%	1.54%	3.90%	4.46%	12.98%
Ratio to the control	1.00	1.21	1.04	2.63	3.01	8.77

	17X								
Post-infection day	3	4	5	6	7	10	12	13	14
spleen/kidney weight (normalized to control)	1.18	1.61	1.97	3.05	4.42	10.09	13.55	14.79	12.60
Estimation of CD19 ⁺ %	1.02%	1.64%	2.53%	2.63%	5.26%	13.79%	17.33%	17.24%	16.43%
Ratio to the control	0.69	1.11	1.71	1.78	3.55	9.32	11.71	11.65	11.10

5.2 BALB/c CD11b-DTR mouse model and parasite infection

Our data suggest that the innate immune responses especially the splenic innate responses determine the disease outcomes in malaria parasite infection. Therefore, we next investigate the parasite growth behaviour in a model when the innate immune system is disrupted.

CD11b, also known as Integrin alpha M, is a member of the Integrin family proteins. It is a type I transmembrane protein that pairs with CD18 to form the on-covalent CR3 heterodimer, which is also named heterodimeric Integrin alpha M beta 2 molecule [268]. CD11b functions to mediate the inflammatory responses through regulating leukocyte adhesion and migration. And it has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation [269-274]. CD11b is expressed on the surface of many leukocytes including subsets of monocytes, neutrophils,

natural killer cells, macrophages and B cells, as well as on some of spleen cells and bone marrow cells. Therefore, knocking down of the CD11b expression would greatly affect the immune system in particular innate immunity.

5.2.1 BALB/c CD11b-DTR mouse model

With the kind gift from Dr. Ruedl (Nanyang Technological University), CD11b-DTR transgenic mice were used to aid the investigation of innate immune responses against the parasite infection. These transgenic mice were generated on BALB/c background, using the CD11b promoter to control the diphtheria toxin receptor (DTR), and a reporter protein dTomato was fused at the C-terminal of the DTR. It is able to conditionally ablate most of the innate immune cells that are CD11b positive upon injection of diphtheria toxin (DT). The cytotoxicity of the DT is strictly dependent on receptor-mediated endocytosis [275]. Moreover, unlike primate cells, murine cells are insensitive to killing by DT [276]. Therefore, only transgenic mice with the transferred DTR are sensitive to DT treatment. In addition, malaria parasites naturally have no DTR expression and DT treatment has been shown to have no impact on parasite growth in both parasite liver stage [277] as well as blood stage [278]. Taken together, this BALB/c CD11b-DTR mouse model facilitates the study to examine the role of CD11b-positive cells in malaria parasite infection.

5.2.1.1 Genotyping of CD11b-DTR mice

The CD11b-DTR mice are heterozygous as the homozygote is not able to survive. Therefore genotyping of the mice was performed prior to each experiment. As shown in Fig5.8, the presence of the band with size corresponding to ~700bp indicated a positive amplification of dTomato, i.e., CD11b-DTR positive mouse,

and vice versa. Positive male mice were selected for the subsequent experiment.

Fig5.8. Genotyping for CD11b-DTR mice. Presence of the DNA band at around 700bp indicates CD11b-DTR positive.

5.2.1.2. Ablation of CD11b positive cells by DT treatment

To verify the knockdown of CD11b positive cells by DT treatment, the cell staining profile was done in both wildtype mice and CD11b-DTR mice with DT treatment. This part of work was done with Dr. Hurst from Prof Peter Preiser's lab (Nanyang Technological University).

First, a standard gating strategy as shown in Fig5.9A was set up. Live cells were sub-gated on the CD11b⁺ lineage (Fig5.9B) and their percentage in wildtype BALB/c mice and DT/DTR mice were quantified and compared (Fig5.9C). DTR mice received three treatments (day-2, -1 and day3 pi as performed in the parallel infection model) and the experiment was done on day3 pi corresponding to the infection timeline. Our data showed that after three DT treatments there was a significant decrease of CD11b⁺ cell population by more than 50% in the DT/DTR mice, indicating a good ablation efficiency of CD11b⁺ lineage cells in

the DT/DTR model.

Fig5.9. Quantification of CD11b-positive cell population in the spleen. A- A standard gating strategy was set up where total mouse splenocytes were prepared as described in Materials and Methods, debris (SSC-A vs. FSC-A) and doublets (FSC-H vs. FSC-A) were excluded and live/dead discrimination was determined using the viability staining solution 7AAD (SSC-A vs. 7AAD); B- CD11b⁺ lineage-cells were gated out (SSC-A vs. CD11b); C- Comparison of CD11b-positive cells in the spleen between wildtype and DT/DTR mice. Data are pooled from 2 individual experiments each with 3 mice. Statistic analysis was done using one way ANOVA with Tukey test. * indicates statistically significant with *p*-value <0.05.

5.2.2 Parasite growth in BALB/c CD11b-DTR mice

Since these transgenic DTR mice can conditionally ablate the CD11b positive population of cells thereby affecting the innate immune system greatly, they were used to examine the virulent and avirulent parasite growth behaviors. Similarly,

mice were infected with 1000 mature schizonts of parasites and parasitemia was monitored daily from day4 pi. Unlike the wildtype BALB/c mice (Fig3.2C), not all the DTR mice after DT treatment could resolve the avirulent 17X infection even with intact spleens; instead, approximately 55% of DT/DTR mice developed long-lasting infection with parasites still detectable beyond 3 weeks after infection (Fig5.10A). In contrast for the DT/DTR mice with the spleen removed, infection with the avirulent 17X parasite was lethal and mice started to die from day8 pi. This indicates that the presence of both the spleen and the innate immune cells are essential in controlling parasite growth. On the other hand, similar to the infection in wildtype BALB/c mice (Fig3.2B), infection with the virulent YM line in DT/DTR mice showed no difference in parasite multiplication regardless of the presence of the host spleen or not (Fig5.10B). When the DT/DTR mice received the mixed strains parasite infection, the complete protection from the pre-infection of the avirulent 17X line was no longer observed. Moreover, the growth suppression of the virulent YM by the presence of the avirulent 17X parasites in the mixed infection was neither observed in the DR/DTR model. Instead, regardless of the presence of the host spleen or not, the parasitemia in the DT/DTR mice with mixed infection was mainly contributed by the YM but not 17X (Appendix III). In addition, it is interesting to note that, the DT/DTR hosts in the mixed infection with intact spleens developed more severe disease with higher parasitemia from day10 pi onwards (Fig5.10C) and appeared to have a lower survival rate than the splenectomized mice (Fig5.10D), suggesting the spleen plays an immunopathological role in the mixed infection when the innate immune system was disrupted.

Fig5.10. *Plasmodium yoelii* growth behavior in BALB/c- CD11b-DTR mice. A-Growth curve for 17X-GFP line in spleen-intact and splenectomized mice, and the proportion of spleen-intact mice with different disease development upon 17X infection; B-Growth curve for YM-Cherry line in spleen-intact and splenectomized mice; C-Growth curve for parasite in mixed infection in spleen-intact and splenectomized mice; D- Survival curve for spleen-intact as well as splenectomized mice with mixed infection. Data are mean $\pm$ SEM results from two to three individual experiments with total 8-15 mice. † denotes death of mice.

5.2.3 Monocyte/macrophage responses upon parasite infection

The growth profile of parasites in the DT/DTR mice clearly indicates that ablation of the CD11b⁺ cell lineage greatly affects the parasite development and disease outcome. Next we investigated in detail the changes of the CD11b⁺ monocyte and macrophage in response to parasite infection. This part of work was done with Dr. Hurst from Prof Peter Preiser's lab (Nanyang Technological University).

5.2.3.1 Quantification of CD11b positive cell population

We first quantified the CD11b⁺ cell population in the early infection period (day1 and day3 pi) in both the spleen as well as the circulating blood (Fig5.11). Prior to the parasite infection (day0), the DTR mice had received two DT treatments following the DT administration schedule (Fig2.1). Wildtype and DT/DTR mice without parasite infection were included as control. As shown in Fig5.11, regardless of the parasite strain used, there were significant increases of CD11b⁺ cells in the spleen in both the wildtype and DT/DTR mice one day after the parasite infection as compared to their respective control. These increased levels were maintained in the wildtype mice in both 17X and YM infections as well as the 17X infections in DT/DTR mice on day3 pi. Only in YM infected DT/DTR mice there was a drop of CD11b⁺ cells in the spleen on day3 pi as compared to that on day1 pi (p -value=0.0513). In contrast, there was a significant decrease of CD11b⁺ cells on day1 pi in the circulating blood in wildtype mice infected with YM which subsequently recovered somewhat by day3 pi (p -value <0.05). In comparison the levels of circulating CD11b⁺ cells decreased much more gradually over day1 and day3 pi in 17X infected wildtype mice. There was a striking difference in circulating CD11b⁺ cells in the DT/DTR mice which showed a rapid drop over the three days after infection irrespective of the parasite strain, suggesting the rapid decline in circulating CD11b⁺ cells in the DT/DTR mice may

be an indication of pathology. Our data suggest a large number of CD11b⁺ cells are rapidly recruited to the spleen in response to parasite infection. Moreover, this finding also supports the idea that the spleen is the major site for parasite clearance. The significant decrease of the total CD11b⁺ cells in the circulating blood of DT/DTR mice upon parasite infection was associated with significant increase in the corresponding splenic CD11b⁺ cell count. This suggests a migration of the CD11b⁺ cells from the circulating blood to the spleen. When the splenic CD11b⁺ cells have been greatly ablated in DTR mice by the DT treatments, the migration of the CD11b⁺ cells to the spleen potentially compensates the loss of the splenic population and facilitates parasite clearance. The total number of splenic CD11b⁺ cells upon parasite infection shows no significant differences on both day1 and day3 pi irrespective of the parasite strains used indicating the total number of CD11b⁺ cells in the spleen alone is not sufficient to predict pathology. This would suggest that either different interaction with the CD11b⁺ cells between the virulent and avirulent parasites or variations in the exact make-up of the CD11b⁺ cells is critical for determining the parasite clearance potential of the spleen. A better interaction of the CD11b⁺ cells would facilitate a better and potentially earlier stimulation of the CD11b⁺ cells by the 17X parasites in the spleen, which allows rapid parasite clearance thereby controlling the disease development. In a situation where the total number of CD11b⁺ cells is greatly reduced (such as in the DT/DTR model), or the stimulation of the CD11b⁺ cells is less effective (YM infection) in the spleen, there is a need for cell migration from the circulating blood to the spleen in order to clear the parasites as is seen in the case of the YM infection and the DT/DTR mice with 17X infection.

Fig5.11. Quantification of CD11b positive cell population in different infection models. A-Quantification of the total CD11b⁺ cell population in the spleen; B- Quantification of the total CD11b⁺ cell population in the circulating blood. Data are mean $\pm$ SEM from two experimental replicates with 3-5 mice. Statistic analysis was done using one way ANOVA with Tukey test. * indicates statistically significant with p -value <0.05 as compared to the respective control unless otherwise specified.

5.2.3.2 Quantification of red pulp macrophages

We individually focused on the CD11b⁺ monocytes and macrophages. Innate immune cells of the monocyte lineage play essential roles during malaria infection. Quantification for monocytes and macrophages in the spleen was carried out as in Fig5.9A and then sub-gated using specific antibodies. Since our data indicates that the early immune responses play a role in controlling the disease outcome of an infection, the splenic monocyte/macrophage responses were investigated on day1 and day3 pi for mice infected with either the virulent YM or the avirulent 17X parasites. Wildtype and DTR mice after two DT treatments (DT/DTR mice) were included for comparison. Red pulp macrophages, as one of the major players in eliminating parasitized RBCs, were studied here and characterized as F4/80^{int}CD11b^{int} (Fig5.12A).

As shown in Fig5.12B, though there seemed to be lower amount of red pulp macrophages in the DT/DTR control compared to the wildtype control, the amount of red pulp macrophages showed similar levels in both wildtype and DT/DTR mice, with infection of either virulent YM or avirulent 17X parasites on day1 pi. They showed similar levels on day3 pi as well, except for the wildtype mice infected with the avirulent 17X parasites (corresponding to a nonlethal infection), which showed a further increase in the red pulp macrophage amount compared to that on day1 pi. This data is consistent with the quantification of CSF1R where infection of 17X in BALB/c mice resulted in significant increase of CSF1R expression on spleen cells compared to the control mice on day3 pi (Fig5.6), supporting the idea that early activation of the red pulp macrophages is indispensable in controlling parasite development. The data also suggest that in wildtype mice with 17X infection the red pulp macrophages make up a larger proportion of the total CD11b⁺ cells on day3 pi as compared to YM infections or 17X infection in DT/DTR mice and this could in part explain the critical differences observed in the clinical outcome of the infection. At this stage it is important to note that the data presented is based on

a single experiment and further work needs to be done to confirm these findings.

Fig5.12. Quantification of red pulp macrophages. A- The standard gating strategy was carried out as in Fig5.9A, and red pulp macrophages were further gated out (F4/80 vs. CD11b); B- Comparison of red pulp macrophages in absolute cell count among mice after parasite infection. Data are mean $\pm$ SD (standard deviation) from 3-5 mice.

5.2.3.3 Quantification of other populations of splenic macrophages and monocytes

Further analysis on splenic macrophages and monocytes was carried out using antibodies specific to Ly6C and F4/80, to characterize three different populations based on Ly6C expression level (Fig5.13). In general, the total number of cells in both Ly6C^{hi} monocyte (CD11b⁺F4/80⁺Ly6C^{hi}) and splenic macrophage (CD11b⁺F4/80⁺Ly6C⁻) population were as expected much higher (more than 2-fold) in the wildtype control as compared to the DT/DTR control, while differentiating monocyte (CD11b⁺F4/80⁺Ly6C^{int}) population showed similar level in both wildtype and DT/DTR control mice. Upon parasite infection, the levels of Ly6C^{hi} monocyte which has pro-inflammatory and antimicrobial roles and would be expected to be rapidly recruited to the site of infection [279-281], did not show any change in the YM infection in both wildtype and DT/DTR mice on day1 pi as compared to their respective control. In addition, the number of Ly6C^{hi} monocyte in YM infection showed to be greatly higher in wildtype mice than the DT/DTR mice on day1 pi but both dropped to very similar levels on day3 pi. In the case of 17X infection there was a gradual decline of Ly6C^{hi} monocytes over the course of the 3 days post-infection in the wildtype mice while the levels showed an initial increase in the DT/DTR mice on day1 pi and then a subsequent reduction on day3 pi. In contrast, splenic macrophages showed an overall reduction in number on both day1 and day3 pi in both 17X and YM infections in wildtype mice while they showed an increase in the DT/DTR mice on day1 pi and then a reduction to about starting levels on day3 pi. The levels of the splenic macrophages in 17X infection appeared to be comparatively similar between the wildtype and DT/DTR mice on day1 pi but not day3 pi. The differentiating monocytes showed an increase in overall numbers in YM and 17X infections in wildtype mice on day1 pi. In wildtype mice, the level of differentiating monocytes dropped again in the YM infection on day3 pi while it continued to increase in 17X infection. In comparison in the DT/DTR mice there appeared to be limited changes in differentiating monocytes upon infection.

Fig5.13. Quantification of splenic monocytes/macrophages. The standard gating strategy was carried out as in Fig5.9A and B, and splenic monocyte/macrophage populations were further gated out (Ly6c vs. F4/80). Different populations were quantified in absolute cell counts and compared. Data are mean $\pm$ SD from 3-5 mice.

The spleen has been reported as a reservoir for monocytes including Ly6C^{hi} monocytes, and Ly6C^{hi} monocytes residing in the spleen red pulp have indistinguishable transcriptomes with those in the circulating blood [282]. While Ly6C^{hi} monocytes can be recruited from the circulating blood, the reduction in the Ly6C^{hi} monocyte population in the spleen on day3 pi following YM infection suggests a rapid reaction of the Ly6C^{hi} monocytes against the parasite infection. This reaction appears to be stronger in the YM infection as the reduction of these monocytes appears to be less pronounced in 17X infection in wildtype mice on day3 pi. In all infections of the DT/DTR mice it appears that the levels of

monocytes on day3 pi are much below those of the control mice on day 0 and this may indicate that it is critical for the mice to maintain a certain level of Ly6C^{hi} monocytes in the spleen to be able to effectively control the parasite.

Another striking observation of this initial study is the changes in the number of differentiating monocytes in different infections in particular the continuing increase of these in the 17X infection in wildtype mice on day3 pi. This pattern is associated with decreased virulence and it would be of importance to establish the exact role these cells have in controlling parasite levels.

In summary, these data showed a general higher amount of macrophages and monocytes in the spleen in 17X-infected wildtype mice. Along with the nonlethal disease outcome associating with the 17X infection in wildtype BALB/c mice, our data indicates that splenic macrophages and monocytes could play an important role in the defense mechanism against *Plasmodium* infection. However, as our data is based on a single experiment, more follow up experiments will be required in order to fully understand its implications.

While F4/80 is commonly used as a general marker for tissue-residing macrophages, there are also marginal zone macrophages and metallophilic macrophages that are F4/80 negative. For these, we examined the CD11b⁺F4/80⁻ cell population in the spleen upon parasite infection (Fig5.14). In general, there were lower amount of cells in both CD11b^{int} F4/80⁻ and CD11b^{hi}F4/80⁻ populations in the DT/DTR mice in contrast to the wildtype mice, regardless of the infection status. It is expected as the DT treatment ablated majority of the CD11b⁺ cells. It appears that infection with YM or 17X has little impact on the

levels of CD11b^{int}F4/80⁻ on day1 and day3 pi in both wildtype and DT/DTR mice. In contrast CD11b^{hi}F4/80⁻ populations showed a great increase in numbers in YM infection on day1 pi before rapidly returning to pre-infection levels on day3 pi. This increase on day1 pi was seen in both the wildtype as well as the DT/DTR mice, albeit at a much lower level. There was no such response observed in the 17X infections in either mouse strain. As already indicated more studies including more mice as well as more cell surface markers to identify specific subset of cells from the CD11b⁺F4/80⁻ population will be required.

Fig5.14. Quantification of CD11b⁺F4/80⁻ cell population in the spleen. The gating strategy was carried out as in Fig5.9A and Fig5.12A. Data are mean ± SD of absolute cell counts from 3-5 mice.

5.2.3.4 Quantification of circulating monocytes

Erythrocytes are the sole host for the erythrocytic stage *Plasmodium* parasites, making circulating blood the major “site” of infection in malaria. Therefore, besides the splenic macrophages/monocytes responses in parasite infection, changes in circulating monocyte were also investigated.

Fig5.15. Quantification of circulating monocytes. A-The standard gating strategy was carried out as in Fig5.9A, and CD11b⁺ lineage cells were gated out (SSC-A vs. CD11b) and circulating monocytes were further sub-gated out (Ly6c vs. Ly6G). B- Comparison of circulating monocytes (CD11b⁺Ly6G⁻Ly6C⁺) in absolute cell count among mice after parasite infection. Data are mean $\pm$ SD from 3-5 mice.

Circulating monocytes were first gated as Fig5.9A, and CD11b⁺ lineage cells were selected and further sub-gated according to their Ly6C and Ly6G expression (Fig5.15A). Ly6G is transiently expressed on monocytes during bone marrow development [283] while Ly6C is typically highly expressed on monocytes [284]. Thus circulating monocytes were characterized as CD11b⁺Ly6G⁻Ly6C⁺. Upon parasite infection, circulating monocytes showed a rapid drop in number as compared to uninfected mice in all the infection models studied, and they were further reduced along with the parasite development from day1 to day3 pi. The drop in number appears to be not as rapid in mice infected with the avirulent 17X parasites as compared to those infected with the virulent YM line, regardless of wildtype mice or DT/DTR mice (Fig5.15B). This suggests that parasite infection leads to a rapid decrease of circulating monocytes possibly by recruiting them to the spleen and/or stimulating monocyte activation and differentiation. This is supported by the observation of increased splenic macrophages and differentiating monocytes in the spleen upon parasite infection. The less rapid drop in circulating monocytes in 17X infections compared to the YM infections implies that circulating monocytes can directly interact with the avirulent 17X parasites in the blood stream potentially to facilitate the parasite clearance.

5.3 Discussion

Infections with the avirulent 17X and virulent YM parasites differentially stimulate the host immune responses, and our study clearly showed that the early immune responses, i.e. the innate immune responses, govern the disease outcomes. Disruption of the innate immune system resulted in mice being unable to completely resolve the originally nonlethal infections with the avirulent 17X

line alone or mixed parasite strains, as demonstrated by the DT/DTR mice model in our study. The importance of the innate immune responses in fighting parasite infection is also supported by the earlier observation of pro-inflammatory cytokines expression in nonlethal 17X infection compared to the lethal YM infection. It is further verified through the mixed infection model which shows similar nonlethal disease outcome as the 17X infection alone. In the mixed infection model, the pre-infection of avirulent 17X parasites followed by subsequent challenge with the virulent YM line induced a very similar splenic cytokine/chemokine profile to what was observed in infection with 17X alone, with even earlier peaks in expression of IFN-gamma and TNF-alpha, indicating that the pre-infection of avirulent parasites is able to prime the host response and stimulate it to be protective against subsequent parasite infection, and that the early stimulation of pro-inflammatory cytokines, in particular IFN-gamma and TNF-alpha, is crucial for the nonlethal disease outcome.

Being part of the innate immune system, monocytes have been reported to be essential in the first line of innate defense against malaria [285, 286], while macrophages in particular the red pulp macrophages have been considered as one of the major players in removing the *Plasmodium* infected cells [193, 194]. A recent report showed that red pulp macrophages are responsible for early sensing of *Plasmodium* infection and secrete a large amount of T1IFNs against parasites [195]. A previous study has also demonstrated that depleting monocytes/macrophages *in vivo* resulted in increased parasitemia levels early in *P. yoelii* lethal and nonlethal infections [192]. Similarly, our ELISA screening on spleen lysate implies that besides the early expression of pro-inflammatory cytokines, the early recruitment of monocytes and macrophages to the spleen is important in the defense mechanism against parasite infection. In line with this, the IHC staining for myeloid cells including monocytes (CD33) showed higher expression in 17X-infected spleens. Furthermore, quantification of CSF1R also

indicates a higher differentiation/activation of macrophages in 17X-infected spleens in early infection stage (day3 pi) as compared to YM-infected spleens. Consistently, our quantification of splenic macrophages and differentiating monocytes by FACs also showed to be elevated in 17X-infected wildtype mice from day1 to day3 pi, and they were higher than those of YM-infected wildtype mice on day3 pi. Moreover, the pro-inflammatory Ly6C^{hi} monocyte population was also higher in 17X-infected wildtype mice as compared to the DT/DTR mice or the YM-infected wildtype mice on day3 pi. It is worth to note that the number of mice included and also the experimental repeats in the quantification of monocytes/macrophages by FACs are not sufficient to make firm conclusions at this stage. Further study will be required to confirm the observed trend is significant. Overall, our data support the idea that monocytes and macrophages play an essential role in controlling parasite development in particular at the early infection stage.

In addition, the trend of rapid decrease in circulating monocytes from day0 to day3 pi associating with the observation of increased splenic macrophages and differentiating monocytes in 17X-infected mice implies that there is rapid recruitment of monocytes and macrophages to the spleen during *Plasmodium* infection, and this recruitment process is important in regulating the disease outcome. It has been demonstrated that in *P. chabaudi* infection model, compared to the wildtype mice, there is sustained acute parasitemia in mice deficient in CCR2, the chemokine responsible for trafficking of CD11b⁺Ly6C⁺ monocytes from the bone marrow to the spleen [287]. Furthermore, the existence of a macrophage migration inhibitory factor (MIF) homolog released by *Plasmodium* parasites, also suggests that preventing monocytes/macrophages from reaching the spleen may favor the parasite development [288, 289]. However, it has been reported that there are splenic reservoir monocytes [282], and there is also evidence showing that monocyte and macrophage are involved

in mediating pathology in malaria [290-292]. Therefore, future studies would be required focusing on the detailed molecules and/or mechanisms for the proper activation of monocyte/macrophage responses.

The spleen is considered a major organ to fight against the *Plasmodium* parasites, while removal of the spleen usually impairs the innate immunity and induction of adaptive immunity [293]. Interestingly, in our DT/DTR model, the presence of the spleen does not seem to protect the mice from the challenge with virulent YM parasites in the mixed infection; instead, the splenectomized host were found to have better survival rate and also lower parasitemia compared to those with the intact spleens. It has been proposed that the spleen has an active role in generating immunopathological reaction during a primary infection in the intact mice, where splenectomy resulted in decreased parasitemia and delayed morbidity of *P. berghei* in certain mouse strains compared to the spleen-intact controls [294]. Disease outcome is largely dependent on the balance between the protective immunity and immunopathology. In our DT/DTR model, with the disruption of innate immunity after DT treatment in the DTR mice, the presence of the spleen favors towards the pathology instead of protection, suggesting a more prominent role of the spleen in the immunological aspect than the mechanical filtration in malaria.

Overall, our data indicates that the highly regulated immune responses, including the expression pattern of cytokines and chemokines as well as the innate immune cells, are important for the disease control. Moreover, the early stimulation of pro-inflammatory cytokines and chemokines as well as their interaction of the innate immune cells are crucial for the nonlethal disease outcome. In our nonlethal infection models, the pro-inflammatory cytokines in general peaked at least one day earlier than the anti-inflammatory cytokines, while in the lethal infection they showed peak expression on the same post-infection day (day6 pi).

Correspondingly, we showed that there were higher macrophages and monocyte amount in general in wildtype mice spleens upon infection of the avirulent 17X parasites. In addition, in the DT/DTR mice with the unbalanced innate immune cell activation, the originally nonlethal mixed infection became lethal despite the presence of the spleen. Taken together, we propose a model here for a protective mechanism against the parasite infection.

When the parasites in the bloodstream encounter certain cells in particular innate immune cells that are CD11b⁺, a pro-inflammatory cytokines will be released, which will trigger activation of the macrophages and monocytes. The red pulp macrophages react rapidly to the parasites trapped in the spleen, and further release cytokines and chemokines. Chemokines stimulate the innate immune cell migration to the spleen, in particular the monocytes, which can differentiate into dendritic cells and macrophages upon stimulation. Macrophages and dendritic cells would further stimulate the pro-inflammatory responses but on the other hand anti-inflammatory cytokines will also be released to control the degree of pro-inflammatory. In addition, they are also important antigen presenting cells that are important for the adaptive immune response. In the case of the virulent YM infection, the interaction with CD11b⁺ cells is less efficient and thus the stimulation of CD11b⁺ cells and the resulting release of pro-inflammatory cytokines are delayed. Thereby the pro-inflammatory and anti-inflammatory responses interfere with each other, leading to an uncontrolled disease development.

Chapter 6

**Potential factors contributing to the
parasite virulence and disease outcomes**

6.1 Innate immunity activation

Our data clearly showed that innate immunity regulates the disease outcome in malaria. We demonstrated that disruption of the innate immune responses has great impact on parasite growth behavior. Next we set out to explore the potential influence of boosting the innate immunity on parasite development.

Vitamin A has been known to be important in homeostatic control of the immune system for more than a decade and its downstream derivative retinoic acid (RA) has been demonstrated to play a role in innate immunity modulation [295-298]. It has been suggested that RA regulates the development and maintenance of a certain subset of splenic dendritic cells that are specialized in MHC class II-restricted antigen presentation [204], and RA treatment enhances the production of cytokines, which favor the Th2-type T cells, by activated macrophages and monocytes [299], thereby promoting the development of anti-inflammatory immune responses. Therefore, here we investigate the effect of RA treatment on *Plasmodium* infection

A preliminary experiment was done with virulent YM infection and the result is shown in Fig6.1A. Mice with pre-treatment of RA (either single dose of 500 µg on day-1 or two doses of 300 µg on day-1 and day+1 each) had a relatively lower parasitemia than the control mice pre-treated with DMSO. However, when comparing the mice received two doses of 300 µg RA to the control, there was no significant difference in parasite load except for day6 pi (p -value <0.05). Though the RA-treated mice showed a delayed mortality, where mice received two doses of 300 µg RA survived 1-3 days longer and mice received a single dose of 500 µg RA survived 1-4 days longer than the control mice (Fig6.1B), there was no statistically significant difference in their survival rates. Our data showed that the RA treatment stimulated a slightly delayed disease development in mice upon

Plasmodium infection. However, the ultimate lethal disease outcome implies that stimulating the innate immunity towards the anti-inflammatory responses at the early stage of parasite infection could not help to protect the mice. As this experiment has been done once only, a technical replicate of the experiment is necessary. Studies using different doses of RA and perhaps also with different RA administration timelines before/during the parasite infection will be required as well for further investigation.

Fig6.1. Growth behavior of YM-GFP line in BALB/c mice with retinoic acid treatment. A-Growth curve for YM-GFP line in mice with pre-treatment of either DMSO (Red) or RA (Green-single dose of 500 μ g each mouse or Blue-two doses of 300 μ g each mouse). Data are mean $\pm$ SD results from 3-6 mice per group. Statistic analysis was done using one way ANOVA with Tukey test. † denotes death of mice; B- Survival curve for mice with pre-treatment of DMSO or RA after YM-GFP infection. Statistic analysis was done using Log-rank (Mantel-Cox) test.

6.2 Red blood cell preference

One well-known difference between the virulent YM and the avirulent 17X parasite lines is their invasion preference to erythrocytes, with the former invading RBC of all ages and the latter one preferentially invading reticulocytes. In fact, red cell preference frequently correlates with the virulence of *Plasmodium* parasites, and it has been demonstrated that reduced parasite virulence is in association with the RBC repertoire switch from normocytes to reticulocytes [148, 152]. Whether this reduction in parasite virulence is a result of changes in RBC interaction with the innate immune cells, or is dependent on parasite genetics, it is still not clear. Therefore, in this study, we investigated the impact of reticulocytes in the disease development, and explored the potential mechanism behind.

6.2.1 Effect of reticulocyte

Reticulocytes are immature erythrocytes and the maturation process takes around 24-36 hr after their expulsion from the bone marrow [157]. Reticulocytes represent 0.6- 2.9% of RBCs in human blood [300] and 2-5% of RBCs in mice [301]. Compared to the mature erythrocyte that is biconcave shape, reticulocyte has an irregular polylobulated or deeply cupped shape, and is larger in size, containing ribosomes and various organelles [302]. During the maturation process, reticulocytes undergo a general streamlining of cellular metabolism [160] coupled with an extensive remodelling of plasma membrane, including the structure as well as the properties. Moreover, organelles are removed during this transition. As a result, the mature RBCs have increased cell density [158, 159], a decreased metabolic profile and a decreased repertoire of carbon sources and other essential

nutrients, as well as decreased surface molecules; some of these components may be important or favored for certain parasite lines to invade [149, 160, 161].

6.2.1.1 Cell deformability changes in association with RBC maturation

Given the differences between the reticulocytes and normocytes, reticulocytes would be expected to have different mechanical properties as compared to normocytes. Therefore, the deformability of reticulocyte was assessed, and compared to that of the normocytes.

Fig6.2. Reticulocyte effect on peripheral blood cell deformability. A- uRBCs of either normocytes (black) or reticulocytes (red); B- 17X-infected mice with reticulocyte-enrichment with >10% in circulating blood; C- YM-infected mice with reticulocyte-enrichment with >10% in circulating blood. Data are pooled from two individual experiments each with three mice per group.

Through repeated bleeding, reticulocytosis was stimulated and reticulocyte count reached 10-30% when the experiment was performed. As shown in Fig6.2A, deformability of reticulocytes had a mean value close to 3, which were significantly lower than those of the normocytes (mean value ~3.7) in the same

mouse samples (p -value <0.05). When these mice with enriched circulating reticulocytes were infected with parasite and reached a parasitemia around 2%, in both 17X and YM infections, uRBCs irrespective of the RBC types, showed similar level as the uninfected normocytes (Fig6.2). The infected reticulocytes regardless with 17X or YM parasites showed dramatic decrease in deformability when compared to the uRBCs as well as the uninfected reticulocytes (p -value <0.05). Moreover, the deformability of the infected reticulocytes had a similar level (velocity ~ 1.3) to those 17X-infected RBCs, but significantly different from that of the YM-infected RBCs (velocity ~ 2) in mice without reticulocytosis stimulation at the similar parasitemia (p -value <0.05).

Fig6.3. Effect of reticulocytes in parasite growth. Blue- Phenylhydrazine-stimulated reticulocytes infected with YM-GFP; Red- un-stimulated RBCs infected with YM-GFP. Data are mean $\pm$ SEM results from two individual experiments each with three mice per group.

Our data demonstrated that reticulocytes have lower deformability than the uRBCs, and infected reticulocytes also have lower deformability comparing to the iRBCs in virulent YM-infected wildtype BALB/c mice, suggesting a higher splenic retention and potentially a higher splenic clearance of reticulocytes and infected

reticulocytes. To investigate the impact of reticulocyte on parasite clearance, mice with phenylhydrazine-stimulated reticulocytosis were used and infected with YM-GFP for the source of infected reticulocytes. Similar to the mixed infection model, wildtype BALB/c mice without any stimulation were first infected with 1000 mature schizonts of 17X line to prime the spleen response. Subsequently, mice were challenged with 5×10^8 YM in either infected normocyte or infected reticulocyte forms. Parasitemia was monitored from 1hr to 72hr pi (Fig6.3). Similar to what has been observed in the YM growth profile in the single infection, YM administered in normal RBCs rapidly replicated and the parasitemia increased dramatically from 24hr pi to 72hr pi. In contrast, YM administered in reticulocytes exhibited relatively lower rate of multiplication as demonstrated by the slopes of the parasitemia curves, and the parasitemia starting from 48hr pi onwards were significantly lower than those of mice receiving the infected normocytes (p -value < 0.05), suggesting a higher clearance rate of infected reticulocytes compared to the infected normocytes.

6.2.1.2 Pre-stimulation of reticulocyte affects the parasite growth

We showed that the infected reticulocytes had a higher clearance rate than the normocytes in the mixed strain infection. Therefore, we hypothesize that limiting the parasite infection in reticulocytes instead of normocytes would help to control the disease development.

To test the hypothesis, mice were stimulated for reticulocytosis through repeated bleeding, and subjected to parasite infection when the reticulocyte count reached ~20%. Fig6.4A showed that the virulent YM parasites had an even faster replication rate in mice with reticulocyte enrichment as compared to the wildtype BALB/c mice without pre-stimulation, and the parasitemia developed to

significantly higher levels than the wildtype control on day6 and day7 pi (p -value <0.05).

Fig6.4. Parasite growth behaviour in mice with reticulocyte enrichment in circulating blood. A- Growth curve for YM-mCherry line in reticulocyte (retic)-enriched mice (Red) or un-stimulated wildtype mice (Blue); B- Parasite growth in retic-enriched mice with modified mixed infection (pre-infection of 1000 YM-GFP and 24 hr later challenged with 1000 YM-mCherry). Data mean $\pm$ SEM results from 5 mice. † denotes death of mice.

Furthermore, in order to check whether the presence of infected reticulocyte would help to accelerate parasite clearance, a modified mixed infection model was established in mice with reticulocyte enrichment (~20% circulating reticulocytes). In this modified mixed infection, mice were first infected with 1000 YM-GFP and

24 hr later challenged with 1000 YM-mCherry. The respective parasite signals as shown in Fig6.3B indicated a rapid replication of YM-GFP in the reticulocyte-enriched environment that was superior to the latter infecting parasite line (YM-mCherry). In contrast to the growth profile of YM-mCherry in the co-infection model with YM-GFP in wildtype mice without reticulocyte stimulation (Fig3.11A), the negligible levels of the YM-mCherry in this model further supports that infected reticulocytes have a greater multiplication rate than infected normocytes. In addition, compared to the complete protection in mice pre-infected with reticulocyte-prone 17X and the partial protection in mice pre-infected with SERA2-KO parasites from the subsequent virulent YM challenge (Fig3.9A and Fig3.10), the modified mixed infection model here had a lethal disease outcome, suggesting that the protection effect in the mixed infection is dependent on parasite genetics. Overall, we demonstrated that artificially increasing the percentage of circulating reticulocytes would worsen the disease development, rather than protecting the host from *Plasmodium* infection. Our data implies that reticulocyte alone is not sufficient to control the disease development in *Plasmodium* infection, and parasite genetics is an important factor modulating the protection in parasite mixed infections.

6.1.2 Exosomes in *Plasmodium* infection

Though the presence of large amount of circulating reticulocytes has been shown to complicate the condition in malaria, recent study showed that a membrane vesicle termed exosome secreted from the avirulent 17X-infected reticulocytes could stimulate the host adaptive immune responses and protect the mice from subsequent virulent parasite infection [166].

Exosomes are membrane vesicles that were initially described in reticulocytes that

act as cargo for eliminating certain proteins during the maturation process to erythrocytes [162]. Maturing reticulocytes have been shown to express LAMP1 and LAMP3 which are then released through secretory vesicles like exosome [303]. The role of exosomes in antigen presentation and immune modulation has been demonstrated in different infection models [304-307]. Here in this study, the role of exosome in stimulating innate immunity in association with parasite infection was investigated.

Fig6.5. Characterization of exosome proteins purified from 17X-infected reticulocytes. 1st lane shows the protein marker and the 2nd lane shows the exosome proteins probed with anti-lamp1 and anti-lamp3.

Exosomes were first purified from plasma samples separating from blood enriched with reticulocytes and infected with avirulent 17X parasite line by ultracentrifugation as described in the Methods and Materials. Purified exosomes

were then lysed and exosome proteins were characterized using antibodies against the exosome surface protein lamp1 and lamp3. Western blotting result as shown in Fig6.5 showed the presence of the both lamp1 (~120 kDa) and lamp3 (30-60 kDa), indicating the successful purification of exosomes.

Fig6.6. Parasite growth behaviour in mice immunized with exosome proteins. A- Growth curve for YM-GFP in control mice receiving DMSO or mice with pre-injection of exosome proteins purified from 17X-infected mouse plasma samples. Data are mean $\pm$ SEM results from 6 mice. † denotes death of mice; B - Survival curve for YM-GFP-infected mice with pre-injection of either DMSO or exosome proteins purified from 17X-infection mouse plasma samples.

Purified exosomes were used to examine the impact on stimulation of immune response against parasite infection. Each mouse received single dose of exosomes immunization equivalent to 10 µg proteins determined by BCA assay. Subsequently mice were subjected to parasite infection with virulent YM line 24 hr after the treatment. A preliminary result revealed a slightly lower parasitemia (Fig6.6A) and one day delayed mortality (Fig6.6B) for mice received exosome immunization when compared to the mice received DMSO treatment, but statistically there was no significant difference in parasite development between these two groups. This data suggests that the dose of the exosomes for immune response stimulation is not enough, or the immunization time before the parasite infection is not long enough for immune response activation, or there is very limited effect of exosomes in modulating innate immune responses *in vivo*. To investigate further on the role of exosome in innate immunity, future study will be required using higher dose of exosome and/or longer immunization gap period that may help to stimulate better activation of the immune responses.

6.3 Discussion

Different *Plasmodium* parasites show distinct preferences for the type of RBC they invade, and this red cell preference has been suggested to correlate with parasite virulence [308]. Moreover, previous studies have also reported that changes in parasite virulence were associated with the switch of host RBC repertoire from normocytes to reticulocytes [148, 152]. It has been shown that there are extensive changes in plasma membrane during the maturation of reticulocytes to normocytes, resulting in different expression of surface molecules on reticulocytes and normocytes [154, 155]. Therefore, interaction with other cells including the innate immune cells would be expected to be different between reticulocytes and

normocytes. Moreover, it has been shown that the spleen retains reticulocytes but not normocytes at normal condition, and this retention is through specific adhesion [309]. Therefore, we hypothesize that the red cell preference of the parasites directs the disease outcome, where parasite lines prone to invade reticulocytes would have comparatively higher splenic retention rate and different interaction with immune cells thereby facilitating accelerated disruption of infected reticulocytes as compared to infected normocytes. Indeed, our data show that reticulocytes and infected reticulocytes (reticulocytosis was stimulated through repeated bleeding) have decreased deformability compared to normocytes and infected normocytes respectively, suggesting a higher spleen retention and potential higher splenic clearance. In line with this, we also show infected reticulocytes have lower replication rate than those infected normocytes when transferred to a new normal mouse, implying increased retention and/or disruption of infected reticulocytes by the spleen. However, the fact that in this experiment the reticulocytes were induced by administration of phenylhydrazine needs to be considered. Phenylhydrazine is an oxidative drug that destroys red blood cells. Though it has been suggested not to affect the immature red blood cell [310], it has been shown to affect red blood cell deformability and also stimulate reticulocytes that are large in size [311]. Therefore we cannot rule out the possibility that the increased retention/disruption observed could be due to increased mechanical trapping due to the increased size or decreased deformability. Further investigation is required to understand the impact of reticulocytes on splenic clearance.

In addition, though reduced parasite virulence has been shown to be associating with host cell switch from normocytes to reticulocytes, our data demonstrate that the virulent YM-infected reticulocytes could not help to accelerate parasite clearance, and presence of large amount of reticulocytes in the circulation actually would complicate the situation in malaria instead of providing protection. A previous study has reported that *P. yoelii* parasite lines are not restricted to their

common host cell types; changes in the host cell environment would lead to switch of host cell types such that virulent YM line would invade reticulocytes while avirulent 17X would normocytes under certain conditions [312]. Therefore, reticulocyte restriction alone is not sufficient to stimulate a protective response in parasite infection, and artificially changing the host cell environment to modulate parasite infection in reticulocytes on its own is not a protective mechanism that can help to control disease development in malaria.

On the other hand, reticulocytes have been shown to secrete exosomes and exosomes released from 17X-infected reticulocytes have been reported to modulate the host adaptive immune responses and protect the subsequent YM infection [166]. Exosomes from different origins have been shown to have different roles in immune modulation, where exosomes derived from dendritic cells showed an immunosuppressive function [313] while exosomes derived from epithelial cells [314] and RBCs [315] exhibited immunostimulatory role and induced secretion of pro-inflammatory cytokines. Overall, exosomes clearly have an impact on immune system. Though in our study the attempt on using exosome to stimulate innate immune system showed limited protective effect on parasite development, there was no adverse effect neither; therefore, it is too early to draw any conclusion now. Further investigation will be required to better characterize the exosomes purified and examine their origin in order to better understand their roles in malaria infection. Red cell surface markers such as CD71 and Integrin $\alpha 4$, together with the antigen presenting cell markers like MHC II and CD86 should be included. Whether there is presence of some secretory proteins like CD11b and CD11c should also be investigated. In addition, more information on the protective function of exosomes isolated from infected reticulocytes could possibly be obtained by modifying the dosage as well as the stimulation timing before the parasite challenge. It is possible that increase in immunization dosage of the exosomes isolated from infected reticulocytes, and/or extension of the stimulation

timing would allow a better and stronger innate immune response to be stimulated, which then can help to protect the host from the subsequent parasite challenge. Moreover, the details on innate immunity modulation towards a proper response against parasite infection are largely unknown, future studies will be required focusing on the specific molecules responsible for activation of the correct immune responses.

Innate immunity acts as the first line of defense against invading pathogens. With its rapid response to the appearance of antigens, it is fundamental in protecting the host from infection. Here we show that stimulating the innate immunity through RA treatment slightly slowed down the disease development with a significant reduced parasitemia in the early infection stage (day6 pi), implying a potential mechanism to regulate the parasite growth through innate immunity modulation. RA has been shown to promote differentiation of immature myeloid cells and prevent them functioning as suppressive cells in immune regulation [316]. RA treatment has also been shown to promote macrophage activation and release of Th2-type cytokines (Kang et al., 2007). However, even with the RA treatment, the infection was eventually lethal to all the mice in our experiment, with no significant difference in the survival rates among the control and the treated groups, suggesting that pre-stimulation of anti-inflammatory immune response could not ultimately help to control the parasite proliferation and protect the host from infection.

Chapter 7

Conclusions and Future Work

Being the largest secondary lymphoid organ in the body and acting as a blood filter, the central function of the spleen is to selectively remove the old or damaged RBCs, microbes as well as other particles from circulating blood, through mechanical trapping and/or the activation of the immune system. Malaria parasite infection stimulates dramatic spleen responses, not only in morphology and architecture, but also in immune cell activation and migration. To gain insight into the dynamic changes in the spleen associating with different parasite virulence, and the relationship to different disease outcomes, we investigate and compare the infections with *P. yoelii* virulent strain YM and avirulent strain 17X.

Here we showed that the spleen controls parasite growth and determines disease outcome in particular the nonlethal parasite infections. The essential role of the spleen in malaria pathogenesis was verified by examining the effect of splenectomy in both virulent and avirulent infections. Removal of the spleen resulted in severe disease outcomes for avirulent 17X parasite infection. This was further confirmed by our mixed infection model, in which pre-infection with avirulent 17X parasites protected the mice from subsequent infection with virulent YM line. This complete protection in parasite mixed strain infections only applies to the hosts with intact spleens, but it is not found in the splenectomized hosts.

The differential spleen remodelling and distinct splenic immune activation in response to virulent and avirulent parasite infections highlight a potential way to control malaria disease development. 17X-infected mice as compared to YM displayed more prominent spleen remodelling as well as earlier activation of pro-inflammatory response, which potentially facilitate a better parasite clearance. Morphologically, there was a significant increase in the spleen size, venous pore size and the splenic vein- to-artery diameter ratio in 17X-infected spleens as compared to YM-infected spleens. This suggests that there is a significant increase in the overall blood volume that is filtered in the spleen and subsequently collected

by the increased venous outflow in 17X infections. In addition, in 17X infection, the earlier parasite deposition and greater preferential splenic iRBC trapping , together with the greater impairment in iRBC deformability indicate that increased retention of iRBC is a key mechanism by which the spleen functions to control nonlethal parasite infections. This was verified by our mixed infection model. In this model, the YM-infected RBCs showed significantly increased preferential splenic trapping, as well as significantly decreased cell deformability as compared to those in the YM single infection. Most importantly, the deformability of iRBCs in the mixed infection was decreased considerably to a similar level of cells in the avirulent 17X single infection. In line with this, our data also showed a broadly similar activation profile of pro-inflammatory cytokines in spleen samples from 17X infections and mixed infections, and this cytokine profile is different from what has been obtained for YM infections. Together with the distinct disease outcomes observed in different parasite infections, our findings suggest that avirulent 17X would induce an “avirulent” spleen response that can protect the mice from displaying severe clinical complications. Our data supports the novel prospect that the strain-specific spleen remodelling is likely to modulate pathological outcome of malaria.

Changes in RBC deformability have been shown to correlate with malaria pathogenesis [178, 180, 181]. In line with this, our finding showed a strong association among the spleen remodelling, RBC deformability and disease outcome, supporting a potential application of RBC deformability as a robust indicator for malaria severity. Moreover, besides the internal factors including the cell size, shape, membrane viscoelasticity, the cytoplasm state, as well as the hemoglobin fluidity, that have been demonstrated to contribute to the altered cell deformability [172], our finding that the spleen and its responses to parasite infection have impact on iRBC deformability is surprising and it indicates that cell external factors also regulate the deformability. A possible explanation is that

iRBC specific factors like circulating antibodies that recognize the surface of the iRBC could contribute to changes in deformability. However, when peripheral blood from either YM- or 17X-infected mice (day7 pi) was collected and incubated in media containing control FBS or antibody-containing serum obtained from 17X-infected mice (day12 pi), our preliminary data (Fig7.1) showed a slightly higher mean deformability of 17X-infected cells when incubated with serum from 17X-infected mice (p -value = 0.036). In contrast, there was no change in the deformability of YM-infected cells regardless of the serum used. All together, these suggest that the presence of circulating antibodies is not the main contributing factor. Moreover, the early effects observed in our data makes it unlikely that the specific antibodies are the main contributing factor as antibodies are unlikely to play a role in an early stage of infection. On the other hand, since there is a large population of monocytes and macrophages residing in the spleen, their recognition may potentially influence the iRBC deformability. It has been shown that infection with the avirulent *P. yoelii* 17X parasites stimulated peritoneal macrophage activation as compared to the virulent strain [317]. Indeed, in our study, we showed a trend of earlier recruitment of macrophages and monocytes to the spleen, as well as significantly higher activation of macrophages in nonlethal infections as compared to the lethal infection. However, how the differential activation of innate immune cells affect the cell deformability is still not fully understood. In addition, whether the repeated passage through the microvasculature of the differently remodeled spleen or the early host cytokine responses would drive the observed changes in deformability also needs to be established. Through injection of appropriate cytokine combinations to mice at the early stage infection may help to reveal the potential impact of cytokine responses on deformability of iRBCs. It would be also interesting to do some *ex vivo* blood perfusion/passage experiment using the differently remodelled spleens from different infected mice, and check whether there is any resulting change in the cell deformability. With this information, we can possibly gain more information on

the structurally differential remodelling of the spleen and speculate the impact of splenic mechanical force towards the cell deformability. On the other hand, the strong association between cell deformability and disease outcome in *P. yoelii* infection provides a potential tool for fast and easy diagnosis and prognosis of malaria. Further application and confirmation on human malaria parasites such as *P. falciparum* will be important to prove the relevance of the cell deformability information as a robust indicator for malaria severity.

Fig7.1. Deformability/velocity of iRBCs with different serum incubation. 17X-se– serum obtained from mice day12 pi with 17X parasites. Ctrl-se- commercial heat inactivated FBS. Red- RBCs infected with YM-GFP; Blue- RBCs infection with 17X1.1-GFP. The black lines indicate the mean value of each population of RBCs. * indicates statistically significant with p-value <0.05.

Interestingly, the presence of reticulocytes in the circulating blood led to further impairment of cell deformability, and infected reticulocytes appeared to have higher splenic retention and clearance rate. This together with our observation that pre-infection of reticulocyte-prone 17X or SERA2-KO parasites provide complete or partial protection against the subsequent virulent YM challenge in mice with intact spleens, suggested that reticulocyte-restriction was a potential mechanism of

protection. However, in our modified mixed infection model, unlike the mixed strain infections with 17X or SERA2-KO parasites, the YM-infected reticulocytes did not convey any protective response and all mice died by day7 pi, similar to what has been observed in YM single infection. We also showed that the presence of large amount of reticulocytes would complicate the disease development in parasite infection instead of protection. This indicates that reticulocyte alone is not sufficient to stimulate a protective splenic response against parasite infection; instead parasite genetic seems to be a critical factor as well contributing to the protection in the mixed infection. However, it would be worth to note that in this study the mice were pre-stimulated for reticulocytosis through repeated bleeding, while we have demonstrated that the presence of large amount of reticulocytes accelerate the parasite multiplication rate. To verify the hypothesis that parasite genetic is a dominant factor regulating the protective response stimulated in the mixed infection, further study will be required using wildtype mice without any pre-stimulation. Moreover, additional information would be obtained potentially through comparing the effect of intact iRBCs and iRBC total lysate in the mixed parasite infections. Our preliminary data (Fig7.2) showed different growth profiles of virulent YM in the mixed infections with pre-infection of different 17X-iRBCs. The blood smear after 4 hr standard parasite culture (Fig7.2A) implied that the heat shock procedure at 45°C for 15 min kills the parasites but retains the iRBC intactness. It was observed that YM parasites in the mixed infection model with heat-killed 17X-iRBCs resembled the growth behavior as in YM single infection, indicating that the complete protection in parasite mixed strain infection relies on the live iRBC but not only the iRBC intactness. However, it is worth to note that the 17X-iRBCs used here were not enough late stage and the expression of surface antigens may not be enough to stimulate a strong immune response. In addition, these iRBCs compared to the control could not progress further after the heat-shock procedure. Therefore, we cannot rule out the possibility that the mechanism behind the observed difference in YM growth profiles in the mixed

infections is enough avirulent parasite antigens that can stimulate a strong innate immune response instead of the live avirulent iRBCs. To test this hypothesis, further investigation can be done using heat-killed late stage 17X-iRBCs as well as protein lysate prepared with late stage 17X-iRBCs to repeat the mixed infection.

A

B

Fig7.2.Mixed infection with pre-injection of live or dead 17X-iRBCs. A. Blood smear of 17X-infected cells after 4 hr standard parasite culture. left panel- control; right panel- parasite culture was incubated at 45°C for 15 min prior to the standard parasite culture; B-Parasitemia curve for YM-mCherry parasites in normal mixed infection (red, with pre-infection of live 17X-iRBCs) or modified mixed infection (blue, with pre-infection of 17X-iRBCs heat-killed by 15 min incubation at 45°C) . Data are mean $\pm$ SEM results pooled from 3-8 mice.

While in both YM and 17X infections the immune system is activated with significant changes in cytokine and chemokine productions as well as innate immune cell activations, it is clearly that 17X infection stimulates earlier pro-inflammatory cytokine activation, monocyte recruitment as well as greater differentiation and activation of monocytes and macrophages. Red pulp macrophages have been reported to be responsible for early sensing of *Plasmodium* infection [195]. Similarly, our findings showed that compared to YM-infected spleens, there were larger amounts of red pulp macrophages present early on (day3pi) and higher macrophage activation and differentiation in 17X infection, corresponding to the observation of earlier parasite deposition and greater preferential trapping of iRBCs in 17X-infected spleens. In addition, the early activation of pro-inflammatory response seems to be important in controlling parasite development, which is reflected through comparison of parasite growth behavior between 17X and mixed infections. Moreover, in contrast to the lethal infection where the pro-and anti-inflammatory cytokines peaked at the same time, the immune response in nonlethal infections seem to be tightly regulated, with a slightly delayed (1 day) activation of anti-inflammatory responses (IL-10) in the nonlethal infections. In line with this, our preliminary data on pre-stimulation of anti-inflammatory response through administration of RA suggests that early activation of anti-inflammatory response could not ultimately control the parasite development and protect the hosts against infection. On the other hand, depletion of the CD11b⁺ populations of innate immune cells resulted in an unbalanced immune response that could no longer protect the host in the mixed infection, even with the presence of an intact spleen. Collectively, our data suggest a highly regulated immune reaction with early activation of pro-inflammatory response is a key factor to fight against the parasite infection. It has also been shown in previous studies that early production (24 hr) of T1IFNs, which has the immunomodulatory function, is associated with control of malaria infections [318, 319]. This

information would help to provide some guidelines to design experiments such as cytokine neutralization or adoptive cell transfer for malaria challenge.

To conclude, we examined the differential splenic response upon virulent and/or avirulent malaria parasite infections and identified a strong correlation across the cell deformability, spleen remodelling and immune cell activation as well as infection severity in malaria. We demonstrated that infection with avirulent 17X parasites induces a more dramatic splenic changes including spleen enlargement, venous vessel dilation and venous branches increase, all facilitating a better blood filtration in 17X-infected spleens. In addition, changes in RBC deformability upon parasite infection were also noted. Removal of the spleen resulted in increased deformability of RBCs in circulating blood upon infection with the avirulent 17X parasites, highlighting the role of the spleen in modulating cell mechanical properties. Reduced RBC deformability is corresponding to higher retention in the spleen, in line with the observation on higher splenic trapping of parasitized RBCs early parasite deposition on red pulp in 17X infection. Furthermore, the preferential trapping of 17X-infected RBCs in the red pulp meshwork allows an early sensing by the red pulp macrophages thus an earlier activation of immune response. This interaction between the iRBC and the red pulp macrophages potentially also stimulates cell migration to the spleen and thus resulting in spleen remodelling. Meanwhile, the better interaction of 17X-infected RBCs with the innate immune cells particularly the CD11b⁺ population facilitates an early stimulation of the innate immune cell activation. The earlier activation of pro-inflammatory response stimulated by the avirulent parasites protects the host from infection and even challenge with virulent parasite lines. This protective response is largely dependent on the presence of the spleen as well as a balanced or highly regulated immune response; removal of the spleen or disruption of the innate immunity through ablation of CD11b⁺ cell population lead to severe parasite conditions in 17X infection and mixed

infections. Therefore, it would be of great value to identify the detailed molecules including specific parasitic proteins that are responsible for the proper activation of innate immune responses, in particular the monocyte/macrophage responses. Surface antigens as well as secretory elements of iRBCs are the main targets to study, since the nature of these molecules allows direct contact with the immune cells. Our finding demonstrated that the host innate immunity in particular the splenic innate immune response governs the disease development in malaria parasite infection, which potentially also takes part in modulating RBC mechanical properties. This leads to an interesting question on the relationship among parasite genetics, host immune response and cell deformability. Future studies can focus more on the early host immune response in particular the protective response against parasite infection. Through a broad screening on early host splenic response upon virulent and avirulent parasite infections, using RNAseq on spleen samples for the detection of the changes in host transcriptome level, would provide valuable information to define a proper protective response against parasite infection. While further investigation on cell deformability of different parasite lines with different virulence may help to answer the question on the relationship between parasite genetic and cell mechanical properties. Last but not least, parasite infection using different mouse models targeting specific molecules in immune system, such as macrophage depletion or IFN-gamma knockout mouse, together with the examination on deformability of the resulting iRBCs, would allow us to get more insight into the malaria pathogenesis.

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Appendix I

Progressive changes in spleen structure upon 17X1.1-GFP parasite infection. H&E stained spleen sections with 20x magnification.

Appendix II

ID	vein 1	vein 2	vein 3	artery 1	artery 2	artery 3	Ratio of vein-to-artery
Control M3	9.24	13.57	12.02	4.35	3.89	4.12	2.24
Control M4	11.88	11.97	13.28	4.27	4.12	2.54	
Control M5	9.51	10.01	11.88	6.95	7.83	8.75	
YM_D3_M1	15.21	15.69	21.19	5.84	7.21	7.65	3.01
YM_D3_M3	23.74	25.1	26.24	8.03	6.15	7.6	
YM_D4_M2	10.38	12.76	10.14	3.67	3.33	2.6	3.21
YM_D4_M3	13.12	13.42	15.27	4.35	5.9	4.48	
YM_D5_M1	7.02	7.06	11.18	2	2.69	3.67	3.54
YM_D5_M2	22.42	22.9	25.71	5.7	5.66	6.26	
YM_D6_M6	18.03	19.56	15.65	4	3.81	3.91	4.26
YM_D6_M2	28.07	31	27.02	7.01	6.71	8.25	
YM_D7_M2	25.34	25.34	25.35	3.35	3.4	3.4	6.94
YM_D7_M1	16.82	19.62	17.27	2.75	3.48	2.33	
17X_D3_M1	25.81	23.11	25.58	7.65	8.51	8.25	2.68
17X_D3_M3	12.78	12.62	14.5	5.41	6.5	5.6	
17X_D4_M3	23.85	21.95	20.88	7.21	7.21	4.22	3.48
17X_D4_M2	21.01	19.91	21	6.52	5.85	7.07	
17X_D5_M1	18.6	19.26	17.33	5.02	4.92	5.39	3.45
17X_D5_M2	22.5	22	24.25	8	6.02	7.16	
17X_D6_M1	18.03	19.56	15.65	4	3.81	3.91	3.96
17X_D6_M2	9.48	8.23	10	2.92	2.33	2.98	
17X_D7_M1	14.16	14.76	13.89	2.85	3.59	3.14	3.61
17X_D7_M2	21.02	20.02	21.63	8	7.07	8.06	
17X_D17_M2	14.08	16.07	13.51	6	4.53	5.02	2.86
17X_D24_M1	18.87	14.56	17.72	5.83	5.83	8.06	
17X_D24_M4	9.71	10.4	9.43	3.81	3.64	4.27	2.59

Raw data of the measurements of vein and artery diameter of the spleen casts collected on different post-infection days with different parasite line infections.

Appendix III

A

Spleen-intact CD11b-DTR mice

B

Splenectomized CD11b-DTR mice

Individual parasite behavior in mixed infection in spleen-intact or splenectomized CD11b-DTR mice. Blue- Growth curve for 17X parasites; Red- Growth curve for YM parasites. Data are represented as mean $\pm$ SEM.

Author's publications

1. Siau, A., **Huang, X.**, Weng, M., Sze, S.K. and Preiser, P.R. (2016) Proteome mapping of Plasmodium: identification of the *P. yoelii* remodelome. *Scientific reports*, **6**, 31055
2. Subramanian, G., C.P. Babu Rajeev, C.D. Mohan, A. Sinha, T.T. Chu, S. Anusha, **H. Ximei**, J.E. Fuchs, A. Bender, K.S. Rangappa, R. Chandramohanadas, and Basappa. 2016. Synthesis and *in vitro* evaluation of hydrazinyl phthalazines against malaria parasite, *Plasmodium falciparum*. *Bioorganic & medicinal chemistry letters*.
3. **Huang X**, Huang S, Ong LC, Lim JC-S, Hurst RJM, Mushunje AT, Matsudaira PT, Han J, Preiser PR. 2015. Differential remodelling associated with different levels of parasite virulence controls disease outcome in malaria parasite infections. *mSphere* 1(1):e00018-15.
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5. Siau, A., **X. Huang**, X.Y. Yam, N.S. Bob, H. Sun, J.C. Rajapakse, L. Renia, and P.R. Preiser. 2014. Identification of a new export signal in *Plasmodium yoelii*: identification of a new exportome. *Cell Microbiol*. 16:673-686.
6. Mok, S., K.Y. Liong, E.H. Lim, **X. Huang**, L. Zhu, P.R. Preiser, and Z. Bozdech. 2014. Structural polymorphism in the promoter of *pfmpr2* confers *Plasmodium falciparum* tolerance to quinoline drugs. *Mol Microbiol*. 91:918-934.
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9. Bapat, D., **X. Huang**, K. Gunalan, and P.R. Preiser. 2011. Changes in parasite virulence induced by the disruption of a single member of the 235 kDa rhoptry protein multigene family of *Plasmodium yoelii*. PLoS One. 6:e20170. **(Co-first author paper)**

Posters and Talks

1. 7th APOCB congress, Singapore. Feb 2014
Poster presentation: “The role of the spleen in mediating pathology in *Plasmodium yoelii*”.
2. 10th Biomalpar conference, EMBL, Heidelberg, Germany. May 2014.
Poster presentation: “The role of the spleen in mediating pathology in *Plasmodium yoelii*”.
3. 6th International Singapore Symposium of Immunology, Singapore. August 2014
Oral presentation: “The role of the spleen in mediating pathology in *Plasmodium yoelii* infection”.
4. BioSyM Annual Workshop and Scientific Advisory Board (SAB) Meeting. Singapore. Jan 2015.
Oral presentation: “Differential spleen remodelling and its interaction with parasite virulence”.
5. The fourth Singapore Malaria Network (SingMalNet) meeting. Singapore. February 2016.
Poster presentation: “Cell deformability associating with spleen response controls the disease outcome in malaria parasite infections”.
6. The fifth Molecular Approaches to Malaria (MAM) Conference . Australia. February 2016.
Poster presentation: “Cell deformability associating with spleen response controls the disease outcome in malaria parasite infections”.