Tissue-Resident CD169+ macrophages as a frontline of host defense in Plasmodium infection

Pravesh Gupta

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defense in *Plasmodium* infection**

Pravesh Gupta

School of Biological Sciences

2017

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**A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of Philosophy
2017**

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Abbreviations

AIDS	AIDS Acquired Immunodeficiency Syndrome
ADCI	Antibody dependent cell inhibition
ADP	Adenosine diphosphate
AKI	Acute kidney injury
ALI	Acute lung injury
ALT	Alanine transaminase
APCs	Antigen presenting cells
Arg	Arginase
AST	Aspartate transaminase
BBB	Blood brain barrier
CD	Cluster of differentiation
Clec9A	C-type lectin domain family 9 member A
CM	Cerebral malaria
cMoP	Committed monocyte progenitor
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
CTLs	Cytotoxic T lymphocytes
CXCR3	Chemokine receptor 3 (in CXC receptor family)
DAMPs	Damage associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
ECM	Experimental cerebral malaria

EF	Elongation factor
ELISA	Enzyme linked immunosorbent assay
EMH	Extra medullary hematopoiesis
EMPs	Erythro-myeloid precursors
Fc	Fragment crystallizable
gbw	Gram body weight
GC	Germinal center
GLURP	glutamate-rich protein
GMCSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte monocyte progenitor
GPI	Glycosylphosphatidylinositol
H&E	Hematoxylin and Eosin
Hb	Hemoglobin
HB-EGF	Heparin-binding epidermal growth factor receptor
HCM	Human cerebral malaria
hDTR	Human diphtheria toxin receptor
HIF	Hypoxia inducible factor
HRP	Horse radish peroxidase
HSCs	Hematopoietic stem cells
hsp	Heat shock protein
Hz	Hemozoin
i.p.	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
iNKT	Invariant natural killer T
iNOS	Inducible nitric oxide synthetase
iRBCs/pRBCs	Infected red blood cells/parasitized red blood cells
LFA-1	Lymphocyte function associated antigen 1
LI	Liver injury
LP	Lamina propria
LPS	Liposaccharide
LXR	Liver X receptor
MA-ARDS	Malaria associated acute respiratory distress syndrome
MARCO	Macrophage receptor with collagenous structure (type I scavenger receptor)
MCP-1	Monocyte chemoattractant protein 1
MDP	MΦ and DC progenitor
MDR	Macrophage disappearance reaction
mg	Milligram
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mL	Milliliter
MMMs	Marginal metallophilic macrophages
MMR	Macrophage mannose receptor
MPS	Mononuclear phagocyte system
MS	Multiple sclerosis
MSP-1	Merozoite surface protein-1
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
MZMs	Marginal zone macrophages

MΦ	Macrophage
ng	Nanogram
NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide binding oligomerization domain
P	Plasmodium
PAMPs	Pathogen associated molecular patterns
PbA	<i>P. berghei</i> ANKA
PbALuc	PbA expressing luciferase enzyme
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator activated receptor
PRRs	Pathogen recognition receptors
PS	Phosphatidylserine
PSR	Picrosirius red
PV	Parasitophorous vacuole
Py	<i>P. yoelii</i> 17X non lethal (NL)
PyLuc	Py expressing luciferase enzyme
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RBCs	Red blood cells
RIG	Retinoic acid-inducible gene
RMCBS	Rapid murine coma and behavioral scale
RNA	Ribonucleic acid
RP	Red pulp
RPMs	Red pulp macrophages

SCS	Subcapsular sinus
SD	Standard deviation
Siglec-1	Sialic acid binding Ig like lectin 1
SMA	Severe malarial anemia
Sn	Sialoadhesin
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
TIM	T-cell immunoglobulin and mucin-domain-containing molecule
TLR	Toll like receptor
TNF	Tumor necrosis factor
T _{regs}	T regulatory
VCAM-1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
WBC	White blood cells
WHO	World health organization
WP	White pulp
WT	Wild type
μl	Microliter

Abstract

Macrophages are professional phagocytic cells that comprise tissue-resident and monocyte-derived subsets with pleiotropic functions ranging from tissue homeostasis, clearance of senescent erythrocytes and cell debris, antigen presentation as well as regulation of innate and adaptive immune responses to pathogens. The complex diversity of macrophage subpopulations across different organs requires that the individual subsets should be considered separately in terms of their functional specializations during inflammation and disease, including malaria. The precise role of tissue-resident macrophages during blood-stage malarial infection and resulting multifaceted malarial syndromes remains obscure. To this end, here we focused on a distinct subset of tissue-resident CD169⁺ macrophages that occupies a privileged micro anatomical location at the blood entry sites, such as marginal zone in the spleen, hepatic sinusoids in the liver, perivascular regions of brain and kidney, with the property to capture blood-borne pathogens in a glycan dependent manner.

In this study, we exploited a CD169-DTR mouse strain and demonstrated that tissue-resident CD169⁺ macrophages are specifically and efficiently ablated in all the tested organs during steady state and *Plasmodium* infection. Using a *P. berghei* ANKA (PbA) infection model we show that the absence of CD169⁺ macrophages results in accelerated mouse mortality due to exacerbated parasite sequestration, hemozoin tissue deposition and vascular leakage. This led to development of overlapping pathologies such as cerebral malaria (CM), acute lung, liver, kidney and heart injury culminating into widespread tissue damage and inflammation, characteristic of malarial syndrome. Except for the development of CM, similar pathologies in different organs were recapitulated in *P. yoelii* non-lethal (Py) infection model as well. Thus the CD169⁺ macrophages play a crucial role in restraining the parasite burden and its sequestration in different tissues and therefore regulate the outcome of *Plasmodium* induced inflammation and pathogenesis.

Functionally, we observed an attenuated T cell effector response in CD169-DTR mice during the *Plasmodium* infection. In addition, parasite specific antibody responses were compromised during Py infection. Furthermore, CD169⁺ macrophages are a predominant constant source of anti-inflammatory cytokine IL-10 during the entire course of PbA infection. Therefore, our results demonstrate that CD169⁺

macrophages possess an intrinsic anti-inflammatory property and are regulators of anti-malarial innate and adaptive immune responses during *Plasmodium* infection.

1. Introduction:

1.1 Global health and economic burden

Malaria is a very complex devastating parasitic disease inflicted by Apicomplexan pathogens of genus *Plasmodium*. Amongst *Plasmodium* parasites, only five species such as *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*, *P. falciparum* cause human malaria and are also associated with high rate of morbidity and mortality. All the aforesaid *Plasmodium* species spread from human to human through bites of female *Anopheles* mosquito, except *P. knowlesi*, which exhibits primarily zoonotic mode of transmission i.e. macaque to human [1]. Malaria occurs primarily in tropical and subtropical regions of the world because of favorable climatic conditions such as temperature, humidity and rainfall that allow the survival and multiplication of *Anopheles* mosquito. *P. falciparum* malaria is mostly found in the African continent and is also the cause of widespread mortality whereas *P. vivax* induced malaria has a larger geographical distribution due to the survival advantage of the parasite at higher altitude and cooler climates. *P. vivax* is more common than *P. falciparum* in many regions outside Africa [2, 3].

Even though the global incidences of malaria fell from an estimated 262 million in 2000 to 214 million in 2015 (Figure 1), it still occupies the center stage in “the big three diseases” alongside tuberculosis and acquired immunodeficiency syndrome (AIDS). In 2015, malaria caused death of 438000 patients predominantly (90%) in the African region and amongst these 70% of the total casualties were children less than five years of age [2, 3].

Despite global efforts in the quest to vanquish and eliminate this parasitic disease since the discovery of parasite in 1880, vaccine-based solution to put an end to the malaria malaise remains elusive. A fundamental understanding of the protective correlates of host’s versatile immune mechanisms might pave way and shed light on intervention strategies in the direction of developing a successful vaccine candidate.

1.2 Life cycle of *Plasmodium* in mammalian host

Malaria is caused by *Plasmodium* and female *Anopheles* mosquito is the culprit for the transmission of the disease. *P. falciparum* is the most threatening parasite which causes acute, chronic malaria and is often associated with severe complications of the disease such as cerebral malaria (CM), hepatic injury culminating into clinical jaundice, severe

malarial anemia (SMA), malaria associated acute respiratory syndrome (MA-ARDS) and renal injury.

Figure 1.1 A map illustrating global distribution of malaria showing confirmed cases per 1000 individuals (Source: WHO World Malaria Report, 2014).

The life cycle of *Plasmodium* comprises a liver stage, blood stage and mosquito stage and is conserved across mammalian hosts (Figure 1.2). The mosquito inoculates the parasite in the form of sporozoites from its salivary gland into the host skin while feeding on a blood meal, which ranges from 10-20 sporozoites per bite. These sporozoites traverse along the blood stream to find its way to the liver within minutes thereby initiating the liver stage of infection [4, 5]. The blood flow slows down in the liver sinusoids, thereby allowing sporozoites to attach to the endothelium through proteoglycans displayed by hepatocytes. After crossing the endothelium, sporozoites translocate through several hepatocytes before settling into parasitophorous vacuole (PV) of one of them. Here they rapidly multiply by a process of asexual replication giving rise to multinucleated exoerythrocytic schizonts containing thousands of merozoites that are released from the PV into the cytoplasm of the host cell [6]. The parasite needs to evade the liver resident macrophages that line the liver sinusoids; they do so by encapsulating itself in a vesicle derived from the hepatocyte cell membrane, thereby avoiding immune recognition. Disruption of the PV membrane results in the host cell death [6, 7]. The merozoites encapsulated in the host cell membrane are called as merozoites, which bud off and are reduced further to smaller 12-18 micron entities by the shear forces in the circulation [8]. The smaller merozoites travel through right side of the heart and subsequently accumulate in capillaries of the lungs, where they rupture, releasing the merozoites to infect red blood cells (RBCs) heralding the onset of blood

stage infection [8]. It is to be noted that recently merozoite-like structures have been reported in a humanized mouse model infected with *P. falciparum* [4] as previously reported in rodent malaria parasites [8, 9]. The liver stage of the infection is non-pathogenic and is asymptomatic [5].

Figure 1.2 Life cycle of the *Plasmodium* in a mammalian host. The mosquito injects sporozoites in the human skin, which reaches liver within minutes via circulation starting the asymptomatic liver stage. The sporozoites transmute through several hepatocytes and finally reside in parasitophorous vacuole of one of them, where they multiply, develop into multinucleated schizont and ultimately encapsulate in host cell membrane to form vesicles called as merozoites which eventually bud off. These merozoites break down into smaller entities by shear forces and reach the lung via right side of the heart. Rupture of the merozoites releases thousands of merozoites that infect RBCs initiating the symptomatic blood stage of the life cycle. Infection of RBCs leads to development of erythrocytic schizonts (containing 10-20 merozoites) that ruptures and invades fresh RBCs. Some of these merozoites are taken up by feeding mosquito to initiate the sexual stages of life cycle in midgut of the mosquito thus termed as mosquito stage. Adapted from [6] and (<http://www.mmv.org/malaria-medicines/parasite-lifecycle>).

After approximately 7-12 days (*Plasmodium species* dependent), rupture of liver exoerythrocytic schizonts results in release of numerous merozoites in the bloodstream. Each merozoite infects the red blood cell (erythrocyte), rapidly multiplies as a result of mitotic divisions to form an erythrocytic schizont (harboring approximately 20 merozoites each). These merozoites continue to infect new erythrocytes with periodic cycles lasting from 48-72 hours (*Plasmodium species* dependent). This marks the onset of the symptomatic phase of the infection [5]. Rupture of infected erythrocytes releases toxic substances in the blood circulation, which are known to cause repeated bouts of fever, chills and profuse perspiration. Unknown factors promote some of these merozoites to develop into male and female gametocytes, which are picked up by the feeding mosquito to generate extracellular gametes. Inside the mosquito midgut, fusion of the gametes results in development of motile zygote (ookinete), which punctures the

mid-gut wall to form oocyst, wherein, by the process of meiosis, haploid sporozoites are generated. Since this occurs in mosquito it is called as mosquito stage [5, 10].

1.3 Animal models, malaria research studies and their relevance

Since human studies on malaria can only be performed on blood samples, biopsies and tissues obtained from patients or samples obtained from post-mortem procedures, it becomes difficult to study the dynamic interplay of parasite virulence and host immune responses. Therefore, animal models such as rodent and non-human primate (NHP) malaria models serve as indispensable tools for novel discoveries [11]. As a matter of fact, since none of the human *Plasmodium* parasites infect animal models, researchers often use rodent and NHP parasites in susceptible animal hosts to emulate different aspects of human disease [12, 13].

Rodent malaria models

Since *P. falciparum* can manifest acute, chronic and even more severe forms of the disease such as CM and in the light that none of the rodent parasites can recapitulate all the aspects of the malaria, researchers employ *P.yoelii* 17XNL, *P. chabaudi* and *P. berghei* ANKA mouse infection models respectively to study the different phases of the host pathogen interactions [12]. Amongst these three mouse infection models, *P.berghei* ANKA represents one of the best available models that share commonality with the clinical malaria but the severity of the infection does not permit the study of humoral immune responses as the mice succumb to infection [14, 15]. However an elegant study has shown that severe malaria caused by *P.berghei* ANKA results in compromise in the germinal center B cell reactions as well as inhibits Tfh differentiation by regulating the severity of malaria that enabled investigation of humoral immunity [16]. Most information regarding humoral immune response has been generated using primarily *P. chabaudi* and to a lesser extent *P.yoelii* 17XNL [12, 17]. The pursuit of understanding prevalent protective host mechanisms against malaria is further complicated by heterogeneous and multifaceted nature of *P. falciparum* inflicted malaria that results in syndromes; CM, ALI, hepatic dysfunction and renal failure which may or may not be coexistent in a malaria patient. To mimic the syndrome nature of the disease different mouse models are available which exhibit one or multiple facets but not all at the same time [12], refer to table 1.1.

One of the major discrepancies in the malaria research is contentious observation in HCM and ECM. A cardinal feature of HCM is sequestration of iRBCs in brain

microvasculature that may be correlated with prevalence of vessel obstruction and micro hemorrhages as observed in most post-mortem studies but in the case of ECM this dogma has divided the researchers with some in favor and others against [12, 14, 18].

Table 1.1 Parasite host combinations used to study multifaceted malarial syndromes and different aspects of the disease. Adapted from [12].

Syndrome/aspect	Parasite/host	Remarks
Cerebral malaria (CM)/ acute and severe infection	<i>P. berghei</i> ANKA/ C57BL/6J or CBA mice	CM + LI + AKI + ALI
Liver injury (LI)/ acute and severe infection	<i>P. berghei</i> ANKA/ C57BL/6J	CM + LI + AKI + ALI
Acute lung injury (ALI) /Acute respiratory distress (ARDS)	<i>P. berghei</i> ANKA DBA/2 and <i>P. berghei</i> NK65/ C57BL/6J	Exclusive model for ALI/ARDS
Acute kidney injury (AKI)/ acute and severe infection	<i>P. berghei</i> ANKA/ C57BL/6J	CM + LI + AKI + ALI
Anemia in acute infection	<i>P. yoelii</i> C57BL/6J or BALB/c	Mainly used to study anemia and humoral immune responses.
Anemia in Acute & chronic infection	<i>P. chabaudi</i> C57BL/6J or BALB/c	

However, recent data provide evidence of iRBC sequestration in organs including brain during ECM [19]. Nonetheless, in both HCM and ECM vessel obstruction is a common feature as a consequence of iRBC sequestration or leukocyte sequestration or both, therefore adjunct therapies to relieve vessel blockade can hence be pursued. Despite the limitations, malaria research in mouse models does not preclude the uncovering of conserved mechanisms that drive the host inflammatory processes, which are correlates of protection as well as pathology [12].

Non-human primate malaria models

One of the major limitations of the rodent malaria models is the absence of chronic malarial infections with the exception of *P. chabaudi*. In addition, some rodent parasite/host combinations are almost completely immune after a single infection thus preventing the mechanistic studies on dynamic host parasite interactions, relapse immunobiology and pathogenesis [12]. NHP malaria models provide an advantage of investigating this aspect due to the availability of relapsing parasite species such as *P. cynomolgi* and *P. vivax* in primate hosts; *Aotus/Saimiri* species and *Macaca mulatta* respectively [13]. In a recent report, *P. coatneyi* has been suggested as an optimal NHP parasite that shares biological characteristics of *P. falciparum* induced severe malaria and a potentially an appropriate model for the studies on physiological and molecular pathways in malaria immune as well as naïve individuals. Experimental infection of *P. coatneyi* in *M. mulatta* resulted in development of anemia, coagulopathy, compromise in kidney function and metabolic dysfunction [20]. *P. coatneyi* infected *M. mulatta*/*M.*

fuscata and *P. fragile* infected *M. mulatta* exhibit certain degree of cerebral sequestration and can be utilized as potential models for HCM [21, 22]. A greater genetic relatedness of NHP host/parasite combinations to human malarial parasites makes NHP malaria models more suitable for the investigative research studies of pathological and immunological mechanisms pertaining to heterogeneous clinical presentations of the disease [12].

The differential infection outcomes, disease progression & pathological patterns, nature of generated immune responses owing to the heterogeneity of disease presentation in different animal malaria models poses a great challenge in extrapolation of these research findings to understand human malaria. Therefore, the most appropriate animal model should be used in line with the research focus in question [11].

1.4 Clinical and pathological features of the disease

Plasmodium falciparum can manifest acute, chronic and severe complications of the disease that result in malarial syndromes causing damage to the vital organs such as brain, liver, lung and kidney and in some cases heart as well.

1.4.1 Cerebral Malaria (CM)

Plasmodium falciparum is the primary contributor to the incidences of human CM (HCM), which is often accompanied with multiple organ dysfunctions. Adult and children show remarkable differences in the intensity of clinical symptoms possibly due to the differential status of immunity. In general, seizures, neurological deficits (ataxia, hemiplegia, cerebral palsies, cognitive impairment) retinopathy, systemic complications such as severe anemia, jaundice, metabolic acidosis, renal failure, respiratory distress syndrome represent the idiosyncratic nature of the disease leading to coma and eventually death in majority of the cases. Mortality in pediatric CM is very rapid with 50% of the subjects succumbing within 24 hours of infection and nearly 100% casualties within 48 hours post admission whereas with adults almost half of the recorded cases perished within 2 days and others died later due other complications including multiple organ dysfunctions [23, 24].

Though the pathogenic mechanisms of CM are poorly understood, some of the factors that contribute to the pathogenesis are iRBC (pRBC; parasitized RBC) sequestration, endothelial cell activation and CD8⁺ T-cell mediated damage, platelet activation, leukocyte sequestration as well as dysregulated cytokine/chemokine responses, which all

together contribute to blood brain barrier (BBB) disruption in *P. berghei* ANKA mouse infection model [23, 25].

The current understanding from ECM (experimental cerebral malaria) studies in mice has established the following model that provides insights into pathogenic mechanisms of the disease (Figure 1.3). Upon *P. berghei* ANKA (PbA: ECM causing rodent *Plasmodium* species) infection Clec9A⁺ dendritic cells (DCs) cross present PbA antigens to prime naïve T cells in the spleen [26]. Cytoadherence of pRBCs to brain endothelium results in the release of inflammatory signals i.e. glycosylphosphatidylinositol (GPI) anchors, hemozoin deposits containing parasite DNA [25]. This triggers the activation of the endothelium. The activated endothelium up regulates adhesion molecules such as ICAM-1, VCAM-1, P-selectin and E selectin that aid in leukocyte and platelet sequestration [27]. The recruitment of activated leukocyte (CD4⁺ and CD8⁺ T cells, monocytes and macrophages) and platelets orchestrate a local inflammatory milieu that resonates with endothelial cell activation, leukocyte, platelets and parasite sequestration [25].

Among the leukocytes, CXCR3⁺CD8⁺ T cells (activated) migrate along the chemokine gradient to the brain where its adhesion is favored by activated endothelium displaying ICAM-1 [28]. IFN- γ produced by NK cells and CD4⁺ T cells stimulate endothelial cells of the brain to phagocytose and cross present large amounts of PbA antigen. CD8⁺ T cells respond to MHC class I loaded PbA epitopes by TCR ligation and secrete effector molecules such as perforin and granzymes that induce apoptotic cell death in the endothelium, thus leading to breach of blood brain barrier [23, 25].

CM is believed to be a consequence of both parasite and immune cell mediated pathologies and the paradox between ECM and HCM is a result of this overlapping school of thoughts. In ECM, leukocyte sequestration is concomitant with parasite sequestration whereas in HCM, latter is more profound with remarkable heterogeneity in the sequestration patterns. However, the findings in humans have been limited to post-mortem studies suggesting the likelihood of such discrepancies could be associated to multiple factors such as pre-existing medical conditions, prior history of antimalarial drugs, time of death and others. Therefore, careful investigation in this direction needs to be done in future to settle this contention [12, 25].

Figure 1.3 Schematic illustrations highlighting pathogenic mechanism of CM. 1) Upon parasite infection, parasite antigens are untaken by Clec9A⁺ DCs, processed and loaded on MHC molecules to prime the T cells. 2) Parasite travels to the brain where it activates the endothelial cells of the brain. 3) Activated endothelium up regulates adhesion molecules and secretes chemokines that facilitate sequestration of leukocytes and platelets 4) Recruitment of activated platelets and leukocytes leads to establishment of pro-inflammatory milieu. 5) Activated effector CD8⁺ T cells migrate along the chemokine gradient to the brain and sequester through ICAM-1 and LFA-1 cognate interactions. 6) IFN- γ stimuli enable endothelial cells to cross present parasite antigen to CD8⁺ T cells. 7) Cytolytic CD8⁺ T cells induce apoptosis of brain endothelial cells leading to compromise in BBB integrity. Adapted from [25].

1.4.2 Liver Injury (LI)

Plasmodium falciparum malaria causes liver pathologies leading to clinical jaundice in a small fraction of patients (3%-5%) in the endemic regions. Hepatic dysfunction in human subjects is mostly correlated with hepatomegaly, high levels parasite load and elevated levels of liver transaminases. Ultrastructure study of *P. falciparum* patients revealed significant correlation between pRBC sequestrations, high levels of AST/ALT, hepatomegaly that corroborated with the status of jaundice in these subjects [29-31]. *Plasmodium* induced hepatic dysfunction and pathogenesis is attributed to parasite driven activation of Kupffer cells (often hyperplastic due to the phagocytosed parasite) that releases inflammatory mediators such as IL-1 β , tumor necrosis factor-- α (TNF- α). These mediators stimulate the endothelium to up regulate adhesion molecules such as ICAM-1 that facilitates parasite sequestration, thus causing congestion in liver sinusoids. Scattered hemozoin pigment deposition in the hyperplastic liver monocytes/macrophages is also a common feature of liver pathology in malaria. Recruited mononuclear cells (inflammatory monocytes, neutrophils), lymphocytes along with sequestered pRBC

contribute to portal tract inflammation [29-32].

Different rodent *Plasmodium* species in various genetic backgrounds have been used to study LI in ECM and non-ECM settings. For instance, a report has demonstrated associations of hemozoin with hepatic inflammation. Herein, non-ECM causing parasites; *P. berghei* NK65 and *P. chabaudi* infection was done in C57BL/6J and BALB/c genetic backgrounds and the mice showed elevated serum liver enzymes indicative of liver injury but the histological changes were not severe, which is in tandem with observations in most human patients. In case of *P. chabaudi* infection massive inflammatory infiltrates, predominantly macrophages and monocytes and to a lesser extent neutrophils, in the livers were observed. These mononuclear cells were often loaded with hemozoin clusters, mostly in the sinusoidal macrophages. In the same study, immunohistochemistry observations on the liver of *P. chabaudi* and *P. berghei* infected mice revealed that the F4/80⁺ macrophages are more widely distributed in the case of *P. chabaudi* rather than *P. berghei* NK65 infection [33]. In another study, *P. berghei* ANKA infection in C57BL/6J mice has been used to study LI in the context of ECM settings. This report suggests that pathogenesis in the liver during infection is independent of pathogenic CD8⁺ T cells instead parasite burdens manifest the pathology in the liver and brain. In the case of ECM authors suggest a parasite and CD8⁺ T cells driven pathology in the brain. This observation led them to conclude that CD8⁺ T cells mediated immunopathology is organ specific [34]. In summary, higher parasite burdens, inflammatory cells, hemozoin, result in unregulated pro-inflammatory immune responses that may collectively culminate into LI. However, the precise mechanisms leading to hepatic injury are poorly understood.

1.4.3 Acute lung injury (ALI)/Malaria associated acute respiratory distress syndrome (MA-ARDS)

ALI and its more severe form MA-ARDS occurs mainly in adults and more often leads to mortality in 80% of the patients even in the circumstances of anti-malarial treatment. MA-ARDS is a very common complication of mostly *P. knowlesi* and *P. falciparum* in humans with prevalence in 60-70% of the patients suffering from severe malaria [35, 36]. Such MA-ARDS patients often show other complications like renal failure, CM, acidosis. ALI/MA-ARDS is characterized by parasite and leukocyte sequestration, severe edema, impaired gaseous exchange and inflammatory infiltrates damage to alveolar capillary membrane and severe hypoxemia [37], refer to Figure 1.4.

Figure 1.4 Illustration showing comparative differences between lungs of non-infected and *Plasmodium* induced ALI/ARDS. The ALI/ARDS lungs show swelling with thickened basement membranes and distended cytoplasmic extensions. The capillaries are occluded with leukocytes, RBCs, iRBCs and occasional bridges with adherent iRBCs and endothelium. The alveolar spaces show oedema, inflammatory infiltrating cells and projections from septum epithelium, thickening of the septum with abundant leukocytes. The layout represents single alveoli of non-infected and malarial lungs. Adapted from [37].

To understand the pathogenesis in ALI/MA-ARDS, *P. berghei* ANKA rodent parasite model is most commonly used to study lung pathology in conjunction with CM in C57BL6/J genetic background but in this setting the mouse perish with CM before pulmonary pathology can develop. Therefore, *P. berghei* ANKA and BALB/c and DBA/2 rodent parasite and mouse combinations are used to study pulmonary pathology [38]. However, in BALB/c, severity of MA-ALI/ARDS is limited upon *P. berghei* ANKA infection, hence DBA/2 mice serve as model of choice with an incidence of 50% [39].

The precise determinants of MA-ALI/ARDS in human and mice remain obscure yet factors such as parasite sequestration, pathogenic CD8⁺ T cells and inflammatory monocytes amongst others contribute to pulmonary pathology in mouse malaria. Similar to *P. falciparum*, *P. berghei* ANKA shows significant sequestration in the lungs and has been shown to partially occur in a CD36 dependent manner at least in mice [40, 41]. In a study pathogenic CD8⁺ T cells play a crucial role in the development of pulmonary pathology in mouse malaria, possibly in a perforin and granzyme mediated fashion, as is the case in ECM [42, 43]. A unique feature of MA-ARDS is abundance of leukocytes in and around pulmonary blood vessels an observation consistent in both mice and humans, which comprise primarily of monocytes, macrophages and lymphocytes and to a lesser

extent neutrophils. On the contrary, non-malarial ARDS, such as sepsis neutrophils comprise the predominant infiltrating inflammatory leukocytic population [44]. Vascular endothelial growth factor (VEGF) has also been implicated in the development of pulmonary edema in *P. berghei* ANKA-infected DBA/2 mice as soluble VEGF receptor-1 mediated neutralization of VEGF conferred protection from MA-ALI [39]. These studies highlight the complex nature of the MA-ALI/ARDS. Therefore, mouse models should be carefully used to prevent over extrapolation of prevalent mechanisms in humans and further harnessing it for translational purposes.

1.4.4 Acute kidney injury (AKI)

Malaria related acute kidney injury is one of the critical life-threatening manifestations of approximately 1-4% and 45% of the hospitalized patients respectively. *P. malariae* has also been incriminated to AKI in some cases [45-48]. The multifactorial pathological associations are not very well understood but current consensus and hypothesis suggests involvement of parasite sequestration, pro-inflammatory responses and oxidative stress induced nephrotoxicity. Patients suffering from AKI may exhibit oliguria, proteinuria, hyperbilirubinemia and elevated plasma creatinine levels [49, 50]. Histopathologically, AKI is characterized by interstitial nephritis, glomerulonephritis, tubular necrosis most likely due to parasite sequestration and inflammatory responses in both human and mice. The interstitium exhibits oedema with significant mononuclear infiltration and pRBCs aggregates in the venules, indicative of interstitial inflammation [48, 51, 52]. In addition, glomerular hyper cellularity shows differential patterns of either pRBCs coupled with monocyte sequestration or pRBCs alone. Glomerular fibrin deposition has also been observed in postmortem analysis of some patients [49, 50, 53]. AKI patients also show higher levels of pro-inflammatory cytokines such as IL-1 β , TNF- α , MCP-1, IL-6 in the plasma [54].

P. berghei ANKA infection in BALB/c mice currently represents a better model to study AKI in the absence of ECM. Such a model exhibits AKI as exemplified by impaired renal function evident by elevated plasma creatinine, blood urea nitrogen, enhanced pro-inflammatory cytokine profile IL-1 β , TNF- α , MCP-1, IL-6 in the renal tissue, enhanced renal endothelial vascular permeability, progressively increased pRBC and leukocyte sequestration including monocytes and neutrophils. In addition, angiogenic such factors such as VEGF decreased in the renal tissue and hypoxia inducible factor-1 α (HIF-1 α) m-

RNA was up regulated in the renal tissue indicative of oxidative stress. The histopathological findings indicated acute tubular interstitial nephritis as well [55].

1.4.5 Severe malarial anemia (SMA)

A higher prevalence of SMA is observed in young children and pregnant women in regions of high malaria transmission and almost all *Plasmodium* species can inflict SMA. According to WHO definition, a hemoglobin (Hb) concentration of <50g/L or hematocrit of <0.15 and a parasitemia >10000 parasites/ μ L is indicative of SMA [56, 57]. Children with SMA show symptoms of fatigue, dyspnea or respiratory distress [58, 59]. The anemia caused due to *P. falciparum* is characteristically normocytic and normochromic with a marked absence of reticulocytes [60]. In the clinical setting, severe anemia is complex as acute infection may result in anemia and/or CM, respiratory distress hypoglycemia and chronic recurrent infection may lead to severe anemia [61]. To study different aspects human malaria associated anemia pathophysiology, tractable mouse models are available such as *P. chabaudi*, which shows tropism to RBCs of all ages, like *P. falciparum* whereas *P. yoelii* which shows preferential reticulocyte tropism serve as likely model to emulate *P. vivax* malarial anemia [12, 61]. A major paradox using rodent parasite models is the patterns of parasitemia wherein most mouse malaria models exhibit high parasitemias, more often beyond 20% during acute infection whereas acute malaria in humans is characterized by lower parasitemia in the range of 1%-2.5% [61]. The plausible mechanistic explanation of SMA could be one or more of the following; firstly, dramatic destruction and/or clearance of iRBCs, secondly, rapid removal of normal RBCs and lastly perturbed erythropoiesis/dyserythropoiesis (production of functionally or morphologically abnormal RBCs) or erythropoietic suppression [62]. All of these mechanisms have been implicated in mouse and human malarial anemia (Figure 1.5). During *Plasmodium* infection destruction of iRBCs takes place by either the maturing parasite or the recognition and phagocytosis by the activated macrophages in both human and mice [63, 64]. In human malarial infection, a large number of normal RBCs are destroyed due to hyperactive macrophages in the spleen and most likely liver as well. In malaria, uninfected RBCs exhibit reduced deformability thus leading to enhanced phagocytic clearance in the spleen [65, 66].

In human SMA, cytoplasmic vacuolation, fragmentation, inter-cytoplasmic bridges, nuclear fragmentation and multicellularity characterized dyserythropoiesis and this was concurrent with impaired reticulocytosis indicative of reduced efflux from the bone

marrow [67, 68]. Similar picture is recapitulated in both non-lethal and lethal mouse malaria. Strikingly in mouse, 40%-70% reduction in bone marrow cellularity relative to normal levels is witnessed at zenith of parasitemia or death whichever is earlier and this reduction is proportionate to the severity of infection which is not mirrored in human malarial anemia [69, 70]. Altogether, the common aspect of inability of bone marrow to appropriately compensate for the loss RBCs due to infection still prevails and results in consequential anemic conditions in both humans and mice [61].

Figure 1.5 Direct and indirect effects of *Plasmodium* on SMA development. SMA is characterized by heavy destruction of pRBCs and phagocytic clearance of both pRBCs and normal RBCs. During infection, alteration membrane protein composition takes place and the resultant RBC associated immune complexes such as RBC:RSP2:Ig are removed by macrophages in the spleen. Hemozoin containing macrophages possibly release inflammatory cytokines and other active soluble mediators (hydroxynonenal; HNE). It is likely that hemozoin or other malaria-associated products can directly inhibit erythropoiesis by affecting growth and differentiation of one or more erythropoietic progenitors. Inadequate reticulocytosis in bone marrow and spleen might be a result of direct or indirect suppressive effects that may result in variable outcome of anemia. Hz indicates hemozoin; GPI, glycoposphatidylinositol anchors of merozoite proteins; Epo, erythropoietin; Epo-R, erythropoietin receptor; MΦ, macrophage; RSP-2, ring surface protein-2; and Ig, immunoglobulin. Adapted from [61].

1.5 Spleen: Center of anti-malarial responses

1.5.1 Importance of spleen in malaria

Splenectomized human and mice infected with *Plasmodium* exhibit impaired innate and adaptive parasite clearance mechanisms that lead to a high peak and a prolonged parasitemia episode [71-74] whereas in mouse *P. berghei* ANKA model, splenectomy conferred protection from ECM [75, 76]. These studies highlight the importance of the

spleen as central lymphoid organ not only in mediating protective immune responses but also promoting inflammatory responses that are detrimental to the host.

After the liver stage, *Plasmodium* infects primarily red blood cells to initiate the blood stage infection and iRBCs continuously passage through the spleen via circulation, thus enabling filtration of the blood. The host spleen essentially performs three distinct functions to control malaria. Firstly, the damaged and iRBCs are eliminated from the circulation [77]. Secondly, spleen serves as site of extra medullary hematopoiesis (EMH) particularly erythropoiesis and myelopoiesis [77-79]. Lastly, parasite specific adaptive immune responses comprising both T and B cells responses are generated in specialized regions of the spleen [80, 81].

1.5.2 Structure of the mouse spleen

The intrinsic structure of the spleen facilitates the execution of specialized functions. The spleen is highly compartmentalized into red pulp (RP; region actively removes senescent and aberrant RBCs), white pulp (WP; generation of adaptive immune responses) and marginal zone (MZ; region with intense phagocytic activity). The blood enters the spleen through the splenic artery, which further branches into arterioles and capillaries.

Figure 1.6 A diagram showing marginal zone and blood flow pattern in the mouse spleen. The blood from the systemic circulation enters the spleen from central arteriole and flows through the terminal arterioles before emptying to marginal sinus and ultimately percolating into the red pulp spaces (open circulation). A larger portion of the blood is drained into collecting veins (closed circulation). The macrophages and the DCs in the marginal zone capture blood-borne pathogens and present it to marginal zone T and B cells for adaptive immune responses. Adapted from [82].

The capillaries terminate into either marginal sinus in the MZ or RP. The larger portion of the blood flows to the draining veins (closed circulation) and the remaining flows

through the RP filtration beds and MZ at a very slow flow rates (open circulation) thus enabling constant proximity interactions to the splenic phagocytic cells including dendritic cells and tissue-resident macrophages and blood-borne materials. This typical open circulation facilitates efficient percolation and filtration of the blood in the extravascular beds of the spleen [83, 84].

The highly structured splenic architecture is seeded with various immune effector cells. The RP comprises of predominantly tissue-resident red pulp F4/80⁺ macrophages (RPMs) that exclusively expresses transcription factor Spi-C, functionally specialized to phagocytose senescent erythrocytes and iron recycling as demonstrated in Spi-C deficient mice, which lacked these functions [85]. The white pulp consists of T and B cells organized into T cell zone and B cell zone respectively zone respectively in a sheath like arrangement surrounding the branching arterial vessels. The T cell zone (inner zone) is populated with T cells, which interact with CD8⁺ DCs and mediate B cell activation for antibody production. The T cell zone is surrounded by the B cells, which are organized in follicles to form the B cell zone (Figure 1.7). Upon receiving T cell help, B cells undergo clonal expansion and Ig isotype switching in an antigen specific manner [86, 87].

Figure 1.7 An illustration showing compartmentalization of mouse spleen and different immune cell populations including tissue-resident macrophages. The mouse spleen is compartmentalized into distinct RP and WP regions separated by MZ. The microanatomical location of the splenic macrophages has been shown at distinct locations in immunofluorescent images; F4/80⁺RPMs involved in recycling of iron (top left, red), SIGN-R1⁺MZMs (bottom left, blue) and CD169⁺ MMMs involved in immune surveillance. The tingible body macrophages in the B cell follicles are involved in apoptotic body clearance resulting from GC reactions. FDC: follicular dendritic cells. Adapted from [88].

The marginal zone (MZ) separates the RP and WP. The MZ is decorated with CD8⁺ DCs and tissue-resident macrophages known as marginal zone macrophages, which are

responsible for the uptake of blood-borne particulate matter [89]. There are two distinct macrophage subsets in the MZ; marginal zone macrophages (MZMs) and marginal metallophilic macrophages (MMMs). The MZMs (forms the outer ring) is characterized by the expression of C-type lectin SIGNR1 and a type I scavenger receptor called macrophage receptor with collagenous structure (MARCO) and the MMMs are characterized by the expression of sialic-acid-binding immunoglobulin-like lectin 1 (Siglec1 also known as sialoadhesin; Sn or CD169) [90-92]. The MZ B cells and Cysteine-rich (CR) domain of the mannose receptor (CR-Fc⁺) DCs subset are juxtaposed with these two macrophage subsets [93, 94]. Thus, the anatomical structure of the spleen, pattern of blood flow, strategic and heterogeneous organization of the cellular arsenal consisting of antigen presenting cells, effector cells, facilitates appropriate immune responses against blood borne pathogens.

1.5.3 Parasite clearance mechanisms in the spleen

The RP and MZ are potential sites for the elimination of iRBCs during *Plasmodium* infection. Mechanical trapping, phagocytosis and generation of adaptive immune responses are critical parasite elimination mechanisms (Figure 1.8). The feature of deformability in RBCs determines the removal of iRBCs and senescent RBCs in organisms with a sinusoidal spleen such as human and rodents. In a very early study, PbA infected rats showed a compromise in splenic trapping ability of iRBCs during precrisis period (characterized by splenomegaly and EMH), followed by restoration in the crisis period (characterized by massive destruction of iRBCs) [95]. Similar to senescent RBCs, iRBCs exhibit decreased deformability; as a result they are trapped in the RP and subsequently phagocytosed and cleared by the RPMs. In addition, squeezing of iRBCs through the reticular network of RP may take place, which results in expulsion of the rigid cytoplasmic particulate matter such as parasite, a process known as ‘pitting’ whereby parasites are pitted out leaving the RBCs intact. Therefore, physical retention of iRBCs in RP and pitting (Figure 1.8) form the basis of mechanical trapping to eliminate the parasites [96-98].

The tissue-resident macrophages such as RPMs, MZMs and MMMs form the trinity of professional phagocytes that prevents dissemination of parasites during blood-stage *Plasmodium* infection by phagocytic mechanism. The RPMs are highly phagocytic as compared to MZMs and MMMs but they are crucial for removal of blood borne-particulate matter [99]. The strategic localization of MZMs and MMMs at the MZ makes

them likely candidates for capture and indirect presentation of malarial antigens in addition to heterogeneous population of professional APCs such as $CD8^+$ DCs. Thus, macrophages serve as major innate immune phagocytic cells [100, 101].

Figure 1.8 Splenic effector mechanisms for the clearance of the parasite. (A) The splenic DCs recognize the PAMPs of parasite via the PRRs displayed on their cell surface. The endocytosed parasite is processed and the antigenic peptides are presented to $CD4^+$ T cells, which secrete $IFN-\gamma$ and $TNF-\alpha$ to activate the macrophages. The activated macrophages phagocytose the parasites and mediate intracellular killing of the iRBCs by reactive oxygen and nitrogen intermediates. (B) Image showing RBC pitted out by the macrophages and the parasite is retained inside by mechanical squeezing process known as pitting. (C) Different cellular sources of $IFN-\gamma$, all contributing to the activation of macrophage and subsequent phagocytic removal of iRBCs. Adapted from [98, 102, 103].

Unlike macrophages, DCs migrate to the T cell areas to stimulate and activate T cells to perform their effector function. The classical DCs; $CD8^+$ DCs and $CD8^-$ DCs both can take up, process and present *Plasmodium* antigens to $CD4^+$ T cell with differential kinetics during the course of the infection. The $CD8^+$ DCs function at the early phase of infection whereby they produce IL-12 to mediate $IFN-\gamma$ producing $CD4^+$ T cell and NK cell responses [104, 105]. In *P. chabaudi* infection, it has been noted that $CD8^+$ DCs undergo apoptosis and $CD8^-$ DCs become the predominant APCs to induce effector $CD4^+$ T cell responses which start to produce additional IL-4 and IL-10 cytokines [105]. At this later phase of the infection, simultaneously follicular DCs and T cell help trigger the

generation of the parasite specific antibodies from plasma B cells that mediate clearance of merozoites. Hence adaptive immune responses also contribute to parasite clearance [106].

1.5.4 Remodeling of splenic architecture during malaria

In *Plasmodium* infection, splenic architecture is dramatically altered which may hamper the developing antimalarial immune responses. Expansion of the RP, transient loss of MZ and disorganization of T cell and B cell zone takes place (Figure 1.9A). Despite these dramatic changes, robust immune responses against the parasite are still efficiently generated [106, 107].

Figure 1.9 Structural remodeling of spleen during malaria in mouse infection model. Immunofluorescence images of spleen showing (A) increase in CD138+plasma cells (green) in the *P. chabaudi* infected mice at day 10-post infection (right). (B) Formation of fibroblastic barrier as evident by FGF8 (red staining) in the *P. yoelii* 17XNL infected spleen at early (left) and late time point (right), F4/80⁺ macrophages in the RP (blue), GFP⁺ parasite (green), nuclei (gray). (C) Model of spleen parasite clearance evasion mechanism in non-lethal malaria. The remodeling of the splenic architecture by the formation of barrier of fibroblastic origin results in channeling of blood from arterioles to smaller veins and cytoadherence of infected reticulocytes (iRet) to the barrier prevents the macrophage mediated phagocytic clearance of the parasitized reticulocytes. V: venous lumen, Ret: reticulocytes, periarteriolar lymphoid sheath (PALS), MS: marginal sinus, BC: FGF8⁺ barrier cells. Adapted from [108, 109].

In *P. yoelii* non-lethal infection, the spleen remodeling leads to transient shift from open circulation to closed circulation due to the development of fibroblastic barriers, which

completely redirect the blood from the arterioles to the veins thereby preventing open circulation in sinuses [79], refer to Figure 1.9B and C. The prevalence of such barriers in the human spleen during malaria remains controversial where remodeling is characterized by expansion of WP and diffused hyper cellularity in the RP as observed in post-mortem studies and patient analysis with spleen rupture cases [110]. Spleen closure renders selective advantage to the parasite as it bypasses the red pulp filtration, thus evading the macrophage-mediated clearance. The splenic closure in contrast renders advantages to the host by restricting the release of toxic and inflammatory mediators produced by the active macrophages in response to *Plasmodium* and thereby sparing the perturbation in the developing adaptive immune responses and also protecting the newly formed reticulocytes [79, 108, 111]. A study has though reported overwhelming destruction of iRBCs despite spleen closure [112].

1.5.5 Comparison of the mouse and human spleen in terms of structure and function

The main differences between human and mouse spleen has been tabulated in Table 1.2 and illustrated in Figure 1.10. The striking anatomical differences comprise the presence of perifollicular zone and the lack of marginal sinus in the human spleen when compared to the mouse spleen. The WP is predominant in the mouse spleen whereas in the human spleen, RP is the major compartment. The presence of venous sinus in the human spleen facilitates mechanical filtration of senescent and deformed RBCs [113, 114]. These structural differences potentially allow better filtration of the blood in the human spleen. However, these differences may contribute to differential degree of the blood filtration but not the filtration function [114].

In humans, blood enters the spleen through the central artery, traversing along the medium sized central artery and further engages into fast/slow microcirculations simultaneously [83]. The RBCs transit from the perifollicular zone to the lumen of venous sinus via direct by passes and are subjected to mechanical filtration similar to the mechanism operating in the capillaries, which comprises the fast/closed microcirculation [115]. The velocity of the RBCs is 20 times greater in the fast as compared to the slow microcirculation. In the slow/open microcirculation, the RBCs navigate in the RP cords before coming to the vascular beds by squeezing mechanism between the cells of the endothelium in the walls of the sinus. The labyrinth like microcirculatory beds are devoid of endothelium unlike channel shaped capillaries and hence the “open” circulation which accounts for 10-20% of the RBC flow in the human spleen [115].

Table 1.2 Differences between the human and mouse spleen in terms of structure, blood circulation.
Adapted from [104].

Human	Mouse
1) Sinusoidal	Non sinusoidal
2) Human spleen consists of a honeycombed reticular meshwork of venous sinuses, in which endothelial cells align longitudinally in a barrel-like shape sustained by a fenestrated basement membrane and adventitial reticular cells. This forces blood to squeeze through the inter endothelial slits before entering the lumen of venous sinuses.	Mouse Spleen consists of flat endothelium, a conventional basement membrane and adventitial reticular cells. Blood flows through open-ended pulp venules, thus offering little impedance to entrance of red blood cells.
3) A clear delimiting marginal sinus is absent in marginal zone (MZ) of human spleen. An additional perifollicular zone surrounds the MZ. Herein, some blood vessels terminate in capillaries that bypass the filtration cords as fast 'closed' microcirculation	A clear marginal sinus is present. Perifollicular zone absent. The blood circulation through the marginal sinus has been detailed in Figure 1.6.
4) Erythropoiesis not reported in human spleen	Site of intense erythropoiesis

Figure 1.10 A diagram highlighting the differences in anatomical structures and blood microcirculation in the human and mouse spleen. Adapted from [104]

The stringency of the inter-endothelial slits on the RBC deformability can result in the retention of the less deformable erythrocytes or elimination of intra-erythrocytic bodies; a process known as pitting [116] and the existence of such a phenomenon remains controversial in the human malarial spleen [115]. However, *ex vivo* studies in malarial

patient's spleen have reported pitting of parasite remnants from the iRBCs as the critical determinant of parasite clearance in the cases administered with artemisinin derivatives [115, 117]. The pattern of the blood circulation in the mouse spleen has been previously described in section 1.5.2.

1.6 The myeloid cell family

In hematopoiesis, a myeloid cell is conventionally defined as any white blood cell (WBC) other than lymphocytes [118]. They are broadly classified as polymorphonuclear and mononuclear phagocytes. Polymorphonuclear cells comprise of eosinophils, basophils, neutrophils and mast cells are generally referred as granulocytes. These granulocytic populations are recruited at the site of injury/disease where they release toxic and inflammatory factors to resolve bacterial and parasitic infections thereby rendering protection to the host [119, 120]. Mononuclear cells comprise of dendritic cells, monocytes and macrophages that are present in various organ systems, where they perform tissue specific functions pertaining to homeostasis, regulation of innate and adaptive immune responses in steady and inflammatory states [88, 121, 122]. In addition, mononuclear cells are further classified into distinct subsets on the basis of cell surface receptor expression, ontogeny, growth factor signaling and function [118, 121, 123]. Thus the myeloid cell family represents a remarkable diversity (Figure 1.11). For simplicity and in line with the scope of this thesis, further discussions will be restricted to the heterogeneous repertoire of monocytes and macrophages seeded in various tissues in the context of inflammation and maintenance of homeostasis.

1.6.1 Historical perspective of mononuclear phagocyte system (MPS)

In the late nineteenth century, the microscopic analysis of starfish larvae by Élie Metchnikoff described the phagocytic activity of the recruited myeloid cells at the site of infection that included the professional phagocytic cells termed as macrophages (macro = large; phage = devouring, derived from Greek) [124, 125] and microphages (small eaters), which are now collectively known as polymorphonuclear phagocytes [126]. This led to the concept of innate cellular immunity as an active host defense mechanism against foreign microorganisms. Later in 1968, *in vitro* and *in vivo* thymidine-³H labeling studies by Ralph van Furth and Zanvil Cohn demonstrated that blood monocytes give rise to most tissue macrophage populations in mouse. This formed the basis of mononuclear phagocyte system [127, 128]. Continuous quest for a cell with a capacity to regulate adaptive immunity responses led to a discovery of dendritic cells in 1973 by

Steinman and Cohn [129]. Hence macrophages, monocytes and dendritic cells formed the trinity of the MPS. The concept of MPS was challenged by studies reporting the evidence of local proliferation of peritoneal, alveolar macrophages in mice depleted of monocytes [130] and *in vitro* studies of human epidermal Langerhans cells suggesting self-renewal capacity [131]. The advent of fate mapping technologies have provided compelling evidence and shed light on the embryonic origins of specific macrophage populations, which maintain themselves through *in situ* proliferation in the tissues, thus leading to departure from the MPS notion [132-134].

1.6.2 Monocytes

Monocytes are irregular shaped cells containing an oval to kidney shaped nucleus that comprise 2-4% of the circulating leukocytes in the mouse blood, bone marrow and spleen while 10% of the leukocytes constitute the human monocytes. They remain in circulation for 1-2 days and subsequently perish if not recruited to sites of inflamed tissue or injury. Monocytes, in general are considered to be the systemic reservoirs of the tissue macrophages and DCs with the exception of the embryonically derived tissue-resident macrophages (eg. microglia), which possess self-renewal capacity [123, 135].

Ontogeny

Hematopoietic stem cell give rise to monocytes in the bone marrow. The monocyte/macrophage lineage is dependent on signaling by colony stimulating factor-1 (CSF-1, also known as M-CSF) and cognate receptor CSF-1R expressed by monocytes, macrophages and their progenitors for differentiation, homeostatic regulation and survival [136, 137]. The monocyte development traverses through a series of progenitor stages; the common myeloid progenitor (CMP), the granulocyte-macrophage progenitor (GMP), CD135⁺ the common macrophage and DC progenitor (MDP) and ultimately the recently discovered CD135⁻ committed monocyte progenitor (cMoP) leading to diverse monocyte subpopulations [138-140]. The heterogeneous monocyte population in mouse comprises Ly6C⁺ (classical), Ly6C⁻ (non-classical) and the poorly characterized LyC^{med} (intermediate) subsets whose development and differentiation is under the control of distinct transcriptional programs. The IRF8-Krüppel-like factor-4 (KLF4) transcription factor cascade has been implicated in the development and differentiation of Ly6C⁺ monocytes also termed as inflammatory monocytes [141, 142]. The deletion of transcription factor nuclear receptor subfamily 4 group A member 1 (NR4A1 or Nurr77) alters the numbers of patrolling Ly6C⁻ in the bone marrow and thus can be regarded as

the master regulator of this subset [143]. A recent fate mapping study has provided evidence that blood-resident Ly6C^- cells derived from the short-lived Ly6C^+ monocytes serve as obligatory steady state progenitors [144].

Heterogeneity and function

Under steady state conditions, *bona fide* monocytes are present in spleen, bone marrow and blood compartment and seed the destined tissues only after receiving appropriate chemotactic signals [145]. A recent study supports such a notion, wherein a circadian gene *Bmal1* has been shown to be involved in regulating the diurnal rhythms of the Ly6C^+ monocyte numbers to facilitate appropriate innate immune responses to dynamic environmental stimuli [146]. Ly6C^+ monocytes fully or partly replenish and contribute to intestinal-resident macrophages [147], dermis-resident [148], lung-resident [149] and cardiac macrophages [150]. The question pertaining to dichotomous contribution to resident macrophages from monocyte dependent and independent pools remain wide open in steady state as well as inflammation. Not much is known about the function of blood resident Ly6C^+ monocytes. The Ly6C^- monocytic subpopulation exhibit a characteristic patrolling phenotype with an inherent phagocytic capacity to ingest vessel endothelium associated foreign particles and subsequent removal of debris. They are activated by TLR7 stimulation in response to danger signals in the environment, produce inflammatory mediators and recruit neutrophils in the vasculature for clearance of the necrotic endothelial and resulting debris. Thus Ly6C^- monocytes are considered as intravascular ‘housekeepers’ [151]. Similar patrolling behavior has been attributed to CD14^{lo} monocytes (human equivalent of Ly6C^- monocytes) via TLR7-8-MyD88 pathway and produced $\text{TNF-}\alpha$, $\text{IL-1}\beta$ against viruses and nucleic acid immune complexes [152]. In mouse, LyC^{med} subset remains poorly characterized, both phenotypically as well as functionally, although $\text{CD14}^+ \text{CD16}^+$ (human equivalent of LyC^{med}) possess inflammatory effector function as they produce high levels of TNF [153, 154].

During inflammation, the tissue-resident macrophages in particular, peritoneal and alveolar macrophages tend to decrease in numbers, often referred as macrophage disappearance reaction (MDR) [155-157]. The homeostatic and compensatory mechanism of the host reinforces the resident-macrophage pool in the tissues by either monocyte-derived macrophages or increased proliferation of self-renewing macrophages to enhance the effector cell numbers to counteract the invading pathogen or inflammation. In a *Listeria monocytogenes* infection model, Ly6C^- monocytes were actively recruited to the tissues to establish early transient inflammatory responses by secretion of $\text{TNF-}\alpha$ and

chemokines to recruit NK and T cells. However, Ly6C⁺ monocytes became the main producers of inflammatory cytokines 8-hour post infection [158].

Figure 1.11 Heterogeneity of mononuclear phagocyte system (MPS) in human and mouse. (A) Human and mouse mononuclear phagocyte cellular network. Note: **/? Denotes unknown murine homologs. (B) Conserved functional specializations of MPS in human and mouse. Adapted from [159].

Similar observations of recruitment of both monocyte subpopulations were also confirmed in myocardial infarction but in this study, the Ly6C⁺ monocytes were the first to be employed to initiate inflammatory responses followed by Ly6C⁻ subset to induce tissue healing by expressing vascular endothelial growth factor and promoting collagen deposition, thus highlighting its angiogenic capacity [160]. In an atherosclerotic model of chronic inflammation both subsets home the activated plaque laden endothelium at the same time and the healing process was followed by monocyte decrement [161]. It is clear that monocytes play an important role in various infections and inflammatory scenarios but whether monocyte derived tissue macrophages share functional similarity to tissue-resident macrophages is yet to be determined.

1.6.3 Macrophages

Macrophages, including both the resident and the monocyte-derived, are professional phagocytic cells. The tissue-resident macrophages comprise 5-15% of the total leukocytes across lymphoid and non-lymphoid organs in adult mammals during steady state, which has the propensity to increase in numbers under inflammatory conditions [162]. In addition to phagocytic clearance of pathogens, debris and tissue surveillance; they exhibit pleiotropic functions in tissues such as tissue homeostasis, immune regulation, angiogenesis and tissue repair. Tissue-resident macrophages are known by

different names according to their tissue location, such as microglia (brain), osteoclasts (bone), histiocytes (connective tissue), Kupffer cells (Liver), alveolar macrophages (lung) Langerhans cells (skin), cardiac macrophages (heart) marginal zone macrophages and red pulp macrophages (spleen), all performing generalized similar functions as well as specialized tissue specific functions despite their differential origins [123, 162].

Ontogeny

All macrophages in fetal and adult tissues arise from the yolk sac (source of some tissue-resident macrophages) or fetal liver (source of fetal liver derived macrophages) or bone marrow (genesis of bone marrow derived resident or monocyte derived macrophages) [123]. Most tissue macrophages are generated during embryogenesis [134]. The exceptions of embryonic origins to tissue-resident macrophages in adult include intestine (half-life of 4-6 weeks), dermis (half-life of 4-6 weeks) and heart (half-life of 8-12 weeks) where adult circulating monocytes differentiate into macrophage during homeostasis, as they are incapable of self-maintenance [150, 163]. Depending on the monocyte input for tissue-resident macrophages adult tissues can be categorized into closed i.e. no recruitment of monocytes (brain, liver, lung and epidermis), open tissue with rapid monocyte recruitment (gut and dermis) and open tissue with slow monocyte recruitment (heart and pancreas) at steady state [164].

Transcription programs governing macrophage development

Almost all the macrophage lineages are dependent and regulated by class III trans membrane tyrosine kinase receptor CSF1R. The defects (skeletal abnormalities and macrophage deficiency) in *csfr1* null mice recapitulates *Csf1^{op} /Csf1^{op}* mice (homozygous for the mutation osteopetrotic) but *csfr1* null mice exhibit a more severe phenotype with the loss of microglia and Langerhans cells [165, 166]. This observation hinted for the presence of another ligand for CSF1R. Targeted ablation of *Il34* resulted in complete loss of microglia and Langerhans cells, while the other tissue-resident macrophages in the bone marrow; spleen and liver remained unaffected [167]. Some tissue macrophages still remain in the *csfr1* null mice suggesting the prevalence of other growth factors and a study using mice deficient in GM-CSF demonstrated its role in regulating the development of only alveolar macrophages [168].

The development and maintenance of heterogeneous populations of tissue-resident macrophages are under selective transcriptional control, which is tissue dependent. A

study on *Myb*^{-/-} embryos highlighted the absence of CD11b^{hi} myeloid cells while F4/80^{hi} macrophages were still present in the tissues of the early embryo [169]. Hence this study illustrates the dependence of fetal macrophages on c-Myb-independent lineage precursors in the yolk sac and hematopoietic cells on the c-Myb⁺ progenitors, thus highlighting the importance of c-Myb transcription factor on development and differentiation of all hematopoietic cell lineages [169]. The loss of SFPI1 (also referred as PU.1), a member of the ETS family results in complete depletion of F4/80⁺ macrophages, including the yolk sac ones along with B cells suggesting its importance but not exclusivity to macrophage lineage [169]. The transcription factor Gata6 regulates the differentiation and proliferation of peritoneum-resident macrophages [170]. The peroxisome proliferator-activated receptor γ (PPAR γ) serves as unique transcriptional regulator exclusive to alveolar macrophages [171]. SPI-C knockout mice are deficient in splenic red pulp macrophages suggesting SPI-C as exclusive transcriptional regulator for the development of red pulp macrophages but not marginal zone macrophages [172]. The SIGNR1⁺ marginal zone macrophages and CD169⁺ marginal metallophilic macrophages in splenic marginal zone are under the transcriptional control of nuclear receptor, liver x receptor α (LXR α) [173]. The capacity of self-renewal of tissue-resident macrophage is dependent on negative regulation of transcription factors MafB and c-Maf and positive regulation of c-Myc and KLF4 as observed in Maf-DKO macrophages *in vitro* [174]. A recent study has investigated the activated enhancer marks histone acetyl transferase p300 and found significant enrichment of this enhancer mark in alveolar macrophages *in vivo* (show naturally low Maf levels) similar to Maf-DKO. Hence lower MafB and c-Maf either experimentally induced or naturally prevalent results in the activation of set of enhancers (macrophage specific) to govern intrinsic self-renewal virtue of the tissue-resident macrophages [175]. Despite the dedicated efforts to understand unique regulatory factors governing the development differentiation of tissue-resident macrophage, the transcriptional programs for other resident macrophages such as liver and brain yet needs to be uncovered.

Heterogeneity and function

Almost all lymphoid and non-lymphoid tissues harbor heterogeneous subpopulations of macrophages characterized by primarily differential and overlapping surface receptor expression of CD11b, F4/80, CD169, Ly6C, MHC class II. Collectively, using such expression profiles, tissue macrophages can be classified as CD11b^{lo/-} F4/80^{hi} CD169⁺,

CD11b^{hi} F4/80^{int} CD169⁻ hereafter referred as F4/80^{hi} CD169⁺ and F4/80^{int} subsets. The F4/80^{int} subset consists of monocyte and macrophage lineage cells with advancing maturational states; Ly6C^{hi} MHC II^{lo} (monocytes), Ly6C^{hi} MHC II^{hi} (transition state) and Ly6C^{lo} MHC II^{hi} (macrophages). The F4/80^{hi} CD169⁺ tissue-resident macrophage subset in the liver (Kupffer cells), lung (alveolar macrophages), spleen (red pulp and marginal metallophilic macrophages) and brain (perivascular macrophages) are derived from the precursors of embryonic origins and possess self-renewal capacity [134, 163]. The heterogeneity of the tissue-resident macrophage and their functions has been summarized in Table 1.3.

Tissue-resident macrophages are equipped with multitude of receptors (PRRs) for recognition of pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). The PRRs expressed on the surface of the macrophage comprise of TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene (RIG)-I like receptors, lectins and scavenger receptors. There is remarkable variation in the usage of these PRRs by tissue-resident macrophages which is primarily governed by the homing tissue coupled with the prevalence of specific tissue microenvironments and insults, if any [176]. After sensing the pathogenic invasion, tissue-resident macrophages along with DCs mediate the recruitment of inflammatory cells such as neutrophils and monocytes that eventually differentiate into monocyte-derived inflammatory macrophages.

During inflammatory response monocyte-derived macrophages supercede the tissue-resident macrophages and become the dominant macrophage population in the inflamed tissue. The role of tissue-resident macrophage as an initiator of inflammatory responses has been highlighted in number of experimental models of inflammation where altered chemokine and neutrophil recruitment was observed when the resident macrophages were depleted [177-179].

In response to tissue insult situations such as peritonitis and influenza infection resident macrophages; peritoneal and alveolar macrophages respectively, acute neutrophil and monocyte derived macrophage influx is observed concomitant with a drastic loss of F4/80⁺Tim4⁺ macrophages, the phenomenon commonly referred as MDR. The disappearance could be possibly due to tissue adherence or emigration to draining lymphatic system or possible apoptosis of the cells, which needs to be investigated by advanced fate mapping approaches [180-183].

During the resolution of inflammation phase, replenishment of the tissue macrophages occur either by self-renewal capacity of tissue-resident macrophages itself or monocyte-derived macrophages in response to the cytokine M-CSF [183]. A replenishment pattern independent of monocyte contribution has also been observed for macrophages seeded in tissues like peritoneum, brain and lung during the resolution phase [181, 184-186].

Table 1.3 Heterogeneity of the tissue-resident macrophages, characterized cell surface markers, transcription factors and their functions in the context of the tissue. Adapted from [88] Note: For the exhaustive reference list, refer to the review [88].

Tissue	Cell type ^a	Functions and notes ^b	Phenotypic markers (tissue-selective transcriptional regulators) ^c
Adipose tissue	'Adipose-associated macrophages'	Involved in control of insulin sensitivity and adaptive thermogenesis	F4/80 ⁺ , CD45 ⁺ (white and brown adipose tissue) (PPAR γ)
Blood	Ly-6C ^{hi} monocytes	Function analogously as 'intravascular housekeepers', clearing endothelial cell debris	CX3CR1 ⁺ , Ly-6C ^{hi} , F4/80 ⁺ , CSF1R ⁺ Nr4a1
Bone	Osteoclasts	Multinucleated cells formed by fusion that resorb bone by disruption of the mineralized matrix	Calcitonin receptor ⁺ (multinucleate)
	Bone marrow macrophages	Support erythropoiesis and maintain hematopoietic stem cells in stem cell niches; this is an independent self-renewing population	CD169 ⁺ , F4/80 ⁺ , ER-IR3 ⁺
Central nervous system	Microglia	Promote neuronal survival and are involved in frontline immune surveillance, removal of dead neurons and synaptic remodeling; derived from yolk sac and maintained in adult and during inflammation independently of the bone marrow	F4/80 ⁺ , CD11b ⁺ , CD45 ^{hi}
	Perivascular macrophages Meningeal macrophages	Immune surveillance Immune surveillance	F4/80 ⁺ , CD11b ⁺ , CD163 ⁺ , CD45 ^{hi} F4/80 ⁺ , CD11b ⁺ , CD45 ^{hi}
Gastrointestinal tract	Intestinal macrophages	Maintenance of intestinal homeostasis and regulation of immune responses to commensals; monocyte-derived	CX3CR1 ^{hi} , F4/80 ⁺ , CD11b ⁺ , CD11c ⁺ , CD64 ⁺
Liver	Kupffer cells (sessile)	Clearance of microorganisms and cell debris from the blood, and clearance of aged erythrocytes; prenatal origins; maintained in the adult independently of the bone marrow	F4/80 ^{hi} , CD11b ^{hi} , CD169 ⁺ , CD68 ⁺ , Galectin-3 ⁺ CD80 ^{hi} PPAR β
	Motile liver macrophages	Immune surveillance	F4/80 ⁺ , CD11b ⁺ , CD80 ^{hi}
Lung	Alveolar macrophages	Immune surveillance of the lung for inhaled pathogens and homeostatic regulation of tissue function; for example, clearance of surfactant; prenatal origins; maintained in adult and during inflammation independently of the bone marrow	F4/80 ^{hi} , CD11b ^{hi} , CD11c ^{hi} , CD68 ⁺ , Siglec F ⁺ , MARCO ⁺ , CD206 ⁺ , Dectin-1 ⁺ Galectin-3 ⁺ PPAR γ
	Interstitial macrophages	Regulates DC maturation and/or activation	F4/80 ⁺ , CD11c ⁺ , CD68 ⁺ , MHC II ⁺
Serosal tissues	Peritoneal macrophages: F4/80 ^{hi} majority	Immune surveillance and regulation of homeostatic environment; apoptotic cell clearance; prenatal origins; maintained in adult and during inflammation independently of the bone marrow	F4/80 ^{hi} , CD11b ^{hi} , Tim4 ⁺ , MHCII ^{hi}
	F4/80 ^{hi}		F4/80 ^{hi} , CD11b ⁺ , Tim4 ⁺ , MHCII ^{hi} , CD11c ^{hi} (this population is most likely heterogeneous, mixed with dendritic cells)
	Pleural macrophages: F4/80 ^{hi} majority	Immune surveillance; maintained in adult and can expand during T _H 2 cell inflammation independently of the bone marrow	F4/80 ^{hi} , CD11b ^{hi} , Tim4 ⁺ ¹⁰⁰
	F4/80 ^{hi}		F4/80 ^{hi} , CD11b ⁺ , Tim4 ⁺ (dendritic cell-macrophage content undetermined; unpublished observations)
Skin	Dermal macrophages	Immune surveillance	F4/80 ⁺ , CD11b ⁺ , CD11c ^{hi} , CD206 ⁺ , MHCII ^{hi} , CD169 ⁺ (in the deep dermis); Dectin-1 ⁺ , CD301 ⁺ , Dectin-2 ⁺
	Langerhans cells	Interaction with T lymphocytes; derived from yolk sac and/or fetal liver and maintained independently of the bone marrow	F4/80 ⁺ , CD11b ⁺ , CD11c ⁺ , Langerin ⁺ Id2 and Runx3
Spleen	Marginal zone macrophages	Immune surveillance of the circulation	CD68 ⁺ , CD209b ⁺ , MARCO ⁺ , Dectin-2 ⁺ Tim4 ⁺ , LXR α
	Metaphilic macrophages	Immune surveillance	CD68 ⁺ , CD169 ⁺ , LXR α
	Red pulp macrophages	Erythrocyte clearance and iron metabolism; prenatal origins; maintained in adult independently of the bone marrow	F4/80 ⁺ , CD206 ⁺ , Dectin-2 ⁺ , Spi-C
	White pulp (tingible body) macrophages	Clearance of apoptotic cells resulting during the germinal center reaction	CD68 ⁺

^aThis table is a simplification; marked heterogeneity is evident in many tissues (for example, bone marrow, gut, spleen, lung and liver) highlighted through fetal macrophage studies and phenotypic variation. ^bOrigin is indicated only where experimentally established. ^cSelect examples of tissue-selective transcriptional regulators involved in cellular development or function are indicated. ^dMarker is expressed by the majority of the indicated cells. Subsets are only listed where distinct anatomical localization, function or origins are reported, and not those that are simply defined by variation in select receptor or antigen expression.

Despite these observations of differential replenishment patterns for tissue macrophages, concrete evidence is lacking in the context of relative contribution of monocyte derived

macrophages and *in situ* self-renewing macrophages due to unavailability of reliable and differentiating markers. The aforesaid observations and interpretations have been primarily derived from mice studies. The property of tissue macrophage proliferation has also been observed in human glomerulonephritis and acute liver failure but the mystery regarding their origin still prevails [187, 188]. During the recovery phase of inflammation, macrophages are employed for continuous removal of apoptotic bodies and cell debris enroute to the reparative process and restoration of homeostasis. The significance and contribution of macrophage to the skin repair and healing process has been illustrated using a conditional depletion using a LysMCre/iDTR mouse model in a time dependent fashion which enabled depletion of peritoneal and tissue-resident macrophages in skin, spleen, and liver and subjecting it to mechanical injury. Early stage (inflammatory phase) macrophage depletion resulted in strikingly reduced vascularized granulation tissue, impairment in epithelialization and minimal scar formation. Depletion at the tissue formation phase resulted in development of hemorrhagic foci in the injured tissue and subsequent perturbation in the wound closure process was observed in the tissue maturation phase. Depletion at the late phase of tissue maturation did not have any noticeable impact on the healing process [189]. However such depletion models do not provide any evidence for the direct role for the macrophages in a reparative process but do provide an evidence for a trophic support to other cell types such as fibroblasts or fibrogenic and adipogenic precursors that play a more direct role in the reparative process [190, 191].

Conventionally and functionally, macrophage has been classified into M1 and M2 depending on the cytokine requirements for their activation and subsequent immune effector response (Figure 1.12). Macrophages that exhibit inflammatory, antimicrobial and tumoricidal response upon stimulation with lipopolysaccharide (LPS), IFN- γ signal by Th1 cells (T cells and NK cells) and GM-CSF are referred as classically activated M1 phenotype [192-194]. The inflammatory activity of M1 macrophages is characterized by up regulation of MHC class II marker, CD80, CD86 co-stimulatory molecules and elevated secretion of pro-inflammatory cytokines IL-1 β , TNF, IL-12 and IL-18. The macrophages under M1 polarized state show augmented phagocytic activity for enhanced clearance of intracellular killing of pathogens mediated by nitric oxide (NO) mediated microbicidal mechanisms [195, 196]. The pro-inflammatory M1 macrophage plays a critical role in controlling *Mycobacterium tuberculosis* or *Listeria monocytogenes*

infection that results in tuberculosis and listeriosis respectively. Mice deficient in components of IL-12 pathway and impaired IFN- γ secretion show enhanced susceptibility to *M. tuberculosis* infection due to uncontrolled growth of mycobacterium in the macrophages [197]. On the other hand, mycobacterial proteins facilitate pathogen evasion strategies to subvert protective M1 macrophage mediated host immune responses. For instance, macrophages permissive for SecA2 deficient mycobacterium secrete elevated levels of TNF, IL-6 and reactive nitrogen species but the mycobacterium protein PE-PGRS62 led to enhanced virulence by down regulation of IL1- β and iNOS transcripts [198, 199]. Thus, these observations illustrate that M1 macrophages are required for appropriate control of mycobacterium infection. Mice lacking IFN- γ and TNF (characteristic of M1 polarized macrophage) or its receptors were unable to clear *Listeria* and eventually succumbed to high loads of the bacteria [200, 201]. Therefore, M1 polarized macrophages are instrumental in killing of intracellular pathogens.

Figure 1.12 M1 and M2 polarized macrophages and their functions. ROI and RNI: reactive oxygen and nitrogen intermediates. M2s: diverse forms of M2 activation (M2a, M2b and M2c). Adapted from [202]

In contrast, CSF-1, IL-4, IL-10, IL-13 and TGF- β stimulation favors polarization to an alternatively activated M2 phenotype, which plays a central role in immune responses against parasites, tissue repair (remodeling), angiogenesis and allergic manifestations [203, 204]. The M2 macrophages are further sub classified as M2a (activation by IL-4 or IL-13), M2b (induced by IL-10) and M2c (activation by LPS coupled with immune complexes) [205]. The CD200R membrane glycoprotein, CD163 in combination with the CMAF transcription factor are signatures of M2 polarized macrophage populations [206, 207]. In mouse, M2 macrophages exhibit distinct genetic profile such as up regulation of

macrophage mannose receptor (MMR, also referred as CD206), arginase 1 (*Arg1*), resistin like molecule α (*FIZZ1*) and chitinase like protein (*Ym1*) [208]. Helminth infections are resolved by Th2 responses as exemplified by accelerated susceptibility of IL-4R α knockout mice due to infection. Exploiting a mouse model of *Strongyloides stercoralis* infection, a study has suggested a killing property of M2 macrophages through collaboration of neutrophils and complement system [209].

Plasticity and adaptability are the cardinal features of macrophages and therefore confinement of the macrophage functions into the dichotomous M1 and M2 model does not hold true in many homeostatic and pathological scenarios such as in the case of cancers, myocardial infarction amongst others [210, 211]. A recent study proposes multidimensional model of macrophage activation wherein human macrophages were subjected to 28 different stimuli illustrating diverse activation states thus reflective of multiple functional states as opposed to fixed M1 and M2 paradigm, thus highlighting the spectrum of activated functional states of the tissue macrophages, whether resident or recruited [212]. Similar spectral nature of macrophage functional states was also evident in mouse [213].

Tissue-resident macrophages play a pivotal role in vascular growth as exemplified by studies on mouse and zebra fish models wherein macrophages act as endothelial cells chaperones directing the development of vasculature in CNS in a vascular endothelial growth factor A (VEGF-A) dependent manner [214]. Defective embryonic vascular development has been witnessed in macrophage deficient embryos. *Spi1*^{-/-} and *CSF1*^{op/op} show reduced vascular branching and complexity at E11.5 [214, 215]. In mice, during development of retina, macrophages induce regression of temporary hyaloid blood vessels by programmed cell death and WNT pathway effector responses [216]. These evidences suggest a unique macrophage vascular interaction in shaping the development of blood vessels. Therefore, the angiogenic property of the tissue-resident macrophages can be harnessed in stimulating vascularization in ischemic scenarios.

Tissue repair and regeneration is yet another functional characteristic of tissue-resident macrophages. In this context, the liver is the best-studied organ with an intrinsic capacity to regenerate post injury as reported in model organisms such as salamander, zebra fish and neonatal mice where macrophages were essential for regeneration [217-219]. During liver injury, Kupffer cells hyperplasia along with rapid expansion occurs to phagocytose the cell debris and subsequent activation of a differentiation program mediated by WNT

ligands results in generation of new hepatocytes from progenitors for reparative cause [220, 221]. The impairment in this regenerative process in the context of hepatocyte differentiation is pronounced in macrophage depletion settings while bile duct regeneration remains unaffected. Liver fibrosis is a common feature observed during injury and inflammation, which is generally considered as a wound healing response to the injury characterized by excessive deposition of extracellular matrix proteins and collagen. This fibrotic reparative mechanism is mediated by monocyte-derived macrophages rather than Kupffer cells as exemplified by reduced fibrosis post-acute liver injury in CCR2 or monocyte chemoattractant protein-1 (MCP-1) deficient mice [222, 223].

Tissue-resident macrophages such as red pulp macrophages and Kupffer cells seeded in spleen and liver respectively plays fundamental roles in maintaining iron homeostasis by recycling iron from the hemoglobin. These resident macrophages phagocytose the senescent RBCs (with altered membrane structures), perform intracellular proteolytic degradation and thereby release the hemoglobin and either stored in the form of ferritin complexes or exported via ferroportin [224]. The released hemoglobin in the circulation is recognized by the scavenger receptor CD163 present on the surface of macrophages in the form of haptoglobin-hemoglobin complex [225]. Another heme clearance system involves formation of heme-hemopexin complex and subsequent recognition by CD91 expressed on the surface of the macrophages [226].

1.6.4 Tissue-resident CD169⁺ macrophages

Sialoadhesin or Siglec1 (also referred as CD169) is a sialic acid binding receptor identified on a subpopulation of macrophage harvested from bone marrow, liver, lymph node and spleen [227, 228], hereafter, referred as CD169⁺ macrophages and its expression is conserved across mammals [229]. The presence of CD169⁺ macrophages is also reported in lymphoid and non-lymphoid organs such as kidney, lungs and intestine amongst others [230-233]. Regardless of their location this macrophage subset is positioned in proximity to the vasculature that justifies their preferential capturing ability of blood borne-pathogens. For instance, in mice spleen CD169⁺ macrophage subset is strategically stationed at the marginal zone area and liver CD169⁺ macrophages within the hepatic sinusoids. Similar parallels can be drawn with respect to anatomical and physiological equivalence of human CD169⁺ macrophage as well, owing to their predominant presence in splenic perifollicular zone [229]. In mice, phenotypically,

CD169⁺ macrophage subset also expresses CD11b, MHCII, CD68, CD11c, and F4/80. However, not all CD169⁺ macrophages are F4/80⁺ and this characterization remains ambiguous in some organs for example kidney. The subcapsular sinus (SCS) macrophages of the lymph node and splenic CD169⁺ macrophage subset are dependent on lymphotoxin α (LT- α) signaling and prone to clodronate liposome mediated depletion [234, 235].

Maintaining tissue homeostasis is functional specialization of tissue-resident macrophages and few studies have addressed the involvement of CD169⁺ macrophages in this direction. An *in vivo* mouse study has shown that depletion of CD169⁺ subset resulted in dramatically impaired erythropoiesis in a phenyl hydrazine induced anemia model although not in steady state conditions suggesting its role in erythropoiesis particularly in conditions of stress [236]. Another study has also shown that G-CSF mobilization and depletion of CD169⁺ macrophage subset via clodronate liposome or DT mediated depletion in CD169-DTR mice respectively, results in blocked erythropoiesis in bone marrow [237]. These reports provide evidences for the role of CD169⁺ macrophages in regulating erythropoietic homeostasis.

The importance of CD169⁺ macrophage subset in regulating adaptive immune responses has been reported in Sn-deficient mice that showed slightly decreased numbers of follicular B cells and significantly impaired IgM titers in response to thioglycolate stimulation [238]. A very recent study has demonstrated antigen targeted (using ova conjugated monoclonal antibody directed against CD169) to CD169⁺ macrophage resulted in high affinity antigen specific antibody responses which was coincident with prolonged persistence of germinal center (GC) B cells and Tfh (follicular Th cells) [239]. Similar antigen targeting approach (formulation of α GalCer; galactosylceramide and Sn targeted liposomes) in a study has illustrated robust invariant natural killer T (iNKT) cell activation in response to lipid antigens in spleen and liver [240]. In an earlier report, using a multiphoton microscopy approach, it was observed that CD169⁺ macrophage in the lymph node SCS mediate stable long lasting CD1d dependent interactions with the iNKT cells that led to their activation as evident by their cytokine secretion ability [241]. These studies are affirmation to the antigen capturing ability of glycan origin and regulating antigen specific responses in a collaborative manner. In yet another study that illustrates this collaborative attribute of CD169⁺ macrophages, it was shown that these macrophages in the spleen capture blood borne adenoviruses and transfer it to splenic

CD8⁺ DCs and cross-presented to prime CD8⁺ T cell responses and this result was supported by direct antigen targeting to the MMMs [242]. The cross-presentation of antigen by CD169⁺ macrophages was again highlighted in a later study. Using a dead tumor cell stimulation model, it was shown that CD169⁺ macrophage deficient mice showed impairment in tumor antigen specific CD8⁺ T cell activation and subsequent anti-tumor immunity, thus concluding this macrophage subset as lymph node resident APCs [243]. A reaffirmation to the CD169⁺ macrophage's priming capacity and inducing cytotoxic T lymphocytes (CTLs) responses was provided by a very recent study which used adenoviral vectors that preferentially accumulate in CD169⁺ macrophages and not DCs, herein, the authors showed that CD169⁺ macrophages was able to elicit antigen specific CTLs in DC deficient mice in response to various epitopes tested [244].

Immunological tolerance is judicious ability of the host immune system to discriminate between self and non-self-antigens thus preventing autoimmune conditions. A couple of studies have showed involvement of splenic CD169⁺ macrophages in maintaining apoptotic cell driven tolerance whereby capture of apoptotic cells results in their activation and secretion of chemokine CCL22 and subsequent accumulation of T_{regs} and CD103⁻ DCs in the follicles. Therefore recruited T_{regs} mediate tolerance [245]. Another study has proposed that splenic and lymph node CD169⁺ macrophages capture exosomes (cell derived vesicles) that serve as source of self-antigens, thus regulating the flux of exosomes to lymphoid organs and decreasing the likelihood of autoimmune responses [246].

Furthermore few studies have shown associations of CD169⁺ macrophages with kidney diseases, antiviral immune responses and intestinal inflammation. In a recent report, it was demonstrated that depletion of kidney resident CD169⁺ macrophages resulted in exacerbated renal injury in ischemia reperfusion injury model [232]. In the context of antiviral responses, CD169⁺ macrophages from the SCS have been shown as the major type I IFN producers and therefore drive NK cell recruitment to exert their antiviral role in recombinant modified vaccinia virus Ankara experimental infection model [247]. Another study has demonstrated antiviral role for CD169⁺ macrophages in vesicular stomatitis virus mice infection model wherein enforced viral replication in the macrophages was prerequisite for the effective induction of adaptive immune responses [248]. With respect to the intestinal inflammation, a recent study has identified a lamina propria (LP)-resident CD169⁺ macrophage subset positioned at bottom end of the LP

microenvironment that regulates mucosal damage. These resident macrophages secrete monocyte-recruiting chemokine CCL8 in response to mucosal injury. Furthermore, specific depletion of CD169⁺ macrophages ameliorates symptoms of colitis in an experimental model [249].

The context dependent roles of the CD169⁺ macrophages in disease, infection and inflammation model is reflective of their plastic behavior and currently available data does not allow distinct characterization of this macrophage into defined and fixed M1 and M2 activation states.

1.7 Role of monocytes/macrophages in malaria

The innate immune cells such as monocytes and macrophages play indispensable role in malaria. Their anti-malarial protective mechanisms/function include opsonic and non-opsonic phagocytosis of iRBCs, a monocyte restricted phenomenon; antibody dependent cell inhibition (ADCI) and establishing appropriate cytokine milieu for the resolution of *Plasmodium* infection. These anti-malarial processes will be discussed later in this section.

1.7.1 Monocytes and malaria

As described previously, monocytes are heterogeneous group of mononuclear phagocytic cells. In humans, the CD16⁺ monocyte subset significantly increases in peripheral blood of malaria-HIV co-infected pregnant women as well as patients suffering from acute complicated malaria [250, 251]. Previous studies and our lab results highlights the prevalence of similar scenario, wherein, enhanced recruitment of Ly6C^{hi} monocytes is witnessed in various tissues (spleen, liver, lung, kidney) of the mice infected with murine *Plasmodium* infection models [252, 253]. Thus, recruitment of inflammatory monocytes is a common feature during inflammation including malaria in both humans and mice. The significance of Ly6C^{hi} monocytes as innate effector cells in the spleen has been previously demonstrated in *P. chabaudi* infection model. Herein, the Ly6C^{hi} monocyte subset was recruited to the spleen from the bone marrow in a CCR2 dependent manner and the activated monocytes readily engulfed iRBCs and showed up regulation of surface markers associated with antigen presentation, thus limiting acute stage parasite load in the peripheral blood. Though the monocytes exhibited antigen presentation potential they show functional inferiority when compared to professional APCs such as DCs [254].

Phagocytosis of the pRBCs is one of effective parasite control strategy along with a unique antibody dependent cell inhibition mechanism (ADCI), characteristic of only monocytes [255], refer to Figure 1.13. The activated monocytes and macrophages secrete high levels of IL-12 and IL-18 to mediate production of IFN- γ during early phase of the infection thus participating in the prevention of complicated forms of malaria [256]. Differential receptor usage results in altered pro-inflammatory cytokine profile. For example, uptake of iRBCs through CD36 receptors does not lead to TNF- α production whereas Fc γ R mediated phagocytic process promotes secretion of pro-inflammatory cytokines. *Plasmodium* associated entities such as GPI can ligate with TLR-2 and CD36 receptors simultaneously to induce activation of mitogen-activated protein kinase pathway for subsequent release of pro-inflammatory cytokines [257-259]. The phagocytic efficiency of the phagocytes is regulated by opsonins such as complement proteins (C3b) and antibodies (IgG1 and IgG3) that facilitate enhanced interaction between the phagocytic receptors (CR1 or CD35) and opsonin-iRBC complex [260, 261]. The expression of Fc γ Rs on various monocyte subsets determines the efficiency of opsonic phagocytosis. The monocytes express three important Fc γ Rs; Fc γ RI (CD64), Fc γ RII (CD32), and Fc γ RIII (CD16). The isoforms Fc γ RI and Fc γ RIIa facilitate uptake of antibody-opsonized iRBCs while Fc γ RIIa functions as inhibitory receptors that can result in decreased phagocytic efficiency and also dampen immune responses [262, 263]. The opsonic phagocytosis are predominant particularly in malaria immune individuals [264]. Non-opsonic phagocytosis constitutes a critical innate protection in non-malaria immune individuals that lack *Plasmodium* specific antibodies (Figure 1.13). This phagocytic mechanism involves direct uptake of pRBCs by the phagocytic receptors independent of opsonins. The scavenger receptor CD36 is the only receptor that has been implicated in the direct non-opsonic uptake of iRBCs. The efficiency of non-opsonic phagocytosis is dependent on parasite's adhesion phenotype and level of CD36 expression on the monocyte. In placental malaria the iRBCs bind to chondroitin sulphate A and not CD36 thus bypassing non-opsonic phagocytic clearance [265].

ADCI is an important monocyte mediated protective mechanism that reduces parasite burden. It involves the acquisition of parasite specific antibodies (IgG1 and IgG3) against surface antigens of merozoites, including merozoite surface protein-1 (MSP-1) block 2 and glutamate-rich protein (GLURP) [266, 267]. Antibody-opsonised merozoite complex activates monocytes and thus release soluble mediators to arrest the growth and

proliferation of surrounding blood stage parasite [255]. Interaction of IgG-merozoite complexes with monocyte FcγRs (FcγRII or FcγRIII) is a prerequisite for ADCI as exemplified by a study wherein blocking FcγRII or FcγRIII and not FcγRI abolished ADCI [268]. A study has reported the involvement of CD14⁺ CCR2⁺ CX3CR1⁺ monocyte subset in ADCI [250]. The monocytes not only phagocytose iRBCs but also can uptake malarial pigment hemozoin (Hz; a detoxification product of free heme) that has consequential effects on up regulation of range of cytokines such as IL-1β, TNF-α and chemokines; macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and monocyte chemoattractant protein-1 (MCP-1). It has also noted that the hemozoin favors monocyte survival via anti-apoptotic protein heat shock protein (hsp 27) [269]. Such situations are detrimental to the host because prolonged monocyte survival with inflammatory phenotype and impaired phagocytic ability results in tissue inflammation culminating into severe malaria as observed in a recent study, which demonstrated immunopathology in ECM can be prevented by depletion of Ly6C^{hi} inflammatory monocytes [253]. In contrast, a recent report has demonstrated exploiting the *P. berghei* ANKA model that sequestration of iRBCs on postcapillary endothelial surfaces in the lung results in ALI and the rapid recruitment of Ly6C^{hi} monocytes from the circulation. These recruited Ly6C^{hi} monocytes differentiate into monocyte-derived macrophages and are instrumental in the phagocytic clearance of sequestered iRBCs [252].

The contrasting protective and pathogenic phenotype of monocyte populations during malaria infection needs to be carefully interpreted in order to harness the potential of monocytes in devising therapeutic strategies. Since monocyte populations represent diversity in subsets, extensive characterization of exclusive subsets that confer protection during *Plasmodium* infection needs to be performed before embarking on to monocyte based therapies.

1.7.2 Macrophages and malaria

In addition to circulating and recruited monocytes, tissue-resident macrophages particularly in the spleen and liver play a crucial role as professional phagocytic cells that mediate direct clearance of iRBCs as well as regulate the cascade of subsequent innate and adaptive immune effector functions essential for resolution of infection and inflammation. Similar to monocytes, macrophages too can perform opsonic and non-opsonic phagocytosis (Figure 1.13).

The liver-resident Kupffer cells are the largest population of tissue macrophages in mammals strategically positioned at the sinusoidal lumen for removal of foreign particulate matter such as sporozoites (liver stage infection) in the case of malaria and induction of portal vein tolerance.

Figure 1.13 Protective monocyte and macrophage mediate anti-malarial mechanisms. Monocytes and macrophages can control parasite numbers by opsonic and non-opsonic phagocytosis of iRBCs. Complement opsonized iRBCs can be recognized by CR1 and subsequently phagocytosed by monocytes/macrophages. Opsonin mediated phagocytosis by FcγRs results in cytokine production in contrast to scavenger receptor CD36 mediated non-opsonic phagocytosis. Another alternative anti-parasitic mechanism is by recognition of parasite associate components such as GPI, hemozoin and microparticles that activate these phagocytes to secrete pro-inflammatory cytokines to facilitate parasite clearance. Furthermore, FcγRs on the monocyte surface can interact with the antibody-opsonized merozoites to stimulate secretion of soluble factors that can be anti-parasitic and limit parasite growth. This monocyte-mediated mechanism is known as antibody dependent cell inhibition. Adapted from [270].

The importance of Kupffer cells in *P. berghei* sporozoite induced malaria is exemplified by a study wherein ablation of these cells resulted in elevated parasitemia [271, 272]. The PRRs on the Kupffer cells binds to PAMPs on the surface of sporozoite such as circumsporozoite protein and thrombospondin related adhesion protein. The phagocytosed sporozoite maintains their integrity despite the encapsulation in endocytotic vesicles and escapes the lysosomal degradation exerted by the Kupffer cells. The sporozoites traverse along Kupffer cells in encapsulated form [271, 273-275]. Tolerance to antigenic epitopes on the sporozoite surface may partly explain poor short-lived protective immunity conferred by parasite specific CD8⁺ T cells [275, 276]. A study has reported the breakdown of such tolerance via immunization with attenuated *P. berghei* sporozoites that resulted in protection on subsequent challenge [277]. Therefore

departure from the tolerant phenotype could be beneficial for the host during liver stage infection. A recent study has identified a triggering receptor expressed on myeloid cells 2 (TREM2) as pathogen sensor on liver macrophages that governs pro-inflammatory macrophage phenotype that mediates parasite clearance, as *Trem2* deficient mice were more prone to liver stage infection as opposed to WT counterparts [278]. Despite continuous efforts in scientific pursuits of deciphering the conclusive role of Kupffer cells in liver stage malaria, a definitive understanding is missing. On the other side, not much is known about the role of Kupffer cells during blood stage infection as well.

Several studies have been undertaken to define the collective role of macrophages, in general, during blood stage *Plasmodium* infection and ensuing pathologies. The role of macrophages in *P. chabaudi* infection has been highlighted in a report wherein, an enhanced parasitemia and susceptibility to an otherwise resistant C57BL/6J mouse in a silica-mediated macrophage depletion system was observed [279]. A study has described macrophage mediated innate immune mechanisms are critical in curtailing the primary wave of *P. yoelii* (Py) parasitemia using clodronate liposome mediated macrophage depletion model (other phagocytic cells such as monocytes are also depleted). Herein, the monocyte/macrophage deficient mice showed significantly reduced ability to contain acute PyL (lethal), PyNL (non-lethal) infection [280]. Using MAFIA mouse model (macrophage/granulocyte depletion system for blood, brain and lungs) a study has been demonstrated that myeloid cells are indispensable for containment of parasite sequestration the outcome of PbA induced ECM but this study does not address the effect of ablation on sequestration and immunopathology in other tissues. Also the MAFIA mice do not represent a macrophage exclusive depletion system [281]. A very recent study has reported acute kidney failure in PyNL induced acute malaria; wherein the clodronate liposome mediated depletion of phagocytic cells including macrophages in mice showed alleviation in peripheral blood parasitemia concomitant with increased parasite sequestration, compromise in vascular endothelial integrity, tissue inflammation exemplified by necrosis, fibrin deposition in liver and kidney and eventually death during acute phase of infection [282, 283]. Therefore, macrophages are key phagocytic cells that facilitate clearance of iRBCs from circulation and elimination of sequestered parasite to restrict inflammation and pathogenesis during blood stage *Plasmodium* infection.

In accordance with the protective role of spleen in malaria, the splenic macrophages have been best-studied phagocytic cells. A recent study has outlined the phagocytic nature of

heterogeneous macrophage subsets seeded in the spleen during erythrocytic stage of malaria and found that only F4/80⁺ red pulp macrophages can efficiently engulf parasites and the remaining macrophage pool such as SIGNR1⁺ marginal zone, CD169⁺ marginal metallophilic, and CD68⁺ tingible-body macrophage subsets appeared to be minimally involved in phagocytosis [283].

1.7.3 Tissue-resident CD169⁺ macrophages and malaria

As previously described tissue-resident CD169⁺ are almost omnipresent in lymphoid and non-lymphoid tissues and the role of CD169⁺ macrophages has so far not been investigated in the context of *Plasmodium* induced malarial syndromes. The CD169⁺ macrophages also express T-cell immunoglobulin and mucin-domain-containing molecule (TIM)-4 and was identified as a phosphatidylserine receptor (PS) [284, 285]. To the best of our knowledge only one report has shown associations of TIM-4⁺ macrophages in PyNL induced malaria. In this study expression of TIM-4 increases in splenic macrophages upon PyNL infection and are readily involved in phagocytosis of iRBCs as dose dependent inhibition of phagocytosis was observed under conditions of antibody mediated blocking of TIM-4 receptor. This study, however, did not address the role of tissue-resident CD169⁺ TIM4⁺ macrophages in malarial syndromes.

1.8 In vivo cell/tissue-resident macrophage depletion systems

One of the best ways to study the cell function is *in vivo* loss of function approach that allows understanding of cellular functional dynamics in the complex environment in the organism that reveals functional specialization of the cells in true sense. The complexity and heterogeneity of immune cells in the organism calls for understanding the function of individual subsets of the cell types in homeostasis and disease so that cell subsets that are beneficial to the host can be exploited for cell based therapies in adverse scenarios.

In the last decade, cell surface receptor phenotyping has uncovered the prevalence of diverse repertoire of macrophage subsets residing in different tissues that perform context dependent specialized functions. These functional studies generally employ use of transgenic mouse models, including transcription factors and immune surface receptors deficient mice and enforced silica, clodronate liposome and antibody mediated depletion of macrophages [286, 287]. Almost all these approaches result in broad macrophage depletion with associated inherent limitations. The transcription factor genetic deletion system such as PU.1, CSF-1R results in broad non-specific macrophage

depletion, plagued by pleiotropic organ defects (example osteopetrosis in CSF-1R mice) and do not survive until adulthood, thus not feasible for experimental studies aimed to study macrophage functions[288]. However, discovery of specific transcriptional factors governing cellular subset development would enable development of transgenic null mouse systems that would facilitate ablation of particular macrophage subsets, for example Spi-C^{-/-} is only deficient in RPMs but such system do not allow study of the functional dynamics over a period of time unlike the conditional ablation systems [85]. The lack of knowledge of unique receptors to define specific macrophage subpopulations hinders the usage of the antibody-mediated approach to achieve targeted ablation. Depletion of resident macrophages can also be achieved by clodronate liposomes but this method leads to ablation of all phagocytic cells that includes all macrophage populations and DCs but not in all organs. Clodronate liposome mediated depletion shows variable consequences upon challenge such as loss of inflammatory mediators and altered inflammatory cell recruitment. Another approach is conditionally knocking out genes that block macrophage maturation by exploiting cre-lox system. The technical limitation of slow turnover of mature macrophages at the time of gene induction prevents the use of this strategy as the choice of depleting macrophages [289].

An inducible suicide gene approach targeted to macrophage specific expression would potentially overcome aforementioned problems because of the following reasons. Firstly, the normal embryonic development would be guided in the presence of appropriate number and milieu of macrophages. Secondly, use of an apt suicide gene would enable systemic ablation of macrophages when suicide gene mechanism is activated. Lastly, withdrawal of suicide activator would result in regulatable, transient and reversible macrophage depletion system, provided suicide gene is not targeted on non-intended cells [290]. In this direction, *Herpes simplex* virus thymidine kinase (HSVtk) gene has been used as a suicide gene in transgenic mice for *in vivo* depletion of various immune cell populations [291]. The activation of HSVtk, the antiviral gancyclovir is converted into a metabolite, resulting in cellular death of mitotically active HSVtk-expressing cells. The HSVtk does not represent an ideal macrophage depletion strategy as it can result in death of actively dividing cells and not resting cells. To address this issue, a suicide gene system that can induce activation of proteins in an apoptosis cascade would be a better fit to deplete resting and dividing cells. To this end, drug-inducible Fas based suicide gene that activates caspase-8 pathway has been used to generate MAFIA mouse model that

enables drug; AP20187 induced apoptosis of resting and cycling cells of the macrophage lineage. However, the MAFIA mouse allows efficient depletion of macrophages in all tissues but it also depletes DCs and is therefore not suitable for functional studies of only macrophages [290]. As of now, the diphtheria toxin receptor-diphtheria toxin (DTR-DT) system, a genetic cell ablation approach demonstrated by Saito *et al.* is the best available system to deplete cells *in vivo* whereby a cytotoxic gene is expressed in transgenic mice under the control of a tissue-specific promoter [292].

1.9 Diphtheria toxin receptor-diphtheria toxin (DTR-DT) system

Corynebacterium diphtheriae produces an exotoxin known as DT. Collier *et al.*, enunciated the structure and mechanism of action of DT, a single chain polypeptide consisting of two subunit (A and B) [293]. Only the subunit B binds to the DTR, a membrane-anchored form of the heparin-binding epidermal growth factor receptor (HB-EGF), and subsequent endocytosis mediated internalization of the DTR results in the entry of the toxin inside the cell.

Figure 1.14 Diphtheria toxin-diphtheria toxin receptor system for conditional ablation of specific cellular subsets *in vivo* in mouse. Since hDTR is 10^5 times more sensitive to DT as compared to mouse DTR, hDTR is expressed under the control of a tissue specific promoter that enables killing of target rodent cell population by DT mediated apoptosis.

Upon entry into the endosome, a trypsin-like protease mediated proteolysis of the peptide and disulphide bonds links the two subunits together. Due to acidic environment of the endosome, the subunit B punches holes in the endosome membrane making it porous and results in subsequent release of subunit A in the cytoplasm. Herein, the subunit A inactivates the elongation factor 2 (EF-2) via ADP ribosylation, thus precluding protein synthesis and eventually apoptosis induced cell death [293]. Since the murine cells are at

least 10^5 times more resistant to DT toxicity than human cells due to differential affinity to DTRs, this property was exploited to develop a robust strategy for *in vivo* cell ablation by engineering the high affinity human DTR (hDTR) in such a way that expression of hDTR is under the control of cell specific promoter in mouse (Figure 1.14). An advantage of developing transgenic mice using this system to target specific cell subsets are that undesired inflammatory responses can be avoided as the DT mediated cell death involves natural apoptosis program. Therefore DTR transgenic mouse models for cell ablation presents a compelling approach for achieving conditional cell ablation *in vivo* to study cell function [292]. However some of the concerns of using DTR transgenic models are development of antibodies against DT in long-term DT administration protocols, which hampers the efficiency of depletion and thus DTR models rarely find utility in such experimental designs [294]. The results obtained from DTR transgenic mice should be carefully interpreted as transient neutrophilia has been reported [295].

Exploiting the DT-DTR system, CD11b-DTR mouse has been generated to investigate the role of tissue-resident macrophages in various inflammation models but all CD11b⁺ cells including monocytes and neutrophils are also depleted [177, 296, 297]. Therefore better choice of cell specific promoters should be used to generate DTR mouse to study specific subsets of macrophages to gain deeper insights into their functions. In a previous study from our lab, Purnama *et al.*, generated a CD169-DTR mouse model which targets the macrophage subpopulation of CD169 expressing cells and demonstrated that transient ablation of CD169⁺ alveolar macrophages resulted in exacerbated virus load in the lungs, pulmonary edema, compromise in blood vessel integrity as evident by increased vascular leakage and lung tissue inflammation and eventually death in a PR8 influenza virus model.

1.10 Objective of the study

In this study, we exploited the transgenic CD169-DTR mouse to characterize the specificity and efficiency of depletion of tissue-resident CD169⁺ macrophages in brain, spleen, liver, lung, kidney and heart at steady state and in murine *Plasmodium* infection. Furthermore we wanted to study the infection course and outcome of two different parasites (*P. berghei* ANKA and *P. yoelii* 17XNL) in absence of CD169⁺ macrophages with the aim to unravel the role of this macrophage subpopulation in controlling parasite propagation/sequestration and in ensuing malarial syndromes such as CM, spleen dysfunction, ALI, liver injury and acute kidney injury.

2. Materials and Methods

2.1 Materials

2.1.1 Mice

Wild type (WT) BALB/c mice were bought from center for animal resources of National University of Singapore or procured from our animal facility. CD169-DTR was developed in BALB/c genetic background as described previously [231] and maintained together with WT BALB/c in the animal facility of Nanyang Technological University of Singapore.

2.1.2 Rodent malaria parasites

Transgenic *Plasmodium berghei* ANKA clone 15Cy1 expressing green fluorescent protein (GFP); PbAGFP [298] or clone 231c11 expressing luciferase and GFP enzyme; PbAluc [41] were used for PbA infections. Transgenic GFP expressing *Plasmodium yoelii* 17X clone1.1 non-lethal strain; Py1.1-GFP) [299] and *P. yoelii* 17XNL expressing luciferase and GFP enzyme; PyLuc (generous gift of Prof. Peter Preiser, School of Biological Science, Nanyang Technological University, Singapore) were used for Py infections.

2.1.3 Antibodies

Anti-mouse commercial antibodies were used as listed in Appendix 6.2.

2.1.4 Media, buffers and solutions

Media, buffers and solutions were used as described in Appendix 6.3.

2.1.5 Reagents, chemicals and kits

All reagents, chemicals and kits are listed in Appendix 6.4.

2.1.6 Equipment

Instruments used in this study are listed in Appendix 6.5.

2.1.7 Computer software

Flow cytometry data was analyzed using FlowJo software (TreesStar Inc, Ashland, OR). GraphPad Prism 5.0 was used to plot graphs and perform statistical significance

(GraphPad Software, La Jolla, CA, USA). Image processing was done by ImageJ 1.48 software (Wayne Rasband, National Institutes of Health, USA). Hz deposits were quantified using the HistoQuest program (TissueGnostics USA Ltd., Tarzana, CA, USA).

2.2 Methods

2.2.1 Mice and ethics statement

Age and sex-matched animals were used for both strains of mice as stated in section 2.1.1. They are referred as WT and CD169-DTR mice throughout the text. 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 IACUC of the Nanyang Technological University of Singapore (Authorization No IACUC SBS/NIE A-0127) approved the protocol.

2.2.2 Depletion of tissue-resident macrophages

WT BALB/c and CD169-DTR mice were injected intraperitoneally (i.p.) at day -2 (48 hours before the day of infection) and -1 (24 hours before the day of infection) with 10 ng/g DT, followed by repeat injections every 3–4 days to ensure that depletion of the target cell subpopulation was maintained throughout the experimental phase.

Note: day 0 refers to the day of *Plasmodium* infection throughout the text.

2.2.3 Cryopreservation of iRBC stock, monitoring of percentage parasitemia and disease scores (Rapid murine coma and behavioral scale; RMCBS)

A set of 3–4 mice was infected (i.p. injection) with 10^6 iRBCs (PbAGFP/PbALuc/PyGFP/PyLuc infected red blood cell), obtained from prior frozen (-196 degree Celsius) stock as per the calculations described in the subsection (iii). At day 6-post infection (PbAGFP/PbALuc infection) the presence of parasite in the blood stream of the mice was verified as percentage GFP⁺ RBC (iRBC) in the case of parasites engineered with GFP by flow cytometry except PbALuc. 1 µl of blood was pipetted by snipping the mouse-tail and suspended in 500 µl of 1X PBS and the percentage of GFP⁺ iRBC was evaluated by flow cytometry using BD FACS Calibur. The stock was prepared on the onset of ECM neurological signs and when the RMCBS scores (as described in subsection (ii) in 2.2.3) were below 12. Upon the onset of neurological signs the mice were retro-orbitally bled and blood was collected in 30 ml of 1X PBS. In the case of Py,

the above steps were repeated and the blood was collected at a time point when parasitemia was in the range of 8-10%. The blood-PBS suspension was centrifuged at 400g for 5 mins, resuspended in 9.5 ml of fresh 1X PBS and subsequently used to count total number of RBCs as described in cell counting section (2.2.4) as well as evaluating the percentage parasitemia by flow cytometry to determine the total number of iRBCs (refer to sub section (iii) in section 2.2.3 for detailed calculation steps). The RBC suspension was centrifuged to obtain RBC pellet and re-suspended in Alsever's solution at concentration of 10^7 iRBC/ml, distributed in 1.2 ml aliquots in cryovials at room temperature (R.T.). These cryovials were transferred to styrofoam boxes and kept at -80 degree Celsius for 24 hours before being transferred to liquid nitrogen for cryopreservation until further use. Frozen vials were removed and immediately thawed at 37 degree Celsius as per the requirement for infections. In the case of PbALuc, Giemsa staining procedure was performed to evaluate the parasitemia as described in sub section (i) in 2.2.3.

(i) Giemsa staining procedure for monitoring parasitemia

15 ml Giemsa stain was filtered with 0.2- μ filter fitted with 20 ml syringe and diluted 1:10 with distilled water in a glass tank. This diluted Giemsa stain is ready for use. A drop of blood (obtained by snipping the tail of the mouse) was smeared on a grease free slide, air-dried, methanol and heat fixed on a hot plate at 50 degree Celsius. The heat fixed slide was brought to R.T. and dipped in the diluted Giemsa stain for 2 hours at R.T. After the staining, the slide was washed five times with distilled water in a glass tank. The stained slides were air-dried and parasites were observed under 100X oil objective (using Nikon Eclipse 80i microscope) and parasitized RBC (iRBC) was manually counted using a cell counter. The slides were scanned under the microscope until 1000 RBC counts. Simultaneously the number of iRBCs was noted, for example 100. The % parasitemia was calculated with following formula [(iRBC/1000) X 100].

Therefore the % parasitemia = [(100/1000)] X 100 = 10.

(ii) Monitoring of RMCBS Scores

After infection with PbAGFP/PbALuc cerebral malaria disease score (RMCBS) was monitored as previously described in [300] and detailed here. The RMCBS monitoring comprises of 10 parameters such as gait, balance, motor performance, body position, limbs strength, touch escape, pinna reflex, toe pinch aggression and grooming. This

scoring system enables the operator to assess the outcome of CM in three minutes/mouse. The scoring range is 0 as minimum/parameter and 2 as maximum/parameter stated above leading to a maximum score of 20 in a completely healthy mouse. In the first 90 seconds, mice were assessed for hygiene-related behavior, gait, body position, exploratory behavior, and balance listed in Table 2.1. A mouse was scored as healthy in the presence of: (i) smooth, preened hair with sheen (ii) a steady gait (iii) full extension of its body as it sat and walked (iv) explored all four corners of the grid box in under 15 seconds and (v) reached the top of the grid box and pulled itself to the edge, lifting its hind legs off the cage floor. In contrast, an ill mouse respectively developed (i) piloerection, and later complete disarray of its hair (ii) ataxia, or no gait (iii) remained in a crouched position, even while walking, and later lay on its side (iv) explored only three, or fewer corners in 90 seconds, and later did not move from the original corner and (v) placed only its forelegs on the cage edge and later ceased to raise the forelegs altogether.

Table 2.1 Cerebral Malaria disease scoring system: Rapid Murine Coma and Behavioral Score. Adapted from [300].

Label	Score	Description
Coordination		
Gait	(0–2)	(none – ataxic – normal)
Balance	(0–2)	(no body extension – extends front feet on wall – entire body lift)
Exploratory Behavior		
Motor Performance	(0–2)	(none – 2–3 corners explored in 90 seconds – explores 4 corners in 15 seconds)
Strength and Tone		
Body Position	(0–2)	(on side – hunched – full extension)
Limb Strength	(0–2)	(hypotonic, no grasp – weak pull-back[front paw grasp only] – strong pull-back[active pull away, jerk away])
Reflexes and Self-Preservation		
Touch Escape	(0–2)	(none – unilateral – instant and bilateral; in 3 attempts)
Pinna Reflex	(0–2)	(none – unilateral – instant and bilateral; in 3 attempts)
Toe Pinch	(0–2)	(none – unilateral – instant and bilateral; in 3 attempts)
Aggression	(0–2)	(none – bite attempt with tail cut – bite attempt prior to tail cut, in 5 seconds)
Hygiene-Related Behavior		
Grooming	(0–2)	(ruffled, with swaths of hair out of place – dusty/piloerection – normal/clean with sheen)

In the next 90 seconds, mice were assessed for reflexes, limb strength, and self-preservation behaviors. A healthy mouse (i) jumped away from the stimulus of a touch to its flank, often on the first offense, and certainly within three attempts made on each side (ii) flicked its ear when the pinna was touched (iii) grasped a 3-mm diameter rod and pulled away (iv) reflexively pulled its hind leg away from a toe pinch while suspended with the front paws grasping the rod and (v) bit at the retention tube and restraining ring when secured for the purposes of tail blood sampling. An ill mouse would respectively demonstrate (i, ii, iv, and v) fewer to no reflexive movements with stimulation, and (iii)

would not pull away when suspended, eventually refusing to grasp the 3-mm diameter rod.

The RMCBS scores were monitored from day 5 to day 14 post infection referred as ECM (Experimental Cerebral Malaria) window in our study and this ECM window is generally from day 5 to day 12 post infection in the case of PbA infected WT C57BL/6 mouse. The ECM window was expanded till day 14 post infection as the PbA infected BALB/c CD169-DTR mouse showed a delay in the onset of ECM symptoms which is not the case with PbA infected WT C57BL/6 mouse (the onset of ECM symptoms occurs at day 7 post infection). The mouse with a disease score lower than 12 and above 12 were considered to be suffering with CM and CM asymptomatic respectively.

(iii) Calculations and counting of iRBCs for cryopreservation of parasite stocks

Total RBCs/ml were counted as described in section 2.2.4.

Assuming this number to be “A/ml”.

Since the RBCs were suspended in 10 ml 1X PBS, therefore the total number of RBC would be 10A.

The percentage iRBCs was calculated either by flow cytometry previously described in this section or Giemsa stained slides (see sub section (i) in 2.2.3).

Assuming this percentage to be “B”.

Total number of iRBCs in 10 ml would be B% of A. Let this value be “X”. This X number of iRBC is pelleted as mentioned above in this section and immediately resuspended in Alsever’s buffer at a concentration of 10^7 iRBCs/ml and finally stored at -196 degree Celsius until further use.

2.2.4 Cell counting

For viable cell counting, 10 µl of cell suspension was mixed with 10 µl of trypan blue and put on a Neubauer chamber. The 4 outer squares of the chamber were counted and number of viable cells was calculated using the following formula:

Total cells in 4 squares/ $4 \times 2 \times 10^4 = \text{cells/ml}$

10 µl of the cell suspension was loaded directly in a Neubauer chamber, the outer 4 squares of the chamber were counted and concentration of the cells was calculated using the formula:

Total cells in 4 squares/ 4×10^4 = cells/ml

2.2.5 Parasite strain, infection protocol, and disease assessment

Transgenic *Plasmodium berghei* ANKA clone 15Cy1 expressing green fluorescent protein (PbAGFP) [298] or clone 231c11 expressing luciferase enzyme (PbAluc) [41] were used for infection. Both WT and CD169-DTR mice (6–10 weeks old) were injected i.p. with 100 μ l of iRBCs (10^6 iRBCs) obtained from freshly thawed cryovial stocks cryopreserved in -196 degree Celsius as described in section 2.2.3. Parasitemia and animal survival were monitored over a period of 25 days. Mice were monitored for experimental cerebral malaria symptoms from day 5-14 post infection as per the scoring system described in section 2.2.3. For studies involving the *in vivo* neutralization of IL-10, 200 μ g/mouse anti IL-10 R blocking antibody (BioXCell, West Lebanon, NH, USA) was injected at day -1 (24 hours before infection) prior PbA infection and subsequently day + 4 and +7 post infection.

2.2.6 Measurement of parasite sequestration

WT and CD169-DTR mice were injected with 10^6 RBCs infected with PbAluc. Parasite sequestration was evaluated from day 6–9 post infection. The mice were anaesthetized with ketamine and xylazine prior to an i.p. injection of 200 μ l of D-luciferin potassium salt (Caliper Life Sciences), reconstituted in PBS at a concentration of 5 mg/mL. Mice were sacrificed after 2 min and then intracardially perfused with PBS. Organs (spleen, brain, liver, lungs, kidney, and heart) were removed immediately for subsequent *ex vivo* imaging using the IVIS Spectrum CT imaging system (Caliper Life Sciences). Imaging was performed with a 13.6 cm field of view, medium binning factor, and auto exposure settings. Bioluminescence was quantified on the regions of interest by calculating the average radiance in p/s/cm²/sr using Living Image software version 4.3.1.

2.2.7 Isolation of leukocytes from spleen, brain, liver, kidney, heart, and lungs

Single cell suspensions from harvested mouse organs (spleen, brain, liver, kidney, heart and lungs) were obtained as previously described in [134, 301] and sub section (i) for spleen and sub section (ii) for other organs such as brain, liver, kidney, heart and lungs.

(i) Isolation of leukocytes from spleen

The mouse spleen was excised after carbon dioxide euthanasia, placed in 2 ml eppendorf tube containing 1ml, 2% IMDM, minced into small pieces followed by 60 min

incubation with 100 µg/ml Collagenase D at 37 degree Celsius. The collagenase-digested spleen was homogenized with syringe piston on a 100-µ metallic wire mesh placed on a petri dish. The single cells thus obtained were washed in 2% PBS and transferred to 15 ml centrifuge tubes for centrifugation at 350g for 5 minutes at 4 degree Celsius. The cell pellet was suspended in RBC lysis buffer (0.89% ammonium chloride) and incubated for 10 minutes at R.T. to get rid of RBC. The RBC free cells were pelleted by centrifugation at 350g, 5 minutes at 4 degree Celsius. The cell pellet was suspended in 2% IMDM to get leukocyte enriched splenic single cell suspension.

ii) Isolation of leukocytes from brain, liver, kidney, heart and lungs

For sequestered leukocyte analysis, mouse was first intracardially perfused with 1X PBS. Brain, lungs, kidneys, heart, and livers were excised, minced and then digested with 100 µg/ml Collagenase D for 60 minutes at 37 degree Celsius. The collagenase-digested tissues were homogenized by passing through a 21 guage needle five times followed by further homogenization using syringe piston on a 100-µ metallic wire mesh placed on a petri dish. The single cells thus obtained were washed in 2% PBS and transferred to 15 ml centrifuge tubes for centrifugation at 350g for 5 minutes at 4 degree Celsius. The resulting pellet was further subjected to 33% PercollTM gradient, as described previously [134] and centrifuged at 700g for 12 minutes at 4 degree Celsius. The resulting pellet was subjected to RBC lysis step and all the downstream processing was identical to the steps described in the sub section (ii) of 2.2.7 to obtain leukocyte enriched single cell suspensions.

2.2.8 Flow cytometry, cell sorting and intracellular cytokine staining

Fluorochrome-labeled anti-mouse -CD45, -CD4, -CD8, -CD11b, -F4/80, -Ly6C, and -Ly6G antibodies were purchased from BioLegend (San Diego, CA, USA). Stained cells were analyzed on an LSRII or Fortessa FACS (BD Biosciences, San Jose, CA, USA) and data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). The cell sorting was carried out on a BD FACS Aria instrument (gating strategies and purity are shown in Fig. S5). For extracellular surface receptor stainings all the antibodies were used at a dilution of 1:600 and incubated with cells at 4 degree Celsius for 20 minutes in dark. After the incubation, the stained cells were washed with 200 µl, 2% PBS and subsequently the washed stained cells were pelleted by centrifugation at 350g, 4 degree Celsius for 5 minutes. These stained cells were suspended in 75-100 µl of 1X PBS and acquisition was done by the aforesaid flow cytometer. For intracellular IFN-γ staining,

isolated spleen, brain, liver, and lung T cells were stimulated with Brefeldin A (at a final concentration of 10 $\mu\text{g/ml}$) for 2 h at 37 degree Celsius and stained with anti-CD45, -CD4, and -CD8 specific antibodies (1:600 dilution) at 4 degree Celsius for 20 minutes in dark. For intracellular IL-10 staining, cells were stimulated 2 hours with PMA/Ionomycin (at a final concentration of 10 ng/ml) before the addition of the Brefeldin A and incubated at 37 degree Celsius for 2 hours in a CO₂ incubator. Subsequently, cells were fixed with 200 μl , 2% paraformaldehyde solution and incubated at R.T. for 45-60 minutes or 4 degree Celsius overnight and the fixed cells were pelleted by centrifugation at 350g, 4 degree Celsius for 5 minutes. Thereafter, the fixed cells were permeabilized by 200 μl , 0.5% saponin solution (permeabilization solution) and stained intracellularly with anti-IFN- γ or anti-Granzyme B antibodies (1:600 dilution), incubated at RT for 20 minutes. The stained cells were washed with 200 μl , 1X PBS and suspended in 75-100 μl of 1X PBS for further acquisition by flow cytometer mentioned above. Gating strategies are shown in Fig. S1-5.

Note: Antibody staining process was performed in 96-well V-bottom plates and cells were stimulated in 96-well round bottom plates.

2.2.9 Histological analysis

Spleen, brain, liver, kidneys, heart, and lungs were dissected from infected WT and CD169-DTR mice and fixed in 4% phosphate-buffered paraformaldehyde for 48 h, dehydrated, and embedded in paraffin. Six-micron sections were stained with H&E, or with picosirius red for collagen visualization, and analyzed by bright field light microscopy at 4 $\times$, 20 $\times$, and 100 $\times$ magnifications. Images were obtained using a Nikon Eclipse 80i microscope.

2.2.10 Picrosirius red (PSR) staining

Deparaffinize the slides with 2 changes of xylene for 5 minutes and rehydrate using following series of washes: 100% ethanol 2x5 min, 95% ethanol 2x2 min, 70% ethanol 2x2 min, 50% ethanol 2x2 min and dH₂O 2x2 min in a glass tank. Apply adequate PSR solution to completely cover the tissue section and incubate for 60 minutes. Thereafter rinse the slide in 10 dips of water. Dehydrate and mount in xylene as follows: rinse in 70% ethanol for 30 seconds, 100% ethanol 2x10 dips and finally, put a coverslip on the slide with DPX mounting medium.

2.2.11 Hematoxylin and Eosin (H&E) staining

Deparaffinize the slides with 2 changes of xylene for 10 minutes and rehydrated through the following steps: 2 changes of 100% ethanol for 5 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes. After washing with distilled water sections were submitted to the following steps: staining with hematoxylin solution for 8 minutes, washing in running tap water for 5 minutes, differentiation in 1% acid ethanol solution, washing in running tap water for 1 minute, bluing in Scott's tap water substitute for 30 seconds, washing in running tap water for 5 minutes, rinsing in 90% ethanol, counterstaining on eosin solution for 30 seconds, dehydration through 90% and 100% ethanol for 5 minutes. Slides were finally mounted using a DPX mounting medium.

2.2.12 Immunofluorescence cell staining

For immunofluorescence staining, various organs spleen, brain, kidney, heart, liver were removed, cryopreserved by embedding in Optimal Cutting Temperature compound (O.C.T. Tissue Tek), and stored at -80°C. Six-micron sections were cut and thawed for 20 min at room temperature, and acetone-fixed for 10 min. For the spleen sections were then incubated with FITC-labeled anti-CD169 (1:200 dilution) and biotinylated anti-SIGN-R1 antibodies (1:200 dilution) or Texas Red-labeled anti-CD169 (1:200 dilution) together with FITC-labeled CD31 (1:300 dilution) at 4°C overnight. After washing with 2% PBS, the sections were stained for an additional hour with Texas Red-labeled Streptavidin, and mounted with DAKO fluorescent mounting medium. The above steps were repeated for other organs but staining was done with anti-CD31-FITC (1:300 dilution) and anti-CD169-Texas red (1:200 dilution. Images were obtained using a Nikon Eclipse 80i microscope at 20× magnifications.

2.2.13 Alanine aminotransferase (ALT) Assay

Liver damage was quantified by measuring ALT in the serum using a MaxDiscovery Alanine Transaminase (ALT) Enzymatic Assay Kit (Bio Scientific, Austin, USA) following the manufacturer's instructions.

2.2.14 ELISA measurement of IgM and IgG in BAL fluid

A sandwich ELISA was used to measure total mouse IgG and IgM levels in BAL samples collected from infected WT or CD169-DTR mice. In brief, 96-well ELISA plates (Nunc, Maxisorp) were coated overnight with 100 µL of a 1:400 dilution of either goat anti-mouse IgG or IgM (Southern Biotech, USA) antibody in PBS. After a blocking

step with 2% bovine calf serum in 1X PBS for 1 hour at R.T., plates were incubated overnight at 4°C with collected BAL samples. A 1:3000 dilution of the appropriate conjugate (HRP conjugated anti-IgG or anti-IgM antibodies; Southern Biotech) was prepared and added to each well for 1 h at room temperature. The plates were washed three times with PBS and incubated with TMB substrate (BioLegend, USA) for 10 min. The water-soluble blue reaction product was measured spectrophotometrically at 370 nm.

2.2.15 ELISA measurement of serum cytokines

Serum IFN- γ , TNF- α , and IL-10 levels were quantified using the appropriate ELISA MAX™ Standard kit (BioLegend, USA) according to the manufacturer's instructions.

2.2.16 Measurement of blood-brain barrier (BBB) integrity

BBB permeability was assessed by intravenously injecting 200 μ L of 1% Evans blue/PBS into PbA-infected WT and CD169-DTR mice. The animals were sacrificed 1 h later and perfused intracardially with PBS. Brains were collected, weighed, photographed, and placed in 1 ml of 100% formamide for 48 h to extract the Evans blue dye from the tissue. Absorbance of the formamide extraction was then measured at 620 nm and normalized by the weight of the tissue.

2.2.17 Hemorrhage and Hz quantification

The numbers of hemorrhagic foci in the brain parenchyma were manually counted per brain section under 20 $\times$ magnification. Hz deposits were quantified using the HistoQuest program (TissueGnostics USA Ltd., Tarzana, CA, USA) as follows: bright field images from at least 10 fields of view were acquired at 100X magnification per organ on Nikon Eclipse 80i. The Hz deposit area was quantified and normalized to total cell area in each image using HistoQuest. Each data point represents these normalized hemozoin deposits area values quantified from single fields of view.

2.2.18 Quantitative real-time PCR (qPCR)

Sorted cells (spleen and liver F4/80^{high}CD11b^{int}, CD4⁺, CD8⁺, CD4⁻CD8⁻ cells and liver F4/80^{high}CD11b^{int} and Ly6C^{high} cells) were lysed in TRIzol reagent (Invitrogen) and the total RNA was recovered with help of RNeasy kit (Invitrogen) according to manufacturer's instructions. The RNase free DNase I (Fermentas) was used to remove traces of genomic DNA from all RNA samples. After quantification and quality assessment, purified RNA was used to synthesise single-stranded cDNA using

oligo dT and M-MLV Reverse transcriptase (NEB) according to manufacturer's instructions. Obtained cDNA was diluted to the concentration of 10ng/ul and used for qPCR (Quantitative PCR).

qPCR was performed on cDNA obtained from the sorted cells using SYBR green chemistry (KabaBiosystem, Woburn, WA, USA). Reactions were run on a real-time qPCR system (Illumina, San Diego, CA, USA). Samples were normalized to β -actin, represent the median of triplicate analyses and are displayed as relative expression. Primer sequences were as follows:

IL-10	Fwd: CAGAGCCACATGCTCCTAGA / Rev: TGTCCAGCTGGTCCTTTGTT
TNF- α	Fwd: AATTCGAGTGACAAGCCTGTAG / Rev: TTGAGATCCATGCCGTTGG
β -Actin	Fwd: AAGGCCAACCGTGAAAAGAT / Rev: CCTGTGGTACGACCAGAGGCATACA

2.2.19 Cell-based ELISA for parasite-specific antibodies

For coating with iRBCs, 100 μ L of blood from an infected mouse having about 20% parasitemia were resuspended in 10 mL of 1X PBS and 100 μ L of the mix were aliquoted in each well of a poly-D-lysine coated 96-well plate. The plate was then spun at 300 g for 5 minutes, the supernatant was discarded and cells were fixed by adding 100 μ L of methanol for 10 minutes. After washing 3 times with 1X PBS, 100 μ L of blocking buffer were added to each well to avoid non-specific binding, and incubated at 4 °C over night. After discarding the blocking buffer, triplicates of 1:50 dilutions of serum samples obtained were added to the plate, incubated for 2 hours and discarded. The plate was then washed 3 times with 1X PBS, and incubated for 1 hour with HRP-labeled secondary antibody diluted in blocking buffer. After discarding the secondary antibody and washing 7 times with 1X PBS, 100 μ L of TMB substrate reagent were added to each well. OD at 370 nm was measured every 5 minutes for 30-40 minutes.

2.2.20 Statistical analysis

Survival was analyzed using the Mantel-Cox log-rank test. Statistical significance between two groups was analyzed using the unpaired student *t*-test or Mann-Whitney U test and for more than two groups using a one-way ANOVA followed by Bonferroni test. Statistical significance is demonstrated in the figures with asterisks: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. GraphPad Prism 5.0 was used to analyze the data.

3. Results

To our knowledge, there is a lack of mouse models that facilitate the functional studies of specific macrophage subsets in homeostasis, immune mechanisms at steady state as well as in the context of disease and inflammation. In this direction our lab generated a CD169-DTR mouse model, specifically aimed to study the function of tissue-resident CD169⁺ macrophages across different lymphoid and non-lymphoid tissues in the context of disease, and in this study, blood stage *Plasmodium* infection.

Thorough characterization of the systemic specificity and efficiency of depletion of CD169⁺ macrophages was performed (section 3.1). Thereafter, functional study of this macrophage subpopulation in acute lethal severe malaria mediated by *P. berghei* ANKA (section 3.2) and acute non-lethal uncomplicated malaria induced by *P. yoelii* 17XNL infection (section 3.3) in the context of multifaceted malarial syndrome was undertaken.

3.1 Characterization of depletion in CD169-DTR mouse

Siglec-1 (sialic acid binding IgG like lectin)/CD169/Sn (sialoadhesin) is a cell surface receptor expressed by a specialized subset of macrophages commonly referred as CD169⁺ macrophages, which are seeded in various lymphoid and non-lymphoid organs. In order to investigate the role of CD169⁺ macrophages in the context of disease and inflammation our lab generated a CD169-DTR mouse model wherein human DTR is expressed under CD169 promoter and hence this mouse model should enable systemic and specific depletion of CD169⁺ macrophages upon diphtheria toxin administration. Therefore, we aimed to characterize the expression patterns of CD169 on the different myeloid subpopulations (Section 3.1.1 and 3.1.4), specificity and efficiency of depletion of CD169 expressing cell populations in the spleen, liver, lung, kidney, brain and heart (hereafter, collectively referred as vital organs) as described in Section 3.1.2, the effect of DT mediated modulation on DC subsets in the spleen, liver and lung (Section 3.1.3) and the effect of depletion on architectural integrity in the tissues (Section 3.1.5).

3.1.1 Expression of CD169 on macrophage populations

Macrophages are a heterogeneous group of phagocytic cells with a repertoire of CD11b⁺, F4/80^{hi} F4/80^{int} populations; (Figure 3.1A) hence, we sought to analyze the expression of CD169 on these various subsets in different organs such as spleen, liver, lung and kidney. Flow cytometry analysis confirmed the exclusive expression of CD169 only on

CD11b^{int} F4/80^{hi} cells (fraction 1) and not CD11b^{hi} F4/80^{int} (fraction 2) cells (Figure 3.1A). Fraction 3, which predominantly comprises of CD11b^{hi}F480⁻Ly6G⁺neutrophils, also did not show CD169 expression (Figure 3.1A and B).

Figure 3.1 Expression of CD169 is restricted to F4/80^{hi}CD11b^{int} cells. (A) Flow cytometric analysis showing representative dot plots and histograms showing CD11b, F4/80 and CD169 expression on the F4/80^{hi}CD11b^{int} (fraction 1), F4/80^{int}CD11b^{hi} (fraction 2) and F4/80^{neg}CD11b^{hi} (fraction 3) on the total leukocytes isolated from the spleen, lungs, liver, and kidney of the uninfected WT BALB/c mouse. Open and closed histograms represent isotype-controls and CD169 stained cells respectively. (B) Representative dot plots showing expression of CD11b, Ly6C, Ly6G in fraction 2 and fraction 3. Data are representative of two independent experiments in (A) and (B) with n=3 mice, 6-8 weeks of age.

3.1.2 Specific and systemic ablation of tissue-resident CD169⁺ macrophages in CD169-DTR mouse in all the vital organs

The specificity and depletion efficiency of the CD169⁺ macrophage subset in the CD169-DTR mouse was assessed upon 2 subsequent DT doses (10ng/gbw) at intervals of 24 hours. Flow cytometric analysis of the stained single cell suspensions and immunofluorescence analysis of tissue sections obtained from organs of DT treated WT and CD169-DTR mouse was performed.

As expected, tissue-resident CD169⁺ F4/80^{hi} cells (namely splenic red pulp, marginal metallophilic macrophage, liver Kupffer cell, brain perivascular macrophage, tissue-resident lung, kidney and heart macrophages) are specifically and efficiently depleted in all the analyzed organs obtained from the DT-treated CD169-DTR mouse (Figure 3.2A and B) sparing the CD11b⁺F4/80^{int} monocyte/macrophage cells and CD11b^{hi}F480⁻Ly6G⁺ neutrophils.

Figure 3.2 Tissue-resident macrophages are effectively ablated in the spleen, liver, lung, kidney, brain and heart of DT-treated CD169-DTR mouse during steady state. (A) Flow cytometric analysis of the stained total leukocyte cells isolated from the spleen, liver, lung and kidney of WT and DT-injected CD169-DTR mice showing effective depletion of F4/80^{hi} CD11b^{int} macrophages in CD169-DTR mouse. WT BALB/c and CD169-DTR mice were injected i.p. at day -2 and -1 with 10 ng/gbw DT and analysed at day 0. Representative pseudocolor plots of stained leukocytic cells showing CD11b and F4/80 expression on the leukocytic cells obtained from the spleen, liver, lung and kidney of WT and DT-injected CD169-DTR mice. Numbers show the percentage of each gated myeloid cell subset (tissue-resident macrophages -

F4/80^{hi} CD11b^{int}, monocyte-derived macrophages -F4/80^{int} CD11b^{hi} - and neutrophils - F4/80^{neg} CD11b^{hi}). The bar graph represents percentage of each myeloid cell subset as explained above in WT (white bar) and DT injected CD169-DTR mice (black bar). Data are represented, as Mean $\pm$ S.D. Statistical significance was determined using an unpaired student *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data and results shown are representative of three independent experiments with *n*=3 mice/group, 6-8 weeks of age. (B) Immunofluorescence staining of SIGN-R1⁺ marginal zone macrophages (MZM) and CD169⁺ marginal metallophilic macrophages (MMM) in the spleen of DT injected (10ng/gbw) WT and CD169-DTR mice. Cryostatic sections (6 μ m) of spleens were stained with biotinylated-anti SIGNR-1/Texas Red-Streptavidin and FITC-anti CD169. The sections of brain, liver, kidney and heart were stained with anti CD31-FITC (endothelial cell marker) and anti CD169-texas red (CD169⁺ tissue-resident macrophage subset). Images were acquired at magnification: 20X. Data are representative of two independent experiment, *n*=2 mice/group, 6-8 weeks of age.

The CD169⁺ macrophages have been reported previously to be positioned at specific anatomical sites such as in the splenic marginal zone [173, 244, 246], sinusoidal regions of the liver [302] that enables capture and clearance of blood borne particulate antigens [244, 246] and sialylated pathogens [229, 303]. The presence of these macrophages in close proximity to vasculature as reported previously [232, 304] and shown in Figure 3.2B in the kidney, brain and heart most likely suggests their involvement in capture of blood borne particulate material/pathogen such as *Plasmodium*.

The CD169-DTR mouse exhibits transient mild monocytosis and neutrophilia (Figure 3.2A), a feature of DTR transgenic mouse as previously reported [295]. This occurs only at earlier stages of the depletion protocol and it is resolved at the later stages (data not shown). It is important to note that the disappearance of the SIGN-R1⁺ CD169⁺ marginal zone macrophage (Figure 3.2B) is due to the structural changes in the spleen and not a direct consequence of DT mediated ablation of cells. The structural changes in the spleen have been previously reported to be associated with maintenance of marginal zone integrity [305]. In addition, chemokine signaling by CC chemokine ligand 21 has been shown to regulate the localization of SIGN-R1⁺ macrophages in the splenic marginal zone [306]. Therefore, these reports suggest crosstalk between splenic cells is important for the destined cellular localization in the spleen. It is possible that depletion of red pulp macrophages (RPMs) results in unidentified aberrant chemokine signaling that led to disappearance of the MZMs. The resultant disappearance of SIGN-R1⁺ macrophages is not DT mediated because in a Clec4a4-DTR mouse model that enables ablation of CD11b⁺ DCs and RPMs (data not shown), we observed a similar disappearance of MZMs but not MMMs (Figure S1). If disappearance of SIGN-R1⁺ macrophages would have been a direct result of DT depletion, then in Clec4a4-DTR strain, MMMs should also be ablated, which is not the case. Therefore, aforesaid studies and our observations in CD169-DTR and Clec4a4-DTR mouse strain leads to a possible interpretation that the

absence of RPM intrinsic unknown factor in CD169 macrophage deficient mouse regulate the maintenance of SIGN-R1⁺ macrophages in the splenic marginal zone.

3.1.3 Depletion of tissue resident CD169⁺ macrophages did not affect the dendritic cell subsets in the spleen, lung and liver of the CD169-DTR mouse

Expression of CD169 has been reported on a subset of myeloid cells expressing high levels of CD11c MHCII, which shows resemblance to CD8⁺ DCs in the lymph nodes (subcapsular sinus and mesenteric) [307] and further confirmed in another subsequent study [308]. Therefore, we sought to evaluate the effect of DT mediated ablation on DC subsets in the lymphoid organ (spleen) and non-lymphoid organs (lung and liver) of DT injected CD169-DTR mouse after two subsequent DT dose of 10ng/gbw at intervals of 24 hours. Flow cytometry analysis of the isolated and stained leukocytes from spleen, lung and liver showed no effect on the DC subsets in the tested organs. There were no significant differences observed on CD8⁺ and CD11b⁺ DCs in the spleen gated on CD45⁺ CD11c⁺ MHCII⁺ cells (Figure S2). Similar results were also observed for the CD103⁺ and CD11b⁺ DC subsets in the lung and liver as well (Figure S2). In conclusion, DT mediated ablation of tissue resident CD169⁺ macrophages did not affect the DC subsets in the tested organs of CD169-DTR mouse and thus provides a CD169⁺ macrophage specific depletion mouse model for the study of their function in inflammation and disease settings.

3.1.4 Expression of CD169 and depletion of CD169⁺ macrophage upon Plasmodium infection

Plasmodium (PbA) infection does not influence the CD169 expression patterns on different myeloid cell subsets such as CD11b^{int} F480^{hi} macrophages (fraction 1), CD11b⁺F4/80^{int} monocyte/macrophage cells (fraction 2), CD11b^{hi}F480⁻Ly6G⁺ neutrophils (fraction 3), mixture of myeloid and lymphoid CD11b^{int} F480⁻ cells (fraction 4 that comprises mixture of DCs, B cells and NK cells; data not shown) and non-myeloid CD11b⁻F480⁻ (fraction 5 that comprises T cells and other immune cells; data not shown) in the liver, lung and kidney at day 4 (Figure S3) and 8 post infection as shown in Figure 3.3A. Therefore, the dynamics of CD169 expression on different myeloid and non-myeloid cellular fractions as mentioned above remains unchanged and CD169 is specifically expressed by CD11b^{int} F480^{hi} subset during the course of *Plasmodium* infection. The depletion patterns remain unperturbed and tissue-resident CD169⁺ macrophage subsets are efficiently depleted during *Plasmodium* infection as evident by

the flow cytometry and immunofluorescence analysis of PbA infected WT and CD169-DTR mouse (Figure 3.3A and B).

Figure 3.3 Unperturbed CD169 expression on different myeloid cell subsets in liver, lungs and kidney and efficient ablation of tissue-resident macrophages in all the tested organs during *Plasmodium* (PbA) infection. (A) Representative dot plots and histograms of the stained leukocytic cells showing CD11b, F4/80, and CD169 expression on different myeloid cell subsets (fraction 1 to 3 based on differential expression of the above cell surface markers), mixture of myeloid and lymphoid cells (fraction 4 comprising DCs, B cells and NK cells; data not shown) non-myeloid CD11b⁺F4/80⁺ cells (fraction 5 comprising T and other immune cells; data not shown) obtained from lungs, liver and kidney of PbAGFP infected and DT treated (10ng/gbw) WT and CD169-DTR mice at day 8-post infection. Blue and red histograms represent isotype-controls and CD169 stained cells respectively. Data are representative of two independent experiments, n=3 mice/group, 6-8 weeks of age. (B) Immunofluorescence images of anti CD31-FITC and anti CD169-texas red stained tissue sections (6 μ m cryostatic sections) obtained from the brain, liver and kidney of PbAGFP infected WT and CD169-DTR mice at day 8-post infection. CD169⁺

macrophages (Red), CD31⁺ endothelial cells (Green). Images were acquired at magnification: 20X. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks of age.

3.1.5 Long term DT administration does not have an effect on architectural integrity of the tissues in the vital organs

In order to evaluate the long term effect of DT mediated depletion of tissue-resident CD169⁺ macrophage subpopulation on the architectural integrity of the tissues (brain, spleen, liver, lung, kidney and heart), WT and CD169-DTR mice were subjected to DT injections every 3-4 days over a period of two weeks and histological analysis was performed by H&E staining. Long term DT mediated depletion of CD169⁺ macrophage did not have any major effect on the architectural integrity (Figure 4B, C and D) including blood brain barrier integrity (Figure 3.4A) in the vital organs of CD169-DTR mouse as observed by H&E stained tissue sections. The infiltration of monocytes and neutrophils (Figure 3.2A) at initial stages of DT administration was also abolished confirming the transient nature of DT mediated effect on cellular infiltrations (Figure 3.4A-D) comprising monocytes and neutrophils, in particular (data not shown).

Notably, ablation of tissue-resident CD169⁺ macrophage resulted in disappearance/collapse of marginal zone but no other changes in the red pulp and white pulp regions of the spleen (Figure 3.4B).

3.1.6 Summary of specificity and depletion efficiency of CD169-DTR mouse model

Here, we confirm that the CD169⁺ macrophages are stationed at specific micro-anatomical location in the marginal zone of the spleen, hepatic sinusoids and close proximity to the vasculature of kidney, brain and heart as previously reported [229, 232, 244, 246, 304]. This privileged micro-anatomical niche, most likely enables them to capture blood-borne pathogens and facilitate subsequent clearance [173, 302, 309]. Furthermore, the CD169-DTR mouse model serves as an efficient tool for specific depletion of CD169⁺ F4/80^{hi} macrophages throughout the system in steady state without affecting the DC subsets in the tested organs. The efficiency and specificity of depletion is also maintained during *Plasmodium* infection thus enabling tissue specific studies pertaining to the role of tissue-resident macrophages in modulating immunity in inflammation and in the context of *Plasmodium* infection. Lastly, long-term depletion of these macrophages does not have any consequence on tissue integrity.

Figure 3.4 Long term DT mediated ablation of tissue-resident CD169⁺ macrophages does not affect the architectural integrity of the tissues in the vital organs during steady state. Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from (A) brain, (B) spleen, (C) liver (left panels) and lung (right panels) and (D) kidney (left panels) and heart (right panels) of DT-treated (10ng/gbw) WT (top panels) and CD169-DTR (bottom panels) at every 3-4 days over a duration of two weeks. Images were acquired at magnification: 4X, 20X. Scale bars are represented on the individual images. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks of age.

3.2 Elucidating the role of tissue-resident CD169⁺ macrophage in *Plasmodium berghei* ANKA (PbA) infection associated malarial syndromes.

Tissue-resident CD169⁺ macrophages are distributed across lymphoid and non-lymphoid organs, therefore we sought to investigate the role of these cells in the outcome of *P. berghei* ANKA (PbA) infection and their implication in the malarial syndromes such as anemia, cerebral malaria as well as acute liver, lung, kidney and heart injury.

3.2.1 PbA infection results in enhanced susceptibility of CD169-DTR mice

The survival and parasitemia was monitored in WT and CD169-DTR mice (BALB/c background) during the entire course of PbA infection to assess the effect of tissue-resident CD169⁺ macrophage on the outcome of malaria (Figure 3.5A). The parasitemia rose steadily in both the groups during early phase of infection (3-7 dpi) with significant differences observed at the middle phase of infection (8-10 dpi) wherein CD169-DTR group showed higher parasite load in the peripheral blood (Figure 3.5A). The parasitemia levels continued to surge in WT group reaching a plateau in the range of 55-60% with continuous decrease in RBC and hemoglobin levels indicative of anemia, the likely reason for their mortality (Figure 3.5A and 3.5E). The dramatically increasing levels of peripheral blood parasitemia beyond day 10 post infection in the PbA infected WT mouse likely resulted in massive destruction of RBCs leading to their anemic phenotype. On the contrary, the parasitemia levels in CD169-DTR mouse began to decline beyond day 9-post infection (Figure 3.5A, right panel). During this phase of infection, CD169-DTR mouse experienced elevated levels of parasite sequestration in different organs tested (Figure 3.5B and 3.5C) and succumbed to PbA infection (Figure 3.5A, left panel). Unexpectedly, PbA infected CD169-DTR mouse did not show anemia despite high levels of parasite load in the organs until day 10 post infection (Figure 3.5E) and anemic parameters could not be measured beyond day 10 post infection as the mouse became dehydrated, moribund and more importantly, increased viscosity of the blood in the cohort of mouse that survived beyond day 10 post infection precluded the withdrawal of requisite amount of blood without clotting for the monitoring of anemia.

In order to evaluate the possible parasite sequestration as the likely reason for decline of parasite load in circulating blood of CD169-DTR group, we exploited PbA expressing bioluminescent luciferase reporter (PbALuc) to investigate the kinetics of iRBC sequestration. Real time *ex vivo* bioluminescence imaging was performed upon PbALuc infection in both the groups from day 6-9-post infection. The bioluminescence in the

organs is reflective of parasite sequestration. CD169-DTR group showed significantly higher iRBC sequestration in brain, spleen, liver and heart at day 8 and 9 post infection (Figure 3.5B-D). This confirmed that the lower iRBCs level in peripheral blood of CD169-DTR was possibly due to parasite sequestration in the vital organs.

Figure 3.5 CD169⁺ tissue-resident macrophage deficient CD169-DTR mice are more susceptible to PbA infection and exhibit exacerbated parasite sequestration. (A) Survival (left) and percentage parasitemia (right) of PbAGFP-infected and DT treated (10ng/gbw) WT controls (white circles) and CD169-DTR mice (black circles). Statistical significance of survival was analyzed using the Mantel-Cox log-rank test at **p < 0.01 and statistical significance of parasitemia was determined using a Mann-Whitney U test at *p < 0.05, **p < 0.01 and ***p < 0.001. Data are cumulative of two independent experiments with n = 6–8 mice/group in each independent experiment, 6–8 weeks of age. (B–C) Quantification of average bioluminescence as a measure of *ex vivo* iRBCs sequestration in the indicated organs of PbALuc-infected and DT treated (10ng/gbw) WT and CD169-DTR mice. (B) Pseudocolor

images of brain, spleen, liver, lungs, kidney, and heart collected from a representative PbA-infected WT and CD169-DTR mouse on day 9. (C) Quantification of daily bioluminescence imaging (from day 6–9 post infection) during PbA infection is represented in form of bioluminescence kinetics graph. Each data point represents the mean average radiance (p/sec/cm²/sr) values (log) $\pm$ S.D. of 6–9 mice/group. White circles: WT; black circles: CD169-DTR mice. Statistical significance was determined using an unpaired student *t*-test. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. Data are cumulative of two independent experiments with *n*=3–5 mice/group/infection time point, 6–8 weeks old. (D) Differences in parasite sequestration in distinct organs between PbA-infected WT and CD169-DTR mice. Fold change in bioluminescence activity is shown for day 8 and 9 post infection as shown in (C). (E) Anemia profile in PbA-infected WT and CD169-DTR mice. Levels of RBC numbers/L (left panel) and hemoglobin in g/L (right panel) in peripheral blood were determined from day 6 until day 10 post infection for CD169-DTR mice and until day 14 post infection for control mice. Data are presented as Mean $\pm$ SD for 4 mice per group. Statistical significance was determined using an unpaired student *t*-test at **p* < 0.05, ***p* < 0.01. Data are cumulative of two independent experiments with *n*=3–4 mice/group/infection time point, 6–8 weeks old.

3.2.2 Exacerbated deposition of hemozoin (Hz) in the vital organs of CD169-DTR mice during PbA infection

Plasmodium infection results in accumulation of brown Hz crystals (a heme detoxification product) in the tissues during the process of iRBCs rupture, which is rapidly internalized predominantly by monocytes and macrophages. High levels of Hz correlate with elevated parasite burden in tissues, tissue inflammation and severity of the pathogenesis during malaria [310].

Figure 3.6 Exacerbated hemozoin deposits in the spleen, brain, liver and lung of PbA-infected CD169-DTR mouse. Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from spleen, brain, liver and lungs of DT-treated (10ng/gbw) and PbAGFP-infected WT (left panels) and CD169-DTR (right panels). Images were acquired at 100X magnification (Scale bar: 50 μ m). Quantification of a series of 4-6 images/organ/mouse/group are represented in the form of scatter plot graph as the ratio of area of the hemozoin deposits over the tissue area as analyzed by Histoquest imaging software. Statistical significance was determined using an unpaired student *t*-test at **p* < 0.05, and *****p* < 0.0001. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks of age.

To investigate the correlation between the enhanced parasite burden as assessed by bioluminescence (Figure 3.5B-C) and the Hz deposition, H&E stained tissue sections obtained from PbA infected WT and CD169-DTR mice were analyzed by bright field microscopy. Gross observation of the magnified images as shown in Figure S4 as well as quantification of the acquired H&E images (Figure 3.6) by the HistoQuest (image processing software) showed exacerbated Hz deposits in all the tested organs of PbA infected CD169-DTR mice. This confirms the direct correlation of the parasite burden and Hz accumulation in the tissues thus suggesting the crucial role of tissue-resident CD169⁺ macrophage in containing the parasite dissemination in the spleen, brain, liver and lungs (Figure 3.6) and to a lesser extent kidney and heart (Figure S4). Notably the hemozoin deposition in the kidney and heart was extremely sparse and therefore not quantified.

3.2.3 Compromised splenic immune effector mechanisms and onset of pathology in CD169-DTR upon PbA infection

Spleen is the central lymphoid organ that not only orchestrates balanced innate and adaptive anti *Plasmodium* immune cascades but also facilitates removal of senescent RBCs through phagocytic action of diverse macrophage subsets in the red pulp and marginal zone. In this context, we sought to understand the contribution of the splenic CD169⁺ MMMs, CD169⁺ RPMs in the process of parasite containment through histology and flow cytometry analysis for the ensuing pathogenesis.

H&E staining was done on the splenic sections obtained from PbA infected WT and CD169-DTR mice at day 8-post infection and subsequent microscopic analysis were performed. In the PbA infected control WT mice, the histological analysis highlighted marginal zone atrophy with a clear zone of white pulp, expansion of red pulp suggestive of extramedullary hematopoiesis (EMH), moderate cellular infiltration, minimal deposition of Hz crystals and distension of splenic sinusoids with blood including few iRBCs (Figure 3.7 A, left panels). On the contrary CD169-DTR mice depicted fusion of white pulp suggestive of a marginal zone collapse, lymphoid hyperplasia, expanded red

pulp with significant infiltration of leukocytes, mild EMH, significantly elevated levels of Hz deposits (3.4 fold higher relative to the infected WT, see Figure 3.7B and 3.6), moderate distension of splenic sinusoids with blood cells containing proportionately higher levels of iRBCs (Figure 3.7A, right panels). Notably, DT mediated ablation of splenic tissue-resident $CD169^{+}$ macrophages resulted in collapse of the marginal zone in $CD169$ -DTR mice in contrast to WT mice during steady state (Figure 3.4B).

Figure 3.7 Histological and cellular analysis of spleens in WT and $CD169$ -DTR mice after PbAGFP infection. (A) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the spleens of WT and $CD169$ -DTR mice on day 8 post PbA-GFP infection. Scale bars: 1 mm (upper panel) and 100 μ m (middle panels). Lower panels show magnification of areas to depict Hz deposits. Data are representative of two independent experiments with $n=2$ mice/group, 6-8 weeks old mice. (B) Dot chart shows the relative fold difference of Hz (from Figure 3.6) in the spleen of infected control

(white circles) and CD169-DTR mice (black circles). (C) Mean of IFN- γ $\pm$ S.D. measured in the serum of WT (white bars) and CD169-DTR (black bars) infected with PbAGFP at day 8 ($n = 4-8$ mice/group, 6-8 weeks old mice). Statistical significance was determined using an unpaired student t -test. $*p < 0.05$. Data are representative of two independent experiments. (D) Augmented numbers of inflammatory Ly6C^{hi} and Ly6G^{hi} cells in the spleen of PbAGFP-infected CD169-DTR mice. Bar charts represent mean absolute numbers $\pm$ S.D. of Ly6C^{hi} and Ly6G^{hi} cells in the spleens of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at days 6 and 8 post infection ($n = 4$ /group, 6-8 weeks old mice). Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at $*p < 0.05$, and $**p < 0.01$. (E) Bar charts represent mean absolute numbers $\pm$ S.D. of CD69⁺CD4⁺ and CD69⁺CD8⁺ T cells (gated on CD3⁺), as well as IFN- γ -producing CD4⁺ T cells and Granzyme B-expressing CD8⁺ T cells, in the spleens of uninfected WT (grey bars), PbAGFP infected WT (white bars), and infected CD169-DTR mice (black bars) at day 8 post infection ($n = 4$ /group, 6-8 weeks old mice). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test at $**p < 0.01$, $**p < 0.001$. Data in (D) and (E) are representative of two independent experiments with $n=3-4$ mice in each independent experiment. Note: NS=Not significant.

Flow cytometry based cell profiling revealed significantly higher proportions and numbers of infiltrating leukocytic cells comprised inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils in the spleens of PbA infected CD169-DTR mouse relative to WT mouse at day 6 and 8 post infection (Figure S5 C, D and 3.7D). Conversely, the numbers of CD8⁺ T cells expressing CD69 (early activation marker) were reduced whereas the numbers of CD4⁺ T cells expressing CD69 remained unaffected in the PbA infected CD169-DTR mouse relative to WT counterparts at day 8 post infection (Figure S5A and 3.7E). On a similar note, the proportion and numbers of IFN- γ secreting CD4⁺, CD8⁺ T cells and Granzyme B expressing CD8⁺ T cells were also significantly reduced in CD169-DTR mouse relative to the WT counterparts (Figure S5B and 3.7E). Therefore, PbA infected CD169-DTR mouse exhibited attenuated T cell mediated antimalarial immune response. The decreased numbers of IFN- γ producing T cells even resulted in alleviated levels of serum IFN- γ in the CD169-DTR mouse (Figure 3.7C).

3.2.4 Tissue-resident CD169⁺ macrophage depletion results in development of ECM in PbA infection

Brain perivascular macrophages express CD169 and since our CD169-DTR mouse enables depletion of CD169⁺ expressing macrophages, we examined the role of these cells in the outcome of ECM development. Cerebral malaria studies are generally performed on ECM susceptible mouse strains (C57BL/6J) with permissive parasite species such as PbA. Parasite and leukocyte sequestration (in particular CD8⁺ T cells) in the brain microvasculature have been reported in several studies to be clearly associated with the onset of neurological syndromes in PbA and C57BL/6J (rodent parasite & host) ECM model [19, 311, 312].

Figure 3.8 PbA infection causes experimental cerebral malaria (ECM) in CD169-DTR mice. (A-B) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from brains of WT and symptomatic CD169-DTR mice on day 8 post PbA-GFP infection. Intravascular sequestration of iRBCs and leukocytes with local hemorrhagic foci are only detected in the brains of CD169-DTR mice. Scale bars: (A) 500 μ m (left panels) and (B) 100 μ m (right panels). Images are representative of two independent experiments with n=2-3 mice/group, 6-8 weeks old mice. (C) Mean number of hemorrhagic foci $\pm$ S.D. per brain in WT and CD169-DTR mice. Statistical significance was determined using an unpaired student *t*-test at ****p* < 0.001. Data represented are cumulative of two independent experiments with n=2-3 mice/group, 6-8 weeks old mice. (D) Dot chart shows the relative fold difference of Hz (from Figure 3.6) in the brains of infected control (white circles) and CD169-DTR mice

(black circles) (E) Severity of ECM determined by RMCBS scoring in WT and CD169-DTR mice ($n = 8$ /group, 6-8 weeks, old mice) infected with PbA-GFP, dash line indicates the threshold scores below which mice show distinct neurological signs characteristic of ECM. RMCBS values of the ECM-susceptible C57BL/6 mouse strain are included. Data represented are cumulative of two independent experiments. (F) Augmented cellular sequestration of total leukocytes in PbA-GFP infected CD169-DTR brains (black bars) compared with uninfected (grey bars) and PbA-GFP infected WT mice (white bars) ($n = 4$ mice/group, 6-8 weeks old mice). Data are represented as mean $\pm$ S.D. Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at $**p < 0.01$ and $****p < 0.0001$. (G) Augmented accumulation of inflammatory $Ly6C^{hi}$ and $Ly6G^{hi}$ cells in the brain of PbAGFP-infected CD169-DTR mice. Total numbers of $Ly6C^{hi}$ and $Ly6G^{hi}$ cells (gated on $CD45^{hi}$ cells) in the brains of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at days 6 and 8 post infection ($n = 4$ mice/group, 6-8 weeks old mice). Data are represented as mean $\pm$ S.D. Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at $**p < 0.01$, $***p < 0.001$ and $****p < 0.001$. (H) Enhanced brain-sequestered effector $CD4^{+}$ and $CD8^{+}$ T cells in PbAGFP-infected CD169-DTR mice. Total numbers of brain-sequestered $CD4^{+}$ and $CD8^{+}$ T cells (gated on $CD3^{+}$), as well as IFN- γ -producing $CD4^{+}$ T cells and Granzyme B-expressing $CD8^{+}$ T cells, in uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at day 8 post infection ($n = 4$ mice/group, 6-8 weeks old mice). Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at $*p < 0.05$, $**p < 0.01$. Data shown in F to H are representative of two independent experiments with $n=3-4$ mice/group. (I) Evaluation of the blood-brain barrier integrity in PbAGFP-infected WT and CD169-DTR mice at the onset of neurological symptoms. At day 8-9 post infection, mice were injected with 0.1% Evans blue dye 60 min prior to dissection. Representative images of brains from PbA-infected WT and CD169-DTR are shown. Evans blue dye was extracted from brains using formamide, and absorption of the extract was measured at 620 nm ($n = 3-6$ mice/group, 6-8 weeks old mice). Data are represented, as mean $\pm$ S.D. Statistical significance was determined using an unpaired student t -test. $*p < 0.05$. Data are representative of two independent experiment with $n=3-6$ mice/group.

Conventionally PbA infected BALB/c WT mice are ECM resistant, as they do not sustain characteristic ECM related neurological symptoms and damages in the brain (Figure 3.8A-C) and die beyond the ECM window (day 5-14 post infection) due to hyperparasitemia and malarial anemia at the later stages of the infection (Figure 3.5A and 3.5E). Histological analysis on the brain at day 8 post infection confirmed the ECM resistant nature of BALB/c WT mice as they did not show any vascular leakage (Figure 3.8I), hemorrhage foci across different anatomical regions in the brain (Figure 3.8A and B, left panels) and congested blood vessels (Figure S6), which are signature hallmarks of ECM.

Conversely, PbA challenged CD169-DTR group surprisingly displayed typical ECM signs exemplified by unique jumping behavior, ataxia, loss of motor reflexes, retinopathy, coma and eventually succumbed to infection. This, in tandem is reflected in the relatively lower RMCBS scores (disease score) in CD169-DTR mice as compared BALB/c WT (Figure 3.8E). For reference the RMCBS scores of ECM susceptible WT (C57BL/6J X BALB/c) is also depicted in Figure 3.8E. The observable ECM neurological signs corroborate well with marked occlusion of vessels (containing iRBCs and leukocytes, see Figure S6), widespread and notable hemorrhage foci observed across different regions in the brain (Figure 3.8A-B, right panels and C) and marked Hz

deposits (7.4 fold difference relative to the infected WT mouse, see Figure 3.8D and 3.6) in the brain of CD169-DTR mouse.

Flow cytometry profiling and analysis of brain cells revealed elevated numbers of sequestered CD45⁺ leukocytes (Figure S7A and 3.8F) subsuming inflammatory cells such as Ly6C^{hi} monocytes and Ly6G⁺ neutrophils in CD169-DTR mice at day 6 and 8 post infection (Figure S7C, D and 3.8G). In addition, numbers of CD4⁺, CD4⁺, IFN- γ secreting CD4⁺, CD8⁺ T cells and Granzyme B expressing CD8⁺ T cells were also significantly increased in CD169-DTR mice relative to the WT counterparts at day 8 post infection (Figure S7A, B and 3.8H).

Parasite sequestration and leukocyte sequestration in the brain vasculature is a consequence of perturbed blood barrier integrity during ECM; hence vascular leakage in the PbA infected brain of both the groups were assessed through Evans blue dye extrusion assay. Consistent with our afore-mentioned observations the brains of PbA infected CD169-DTR mice showed exacerbated vasculature leakiness suggesting a breach of blood brain barrier (Figure 3.8I). In conclusion, ablation of tissue-resident CD169⁺ macrophage renders PbA challenged CD169-DTR mice susceptible to ECM suggesting their pivotal role in maintenance of blood brain barrier integrity.

3.2.5 Ablation of CD169⁺ Kupffer cells in liver causes acute liver injury in CD169-DTR mice during PbA infection

Liver-resident CD169⁺ (Kupffer) cells play an important role in exerting anti *Plasmodium* immune responses through phagocytic clearance of iRBCs, hence, we aimed to investigate the role of these macrophages in the outcome of liver pathology in CD169-DTR mice. Consistent with the observations in the spleen, flow cytometry based cell profiling indicated that the numbers of CD4⁺ and CD8⁺ T cells expressing CD69 (an early activation marker) were reduced (Figure S9A and 3.9A) in PbA infected CD169-DTR mouse. Likewise, the proportion and numbers of IFN- γ secreting CD4⁺ and CD8⁺ T cells and Granzyme B expressing CD8⁺ T cells were also significantly reduced in CD169-DTR group relative to the WT controls at day 8 post infection (Figure S9B and 3.9A). Thus, PbA infected CD169-DTR mice exhibit attenuated T cell mediated antimalarial effector mechanisms. Conversely, significantly higher numbers of infiltrating inflammatory cells comprising of inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils were detected in the liver of PbA infected CD169-DTR mice relative to WT mice at day 6 and 8 post infection (Figure S9C, D and 3.9B).

Figure 3.9 Depletion of Kupffer cell leads to malaria-induced liver damage. (A) Liver effector CD4⁺ and CD8⁺ T cells in PbAGFP-infected WT and CD169-DTR mice. Bar charts represent absolute numbers as mean $\pm$ S.D. of CD69⁺CD4⁺ and CD69⁺CD8⁺ T cells (gated on CD3⁺), as well IFN- γ -producing CD4⁺ T cells and Granzyme B-expressing CD8⁺ T cells, in the liver of uninfected WT (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at day 8 post infection. (n = 4 mice/group, 6-8 weeks old mice). Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at *p < 0.05, ****p < 0.0001. (B) Augmented infiltration of inflammatory Ly6C^{hi} and Ly6G^{hi} cells in the livers of PbAGFP-infected CD169-DTR mice at day 6 and 8-post infection. Bar chart represents mean total numbers $\pm$ S.D. of Ly6C^{hi} and Ly6G^{hi} cells in the livers of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) (n= 4 mice/group, 6-8 weeks old mice). Statistical significance was determined using an one-way ANOVA followed by Bonferroni test *p < 0.05, ****p < 0.0001. Data shown in A and B are representative of two independent experiments with n=3-4 mice/group in each experiment. (C) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the livers of PbA-infected WT and CD169-DTR mice at day

8-post infection (n=2-3 mice/group, 6-8 weeks old mice). Scale bars: 500 μ m (left panels) and 10 μ m (right panels). Pictures on the right-hand side show magnification of areas to depict Hz deposits. Dot chart shows the relative fold difference of Hz (from Figure 3.6) in the livers of infected control (white circles) and CD169-DTR mice (black circles). Data are representative of two independent experiments with at least 2 mice/group in each experiment. (D) Serum alanine transaminase (ALT) levels of WT (white circles) and CD169-DTR mice (black circles). Values represent mean ALT levels $\pm$ S.D. in units/L (n = 7/group, 6-8 weeks old mice). Statistical significance was determined using an unpaired student *t*-test at $**p < 0.01$. Data shown are cumulative of two independent experiments with n=3-4 mice/group in each independent experiment. (E) Representative bright field microscopic images of picrosirius red stained paraffin tissue sections (6 μ m) for collagen visualization obtained from the livers of PbAGFP-infected WT and CD169-DTR mice (n=2 mice/group, 6-8 weeks old mice). Scale bars: 50 μ m. Data shown are representative of two independent experiments with n=2 mice/group in each independent experiment. (F) Vascular permeability in the liver is increased in PbAGFP-infected CD169-DTR mice. Values in the dot chart represent the mean optical density $\pm$ S.D. of Evans blue dye extracts measured at 620 nm (n = 3-6/group). Statistical significance was determined using an unpaired student *t*-test at $*p < 0.05$. Data shown are representative of two independent experiments with n=3-6 mice/group in each independent experiment. Note: NS means not significant.

Histological findings depict enhanced cellular infiltrates in the hepatic sinusoids of PbA-infected CD169-DTR mice (Figure 3.9C, lower panels) relative to the WT controls (Figure 3.9C, upper panels). An exacerbated Hz deposition is observed within the hepatic sinusoids (Figure S8) as well as tissue parenchyma of the infected CD169-DTR mice in comparison to the WT mice (Figure 3.9C). The hemozoin deposits in the livers of PbA-infected CD169-DTR mice was quantitatively 1.4 fold higher relative to the WT counterparts (Figure 3.9C and 3.6). Notably the blood vessels (Figure 3.9C) and hepatic sinusoids of PbA-infected CD169-DTR mice were remarkably occluded (Figure S8) when compared to the infected WT mice.

Higher parasite and Hz burdens leads to inflammation and tissue injury, therefore we evaluated serum alanine transaminase (ALT) levels as a measure of the degree of liver injury. Expectedly, PbA-infected CD169-DTR mice showed significantly higher ALT levels when compared to infected WT mice at day 6-post infection, an evidence of acute hepatic injury in the mice lacking tissue-resident CD169⁺ macrophages (Figure 3.9D). Since accumulation of the collagen is an indicator of tissue fibrosis and injury we performed picrosirius red (PSR) staining on the liver tissue sections to probe collagen deposition. PSR staining revealed mild collagen deposition around the blood vessels as well as the hepatic parenchyma of the infected CD169-DTR mice suggesting liver fibrosis (Figure 3.9E, left panels), this was not the case in the livers collected from WT counterparts (Figure 3.9E, right panels). In addition, infected CD169-DTR mice showed significantly higher vascular permeability as observed by Evans blue extrusion assay (Figure 3.9F). All these evidences suggest that liver CD169⁺ Kupffer cells play a pivotal role in preventing acute liver injury during PbA infection.

3.2.6 Absence of CD169⁺ alveolar macrophages in lung causes acute lung injury (ALI) in CD169-DTR mice during PbA infection

ALI is a pathological manifestation of severe malaria and its underlying cellular and pathological mechanisms remains elusive. Therefore we sought to investigate the role of CD169⁺ alveolar macrophages by utilizing our CD169-DTR mouse model, which enables us to specifically ablate this macrophage subset. We exploited this mouse model to study the impact of CD169⁺ deficiency on the resulting ALI, if any.

Figure 3.10 PbA infection causes lung inflammation and pathology in CD169-DTR mice. (A) Macroscopic view of lungs collected from PbAGFP-infected WT and CD169-DTR mice (day 8 post infection) (B) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μm) obtained from the lungs of PbAGFP-infected WT and CD169-DTR mice at day 8-post infection (n=2-3 mice/group, 6-8 weeks old mice). Scale bars: 10 μm. Dot chart shows the relative fold difference of Hz (from Figure 3.6) in the lungs of infected control (white circles) and CD169-DTR mice (black circles). Data are representative of two independent experiments with at least 2 mice/group in each experiment. (C) Augmented infiltration of inflammatory Ly6C^{hi} and Ly6G^{hi} cells in the lungs of PbAGFP-infected CD169-DTR mice. Bar chart represents mean total number ± S.D. of Ly6C^{hi} and Ly6G^{hi} cells in lungs of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at days 6 and 8 post infection (n = 4 mice/group, 6-8 weeks old mice). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test at **p < 0.01, ***p < 0.001 and ****p < 0.0001. (D) Lung effector CD4⁺ and CD8⁺ T cells in WT and CD169-DTR mice. Bar charts represent mean total numbers ± S.D. of CD69⁺CD4⁺ and CD69⁺CD8⁺ T cells (gated on CD3⁺), as well as IFN-γ-producing CD4⁺ T cells and Granzyme B-expressing CD8⁺ T cells, in lungs of uninfected WT (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at day 8 post infection. (n = 4 mice/group, 6-8 weeks old

mice). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test at $**p < 0.01$, $***p < 0.001$. Data shown in C and D are representative of two independent experiments with $n=3-4$ in each independent experiment. (E) Determination of IgM and IgG levels by ELISA in bronchoalveolar lavage (BAL) fluid as biomarkers for pulmonary vascular leakage. BAL was collected from PbAGFP-infected WT (white circles) and CD169 DTR mice (black circles) at day 6-post infection. Data are represented as mean optical density $\pm$ S.D. ($n \geq 7/\text{group}$). Statistical significance was determined using an unpaired student *t*-test. $**p < 0.01$, $***p < 0.001$. Data shown are cumulative of two independent experiments with $n=3-4$ in each independent experiment. Note: NS means not significant.

The lungs of PbA infected CD169-DTR mice were strikingly darker indicating massive H₂O₂ deposition relative to the WT mice (Figure 3.10A) at day 8 post infection which is very well reflected in the microscopic analysis of H&E stained lung sections and quantified H₂O₂ deposits (Figure 3.10B, Figure 3.6). The hemozoin deposits in the lungs of PbA-infected CD169-DTR mice was quantitatively 3.8 fold higher relative to the WT counterparts (Figure 3.10B and 3.6). Histology analysis revealed enhanced infiltration of leukocytes in the lung tissue of PbA infected CD169-DTR mice (Figure 3.10B), which was confirmed by flow cytometry analysis revealing increased numbers of infiltrating inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils at day 6 and 8 post infection (Figure S10C, D and 3.10C). Similar to the spleen and liver, the numbers of IFN- γ secreting CD4⁺ CD8⁺ and T cells and Granzyme B expressing CD8⁺ T cells in the lungs were significantly reduced in the CD169-DTR mouse relative to the WT controls at day 8 post infection (Figure S10A, B and 3.10D). In addition, IgG and IgM analysis of the BAL fluid in PbA infected CD169-DTR mice showed elevated levels of these immunoglobulins relative to the WT counterpart at day 6-post infection, an indication of a compromise in alveolar capillary membrane barrier (Figure 3.10E). Therefore, our results demonstrate that lung CD169⁺ alveolar macrophages are frontline cells in determining the outcome of ALI during PbA infection.

3.2.7 Tissue-resident CD169⁺ macrophages prevent acute renal and heart injury

Since CD169⁺ macrophages are present in the kidney and heart as well, we sought to investigate the role of these cells in the outcome of pathologies as seen through CD169-DTR mouse model. Consistent with our previous findings in the other organs, pathologies in kidney and heart of PbA infected CD169-DTR mice were no exception. Histological findings revealed CD169⁺ macrophages depleted mice showed pronounced cellular infiltration in comparison to WT counterparts at day 8-post infection (Figure 3.11A). Flow cytometry analysis revealed significantly elevated numbers of infiltrating inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils at day 8-post infection (3.11B). Compromise in vascular integrity was observed in PbA infected CD169-DTR mouse

relative to the WT counterpart as evident by Evans blue extrusion assay (3.11C). Thus, kidney and heart resident $CD169^{+}$ macrophages prevent tissue injury during PbA infection in the kidney and heart respectively.

Figure 3.11: Severe kidney and heart damages sustained by CD169-DTR mice following PbA infection. (A) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the kidney and heart of PbAGFP-infected WT and CD169-DTR mice at day 8-post infection (n=2-3 mice/group, 6-8 weeks old mice). Scale bars: 100 μ m. Data are representative of two independent experiments with at least 2 mice/group in each experiment (B) Augmented infiltration of inflammatory $Ly6C^{hi}$ and $Ly6G^{hi}$ cells in the kidney and heart of PbA-infected CD169-DTR mice at day 8 post infection. Bar chart represents mean total numbers $\pm$ S.D. of $Ly6C^{hi}$ and $Ly6G^{hi}$ cells in uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) (n = 4 mice/group, 6-8 weeks old mice). Statistical significance was determined using an one-way ANOVA followed by Bonferroni test at * $p < 0.05$, ** $p < 0.01$. (C) Perturbed vascular permeability in the kidneys and hearts of CD169-DTR mice. Values represent the mean optical density $\pm$ S.D. of Evans blue dye extracts measured at 620 nm (n = 3–6 mice /group, 6-8 weeks old mice). Statistical significance was determined using an unpaired student *t*-test at ** $p < 0.01$, **** $p < 0.0001$. Data shown are representative of two independent experiments with n=3-6 mice/group in each independent experiment.

3.2.8 Tissue-resident $CD169^{+}$ macrophages exhibit anti-inflammatory attributes

Our findings suggest an onset of systemic inflammation and tissue injury in $CD169$ -DTR mice during PbA infection that led us to profile serum cytokines to gain insights into reasons of this inflammatory phenotype. The infected WT mice showed consistently elevated levels of anti-inflammatory IL-10 in comparison to the $CD169^{+}$ macrophage deficient $CD169$ -DTR mice (Figure 3.12A, left). Conversely, infected $CD169$ -DTR mice depicted escalated levels of pro-inflammatory $TNF-\alpha$ relative to WT mice (Figure 3.12A, right). Thus, deregulated IL-10/ $TNF-\alpha$ ratio in the $CD169$ -DTR hinted towards the probabilistic cues for the systemic inflammation.

Figure 3.12: $CD169^{+}$ macrophages produce immunosuppressive IL-10 cytokine throughout the entire course of PbA infection. (A) Serum IL-10 and $TNF-\alpha$ levels during the course of PbA infection. Mean of IL-10 and $TNF-\alpha$ $\pm$ S.D. measured in the serum of WT and $CD169$ -DTR infected with PbA at day 6 and 8 (n = 6–11 mice/group). Statistical significance was determined using an unpaired

student *t*-test. **p* < 0.05 and ***p* < 0.01. Data are cumulative of two independent experiments. (B) qPCR analysis of *IL-10* expression in F4/80^{high} macrophages, CD4⁺, CD8⁺ and CD4⁺CD8⁺ cells isolated from pooled spleens obtained from three uninfected mice. *IL-10* and β -actin mRNA levels were measured using real-time PCR. Data were normalized to β -actin and are expressed as fold induction and represented as means of triplicates $\pm$ SD. (C) qPCR analysis of *IL-10* and *TNF- α* expression in F4/80^{high} macrophages and Ly6C^{high} monocytes obtained from livers of control WT mice and PbA-infected mice (day 8). Data are representative of cells collected from three livers in each group. (D) Time-course analysis of *IL-10* expression in F4/80^{high} macrophages, CD4 and CD8 T cells obtained from 3 pooled livers of uninfected (day 0) and infected mice (day 4 and 8). *IL-10* mRNA was determined as described above. Data are expressed as means of triplicates $\pm$ S.D. Data shown in (B, C and D) are representative of two independent experiments. (E) Detection of *IL-10* secreting CD4⁺ T cells obtained from livers of PbAGFP infected WT or CD169-DTR mice. Intracellular staining of *IL-10*/IFN- γ is shown in representative dot plots (left panel) and in a bar chart (right panel). Data in bar chart are shown as percentage mean $\pm$ SD, *n*=3 mice/group, 6-8 weeks old. Statistical significance was determined using an unpaired student *t*-test at **p* < 0.05. Data are representative of two independent experiments with *n*=3 in each independent experiment. (F) WT BALB/c mice (*n*=4) were i.p. injected with anti-*IL-10* receptor monoclonal antibody (α IL-10R) or isotype-matched IgG control antibody day -1 before PbA and at day + 1, 4 and 7. In parallel, a group of 4 DT-injected CD169-DTR mice was included. Parasitemia (left panel), survival (middle panel) and severity of ECM determined by RMCBS (right panel) were evaluated. Statistical significance of survival was analyzed using the Mantel-Cox log-rank test. ***p* < 0.01. Data shown are representative of two independent experiments.

Following the cues, we sought to investigate the intrinsic capacity of CD169⁺F480⁺ macrophages, monocytes and lymphocytes in the production of *IL-10*. In the steady state, CD169⁺F480⁺ macrophages relative to monocytes and lymphocytes are the dominant cellular source of *IL-10* advocating its anti-inflammatory properties. On the contrary the *TNF- α* m-RNA levels are reduced in CD169⁺F480⁺ macrophages and enhanced in monocytes isolated from uninfected mice (Figure 3.12B). During the course of PbA infection, CD169⁺F480⁺ macrophages maintain this intrinsic anti-inflammatory property as evident by the relative *IL-10* m-RNA expression by these cells obtained from infected WT livers quantified by real time quantitative PCR at day 4 and 8 post infection (Figure 3.12C and D). At the later stages of infection activated CD4⁺ T cells also become substantial producers of *IL-10*, which is not the case with activated CD8⁺ T cells (Figure 3.12D).

The recruited inflammatory monocytes are the predominant source of *TNF- α* relative to tissue-resident CD169⁺F480⁺ cells as observed by their sustained m-RNA levels during PbA infection (Figure 3.12C, right). To strengthen our finding that the decrease in serum *IL-10* of PbA infected CD169-DTR mice was due to the absence of CD169⁺F480⁺ macrophages and not CD4⁺ T cells (another cellular source for *IL-10*), we performed flow cytometry analysis to find out differences in *IL-10* secretion by CD4⁺ T cells isolated from the infected WT and CD169-DTR at day 8 post infection. Nonetheless, proportion of *IL-10*⁺ CD4⁺ T cell were similar in both the groups (Figure 3.12E), thus reinstating the fact that lower serum *IL-10* levels were probably due to the absence of CD169⁺F480⁺ macrophages in CD169-DTR mice. To revalidate our understanding that

lower levels of IL-10 due to absence of CD169⁺ macrophages in CD169-DTR mice contributes to PbA infection mediated immunopathology, we adopted blocking IL-10R approach by treating the WT mice with anti-IL10R antibody to test if neutralization of IL-10 in WT phenocopied CD169-DTR mice in the outcome of PbA infection. Indeed, IL-10R blocking approach in WT mice phenocopied CD169-DTR mice in terms of parasitemia profile, development of characteristic ECM signs and rapid lethality (Figure 3.12F).

3.2.9 Summary of the pleiotropic functional attributes of tissue-resident CD169⁺ macrophage in PbA infection

Tissue-resident CD169⁺ macrophages regulate the outcome of PbA infection and exhibit pleiotropic functions during PbA infection such as parasite control, T cell priming, maintenance of vasculature integrity and the most important intrinsic anti-inflammatory property characterized by IL-10 production. Compromise in these pleiotropic functional attributes due to absence of this macrophage subset, renders CD169-DTR mice prone to multifaceted malarial syndromes such as CM, hepatic injury, ALI, renal dysfunction and acute heart injury culminating into widespread tissue damages in the vital organs. Thus, tissue-resident CD169⁺ macrophages can be regarded as important frontline of host defense in PbA infection.

3.3 Understanding the role of tissue-resident CD169⁺ macrophage in acute malaria mediated by in *Plasmodium yoelii* 17XNL (Py) infection

Py infection causes self-resolving acute malaria in BALB/c or C57BL/6J mouse strains that are commonly employed to study non-complicated malaria and anemia. Here we utilized BALB/c WT and CD169-DTR mice model to compare the outcome of this form of malaria in the absence of tissue-resident CD169⁺ macrophages and ensuing pathologies, if any.

3.3.1 Tissue-resident CD169⁺ macrophage deficient CD169-DTR mice succumbed to non-lethal Py infection

The survival and parasitemia was monitored in WT and CD169-DTR mice (BALB/c background) during the entire course of Py infection to assess the effect of tissue-resident CD169⁺ macrophage on the outcome of non-lethal malaria (Figure 3.13A). The parasitemia rose steadily (3-7 dpi) with an exponential transition (8-10 dpi) reaching the peak levels (10 dpi) followed by decline phase (11-17 dpi) and eventually iRBCs were undetectable (18 dpi onwards) in WT mice (Figure 3.13A, left panel). Strikingly, the parasitemia profile of CD169-DTR showed significant differences in the early phase (5-8 dpi) with a transient fluctuating recovery (9-11 dpi) to an exponential rise (12-15 dpi) and followed by sharp decline in parasitemia (16-21 dpi) as shown in Figure 3.13A, left panel. The CD169-DTR group continuously perished during different phases of infection with a survival of 10%, while the WT mice did not (Figure 3.13A, right panel).

In order to understand the parasite sequestration patterns in different organs during Py infection we exploited a Py expressing bioluminescence luciferase reporter (PyLuc) parasite to understand the dynamics of parasite sequestration in the tissues. Real time *ex vivo* bioluminescence imaging was performed upon PyLuc infection in WT and CD169-DTR mouse at time points when parasitemia levels were significantly different (day 7 post infection and not significant (day 9 post infection). CD169-DTR mice showed higher iRBC sequestration in brain, spleen, liver and heart at day 7-post infection unlike day 9-post infection (Figure 3.13B-C).

In order to investigate the anemic phenotype in CD169-DTR mice, which showed significantly higher levels of parasitemia at early time points of the infection, we performed complete blood count (CBC) analysis. The CD169⁺ macrophage deficient

mice showed significantly lower levels of the RBC, hemoglobin and hematocrit at day 8-post infection relative to WT mice (Figure 3.13D) pointing to an anemia phenotype.

Figure 3.13: CD169⁺ tissue-resident macrophage deficient CD169-DTR mice succumb to self-resolving non-lethal *P. yoelii* (Py) infection. (A) Survival (left) and parasitemia (right) of PyGFP-infected WT controls (white circles) and CD169-DTR mice (black circles) (n = 12-16 mice/group, 6-8 week old mice). Statistical significance of survival was analyzed using the Mantel-Cox log-rank test and statistical significance of parasitemia was determined using a Mann-Whitney U test at *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Data are cumulative of two independent experiments with n=6-8 mice/group in each independent experiment. (B-C) Quantification of the average bioluminescence as a measure of *ex vivo* iRBCs sequestration of Py-infected WT and CD169-DTR mice in the indicated organs. (B) Pseudocolor images of brain, spleen, liver, lungs, kidney, and heart collected from a representative Py-infected WT and CD169-DTR mouse on day 7. (C) Bioluminescence imaging during Py infection at day 7 and 9 post infection. Each point represents the mean average radiance (p/sec/cm²/sr) values (log) ± S.D. of 3-4 mice/group. White circles: WT; black circles: CD169-DTR mice. Statistical significance was determined

using an unpaired student *t*-test at **p* < 0.05, ***p* < 0.01 Data are representative of two independent experiments. (D) Anemia profile in Py-infected WT and CD169-DTR mice. RBC numbers (left panel), hemoglobin (middle panel) and hematocrit (right panel) levels in peripheral blood were determined day 8 post infection for WT and CD169-DTR mice. Data are presented as mean ± SD for 6 mice per group, 6-8 weeks old. Statistical significance was determined using an unpaired student *t*-test at ***p* < 0.01. Data shown are cumulative of two independent experiments with *n*=3 mice/group.

3.3.2 Exacerbated deposition of hemozoin (Hz) in the vital organs of CD169-DTR mice during Py infection

High levels of Hz correlate with elevated parasite burden in tissues, tissue inflammation and severity of pathogenesis during malaria. To investigate this possibility in Py infection, H&E stained tissue sections obtained from Py infected WT and CD169-DTR mice were analyzed by bright field microscopy.

Figure 3.14: Exacerbated hemozoin deposits in the spleen, lung and liver of Py-infected CD169-DTR mouse. Representative bright field microscopic images of H&E stained paraffin tissue sections (6 µm) obtained from spleen, lungs and liver of DT-treated (10ng/gbw) and PyGFP-infected WT (left panels) and CD169-DTR (right panels). Images were acquired at 100X magnification (Scale bar: 50 µm). Quantification are represented in the form of scatter plot graph as the ratio of area of the hemozoin deposits over the tissue area as analyzed by Histoquest imaging software. Statistical significance was determined using an unpaired student *t*-test at **p* < 0.05, and *****p* < 0.0001. Data are representative of two independent experiment, *n*=2 mice/group, 6-8 weeks of age.

Exacerbated Hz deposits in spleen, lung and liver of CD169-DTR mice relative to the WT counterparts were observed (Figure 3.14 and S12). The brain, kidney and heart showed minimal Hz deposits in selected regions of the tissue as compared to above organs and therefore not quantified. Gross observation of these selected regions in the brain kidney and heart of the Py infected CD169-DTR mice show relatively more Hz deposition than the WT counterparts in these organs as well (Figure S12). Our results therefore highlight a crucial role of tissue-resident CD169⁺ macrophages in containing the Py parasite similar to the PbA infection.

3.3.3 Anti-malarial splenic immune responses are impaired in CD169-DTR mice during Py infection

Histological analysis of the splenic tissue sections revealed similar pathophysiological scenario in Py infected WT and CD169-DTR group (Figure 3.15A) as observed during PbA infection described in section 3.2.3. Notably, collapse of MZ was evident in Py infected CD169-DTR mice (Figure 3.15A). Hz deposits were 3.7 fold higher in the spleens of the infected CD169-DTR mouse relative to the infected WT counterparts (Figure 3.15A and S12). Flow cytometry analysis indicated that Py infected CD169-DTR mice exhibit significantly increased numbers of infiltrating inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils relative to WT already at day 6-post infection in the spleen (Figure 3.15B). Histological analysis revealed the enhanced cellular infiltration in Py infected CD169-DTR mice relative to the WT counterparts at day 11 post infection (Figure 3.15A). The proportion and number of effector CD8⁺ T cells were also significantly reduced as evident by attenuated Granzyme B expression in CD169-DTR group relative to the WT counterparts at day 6-post infection (Figure 3.15C).

During blood stage of Py infection, parasite specific IgG responses play a significant role in parasite clearance; therefore we evaluated this aspect of splenic immunity by measuring the serum IgG levels at different time points before CD169-DTR began to perish. Tissue-resident CD169⁺ macrophage do have a role in modulating parasite specific anti-*Plasmodium* responses as evident by alleviated levels of serum IgG in CD169-DTR mice in comparison to WT group at day 11 and 14 post infection (Figure 3.15D). Not only total serum IgG but also Ig subtypes IgG1 and Ig2a were significantly reduced in the CD169⁺ macrophage deficient CD169-DTR mice at day 14-post infection. Thus, our preliminary results suggests that both splenic T-cell mediated and antibody mediated anti-malarial effector control mechanisms are compromised in the absence of

CD169⁺ macrophages, the likely reason for ineffective parasite control in the spleen and probably in other organs as well. Future experiments will enable us to dissect the role CD169⁺ macrophages in regulating the T and B cell axis, possibly uncovering the underlying cellular mechanisms in mediating protective anti-*Plasmodium* immune responses.

Figure 3.15: Histological and cellular analysis of spleens in WT and CD169-DTR mice during Py infection. (A) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the spleens of WT and CD169-DTR mice on day 11 post Py-GFP infection. Scale bars: 1 mm (left panels) and 100 μ m (middle panels) and 10 μ m (right panels). Data are representative of two independent experiments with n=2 mice/group, 6-8 weeks old mice. Dot chart shows the relative fold difference of Hz (from Figure 3.12) in the spleen of infected control (white circles) and CD169-DTR mice (black circles). (B) Augmented numbers of inflammatory Ly6C^{hi} and Ly6G^{hi} cells in the spleen of Py-infected CD169-DTR mice. Representative flow cytometry dot plots showing CD11b, Ly6C and LyG expression on the stained total leukocytes obtained from spleens of uninfected, PyGFP infected WT and CD169-DTR mice at day 6 post infection. Bar charts represent mean absolute numbers $\pm$ S.D. of Ly6C^{hi} (monocytes) and Ly6G^{hi} neutrophils in the spleens of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at days 6 post infection (n = 4 mice/group, 6-8 weeks old mice.). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test. *p < 0.05, **p < 0.01. (C) Representative flow cytometry dot plots showing Granzyme B expressing CD8⁺ T cells in the spleens of uninfected, PyGFP infected WT and CD169-DTR mice. Bar charts represent mean absolute numbers $\pm$ S.D. of Granzyme B-expressing CD8⁺ T cells, in the spleens of uninfected WT (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at day 6 post infection (n = 4/group, 6-8 weeks old mice). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test at *p < 0.05. Data shown in (B and C) are representative of two independent experiments with n=3-4 mice/group in each independent experiment. (D) Dot graph represents serum levels of total IgG (left) at day 11 and 14 post infection and IgG1 (middle) and IgG2a (right) at day 14-post infection in Py infected WT (white circles) and CD169-DTR (black circles). Data is represented as mean $\pm$ S.D. n=7 mice/group. Statistical significance was determined using an unpaired student t-test at **p < 0.01, ***p < 0.001. Data are representative of two independent experiments with n=3-4 mice/group in each independent experiment.

3.3.4 Tissue-resident CD169⁺ macrophage regulate the outcome of ALI in Py infection

To assess the possibility of ALI during Py infection, flow cytometry analysis was done for the lung cells obtained from WT and CD169-DTR mice. The scenario was no different as observed during PbA infection described in section 3.2.6, wherein increased numbers of infiltrating cells comprising of inflammatory Ly6C^{hi} monocytes and Ly6G⁺ neutrophils was observed in CD169-DTR mice relative to the WT at early phase, day 6 post infection (Figure 3.16B). Similar picture of increased cellular infiltration in the lungs of CD169-DTR mice relative to WT mice was observed at day 11-post infection as evident by histological analysis. In addition, Py infected CD169-DTR mice showed highly congested vessels with iRBCs (Figure 3.16A bottom right panel) and Hz deposits (11 fold higher) when compared to WT counterparts (Figure 3.16A and S12).

In addition, IgG and IgM analysis of the BAL fluid in Py infected CD169-DTR showed elevated levels of these immunoglobulins relative to WT at day 6-post infection an indication of compromise in alveolar capillary membrane barrier (Figure 3.16C). Therefore lung CD169⁺ alveolar macrophages also regulate the outcome of ALI during Py infection.

Figure 3.16: Py infection causes lung inflammation and pathology in CD169-DTR mice. (A) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μm) obtained from the lungs of WT and CD169-DTR mice on day 11 post Py-GFP infection. Scale bars: 1 mm (left panels) and 10 μm (middle and right panels). Data are representative of two independent experiments with n=2 mice/group, 6-8 weeks old mice. Dot chart shows the relative fold difference of Hz (from Figure 3.12) in the lungs of infected control (white circles) and CD169-DTR mice (black circles). (B) Augmented numbers of inflammatory Ly6C^{hi} and Ly6G^{hi} cells in the lungs of Py-infected CD169-DTR mice. Representative flow cytometry dot plots showing CD11b, Ly6C and LyG expression on the stained total leukocytes obtained from the lungs of uninfected, PyGFP infected WT and CD169-DTR mice at day 6-post infection. Bar charts represent mean absolute numbers ± S.D. of Ly6C^{hi} and Ly6G^{hi} cells in the lungs of uninfected (grey bars), infected WT (white bars), and infected CD169-DTR mice (black bars) at days 6 post infection (n = 4 mice/group, 6-8 weeks old mice). Statistical significance was determined using a one-way ANOVA followed by Bonferroni test at ***p < 0.001, ****p < 0.0001. Data shown in (B) are representative of two independent experiments with n=3-4 mice/group in each independent experiment. (C) Determination of IgM and IgG levels by ELISA in bronchoalveolar lavage (BAL) fluid as biomarkers for

pulmonary vascular leakage. BAL was collected from Py-infected WT (white circles) and CD169 DTR mice (black circles) at day 6-post infection. Data are represented as mean optical density $\pm$ S.D. ($n \geq 7$ /group). Statistical significance was determined using an unpaired student *t*-test, * $p < 0.05$. Data shown are cumulative of two independent experiments with $n=3-4$ mice/group in each independent experiment.

3.3.5 Tissue-resident CD169⁺ macrophages modulate pathogenesis in liver, kidney and heart during Py infection

Histopathology examination of tissue sections depicted increased numbers of infiltrating leukocytic cells compounded with pronounced tissue injury in CD169-DTR mice in comparison to WT mice in liver, kidney and heart (Figure 3.17A, C and E). We also evaluated ALT levels as a measure of liver injury and expectedly infected CD169-DTR mice showed significantly higher ALT levels when compared to WT mice at day 6 post infection, an evidence of acute hepatic injury (Figure 3.17B, lower panel). Hemozoin deposits in liver were comparable in Py infected CD169-DTR mice and WT mice (Figure 3.17B and S12). In kidney, the hemozoin deposition was seen selectively in the glomerular region and in higher proportions in CD169-DTR mice as visualized in Figure 3.17C (bottom right). Similar to kidney, the heart of Py infected CD169-DTR mouse showed enhanced Hz deposits only in very selective region as exemplified in Figure 3.17E (bottom right).

Widespread systemic tissue damage provided cues to profile serum cytokines in order to gain insights into reasons of this inflammatory phenotype. CD169⁺ macrophage sufficient infected WT mice showed consistently elevated levels of anti-inflammatory IL-10 in comparison to the CD169⁺ macrophage deficient CD169-DTR group (Figure 3.17D, top panel). Conversely, infected CD169-DTR depicted escalated levels of pro-inflammatory TNF- α relative to WT mice (Figure 3.17D, bottom panel). Thus, deregulated IL-10/TNF- α ratio in the CD169-DTR could be the likely reason for the systemic tissue inflammation.

3.3.6 Summary of the role of tissue-resident CD169⁺ macrophages in Py infection

Tissue-resident CD169⁺ macrophages deficient CD169-DTR mice succumb to non-lethal Py infection due to widespread tissue damage, showing striking similarity with the multifaceted pathogenesis observed during PbA infection described in section 3.2 with the exception of appearance of CM. Here, this macrophage subset can be implicated in mediating parasite specific antibody responses as well. Thus, the tissue-resident CD169⁺ macrophages can be generalized as frontline sentinel cells that regulate the outcome of *Plasmodium* infection.

Figure 3.17: CD169⁺ tissue-resident macrophage regulates pathogenesis in the liver, kidney and heart during Py infection. (A) Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the livers of WT and CD169-DTR mice on day 11 post Py-GFP infection. Scale bars: 1 mm (left panels) and 10 μ m (right panels). Data are representative of two independent experiments with n=2 mice/group, 6-8 weeks old mice. (B) Dot chart shows the relative fold difference of Hz (from Figure 3.12) in the livers of infected control (white circles) and CD169-DTR mice (black circles), top panel. Serum alanine transaminase (ALT) levels of WT (white circles) and CD169-

DTR mice (black circles), bottom panel. Values represent mean ALT levels $\pm$ S.D. in units/L ($n \geq 6$ mice/group, 6-8 weeks old mice). Data shown are cumulative of two independent experiments with $n=3-4$ mice/group in each independent experiment. (C) Representative bright field microscopic images of H&E stained paraffin tissue sections ($6 \mu\text{m}$) obtained from the kidneys of WT and CD169-DTR mice on day 11 post Py-GFP infection. Scale bars: 1 mm (left panels) and $10 \mu\text{m}$ (right panels). Data are representative of two independent experiments with $n=2$ mice/group, 6-8 weeks old mice. (D) Serum IL-10 (top panel) and TNF- levels (bottom panel) measured in the serum of WT and CD169-DTR infected with PyGFP at day 6 post infection ($n \geq 5$ /group). Data are represented as mean of IL-10 and TNF- $\alpha \pm$ S.D. ($n \geq 5$ /group, 6-8 weeks old mice). Statistical significance was determined using an unpaired student *t*-test at $*p < 0.05$. Data are cumulative of two independent experiment with $n=2-4$ mice in each independent experiment. (E) Representative bright field microscopic images of H&E stained paraffin tissue sections ($6 \mu\text{m}$) obtained from the hearts of WT and CD169-DTR mice on day 11 post Py-GFP infection. Scale bars: 1 mm (left panels) and $10 \mu\text{m}$ (right panels). Data are representative of two independent experiments with $n=2$ mice/group, 6-8 weeks old mice.

4. Discussion

The mononuclear phagocyte network comprises of a heterogeneous population of DCs, monocytes and macrophages including both the ones recruited from the circulation and tissue-resident subsets that are involved in the antigen presentation and regulation of innate and adaptive immune responses to pathogens, vaccines, tumor as well as maintenance of tolerance to self-antigens [159]. The cells of the MPS exhibit differential phagocytic and antigen presentation ability. The differential phagocytic attributes of these cells results in altered functional outcomes. DCs are considered as professional antigen presenting cells (APCs) because endocytic proteolysis in DCs results in partial degradation/processing of proteins, a process aimed to produce peptides, a functional adaptation that allows antigens to be presented on their surface and are therefore capable to efficiently stimulate adaptive immune responses. Unlike DCs, monocytes and macrophages on the other hand are regarded as professional phagocytic cells as they scavenge and destroy the ingested particles completely and thus are referred as key sentinel cells of the innate immunity [313]. Unlike DCs, macrophages to a greater extent also play a vital role in mediating balanced pro and anti-inflammatory responses during infectious diseases such as malaria, tissue homeostasis, angiogenesis and tissue repair [88].

4.1 Role of the monocytes and macrophages in malaria

Monocytes, in addition to phagocytosis also perform ADCI to limit parasite dissemination only in malaria immune hosts [255, 270]. Several reports have highlighted the importance of generalized innate immune mechanisms of macrophages in the control of *Plasmodium* infection [280, 282, 283, 314, 315] whereas others have associated them with severe malarial pathogenesis [252, 316], thus, highlighting that balanced immune responses are the key to protection against *Plasmodium*, which otherwise might result in malaria associated pathophysiological consequences that culminate into a spectrum of malarial syndromes. These phagocytic cells, in general, have so far been conventionally implicated in an opsonin independent and antibody dependent phagocytic parasite clearance mechanisms at early and later stages of *Plasmodium* infection, respectively [317-320].

Macrophages show remarkable heterogeneity in tissues. Tissues harbor phenotypically and functionally diverse repertoire of tissue-resident macrophage subsets characterized

by overlapping receptor expression patterns of CD11c, CD11b, F480, CD64, CD169, CD163 and MHC class II whose exclusive functions are unclear pertaining to various diseases and in particular, *Plasmodium* infection [88]. For instance, the spleen, which plays a central role in *Plasmodium* control, is seeded with diverse populations of tissue-resident macrophages such as RPMs, MMMs and MZMs as well as monocyte and monocyte-derived macrophages. Lack of specific mouse models that would enable conditional and reversible depletion of the specific subsets of macrophages *in vivo* have precluded the functional studies of these subsets during the course of dynamic malarial infection as well as other blood-borne diseases [100]. Similar to the spleen, distinct subsets of macrophages are also present in the other organs such as brain, liver, lung, kidney whose functions are not yet understood in the context of the malaria.

4.2 Tissue-resident macrophages and their functions

Plasmodium infection results in multifaceted overlapping pathologies (malarial syndromes) such as CM, acute liver injury, MA-ALI/ARDS, AKI and SMA which may or may not be coexistent in both the human and mouse hosts [12, 61]. Few studies have highlighted the role of macrophages in the outcome of malarial syndromes using macrophage depletion models that do not show exclusivity to only macrophages but ablate other phagocytic cells as well. For instance, clodronate liposome mediated depletion of macrophages and subsequent challenge with non-lethal Py infection resulted in renal and hepatic dysfunction in a recent mouse study [282]. Using a similar ablation strategy a study had earlier reported failure in the control of primary wave of parasitemia during Py infection [280]. The aforementioned studies have employed broad macrophage depletion strategies; therefore, to our knowledge the specialized functions of distinct subsets of macrophages remain obscure in relation to the malarial syndromes.

To address this aspect, here in this study we set out to investigate the role of tissue-resident macrophages that expresses high levels of CD169 (sialoadhesin) and F4/80 and are commonly referred as F4/80^{hi} macrophages. The CD169⁺ tissue-resident macrophages are widely distributed in different lymphoid tissues such as the lymph nodes (SCS macrophages) and spleen (MMMs, RPMs) and non-lymphoid tissues such as brain (perivascular macrophages), liver (Kupffer cells), lungs (alveolar macrophages), heart (cardiac macrophages), bones (osteoclasts) and kidney-resident macrophages in the kidney [88, 229, 231, 232]. This subset of macrophages is present across different mammalian species at least in lymphoid organs and the presence of this macrophage

subset in other organs still needs to be uncovered. Unlike, monocyte-derived macrophages that are rapidly recruited during inflammation, tissue-resident macrophages possess self-renewal capacity and replenish their own populations by *in situ* local proliferation and this proliferation capacity increases during inflammatory insults to promote resolution of inflammation and restoration of homeostasis [88, 183, 184].

The CD169⁺ macrophages are positioned at strategic micro anatomical location such as proximity to the blood vessels in brain, kidney and heart, marginal zone of the spleen and hepatic sinusoids and this privileged localization enables capture of blood-borne pathogens in a glycan dependent manner [229]. The function of the CD169⁺ macrophages has thus far been studied in relation to erythropoiesis [236, 237], cross presentation of antigens to CD8⁺ T cells [243, 244], apoptotic cell driven tolerance [321] and regulation of germinal center B cell responses [239]. Despite such functional revelations, to the best of our knowledge, no study till date has been done to dissect the role of CD169⁺ macrophages in malaria. In this direction our lab generated a CD169-DTR mouse model [231], specifically aimed, in this study, to understand the role of the tissue-resident CD169⁺ macrophages in protection and pathology to severe lethal malaria inflicted by PbA infection and acute uncomplicated non-lethal malaria caused by Py infection.

4.3 CD169-DTR mouse model: Robust system to study the role of tissue-resident CD169⁺ during the steady state and *Plasmodium* infection

Here, we show that CD169-DTR mouse model enables specific and efficient depletion of the CD169⁺ macrophages in the spleen, brain, liver, lung, kidney and heart during steady state and malarial infection. In addition, we did not observe any DT mediated modulation on the DC subsets in the lymphoid (spleen) and non-lymphoid organs (liver and lung). This was necessary as CD169 expression has been previously reported on a DC subset (CD8⁺ DCs) [307] and also to rule out the possibility of DC mediated effects on our findings. Modulations of CD169 expression on monocytes have been previously reported during the course of HIV and SIV infection [322] but not in malaria, hence evaluation of CD169 expression on different myeloid and non-myeloid cellular fractions during malarial infection was performed. Notably, CD169 expression patterns remained unperturbed during *Plasmodium* infection.

Therefore, here, we present a suitable mouse model that enables reversible and conditional depletion of a specialized subset of macrophage that permits simultaneous functional study across different organs during steady state and in the context of the

disease. Additionally, the CD169-DTR model does not result in the depletion of other non-targeted immune cell populations, unlike the other conventional macrophage depletion strategies that result in broad macrophage ablation. Hence this mouse model serves as optimal system to elucidate the role of CD169⁺ macrophages in regulating the outcome of diverse organ pathologies associated with malarial syndromes.

4.4 Tissue-resident CD169⁺ macrophages regulate the outcome of malarial syndromes during PbA infection

4.4.1 CD169⁺ macrophage deficiency in mouse result in accelerated mortality due to higher parasite burdens in PbA infection

Strikingly, we observed CD169⁺ macrophages deficient CD169-DTR mice showed higher iRBC levels in blood during 8-10 days post infection followed by the decline in parasitemia, which was coincident with higher parasite sequestration and Hz deposition in the tissues that possibly resulted in premature death of these mice during PbA infection. On the contrary, PbA infected WT mice showed continuous exponential increase in parasitemia that led to dramatically higher iRBC levels in the blood during the course of infection, which correlated, with their anemic phenotype, lower parasite sequestration and minimal Hz deposition and relatively improved survival when compared to CD169-DTR mice. Thus, CD169-DTR mice exhibited higher parasite burdens in the tissues and conversely WT mice showed higher iRBC levels in the blood succumbing to the complications of hyperparasitemia; SMA. The higher parasite and Hz burdens in the tissues could be a result of absence of direct or indirect parasite control mechanisms mediated by CD169⁺ macrophages in the CD169-DTR mouse.

4.4.2 Increased recruitment of inflammatory monocytes and neutrophils in response to the higher parasite burdens in CD169-DTR mouse during PbA infection

The exacerbated parasite and Hz loads in all the tested organs resulted in abundant recruitment of monocytes and neutrophils in CD169-DTR mice to facilitate clearance of the parasite and parasite-associated products. The monocytic cells and to a lesser extent neutrophils have been shown to phagocytose iRBCs and free parasites and kill them by oxidative burst mechanism [323, 324]. These inflammatory responses during *Plasmodium* infection needs to be tightly regulated which might otherwise result in multiple organ injuries leading to CM, hepatic and renal dysfunction [253, 282]. To counter the hyper inflammatory responses, the highly plastic tissue-resident macrophages

polarize and display anti-inflammatory phenotype in the context of inflamed tissue to strike a balanced immune response to override the infection [325]. More likely, the absence of CD169⁺ macrophages would lead to the failure of regulatory mechanisms of inflammation.

4.4.3 Sequestration of parasite and recruited leukocytes led to occluded vasculature and compromise in vascular integrity in CD169-DTR mouse during PbA infection

The sequestration of the parasite and the recruited leukocytes led to congestion in the blood vessels and in addition pronounced vascular leakage including a breach in the blood brain barrier (BBB) integrity was witnessed in all the tested organs of CD169⁺ macrophage deficient mouse during PbA infection. This observation was indicative of widespread tissue damage in all the analyzed organs and the likely cause for their premature mortality.

A neurovascular unit comprises of cerebral endothelial cells (CECs), basal lamina, astrocytic endfoot processes, pericytes and neurons which is surrounded by microglia, perivascular macrophages and infiltrating macrophages in a healthy intact brain [326]. Pericytes and perivascular macrophages are in proximal contact with the blood vessels and therefore form a critical structural support for the maintenance of BBB function. The disruption of BBB is a very common feature in a spectrum of diseases affecting the brain including CM [326]. In the context of ECM, compromise in BBB integrity has been hypothesized to be a result of alterations in tight junction proteins (TJPs) of CECs and a study has demonstrated that antigen specific CD8⁺ T cells disrupt TJPs and initiate vascular permeability in a perforin dependent fashion in the CNS [327]. Monocyte-derived macrophages have been shown to constantly express TJPs in an *in vitro* model in response to particulate antigen [328]. Therefore, there is likelihood that vascular-associated CD169⁺ macrophages have a critical role in the maintenance of functional BBB through TJPs. Thioglycolate stimulated murine peritoneal macrophages (characterized by F4/80^{hi} Tim4⁺ CD169⁺ expression) have been shown to possess angiogenic property as exemplified by their constitutive VEGF production ability under hypoxic conditions [329]. In a recent study, PbA induced endothelial damage in a ECM model was reversed by VEGF treatment and conversely, neutralization of VEGF receptor resulted in exacerbated cerebral pathology, thus implying that VEGF plays a critical role in preserving blood vessel integrity. In this direction there is a possibility that CD169⁺ macrophages are involved in maintenance of endothelial integrity in a VEGF dependent

manner [330]. As other examples, in the ear and eye vascular associated myeloid cells that comprise of perivascular macrophages CD169⁺ macrophages have been reported to maintain the integrity of intrastrial fluid-blood barrier in ear and immune surveillance function in the eye [304, 331]. Another very recent study has reported that the kidney-resident F4/80^{hi} macrophages are present between the capillary endothelium and basement membranes of the tubules and Bowman capsule, located in the immediate vicinity of the microvasculature. This constitutes the anatomical and functional unit that regulates trafficking of proteins and particulate matter in a size selective manner, which also facilitates rapid detection of potential threats [332]. In summary, these studies highlight a conserved anatomical-functional association of tissue-resident CD169⁺ macrophages in many organs, which can possibly be implicated as crucial players in establishing a microenvironment for maintenance of blood vessel integrity during homeostasis and inflammation.

4.4.4 CD169⁺ macrophages regulate the outcome of malarial syndromes during PbA infection

The deficiency of CD169⁺ macrophages resulted in hepatic dysfunction as evident by elevated serum ALT levels, mild collagen deposition around the blood vessels and occasionally in the tissue parenchyma indicative of liver fibrosis during PbA infection. More strikingly, the CD169-DTR mouse developed ECM in a CM resistant BALB/c genetic background as exemplified by typical neurological ECM behavioral phenotype, parasite and leukocyte sequestration, prevalence of hemorrhage foci and breach of BBB integrity. However we could observe, that the neurological ECM symptoms observed in CD169-DTR were less pronounced as compared to the symptoms seen in ECM sensitive C57BL/6J genetic background. MA-ALI was yet another pathological manifestation sustained in CD169-DTR mouse which was concurrent with AKI and acute heart injury. Exacerbated parasite and leukocyte sequestration along with vasculature leakage and damaged tissue architecture was a common feature across analyzed tissues pointing to multiple organ damages in CD169-DTR mouse during PbA infection. A very recent study has reported the induction of acute renal failure in a *P. yoelii* non-lethal infection by depleting the phagocytic cells. In this study phagocytic cells deficient mice showed overlapping kidney and liver dysfunction characterized by the increased pRBC sequestration, fibrin deposition, compromised endothelial integrity and inflammation, thus phagocytic cells such as macrophages play a crucial role in determining the outcome

of malaria associated pathologies [282]. However, the results of this study are compounded by the clodronate liposome mediated broad macrophage depletion strategy, which also results not only in the ablation of tissue-resident CD169⁺ macrophages but also affects other phagocytic cells, such as monocyte derived macrophages and neutrophils. Therefore, our results show compelling evidence for the exclusive involvement of the tissue-resident CD169⁺ macrophages in regulating the outcome of multifaceted malarial syndromes.

4.4.5 Tissue damage in CD169-DTR mouse is independent of pathogenic T cells during PbA infection

Except for the brain, the pathogenesis in the organs of CD169⁺ macrophage deficient mice seems to be independent of activated and pathogenic T cells because the numbers of IFN- γ ⁺ CD4⁺ and CD8⁺ T cells in the spleen, liver and lung were in fact comparable or even reduced relative to the WT counterparts. This suggests a compromise in the T cell effector function, which was also reflected in the serum IFN- γ as well. Several reports have highlighted the protective role of IFN- γ in parasite control [333-335]; hence systemic decrease in IFN- γ might have contributed to uncontrolled parasite dissemination in different organs. Haque *et al.*, have previously demonstrated that the liver damage in PbA infection is due to high parasite burdens and independent of CD8⁺ T cell, in contrast to pathogenesis in the brain where parasite and CD8⁺ T cell, both contribute to the cerebral damage [34]. The results of this study and our own findings support parasite mediated damage in the peripheral organs such as liver, lung and kidney. The attenuation in cytokine producing ability of T cells in CD169-DTR mouse could be due to putative antigen presentation ability of CD169⁺ macrophages [243, 244, 283]. Our findings support the involvement of CD169⁺ macrophages in antigen presentation and generation of T-cell mediated adaptive immune responses against PbA because the DCs, which are professional antigen presenting cells, were not affected in both the lymphoid (spleen) and non-lymphoid organs (liver and lung) due to the DT mediated depletion of the CD169⁺ macrophage subset in the CD169-DTR mouse. Only in the brain, we observed enhanced numbers of recruited and activated T cells, which could have migrated in response to enhanced parasite sequestration in brain endothelium, possibly resulting in the pathogenesis as seen in ECM.

4.4.6 Exacerbated hemozoin (Hz) deposition in organs of CD169-DTR mouse: driver of inflammatory response and pathology

Hz is a by-product of heme detoxification released from the digestive vacuole of the parasite and has been described to sequester in different organs such spleen, lung and liver and to a lesser extent brain [33, 336, 337]. The amount of sequestered Hz in liver and lungs has been shown to directly correlate with the degree of inflammation and the pathogenesis resulting in ALI and hepatic injury [33, 337]. Monocytes, macrophages and neutrophils rapidly ingest the released Hz and the numbers of Hz loaded mononuclear cells show significant correlation with the severity of the malaria. For instance, in humans and mice hosts, abundance of Hz-laden mononuclear cells has been seen in CM as opposed to uncomplicated malaria [324, 338]. Hz stimulates monocytes and macrophages to release pro-inflammatory mediators TNF- α and MCP-1 [339]. Hz also activates NLRP-3 inflammasome that results in release of IL-1 β , which recruit neutrophils [340]. Consistent with these observations, exacerbated Hz deposition in all the tested organs of the CD169-DTR mouse could be one of the likely reasons for the resulting inflammation and development of malaria syndromes during PbA infection.

4.4.7 Absence of tissue-resident CD169⁺ macrophages resulted in deregulated cytokine responses during PbA infection

The observed inflammatory phenotype and multifaceted malarial syndromes sustained by CD169⁺ macrophages deficient mice provided cues of deregulated cytokine milieu. Further investigation in this direction confirmed the imbalance between pro-inflammatory and anti-inflammatory cytokine wherein CD169-DTR mice exhibited a high TNF- α /IL-10 ratio thus revealing an inherent anti-inflammatory attribute of this macrophage subset. Hz (purified from different *Plasmodium* species and synthetic) stimulates human monocytes and murine macrophages to produce TNF- α MIP-1 α , and MIP-1 β *in vitro*. Therefore, the high levels of TNF- α in the serum could be most likely due to the elevated numbers of recruited and activated monocytes that could be another driver of the local tissue inflammation in the PbA-infected CD169-DTR mouse. Another possibility of uncontrolled inflammation in CD169-DTR could be reduced levels of IL-10, which is an anti-inflammatory cytokine that regulates inflammatory responses that in unregulated scenario may be detrimental to the host and can cause inflammation induced tissue damage [341].

4.4.8 Tissue-resident CD169⁺ macrophages are constant source of IL-10 production throughout the course of PbA infection and exhibit anti-inflammatory properties

Our results show that CD169⁺ macrophages are a predominant source of IL-10 early in the infection with activated CD4⁺ T cells being another contributor to the systemic IL-10 during later phases of the PbA infection. Hence, CD169⁺ macrophage subset serves as the dominant cellular source for IL-10 during the entire course of PbA infection. During malaria, several studies have implicated IL-10 as an anti-inflammatory and immunoregulatory cytokine that ameliorates inflammation and associated pathogenesis [342-344] by limiting sequestered parasite load [345, 346], counter regulating pro-inflammatory cytokine responses [347] and the resulting disease severity. Thus, intrinsic IL-10 producing capacity of CD169⁺ macrophages regulates the exacerbated inflammation and associated multifocal organ pathologies during severe and complicated forms of malaria induced by PbA infection.

4.5 Tissue-resident CD169⁺ macrophages regulate the outcome of malarial syndromes during non-lethal Py infection

Plasmodium infection results in SMA, which is a consequence of combination of the destruction of RBCs and erythropoietic inhibition of proliferative erythroid precursors [348-350]. To extend our understanding to the role of CD169⁺ macrophages in SMA and antibody mediated adaptive immune responses we exploited Py parasite model, which facilitates the studies of immune mechanisms in this aspect.

4.5.1 CD169⁺ macrophage deficiency in mouse result in lethality due to higher parasite burdens and possibly anemia in a non-lethal Py infection

Previous studies using broad macrophage depletion strategies (clodronate liposome mediated) have demonstrated that ablation of phagocytic cells results in significantly prolonged and greater peak parasitemia in different non-lethal parasite models such as *Babesia microti*, PyNL and lethal infection models such as PyL, thus highlighting the importance of macrophages in controlling the parasite [280, 351]. The lack of macrophage specificity and exclusivity in the models used in these studies could only ascertain the collective role of phagocytic cells in containment of the parasite and not a macrophage specific phenomenon. In this study we emphatically show that CD169⁺ macrophages are specifically involved in the control of parasitemia. The CD169-DTR mice challenged with Py depicted exponentially higher levels of peripheral parasitemia at

early stages with a transient recovery during the middle phase and eventually succumbed to non-lethal infection with significantly higher levels of parasite load in the blood. The enhanced parasite load in the blood possibly resulted in significant destruction of RBCs, which was concurrent with the anemic phenotype at early stages of the infection observed in CD169-DTR mice. The anemia in CD169-DTR mouse could be possibly explained by perturbed erythropoiesis as bone marrow CD169⁺ macrophages have been shown to regulate erythropoiesis in a PHZ induced hemolytic anemia model [236]. This aspect needs to be further investigated.

A very recent study has demonstrated dramatically higher parasitemia concurrent with significant weight loss and accelerated mortality in the CD169⁺ macrophage deficient mouse, particularly in the recrudescence phase of an acute and chronic *P. chabaudi* infection model [352]. These documented observations are in line with our unpublished data on *P. chabaudi* and acute PyNL infection, which show significantly higher parasitemia as reported in this above study and throughout the course of infection respectively. Therefore, the CD169⁺ macrophages can be regarded as key sentinel cells that control the murine *Plasmodium* parasite irrespective of the species in question as per the published studies [352, 353] and our unpublished work on PyNL and *P. chabaudi*.

4.5.2 CD169-DTR mouse recapitulates malarial syndromes with the exception of CM during Py infection

Similar to PbA infection, Py infected CD169-DTR mice sustained considerable parasite induced tissue damages in the lungs, liver, kidney and heart as evident by enhanced H₂O₂ deposits, increase in lung alveolar capillary membrane permeability, elevated ALT levels. Similar pathophysiological damages were recapitulated in Py infection as seen during PbA infection thus again indicative of the conserved parasite control mechanisms of CD169⁺ macrophage subset and highlighting its regulatory role in curbing inflammation and pathogenesis.

4.5.3 Tissue-resident CD169⁺ macrophages regulate anti-malarial adaptive T and B cell immune responses during Py infection

The capacity of the CD169⁺ macrophages to present antigens and trigger T and B cell mediated adaptive immune responses has been demonstrated by various studies [234, 239, 243, 244]. An increasing body of evidence suggests that CD169⁺ macrophages are capable of priming cytotoxic T lymphocyte (CTL) responses [242-244]. Lymph node

CD169⁺ macrophages have been shown to engulf dead tumor cells and subsequently cross present dead cell associated antigens to generate tumor specific CTL responses. In this study specific depletion of CD169⁺ macrophages resulted in impaired tumor antigen specific CTL responses [243]. In another study, splenic CD169⁺ macrophages (MMMs) have been shown to collaborate with CD8⁺ DCs to generate effective CTL responses in CD169 directed ova antigen and adenovirus immunization model [242]. A very recent report has shown that normal CTL responses can be generated even in the absence of DCs suggesting a presence of yet unidentified APC population capable to cross-present antigens. Herein, authors show that cross priming DCs have limited specificities only for very strong epitopes thus generating limited CTL repertoires. Using a Δ -DC mouse model, which lacks DCs, Bernhard *et al.*, demonstrate that presence of infected CD169⁺ macrophages is sufficient for CTL priming to all the epitopes tested including the weaker epitopes [244].

In the context of B cell responses, an earlier study has shown that SCS CD169⁺ macrophages efficiently capture viral particles, translocate the bound viruses and present them to the migrating B cells and demonstrated that depletion of these macrophages resulted in impaired antiviral humoral immune responses [234]. In a similar direction, a recent study has reported antigen targeting to splenic CD169⁺ results in induction of high affinity isotype switched antibody responses in addition to increase in Tfh numbers [239]. Therefore, all these studies provide evidence for the involvement of CD169⁺ macrophages in regulating adaptive immune responses.

In Py infection, antibody mediated immune mechanisms play a central role in the limiting the parasite. Parasite specific IgG responses, in particular of the IgG2a isotype, control the course of parasitemia and definitive as the protective correlates in both mouse (Py and *P. chabaudi* infection) and human (*P. falciparum* infection) malaria [354-358]. Similar to PbA infection we observed a decrease in the number of effector CD8⁺ T cells in the spleen of Py infected CD169-DTR mice. Not only T cell responses were compromised but also parasite specific IgG responses were perturbed in CD169⁺ macrophage deficient mice. Our preliminary observations suggest a possible functional deficit in either the inappropriate T cell help to B cell or perturbed inherent capacity of B cells to produce parasite specific antibodies, which requires further investigation and will be addressed in future studies.

4.6 Summary

In summary, our findings suggest, tissue-resident CD169⁺ macrophage subset acts as a key sentinel that plays an instrumental role not only in innate immunity but also orchestrating appropriate adaptive immune responses during *Plasmodium* infection. This study highlights pleiotropic functional attributes of the CD169⁺ macrophage subset in controlling parasite dissemination, triggering optimal T and B cell responses and maintenance of vascular integrity. More importantly, intrinsic capacity of CD169⁺ macrophages to produce IL-10 throughout the course of *Plasmodium* infection uncovers an immunoregulatory role whereby they regulate balance between pro and anti-inflammatory immune milieu for resolution of inflammation and limiting pathogenesis, which otherwise results in multiple organ pathologies observed in overlapping multifaceted malarial syndromes. Hence, the tissue-resident CD169⁺ macrophage subpopulation can be considered as a frontline of host defense in *Plasmodium* infection. Since the CD169⁺ macrophage subset is also present in the perifollicular zone of the human spleen in proximity to the blood vessels [229], there is a likelihood of similar functional characteristics as seen in murine malaria. The clinical measures that provide functional enhancement of these macrophages should be exploited for therapeutic interventions to alleviate the pathologies and resulting morbidity during malaria.

4.7 Significance of the current study and prospective impacts on the basic and translational malarial research

To the best of our knowledge, this study is a first *in vivo* demonstration of pleiotropic functional attributes of a CD169⁺ macrophage subset in a natural blood borne infection model i.e. *Plasmodium* species which comprehensively and simultaneously attempts to dissect the role of this specialized macrophages in the outcome of multifaceted malarial syndromes. The results documented in this study presents the murine *Plasmodium* infected CD169-DTR mouse model as a unique system that mirrors most of the pathological consequences as seen in human *P. falciparum* infection. In addition, the present study sheds light on the exclusive functional capacities of a cell subset of macrophage family, which was previously unappreciated due to either absence of such a cellular subset depletion system or lack of such endeavors on the studies in malaria.

This study highlights the critical role of tissue-resident CD169⁺ macrophages in the outcome of murine severe/complicated malaria (*P. berghei* ANKA infection), acute non-lethal malaria (*P. yoelii* infection) and the same holds true also in the case of relapsing

malaria (*P. chabaudi* infection) as per the unpublished data of our lab and preliminary observations in this very recent study [352]. The accounted functional deficits in parasite infected CD169-DTR mouse implicates the CD169⁺ macrophages as mediators of T cell and B cell driven downstream antimalarial effectors in addition to their intrinsic anti-inflammatory capacities. This opens future research avenues to identify the CD169⁺ macrophage centered anti-*Plasmodium* cellular and molecular mechanisms in the context of generation of appropriate parasite specific antibody responses and T cell mediated cytotoxic responses.

Another key finding of the present study implicates CD169⁺ macrophages in maintenance of the vascular integrity including blood-brain barrier during PbA infection. This leads to an obvious research question; the identification of the inherent CD169⁺ macrophage centered angiogenic factors, which might play a role in blood vessel maintenance.

Lately, vaccination approaches have been reoriented to target antigens to APCs in order to induce robust downstream cellular and humoral immune responses. In this context, antigen targeting to CD169⁺ macrophage have been reported to elicit desired cellular responses in the liver and spleen by administering nanogram amounts of the lipid antigen [240]. In the same direction, another study has demonstrated antigen targeting to the splenic CD169⁺ macrophages results in the induction of robust antigen specific germinal center B cell mediated high affinity antibody production. In addition, this study also reports, macrophages mediate establishment of apt cytokine milieu that result in these antigen specific antibody responses [239]. These evidences suggest CD169⁺ macrophages can be the likely APCs that can be selectively targeted with antimalarial vaccine formulations to induce robust cellular and humoral effectors along with establishment of desired cytokine milieu in a case dependent manner. The results documented in this study also strongly support such a possibility in future translational malaria research and healthcare. Since, we present CD169⁺ macrophages as anti-inflammatory cells in malaria and given the fact the macrophages are highly plastic in nature and differentiate into pro/anti-inflammatory phenotypes in response to tissue specific molecular and cytokine signals; strategies aimed to direct differentiation of monocytes into anti-inflammatory macrophages can be explored. This approach could be highly beneficial to ameliorate *Plasmodium* induced pathologies and the resulting morbidity.

5. References

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6. Appendix

6.1 Supplementary Figures

Figure S1: Diphtheria toxin mediated depletion of marginal zone macrophages (MZMs) in Clec4a4-DTR mouse. Immunofluorescence staining of SIGNR-1⁺ marginal zone macrophages (MZMs; red) and CD169⁺ marginal metallophilic macrophages (MMMs; green) in the spleen of DT injected (10ng/gbw) of WT and Clec4a4-DTR mice. Cryostatic sections (6 μ m) of spleens were stained with biotinylated-anti SIGNR-1/Texas Red-Streptavidin and FITC-anti CD169. Images were acquired at magnification: 20X. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks of age.

Figure S2: Diphtheria toxin mediated ablation of CD169⁺ macrophages did not affect the CD8⁺/CD103⁺ and CD11b⁺ DC subsets in spleen, lung and liver of CD169-DTR mouse. (A) Representative flow cytometry dot plots of stained leukocytic cells showing percentage of CD8, CD103 and CD11b expressing DC subsets gated on CD45 CD11c MHCII cells obtained from spleen, lung and liver of DT injected (10ng/gbw i.p.) WT and CD169-DTR mouse. The above organs were extracted after 2 subsequent DT injections at the interval of 24 hours. (B) Bar charts represent percentage mean $\pm$ S.D. of CD8⁺ & CD11b⁺ DC subsets obtained from spleen and CD103⁺ & CD11b⁺ subsets as explained in (A) of DT injected WT (white bar) and CD169-DTR (black bar). Statistical significance was determined using an unpaired student *t*-test. NS= Not significant at $p < 0.05$. Data in (A) and (B) are representative of two independent experiments, $n=3$ mice/group, 6-8 weeks old mice.

Figure S3: Unperturbed CD169 expression on different myeloid cell subsets in liver, lungs and kidney and efficient ablation of tissue-resident macrophages in all the tested organs during *Plasmodium* (PbA) infection. (A) Representative dot plots and histograms of the stained leukocytic cells showing CD11b, F4/80, and CD169 expression on different myeloid cell subsets (fraction 1 to 3 based on differential expression of the above cell surface markers), mixture of myeloid and lymphoid cells (fraction 4 comprising DCs, B cells and NK cells; data not shown) non-myeloid CD11b⁺F480⁻ cells (fraction 5 comprising T and other immune cells; data not shown) obtained from lungs, liver and kidney of PbA infected and DT treated (10ng/gbw) WT and CD169-DTR mice at day 8-post infection. Blue and red histograms represent isotype-controls and CD169 stained cells respectively. Data are representative of two independent experiments, n=3 mice/group, 6-8 weeks of age.

Figure S4: Hemozoin deposits in the brain, spleen, liver, lung, kidney and heart sections of PbA infected WT and CD169-DTR mice at day 8-post infection. Magnified images of H&E stained sections (original magnification 100X). Scale bars: 5 μ m. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks old mice. Note: These images show selected areas of intense Hz deposits in different organs for visualization only and were not used for quantification.

Figure S5: Representative flow cytometry gating strategy of splenocytes for the data shown in Figure 3.7D and 3.7E (A) Representative dot plots showing the percentage of CD4⁺, CD8⁺, CD69⁺CD4⁺, and CD69⁺CD8⁺ T cells (gated on CD3⁺) and (B) IFN-γ-producing CD4⁺ and CD8⁺ T cells and Granzyme B-expressing CD8⁺ T cells in the spleen of naïve (left), infected WT (middle), and infected CD169-DTR mice (right). (C) Gating strategy for analysis of myeloid cells showing FSC, SSC, CD11b, Ly6C, and Ly6G expression in naïve mice. (D) Representative dot plots showing the percentage of CD11b⁺, Ly6C⁺, and Ly6G⁺ splenocytes of naïve (left), infected WT (middle), and infected CD169-DTR mice (right).

Figure S6: PbA infection results in vascular occlusion in the brain of CD169-DTR mouse. Representative bright field microscopic images of H&E stained paraffin tissue sections (6 μ m) obtained from the brains of PbAGFP-infected WT and CD169-DTR mice at day 8-post infection (n=2-3 mice/group, 6-8 weeks old mice). Scale bars: 50 μ m. Data are representative of two independent experiments with at least 2 mice/group in each experiment.

Figure S7: Representative flow cytometry gating strategy of the leukocytes in the brain for the results shown in Figure 3.8G and 3.8H. (A) Representative dot plots showing the percentage of CD45⁺, CD3⁺, CD4⁺, CD8⁺ (gated on CD3⁺) and (B) IFN- γ -producing CD4⁺ and CD8⁺ T cells and Granzyme B-expressing CD8⁺ T cells in the brain of naïve (left), infected WT (middle), and infected CD169-DTR mice (right). (C) Gating strategy for analysis of myeloid cells showing FSC, SSC, CD11b, Ly6C, and Ly6G expression in naïve mice. (D) Representative dot plots showing the percentage of CD11b⁺, Ly6C⁺, and Ly6G⁺ leukocytes of naïve (left), infected WT (middle), and infected CD169-DTR mice (right).

Figure S8: PbA infection results in occlusion of hepatic sinusoids in the CD169-DTR mouse. Representative bright field microscopic images of picosirius red stained paraffin tissue sections (6 μ m) obtained from the livers of PbAGFP-infected WT and CD169-DTR mice at day 8-post infection (n=2-3 mice/group, 6-8 weeks old mice). Images show marked hemozoin deposits in the sinusoids of infected CD169-DTR mouse. Scale bars: 50 μ m. Data are representative of two independent experiments with at least 2 mice/group in each experiment.

Figure S9: Representative flow cytometry gating strategy of the leukocytes in the liver for the results shown in Figure 3.9A and 3.9B. (A) Representative dot plots showing the percentage of CD4⁺, CD8⁺, CD69⁺CD4⁺, and CD69⁺CD8⁺ T cells (gated on CD3⁺) and (B) IFN-γ-producing CD4⁺ and CD8⁺ T cells and Granzyme B-expressing CD8⁺ T cells in the liver of naïve (left), infected WT (middle), and infected CD169-DTR mice (right). (C) Gating strategy for analysis of myeloid cells showing FSC, SSC, CD11b, Ly6C, and Ly6G expression in naïve mice. (D) Representative dot plots showing the percentage of CD11b⁺, Ly6C⁺, and Ly6G⁺ in the liver leukocytes of naïve (left), infected WT (middle), and infected CD169-DTR mice (right).

Figure S10: Representative flow cytometry gating strategy of the leukocytes in the lung for the results shown in Figure 3.10C and 3.10D. (A) Representative dot plots showing the percentage of CD4⁺, CD8⁺, CD69⁺CD4⁺, and CD69⁺CD8⁺ T cells (gated on CD3⁺) and (B) IFN- γ -producing CD4⁺ and CD8⁺ T cells and Granzyme B-expressing CD8⁺ T cells in the lungs of naïve (left), infected WT (middle), and infected CD169-DTR mice (right). (C) Gating strategy for analysis of myeloid cells showing FSC, SSC, CD11b, Ly6C, and Ly6G expression in naïve mice. (D) Representative dot plots showing the percentage of CD11b⁺, Ly6C⁺, and Ly6G⁺ in the lung leukocytes of naïve (left), infected WT (middle), and infected CD169-DTR mice (right).

Figure S11: Sorting strategy and cell purity obtained for $F4/80^{high}CD11b^{low}$ and $Ly6C^{high}$ cell populations for the results shown in Figure 3.12B, C and D.

Figure S12: Hemozoin deposits in the brain, spleen, liver, lung, kidney and heart sections of Py infected WT and CD169-DTR mice at day 11-post infection. Magnified images of H&E stained sections (original magnification 100X). Scale bars: 5 μ m. Data are representative of two independent experiment, n=2 mice/group, 6-8 weeks old mice. Note: These images show selected areas of intense Hz deposits in different organs for visualization only and were not used for quantification.

6.2 List of commercial antibodies

Anti-mouse antibody	Label	Clone	Company
CD45.2	APCCy7	30F11	Biolegend
CD45.2	PE-Cy7	104	eBioscience
CD3	BUV737	17A2	BDbioscience
CD4	FITC	H129.19	Biolegend
CD4	BUV395	GK1.5	BDbioscience
CD8	BUV395	53-6.7	BDbioscience
CD8	PerCp-Cy5.5	53-6.7	eBioscience
CD8	APC	53-6.7	eBioscience
CD69	PE	H1.2F3	ebioscience
IFN- γ	PE	MP6-XT22	Biolegend
IFN- γ	PE-CF594	XMG1.2	BDbioscience
Granzyme B	Pacific Blue	GB11	Biolegend
Granzyme B	PE	NGZB	ebioscience
IL-10	PECy7	JES5-16E3	Biolegend
CD11b	APC-Cy7	M1/70	Biolegend
CD11b	PerCp-Cy5.5	M1/70	Biolegend
CD11b	FITC	M1/70	Biolegend
CD11b	BUV395	M1/70	BDbioscience
F4/80	PE	BM8	eBioscience
Ly6C	PerCp-Cy5.5	HK1.4	ebioscience
Ly6G	APC	1A8	Biolegend
Ly6G	BUV395	1A8	BDbioscience
I-A/I-E	Pacific Blue	M5/114.15.2	Biolegend
CD169	PE Texas Red	Ser4	Biolegend
CD169	APC	Ser4	eBioscience
CD31	FITC	390	eBioscience
SIGNR-1	Biotin	ER-TR9	abcam
Strep	PE Texas Red	N.A.	Biolegend
IgG	HRP	N.A.	Southern Biotech
IgG1	HRP	N.A.	Southern Biotech
IgG2a	HRP	N.A.	Southern Biotech

Dye	Label	Company	Dye
Live/Dead® Fixable Violet Dead Cell stain	Violet AA	Invitrogen, Paisley, UK	Live/Dead® Fixable Violet Dead Cell stain
Zombie aqua™ fixable viability dye	BrilliantViolet 510	BioLegend, San Diego, CA, USA	Zombie aqua™ fixable viability dye
Propidium iodide (PI)	Max emission: 617	Sigma, St. Louis, MO, USA	Propidium iodide (PI)

6.3 Media, buffers and solutions

1% acid ethanol solution

Ethanol

1% HCl

Alsever's solution

For 500 mL:

10.25 g D-(+)-Glucose

3.95 g trisodium citrate·2H₂O

2.1 g NaCl

50 mL glycerine

450 mL distilled H₂O

pH adjusted to 6.1

Blocking buffer

1X PBS

10% FCS

Cell fixation buffer

1X PBS

1% paraformaldehyde

pH adjusted to 7.4

Diphtheria Toxin (DT)

1X PBS

1% mouse serum

2 ng/μl DT

ELISA wash buffer

1X PBS

0.05% Tween-20

ELISA assay buffer

1X PBS

1% Bovine Serum Albumin (BSA)

IMDM 2%

Iscove's Modified Dulbecco's Medium (IMDM)

2% Fetal Calf serum (FCS)

Luciferin substrate solution

D-Luciferin-K⁺ Salt 1g

Dissolve in 66 ml of 1X D-PBS to get concentration of 15mg/mL

Organ fixation buffer

1X PBS

4% paraformaldehyde

pH adjusted to 7.4

PBS 2%

1X PBS

2% FCS

Permeabilization Buffer

PBS 2%

0.5% saponin

70% Percoll solution

10X PBS, 35 ml

Percoll, 315 ml

IMDM, 150 ml

40% Percoll solution

10X PBS, 20 ml

Percoll, 180 ml

IMDM, 300 ml

Picrosirius Red Stain

For 200ml

0.1% Direct Red (0.2g)

0.1% Fast Green FCF (0.2g)

1.2% Picric acid (2.4g Picric acid dissolved in 200 ml water)

Red blood cells (RBC) lysis buffer

0.89% ammonium chloride

Distilled H₂O

3% Sodium Citrate Solution

70 µl of 20% Sodium Citrate solution (Distilled H₂O)

500 µl 1 X PBS

Scott's tap water substitute

For 500 mL:

1 g sodium bicarbonate

10 g magnesium sulphate

Distilled water

6.4 Reagents, chemicals and kits

Name	Manufacturer
Acetone	Fisher Scientific, Longhborough, UK
Ammonium chloride	Sigma, St. Louis, MO, USA
Alanine Transaminase (ALT Kit)	Bioo Scientific, Austin, TX, USA
Brefeldin A	Sigma, St. Louis, MO, USA
BSA	Sigma Aldrich, St. Louis, MO, USA
Collagenase D	Roche, Basel, Switzerland
D-(+)-Glucose	Sigma Aldrich, St. Louis, MO, USA
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich, St. Louis, MO, USA
Direct Red 80	Sigma Aldrich, St. Louis, MO, USA
DMEM	Gibco, Grand Island, NY, USA
DPX Mountant for histology	Sigma Aldrich, St. Louis, MO, USA
DT	Sigma, St. Louis, MO, USA
EDTA	USB, Cleveland, OH, USA
Eosin	Merck KGaA, Darmstadt, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Evans Blue	Sigma Aldrich, St. Louis, MO, USA
Fast Green FCF	Sigma Aldrich, St. Louis, MO, USA
FCS	Chemicon International, CA, USA
Fluorescence mounting medium	Dako, Golstrup, Denmark
Geimsa stain	Sigma Aldrich, Steinheim, Germany
Glycerine	Affymetrix, Santa Clara, CA, USA
HCl	Merck KGaA, Darmstadt, Germany
Hematoxylin	Merck KGaA, Darmstadt, Germany
Ionomycin	Sigma Aldrich, St. Louis, MO, USA
IMDM	Gibco, Grand Island, NY, USA
Live/Dead violet stain	Invitrogen, Paisley, UK
Luciferin-(D) K ⁺ salt	Perkin Elmer, Caliper Life Sciences USA
Magnesium sulfate	Sigma Aldrich, St. Louis, MO, USA
Methanol	Sigma Aldrich, St. Louis, MO, USA
Mouse IFN- γ ELISA MAX	BioLegend, San Diego, CA, USA
Mouse IL-10 ELISA MAX	BioLegend, San Diego, CA, USA
Mouse TNF- α ELISA MAX	BioLegend, San Diego, CA, USA
NaCl	Merck KGaA, Darmstadt, Germany
NaOH	Merck KGaA, Darmstadt, Germany
N, N-dimethyl formamide	Sigma Aldrich, St. Louis, MO, USA
Paraffin	Leica Microsystems, Wetzlar, Germany
Paraformaldehyde (PFA)	Sigma Aldrich, St. Louis, MO, USA
PBS	Gibco, Grand Island, NY, USA
Percoll TM	GE Healthcare, Uppsala, Sweden
Picric acid	Sigma Aldrich, St. Louis, MO, USA
PMA	Sigma Aldrich, St. Louis, MO, USA
Saponin	Fluka Chemie AG, Buchs, Switzerland
Sodium bicarbonate	Sigma Aldrich, St. Louis, MO, USA
Sodium citrate	Sigma Aldrich, St. Louis, MO, USA
Tissue-Tek OCT compound	Sakura Finetek, Torrance, CA, USA

TMB Substrate Reagent Set	BioLegend, San Diego, CA, USA
Trisodium citrate·2H ₂ O	Fluka Chemie AG, Buchs, Switzerland
Trypan blue	Sigma Aldrich, St. Louis, MO, USA
Tween 20	Sigma Aldrich, St. Louis, MO, USA
Xylene	Sigma Aldrich, St. Louis, MO, USA

6.5 List of equipments

BD FACSCalibur Becton Dickinson, Franklin Lakes, NJ, USA

BD LSR II Becton Dickinson, Franklin Lakes, NJ, USA

BD LSR Fortessa X-20 Becton Dickinson, San Jose, CA, USA

BD FACSAria™ Ilu Becton Dickinson San Jose, CA, USA

Perkin Elmer IVIS Spectrum CT Imaging System, Caliper Life Sciences, USA

Abaxis VetScanR HM2 Hematology System, MA, USA

Illumina ECOTM Real Time PCR System, San Diego, CA, USA

Leica CM3050 S - Cryostat Leica Microsystems, Wetzlar, Germany

Leica EG1150 C - Embedding Leica Microsystems, Wetzlar, Germany

Leica EG1150 H - Embedding Leica Microsystems, Wetzlar, Germany

Leica RM2265 - Microtome Leica Microsystems, Wetzlar, Germany

Microscope Nikon Eclipse 80i Nikon Instruments, Melville, NY, USA

Multiskan Spectrum Thermo Scientific, Waltham, MA, USA

Thermocycler T3000 Biometra GmbH, Goettingen, Germany

7. Author's publication

Gupta P, Lai SM, Sheng J, Tetlak P, Balachander A, Claser C, Renia L, Karjalainen K, Ruedl C. *Tissue-Resident CD169⁺ Macrophages Form a Crucial Front Line against Plasmodium Infection*. Cell Reports. 2016. S2211-1247 (16) 30896-8.

8. Oral and Poster Presentations

1. Nominated as **chairperson** and selected for **oral presentation**, “*Tissue-resident CD169⁺ macrophages as a frontline of host defense in Plasmodium infection*”, at ICI 2016 (International Congress of Immunology), Melbourne, Australia, August 2016.
2. **Poster presentation**, “*Tissue-resident CD169⁺ macrophages as a frontline of host defense in Plasmodium infection*” at 8th International Singapore Symposium of Immunology, Singapore, May 2016.
3. **Oral presentation**, “*Tissue-resident macrophages as a frontline of host defense in Plasmodium infection*”, at 4th Singapore Malaria Network Meeting”, Nanyang Technological University, Singapore, February 2016.
4. **Poster presentation** on “*Tissue-resident macrophages prevent inflammation in murine Plasmodium infection*” at FIMSA (Federation of Immunological Societies of Asia-Oceania) Meeting, Singapore, July 2015.
5. Nominated for a **talk** on “*CD169⁺ macrophages prevent severe immunopathology in murine Plasmodium infection*” in Joint Singapore and Turkey Workshop at Bilkent University Ankara, Turkey, May 2015.
6. **Poster presentation** on “Importance of distinct myeloid cells subsets in protection and pathology to malarial parasite” at 4th SGSI PhD Student Retreat-2014, Singapore.