IMPORTANCE OF DISTINCT DENDRITIC CELL SUBSETS IN MALARIA IMMUNITY AND PATHOLOGY

LUCIA PIVA
SCHOOL OF BIOLOGICAL SCIENCES
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<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>cDCs</td>
<td>Conventional Dendritic Cells</td>
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<tr>
<td>CDP</td>
<td>Common Dendritic cell Progenitor</td>
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<td>CLP</td>
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<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>Diphtheria Toxin Receptor</td>
</tr>
<tr>
<td>DTR</td>
<td>Diphtheria Toxin Receptor</td>
</tr>
<tr>
<td>ECM</td>
<td>Experimental Cerebral Malaria</td>
</tr>
<tr>
<td>ECM</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Fetal Calf Serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Flt3</td>
<td>Fms-like thyrosin kinase 3 receptor</td>
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<td>Flt3L</td>
<td>Fms-like thyrosin kinase 3 Ligand</td>
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NaOH  Sodium Hydroxide
NK    Natural Killer
NKT   Natural Killer T
NO    Nitric Oxide
OCT   Optimal Cutting Temperature
OD    Optical Density
p.i.  Post infection
pA    poly Adenylation
PAMPs Pathogen-Associated Molecular Patterns
PbA   Plasmodium berghei ANKA
PbA-GFP Plasmodium berghei ANKA-GFP
PBMCs Peripheral Blood Mononuclear Cells
PBS   Phosphate Buffer Saline
PCR   Polymerase Chain Reaction
pDCs  Plasmacytoid Dendritic Cells
PfEMP-1 P. falciparum erythrocytes membrane protein-1
PMS   Pregnant Marel Serum
PP    Peyer’s patches
Py1.1-GFP Plasmodium yoelii 1.1-GFP
RBCs  Red Blood Cells
RFP   Red Fluorescence Protein
RMCBS Rapid Murine Coma Behaviour Scale
TCR   T Cell Receptor
TGF-β Transforming Growth Factor beta
TLR   Toll-Like Receptor
TMB   3, 3', 5, 5' tetramethylbenzidine
TNF   Tumor Necrosis Factor
TNFR1 Tumor Necrosis Factor Receptor 1
TNFR2 Tumor Necrosis Factor Receptor 2
Tregs Regulatory T cells
WBCs  White Blood Cells
WHO   World Health Organization
wt    Wild type
YM-GFP  \textit{Plasmodium yoelii} YM-GFP
\(\alpha\beta\)  Alpha Beta
\(\gamma\delta\)  Gamma Delta
SUMMARY

Plasmodium species infections trigger strong innate and acquired immune responses, which are often associated with severe pathology, like in the case of cerebral malaria. Dendritic cells (DCs), a heterogeneous family of antigen presenting cells, constitute the most important myeloid lineage in orchestrating the host immune responses against the parasite. However, due to the cellular complexity of the innate immune system, the contribution of distinct DC subsets has remained elusive. In order to untangle the roles of different DC subsets in protection against Plasmodium parasites and in parasite induced pathology, we have generated three independent mouse strains (Siglec-H−, Clec9A− and Clec4a4-DTR mice) which allow us to specifically ablate in vivo plasmacytoid DCs, CD8+ Clec9A+ and CD8−CD11b+ DCs, respectively. The aim of this thesis was to study the consequences of the absence of these myeloid cell subtypes during blood stage malaria, using the murine P. yoelii and P. berghei ANKA experimental models.

In infection with a P. yoelii 17X clone 1.1 non lethal strain, DT treatment of both Siglec-H− and Clec9A-DTR strains resulted in an improved control and clearance of the parasite, whereas Clec4a4 DTR mice did not control parasite growth and succumbed to infection after 12 to 16 days. When compared to Siglec-H−, Clec4a4-DTR and wild type (wt) mice, Clec9A-DTR mice had significantly reduced serum levels of the Th1 cytokine Interferon gamma (IFN-γ). Consistently, ablation of Clec9A+ DCs resulted in a switching towards Th2 immune responses and in the generation of a clear isotype profile of parasite-specific antibodies (IgG1 > IgG2a). When injected intra peritoneally, these IgG1 antibodies reduced blood levels of non lethal P. yoelii, however did not protect mice when infected with a lethal strain. The higher susceptibility of the Clec4a4-DTR mice to the non lethal P. yoelii strain could possibly be explained by the lack of F4/80+ red pulp macrophages which, together with the CD8−CD11b+ DCs, are also affected upon DT injection.
Using the *P. berghei* ANKA mouse model of experimental cerebral malaria, we have shown that the Clec9A⁺ subset, but not the Clec4a4⁺ subset, of conventional DCs, are the antigen presenting cells responsible for priming and activating cytotoxic CD8⁺ T cells involved in ECM pathogenesis. All of these findings contribute to a better understanding of malaria immunity and pathology, and point the way for future investigations, which might lead to new applications in vaccine development and malaria immunotherapy.
1. INTRODUCTION

1.1 Dendritic cells
DCs are rare hematopoietic cells belonging to the antigen presenting cell (APC) family, playing a pivotal role as coordinator of the immune system. DCs were first isolated from the mouse spleen in 1973 by Ralph Steinman and colleagues, who identified them as a novel population of cells, distinct from the monocytes/macrophages system [1]. However, due to the rarity of DCs in vivo, the lack of markers to unambiguously discriminate them from monocytes and macrophages, and the difficult isolation, understanding of DC functions proceeded very slowly for about 2 decades, until the 1990s, when novel methods for purification and generation of DCs from blood and bone marrow (BM) led to progressive interest and knowledge on DCs [2-4]. According to the ‘Langerhans paradigm’ elaborated on Ralph Steinman’s studies, and the observations done by Paul Langerhans one century earlier on Langerhans cells (LCs), DCs have been described as immune cells residing in peripheral tissues in an immature state, equipped with a large variety of pattern recognition receptors to sample the local environment. Upon activation they migrate to draining lymph nodes (LNs), where they are responsible for activating naïve T cells thanks to their ability to express high levels of antigen-loaded major histocompatibility complex (MHC) molecules, together with the required co-stimulatory signals [5-7]. Since the enunciation of the ‘Langerhans paradigm’, new evidence has shown that, beside activating immune responses, DCs are also involved in establishment of tolerance to self antigens and non-pathogenic foreign antigens. Moreover, analysis of DCs from various lymphoid and non-lymphoid tissues has revealed a considerable heterogeneity among the DC population, with different subsets specializing in particular functions, and with subsets diverging from the ‘Langerhans paradigm’ [8].

1.1.1 DC heterogeneity and location
Phenotypically, murine DCs express CD45, a marker shared by all white blood cells (WBCs) of the hematopoietic system, CD11c, an integrin
common to all DCs and found at lower levels also on monocytes, macrophages and neutrophils, and constitutively express MHC class II. Although all DCs are capable of antigen uptaking, processing and presentation to naïve T cells, different subsets differ in transcriptional programs and cytokines required for their generation, migratory pathways, detailed immunological function and location, with more than one subset being found within one organ or tissue (Figure 1.1).

Figure 1.1. Mouse DC populations, locations and turnover in steady state. Frequencies are given as percentage of total nucleated hematopoietic cells. Time to approximately 50% renewal in steady state is given in days (d). *skin-draining LNs; **epidermis; +present but exact number not known; ↑present in inflammation. (Adapted from [9]).

A first important distinction is between plasmacytoid DCs (pDCs) and conventional DCs (cDCs).

pDCs are relatively long-lived, round cells with a non-dendritic morphology that circulate in the blood and can be found in the steady state mainly in BM, spleen, thymus, LNs, liver and in mucosal-associated lymphoid tissues. Murine pDCs do not express CD11b, an integrin found on most monocytes, macrophages and granulocytes. They express low levels of MHC II, CD11c and lineage markers B220 and Ly6C, and express PDCA-1 (also called Bst-
2), and Siglec-H, a lectin recently characterized and which has become a marker for this specific subset of cells, due to its selective presence on pDCs [10]. Upon activation, they can differentiate into cDCs and acquire the ability to present antigens. However, pDC primary function is to produce high levels of type I interferons [11]. A human counterpart of murine pDCs exists, which expresses low levels of CD11c, CD45, CD4, and the lectins BDCA2 and BDCA4.

cDCs form a heterogeneous compartment of cells, and can be further distinguished in non-lymphoid tissue migratory DCs and lymphoid tissue-resident DCs. Tissue migratory cDCs are present in most tissues in the steady state. They follow the classical paradigm, constantly sampling the environment for antigens in peripheral tissues, and then migrating through the lymphatic vessels to the LN. The Langerhans cells (LCs) in the epidermis represent the model of migratory DCs. Distinct from tissue cDCs, lymphoid tissue-resident cDCs do not migrate, but they are found in lymphoid organs, where they directly collect and present antigens locally. Compared to migratory tissue cDCs, which present a mature phenotype when reaching the LN, lymphoid tissue-resident cDCs have an immature phenotype and are active in antigen uptaking and processing [11]. Because different subsets of lymphoid and non-lymphoid cDCs exist in different organs, they will next be described in detail according to their location.

**Lymphoid tissue-resident cDCs**

In the mouse spleen two main subsets of cDCs can be distinguished, which have been shown to be specialized in different functions [11, 12]. One is the CD8<sup>+</sup>Clec9A<sup>+</sup> cDC subset, localized mainly at the T cell area and marginal zone [13]; CD8<sup>-</sup>CD11b<sup>+</sup> cDCs, instead, are found primarily in the red pulp and bridging channels [12]. Like all cDCs, both subsets are characterized by high levels of CD11c on their surface. However, CD8<sup>+</sup>Clec9A<sup>+</sup> cDCs also express the lectin-like receptors CD205 and Langerin, but are negative for CD11b, the alpha chain of the macrophage-1 antigen (Mac-1); on the contrary CD8<sup>-</sup>CD11b<sup>+</sup> cDCs do not express CD205 and Langerin, but are positive for CD11b and, a small subset, also for CD4. Both subsets move to the T cell area of the spleen upon activation, however CD8<sup>+</sup>Clec9A<sup>+</sup> cDCs
are specialized in MHC I cross-presentation of antigens [14-16], whereas CD8^−CD11b^+ cDCs are more efficient in MHC II processing and presentation of blood-borne antigens [12]. Moreover, the former subset can produce high levels of interleukin 12 (IL-12), which results in activation of a Th1 response, whereas the latter can produce the Th2 cytokines IL-4 and IL-10 [17]. CD8^+Clec9A^+ and CD8^−CD11b^+ cDCs are present also in LNs, where they account only for half of the cDC population, because migratory cDCs are also found here, which enter via lymphatic vessels, and can transfer antigens from the periphery to the resident cDCs [18]. The majority of cDCs in the thymus are CD8^+Clec9A^+, even though a minority of CD8^− cDCs is also present. Negative selection in the thymus is highly dependent on the presentation of self antigens by these cDCs.

**Non-lymphoid tissue migratory cDCs**

Both skin epidermis and dermis contain DCs. LCs are found in the epidermal layer and are characterized by high expression of Langerin, intermediate CD11c levels, CD11b, and the macrophage marker F4/80. They can sense a variety of stimuli such as lipopolysaccharide, and acquire strong T cell stimulatory properties. In the dermis, two main subsets are present: CD11b^loLangerin^+ cDCs, which express also the E-cadherin ligand CD103, and CD11b^hiLangerin^− cDCs [19]. Lung cDCs are found in the airways epithelia and lung parenchyma. Those present in the airways express Langerin and CD11b and are similar to LCs [20, 21]. Parenchymal cDCs can be divided in two subsets: CD103^+CD11b^− cDCs and CD103^−CD11b^+ cDCs [22]. The former is also Langerin^+, whereas the latter expresses the cell surface molecule CX3CR1, involved in leukocyte adhesion and migration. Both DC populations are present also in liver and kidney. CD103^+CD11b^− cDCs in all these organs are developmentally and functionally related to CD8^+Clec9A^+ cDCs in the spleen [23, 24].

In the intestine, three populations of cDCs can be distinguished: CD103^+CD11b^lo cDCs are found in the Peyer's Patches, whereas CD103^+CD11b^+ and CD103^−CD11b^hi cDCs are in the lamina propria [25,
CD103^+CD11b^{hi} cDCs also express CX3CR1, which is absent on the other two subsets. Expression of CX3CR1 has been proposed to play a role in dendrites movements for lumen sampling [27]. The CD103^+CD11b^{hi} subset has been shown to be able to migrate to the mesenteric LNs, carrying pathogenic bacteria from the intestine [26]. Even though all cDCs can uptake, process and present antigens, specific subsets have different antigen presentation properties in vivo. Although MHC I and MHC II presentation are operational in both CD8^+Clec9A^+ and CD8^-CD11b^{hi} cDCs, different molecules are available on their surface for antigen capture, which may determine the mechanism of antigen presentation and, ultimately, the functional specialization. For example, CD8^+Clec9A^+ cDCs are the most efficient at phagocytosis of dead cells, and therefore, at presenting cellular antigens in both MHC I and II [14, 28, 29]. On the other hand, CD8^-CD11b^{hi} cDCs seem to be more efficient at MHC II presentation of exogenous phagocytosed antigens [30] or captured by C-type lectin receptors [12, 31, 32]. Moreover, CD8^+Clec9A^+ cDCs are equipped with specialized mechanism required for cross-presentation, which are absent in CD8^-CD11b^{hi} cDCs [30]. It has been shown that CD8^+ cDCs have higher expression of proteins associated with MHC I processing, like Tap1, Tap2, calreticulin and calnexin, whereas CD8^- cDCs, which are more efficient in MHC II presentation, express more of proteins necessary for MHC II presentation, such as cathepsin C, H and Z, H2-M\beta 1 and GILT [12].

1.1.2 DC development and homeostasis

DCs originate from hematopoietic stem cells (HSC) in the BM. Understanding of DC development has been controversial for many years. However, recently, early committed progenitors have been isolated and defined, and details on DC differentiation programs are starting to be uncovered. According to the current model, a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP) develop from HSC: CLPs give rise to NK, B and T cells, whereas DCs, monocytes, macrophages, granulocytes, erythrocytes and megakaryocytes develop from CMPs [33-36]. It has previously been suggested that CD8^+Clec9A^+ cDCs originate
from CLPs and CD8<sup>−</sup>CD11b<sup>+</sup> from CMPs [37]. However later studies have shown that both progenitors have comparable potential to give rise to both cDC subsets, but CMPs are much more frequent than CLPs in the BM, therefore most DCs originate from myeloid precursors [36, 38]. CMPs give rise to a macrophage and DC progenitor (MDP) which can differentiate into pDCs, DCs, monocytes and macrophages, but has lost the potential to develop into granulocytes, erythrocytes and megakaryocytes [39, 40]. Macrophage and DC fate separates when MDPs give rise to common DC progenitors (CDPs), which can differentiate into pDCs and DCs, but not monocytes and macrophages [41-43]. CDPs produce pDCs and pre-DCs, committed precursors which can give rise only to cDCs, but not pDCs [43, 44]. Pre-DCs finally exit the BM and migrate through the blood to lymphoid organs and peripheral tissues (Figure 1.2).

Figure 1.2. DCs, pDCs and monocytes development. (Adapted from [45]).

Pre-DCs are characterized by a very short life in the blood [46], which, together with their paucity, has made for the difficult isolation of these progenitors. cDC homeostasis requires constant replacement with new cells, and circulating pre-DCs in the blood are in equilibrium with cDCs in lymphoid organs and tissues. In particular, pre-DCs give rise to cDCs in spleen, resident cDCs in LNs, all CD103<sup>+</sup> cDCs and some CD103<sup>−</sup> DCs in gut, lung, kidney and skin [43]. The CD103<sup>−</sup> cDC subset appears to be in general more heterogeneous, and monocytes also contribute to their origin.
cDCs have a half-life of about 5 to 7 days in lymphoid organs (CD8\(^{+}\)Clec9A\(^{+}\) DCs have a turnover time of about 3 to 4 days in the spleen, and about 10 days in the thymus [47]), as well as liver and kidney, 7 to 13 days in the intestine, whereas lung cDCs can live for more than 20 days [46]. Although it has been shown that 5% of pre-DCs or cDCs in lymphoid organs are dividing at any given time [48], parabiosis experiments have proven that DC progenitors do not self-renew in situ and need continuous replacement by blood circulating pre-DCs [38, 46, 49].

Distinct from other cDCs, LCs in the epidermis are not generated from a BM derived blood progenitor, but are self-renewing in situ from precursors which colonize the dermis at the embryonic stage [50, 51]. Because monocytes can easily be differentiated into DCs in vitro [4], it has been suggested that they could also function as direct DC precursors in vivo. However, when fluorescein isothiocyanate (FITC)-labeled microspheres were injected in mice intracutaneously, FITC\(^{+}\) monocytes found in the draining LNs 4 days later expressed high levels of MHC I and II and CD86, but low levels of CD11c and CD8, therefore they did not become classical DCs [52]. Nonetheless, during inflammation Ly6C\(^{+}\) activated monocytes expressing MHC II as well as CD11c can be found in the spleen. These cells produce tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS), and have thus been named Tip-DCs [53].

**1.1.3 A combined differentiation-function DC classification**

DCs can be further classified according to their differentiation program, cytokines required for development and homeostasis, and function [54] (Figure 1.3).

**The Batf3-IRF8-Id2-Dependent cDC lineage**

The Batf3-IRF8-Id2 cDC lineage includes lymphoid tissue CD8\(^{+}\)Clec9A\(^{+}\) cDCs and non-lymphoid tissue CD103\(^{+}\)CD11b\(^{-}\) cDCs. Both subsets have been shown to require Batf3 and IRF8 transcription factors and Id2, a DNA inhibitor protein, for their development [22-24, 55-58]. Moreover, as pDCs, they both express the fms-like thyrosin kinase 3 receptor (Flt3) and depend on its ligand (Flt3L) for their development. Flt3L is a cytokine secreted by
stromal and endothelial cells and by activated T cells [59, 60]. Flt3 is expressed on progenitors very early in hematopoiesis, however it is retained only by pre-DCs and cDCs in lymphoid organs, as well as CD103−CD11b− DCs in non-lymphoid tissues [9]. Flt3L has been shown to control proliferation and homeostasis of these cell subsets [46, 61], and absence of either Flt3L or its receptors results in reduced numbers of Batf3-IRF8-Id2-Dependent cDCs [61, 62]. On the other hand, these cells do not express colony stimulating factor 1 (Csf-1) receptor, which is instead required for macrophage development [63].

When purified tissue CD8+Clec9A+ cDCs and non-lymphoid tissue CD103−CD11b− cDCs were analyzed for their transcriptional profile, it was shown that they have comparable gene expression patterns [23]. Interestingly about 50 to 70% of CD8+Clec9A+ cDCs in the spleen co-express CD103 [64, 65]. Moreover, they have a similar sensing receptor repertoire on their surface. For example, even though all DCs express Toll-like receptor 9 (TLR-9), both subsets express TLR-3 and TLR-11, which is missing from cDCs from the Batf3-IRF8-Id2-Independent lineage, and lack TLR-7 [66, 67], which is present on Batf3-IRF8-Id2-Independent cDCs. They also express the lectin-like receptors CD205, Langerin (at least in lymphoid organs, skin and lung), Clec9A, a molecule specialized in sensing apoptotic or necrotic cells, and CD36, another molecule believed to be involved in recognition of dead cells [68], which are absent or expressed at low levels on the CD8−33D1+ and CD103−CD11b+ subsets [12, 69-71].

Beside sharing origin, differentiation program and sensing receptors, both CD8+Clec9A+ cDCs and CD103−CD11b− cDCs are very potent IL-12 producers upon activation, they specialize in cross-presentation of self or foreign antigens to CD8+ T cells and can induce their differentiation [18, 70, 72-74]. CD8+Clec9A+ cDCs are also involved in elimination of self-reactive T cells in the thymus. Together with CD103−CD11b− cDCs, they exert regulatory functions by producing TGF-β and inducing development of regulatory T cells (Tregs) [75, 76], and are involved in maintenance of peripheral self tolerance through their ability to acquire peripheral antigen from migratory DCs [77].
A population of human DCs, defined as CD141\(^+\)CD1c\(^-\) or BDCA3\(^+\) DCs, has been shown to have similar properties to the Batf3-IRF8-Id2-Dependent cDCs. These cells express TLR3, Clec9A, can produce high amounts of IL-12 and cross-prime CD8\(^+\) T cells [78-82].

The Batf3-IRF8-Id2-Independent cDC lineage

Batf3-IRF8-Id2-Independent cDC subsets are more heterogeneous than Batf3-IRF8-Id2-Dependent cDCs. All of them express CD11b and are CD8\(^-\); however they are heterogeneous for CD103, F4/80 and CX3XR1 expression [22, 71]. In lymphoid organs, differentiation of CD8\(^-\)CD11b\(^+\) cDCs have been shown to partly depend on RelB, IRF2, IRF4 and RBPJ transcription factors [83-85] However, the differentiation program for Batf3-IRF8-Id2-Independent cDCs in non-lymphoid tissues is yet to be elucidated.

*Lamina propria* CD103\(^-\)CD11b\(^+\) and CD103\(^-\)CD11b\(^{hi}\) cDCs represent the best known example of Batf3-IRF8-Id2-Independent cDC heterogeneity. The CD103\(^-\)CD11b\(^{hi}\) subset develops from circulating monocytes, requires Csf-1 but not Flt3 for their differentiation, expresses the macrophage markers CD172a, CX3CR1 and Csf-1 receptor, and migrates poorly to draining LNs [25, 26, 86]. On the other end, CD103\(^+\)CD11b\(^+\) cDCs require Flt3L and granulocytes monocytes colony stimulating factor (GM-CSF) for their development, express only low levels of macrophage markers and can efficiently migrate to draining lymph nodes [25, 26, 86].

In the spleen, CD8\(^-\)CD11b\(^+\) cDCs share their Flt3L dependence with CD8\(^+\)Clec9A\(^+\) cDCs, however injection of this cytokine enhances the number of CD8\(^+\)Clec9A\(^+\) cDCs, but not CD8\(^-\)CD11b\(^+\) cDCs [87].

In lung and intestine, CD103\(^-\) cDCs have been shown to secrete more cytokines than CD103\(^+\) cDCs, to be more efficient in antigen clearance and leukocytes recruitment [64, 88].

**pDCs**

Upregulation of the basic helix-loop-helix transcription factor (E protein) E2-2 has been shown to mark the commitment to pDC development [89]. However, more yet unknown transcription factors are likely to be involved in pDC fate regulation. pDCs require Flt3 signaling for their development and
homeostasis [89]. They express TLR7 and TLR9, which allows them to sense viral nucleic acids. They are crucial during viral infection because they can produce large quantities of type I interferons [11] prior to differentiating into mature DCs, able to prime T cells against viral antigens. Moreover, recent evidence suggests a role for pDCs in tolerance by promoting $T_{reg}$ differentiation [90].

**Langerhans cells**

Consistent with their peculiar ontogeny, LCs have distinct differentiation requirements, compared to other DC subsets. In fact, LCs rely on signaling through the Csf-1 receptor for their development, but do not require Csf-1 or Flt3L [91]. LCs are highly phagocytic in the epidermis, but become indistinguishable from other migratory DCs upon reaching the LNs [19]. They have been shown to be, as Batf3-IRF8-IId2-Dependent cDCs, highly efficient in cross-presentation and T cell activation *in vitro* [92, 93].

**Tip-DCs**

The differentiation program and cytokines required by monocyte-derived DCs have yet to be uncovered. Some experiments suggest that GM-CSF might be involved in inflammatory DCs development [94].

*Figure 1.3. The Batf3-IRF8-IId2-Dependent and Independent DC lineages.* Different transcription factors and cytokines are involved in the development and homeostasis of different DC subsets (Adapted from [54]).
1.2 Studying the function of DCs

DCs are ideally located in strategic sites of the body such as skin, lung, spleen, gut, where they can detect invading pathogens and initiate immune responses. Their role as key coordinator of the immune system makes them a possible target for immunotherapy. A deep understanding of DC functions in vivo is a necessary pre-requisite in order to effectively exploit their properties. However, attempts to unambiguously characterize DC functions have been challenged by their heterogeneity and the diverse specialized functions of different subsets. Until a few years ago, much of our knowledge about DC biology was mainly based on in vitro assays and in vivo transfer studies involving bone marrow (BM) and cord blood culture-derived DCs. More recently, the use of murine infection models and genetic mouse models, including transcription factors and immune receptors deficient mice, as well as mice lacking specific cell populations, has helped to advance the understanding of DC functions. Whereas the absence of transcription factors or receptors is useful to investigate the role of those particular elements, a mouse model lacking a specific cell population is a useful tool to determine the in vivo role of that particular population in the steady state, infection, or other scenarios such as tumor, allergy and autoimmunity.

The lack of markers to uniquely define particular DC subsets has limited the application of conventional strategies, like depleting antibodies, for DCs ablation in vivo. The injection of liposomal clodronate, which blocks the cell metabolism and causes cell suicide, has been the only applicable method. However, this technique is not specific since liposomes uptaking occurs via phagocytosis, and therefore macrophages are also affected. Currently, the most efficient method to deplete cells in vivo is the diphtheria toxin receptor-diphtheria toxin (DTR-DT) system, a genetic cell ablation approach described for the first time in 2001 by Saito et al. [95], in which a cytotoxic gene is expressed in transgenic mice under the control of a tissue-specific promoter.
1.2.1 The DTR-DT system

DT is produced by Corynebacterium diphtheriae, a Gram positive bacterium responsible for causing diphtheria. DT structure and mechanism of action were described for the first time by Collier in 1975 [96]. The DT molecule is composed of two subunits: the subunit B is responsible for binding to the DTR, a membrane-anchored form of the heparin-binding epidermal growth factor receptor (HB-EGF), thus allowing the entry of the toxin inside the cell by endocytosis. Once inside the endosome, a trypsin-like protease breaks the peptide and disulphide bonds linking the two subunits together, and due to the acidity of the endosome, the subunit B creates pores in the endosome membrane. The subunit A is then released in the cell cytoplasm where it can inactivate the elongation factor 2 (EF-2) via ADP ribosylation, thus preventing protein synthesis and causing cell death via apoptosis [97, 98] (Figure 1.4(a)).

![Diagram of the DTR-DT system](image)

**Figure 1.4. The DTR-DT system.** (a) ADP ribosylation caused by DT prevents protein synthesis and thus results in apoptosis (Adapted from http://brainboxes2.wikispaces.com). (b) As rodent cells are $10^5$-$10^6$ times more resistant to DT compared to humans or other primate cells, expression of the high affinity human DTR under the control of a gene specifically expressed on a target rodent cell population renders only that population susceptible to DT-mediated apoptosis (Adapted from [95]).

In particular, DT is a potent toxin in humans and primates due to the high affinity of their DTR to the toxin. The extracellular region of rodent DTR presents a change in three aminoacids in a region important for making
contact with the B subunit of DT. Such a change is responsible for decreasing the DTR affinity to DT, thus making rodent cells $10^3$-$10^5$ times more resistant to DT, compared to human or other primate cells. Based on this, a powerful strategy for in vivo ablation in mice has been designed by expressing the high affinity human DTR under the control of a gene specifically expressed on a target rodent cell population (Figure 1.4(b)). One major advantage of this technique is that DT causes cell death via apoptosis, and therefore it does not cause inflammation.

The CD11c-DTR transgenic mouse was the first DTR mouse for ablation of DCs, generated by Jung et al. in 2002 [99]. CD11c is an integrin expressed at various levels on all DCs. The authors used the CD11c promoter to drive the human DTR expression. Upon a single DT injection, within 24 hours DCs were efficiently ablated in spleen, BM, colon, LNs and lung. On their first report describing the generation of the CD11c-DTR mouse, the authors demonstrated the role of CD11c$^{hi}$ DCs in cross-presentation to CD8$^+$ T cells [99]. Since the CD11c-DTR mouse has become available among the scientific community, several studies have been performed which have helped the understanding of DC roles in vivo. For instance, different experiments with bacterial and viral infections have demonstrated the key role of DCs in controlling pathogen spread, and in initiation of antigen-specific CD4$^+$ and CD8$^+$ T cell expansion [100-104]. Moreover, the CD11c-DTR mouse has been successfully used to study the development of DCs in vivo [44]. However, this DTR-transgenic mouse has a few limitations: (i) repeated administration of DT causes death, due to ectopic expression of the DTR in non hematopoietic cells, thus limiting the time frame to study the effect of the ablation. (ii) Beside being expressed on DCs, CD11c is also found in other cell populations, such as monocytes and macrophages. Indeed, marginal zone and metallophilic macrophages in the spleen, as well as alveolar macrophages, have been shown to be ablated in DT treated CD11c-DTR mice [105, 106]. Moreover, activated CD8$^+$ T cells and plasma cells are also sensitive in these mice, and other cells, like natural killer (NK) cells, have been shown to express CD11c [107-109]. This aspecific effect should be carefully considered when interpreting results obtained with this model and when assigning functions to DCs. To overcome this issue, in
2012 Nussenzweig's group generated a DTR mouse using the zinc finger transcription factor zDC (Zbtb46) to drive the DTR expression. zDC has been shown to be selectively expressed only by cDCs and committed cDC precursors, but not by monocytes, pDCs, or other immune cell populations. Indeed, injection of DT in the zDC-DTR mouse causes cDC ablation, leaving intact pDC, monocyte, macrophage, and NK cell compartments \[110\]. (iii) Given the considerable heterogeneity of the DC compartment, and given that, even though details on the function of different subsets have started to be uncovered, we are still far from fully understanding their parts in different scenarios, DTR models for ablation of defined specific subsets would be more useful and efficient tools.

1.2.2 DC markers for specific subset ablation
The selection of a cell-specific promoter to drive the DTR expression is crucial in the generation of cell depleting transgenic mice using the DTR-DT system, and the identification of specific markers remains one of the major limitations. In fact, most receptors are usually expressed on different leukocyte populations and only a few are restricted to DC subsets. Recently a few molecular markers have been described which identify particular subsets of DCs.

1.2.2.1 Siglec-H
Siglec-H is a novel described murine molecule belonging to the family of siglec proteins \[10\]. Siglec proteins are immunoglobulin-like lectins, mainly expressed in cells of the immune system. They bind to sialic acid, through which they exert both adhesive and signaling functions. An interesting characteristic of siglec proteins is that their expression is generally restricted only to certain cell lineages. For example, Siglec-1 is specifically expressed only by a distinct population of macrophages in the marginal zone of the spleen \[111\]. Similarly, eosinophils selectively express Siglec-8 \[112\], and other such examples exist. In particular, Siglec-H belongs to a particular subset of molecules which are principally expressed in cells of the innate immune system, the CD33-related siglecs, which comprises 5 murine members. Three of them, designated as Siglec-E, Siglec-F and Siglec-G,
contain the conserved immune receptor tyrosine-based inhibitory motif (ITIM) and ITIM-like motif, and are involved in recruitment and activation of protein tyrosine phosphatases SHP-1 and SHP-2 [10]. The other two murine CD33-related siglecs are the mouse ortholog of the human CD33/Siglec-3 expressed on neutrophils [113], and the recently described Siglec-H. Siglec-H is a type I membrane protein of 309 amino acids. Because the cytoplasmatic tail does not contain any ITIM-related sequence, Siglec-H does not function as an inhibitory receptor. Moreover, since no evidence for carbohydrate recognition has been observed, Siglec-H seems not to be a sialic acid-binding lectin. Instead, it has been shown to work as an endocytic receptor [10].

![Figure 1.5. Siglec-H is expressed specifically on pDCs. Single-cell suspensions from bone BM, spleen, and blood. (Adapted from [10]).](image)

Analysis of Siglec-H expression in BM, spleen and LNs of different mouse strains has shown that it is expressed exclusively on pDCs, defined as B220⁺CD11c⁻⁻⁻ [114]. In particular, about 1%, 0.5% and 0.1% of cells are Siglec-H⁺ cells in BM, spleen and blood, respectively (Figure 1.5). Siglec-H is also expressed intracellularly in ER-TR9⁺ marginal zone macrophages (MZMs) in the spleen, and on medullary cord macrophages in LNs [10].

### 1.2.2.2 Clec9A

Clec9A belongs to a family of C-type lectin-like molecules encoded on chromosome 6 in mice. In particular, the mouse gene encodes a type II transmembrane protein of 264 amino acids with a single C-type lectin-like domain in its extracellular region, a cytoplasmic tail and a transmembrane
region containing a potential signaling motif. Targeting of antigens to Clec9A via a single injection of anti-Clec9A antibody has been shown to lead to enhanced antibody response, which is entirely dependent on T cells [115]. Moreover, Clec9A has been recently shown to be required for cross-presentation of antigens from necrotic cells to CD8⁺ T cells [69]. In particular, Clec9A recognizes a filamentous form of actin in association with particular actin-binding domains of cytoskeletal proteins, a conserved signal exposed on dying cells when the membrane is damaged [116]. Sensing through Clec9A mediates recruitment and activation of the tyrosine kinase Syk, which is essential for cross-presentation, via a key tyrosine residue within the intracellular tail of the Clec9A molecule.

Figure 1.6. Surface expression of mClec9A protein on DCs and other hematopoietic cells. DCs from spleen, thymus and BM were purified and labeled by 4-color immunofluorescent staining. An enriched preparation of blood DCs was stained. Clec9A is expressed on CD8⁺ cDCs and, at lower levels, on pDCs in spleen, LNs, and thymus. Blood DCs do not express CD8, but do express CD24. Similar to splenic DCs, blood DCs expressing CD24 also co-express Clec9A. pDCs from the blood, like their splenic counterpart, express low levels of Clec9A. (Adapted from [115]).

Many lectin-like molecules are expressed on the surface of cells of the hematopoietic system. Interestingly, Clec9A shows a more restricted expression on DCs than other C-type lectins. In fact, analysis of its expression on a panel of hematopoietic cells has shown that Clec9A is not expressed on monocytes, macrophages and T cells. Although NK cells express some mRNA for Clec9A, no surface protein is detectable. Instead,
Clec9A expression is limited to DCs and in particular to the CD8⁺ cDCs, and at lower levels, on pDCs in spleen, LNs, and thymus, and on CD103⁺ cDCs in peripheral tissues. In blood, Clec9A is expressed on the small subset of CD24⁺ cDCs, which is an early marker of the CD8⁺ DC lineage (Figure 1.6). On the contrary, the CD8⁻ lymphoid tissue cDCs and the monocyte-derived inflammatory DCs are predominantly negative for Clec9A. However, a very small proportion of B cells have shown positive staining for Clec9A [115]. Interestingly, a human ortholog of mouse Clec9A, hCLEC9A, has been identified, which is selectively expressed on BDCA3⁺ cDCs, the proposed equivalent of mouse CD8⁺ cDCs, but not on BDCA4⁺ DCs, the human counterpart of mouse pDCs, or other human blood cells [115].

1.2.2.3 Clec4a4
Clec4a4, or Dendritic cell Inhibitory Receptor 2 (DCIR2), is a type II transmembrane protein belonging to the C-type lectin superfamily receptors. Clec4a4 carries an immune ITIM in its cytoplasmic tail, predicting inhibitory functions for this molecule [117].

![Figure 1.7. Clec4a4 is expressed on CD8⁻CD11b⁺ but not on CD8⁺DEC205⁺ DC subsets in spleen. (a) Dot plot showing staining of CD11c^{high} splenocytes. (b) The histograms show extracellular anti DEC205 or 33D1 antibodies on CD8⁻CD11b⁺ and CD8⁺DEC205⁺ DCs 30 minutes after intravenous injection of 10 μg of αDEC205-OVA, 33D1-OVA, or Iso-OVA control. (Adapted from [12]).](image)

Clec4a4 is recognized by the antibody 33D1. In particular, expression analysis of Clec4a4 has shown that it is specifically expressed on CD11b⁺CD8⁻ cDCs in spleen, LNs and Peyer’s patches (PP), but not on CD8⁺DEC205⁺ DC subsets. Therefore, the 33D1 antibody allows to
distinguish between the CD8\(^{-}\)CC11b\(^{+}\) and the CD8\(^{+}\)DEC205\(^{+}\) cDC subsets in spleen (Figure 1.7) [12].

1.3 Malaria

1.3.1 General concepts about malaria
Malaria still remains a devastating infectious disease, causing about 200-300 million new cases every year in more than 100 countries, and 1 to 2 million deaths. According to the 2012 World Health Organization (WHO) world malaria report [118], in 2011 about 3.3 billion people were at risk of malaria infection. 80% of cases and 90% of deaths were estimated to occur in the sub-Saharan Africa region, and children under the age of five, as well as pregnant women, are the most severely affected. Beside remaining an important global health problem, malaria is a serious economic burden, standing as a main cause of poverty in many affected countries [119]. Despite years of research, at present a vaccine to prevent malaria infection has not yet been developed. One of the main reasons is the limitation of our knowledge on how to stimulate efficient immune responses to control the parasite spread, without inducing pathology. Even though effective drugs are available for malaria treatment, access to early treatment is limited in many endemic areas. Moreover, due to the enormous plasticity of the malaria parasite genome, the prevalence of parasites resistant to the most commonly used drugs is increasing.

Malaria immunity and pathogenesis are complex and still poorly understood. A deep understanding of immune and pathogenic mechanisms acting during malaria infection would allow a better basis for vaccine development, as well as to design effective strategies to prevent or treat malaria associated pathogenesis.

1.3.2 Plasmodium life cycle in the mammalian host
Malaria infection is caused by the apicomplexan protozoa of the genus *Plasmodium*, and is transmitted by infected female *Anopheles* mosquitoes.
Six species have been reported to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, which comprises two distinct species *P. wallikeri* and *P. curtisi*, and *P. knowlesi* [120, 121]. Among them, *P. falciparum* is by far the most dangerous, being implicated in most of the severe and lethal cases. However, *P. vivax* is also responsible for a significant burden of disease [122]. The life cycle of *Plasmodium* parasites is conserved between mammalian species.

![Life cycle of mammalian Plasmodium](image)

Figure 1.8. Life cycle of mammalian Plasmodium. When an infected female *Anopheles* mosquito takes a blood meal, sporozoites injected into the host reach the liver, where they infect hepatocytes and replicate for a few days to give rise to schizonts containing exoerythrocytic merozoites (liver stage). Thousands of merozoites are then released into the blood circulation and can invade erythrocytes, were they replicate to form mature erythrocytic schizonts. Upon rupture of the schizonts, released merozoites can infect new erythrocytes, thus repeating the cycle (blood stage). However, some of them can differentiate into gametocytes, which can be taken up by a feeding mosquito, and develop to form sporozoites (mosquito stage) (Adapted from [123]).

The infection begins when an infected mosquito takes a blood meal, thus injecting parasites in the form of sporozoites in the host dermis (Figure 1.8). When sporozoites reach the blood-stream, they are carried to the liver where they invade hepatocytes and replicate asexually to produce schizonts containing exoerythrocytic merozoites. The replication phase in the liver
proceeds for about 2 to 14 days, depending on the *Plasmodium* species, it is clinically asymptomatic and is called liver stage. Rupture of liver schizonts releases thousands of merozoites into the blood stream, each of which can invade an erythrocyte and replicate asexually through different stages: ring, trophozoite, and finally, exoerythrocytic schizont containing up to 20-30 merozoites. When schizonts burst destroying the host erythrocyte, the newly formed merozoites are free to infect new erythrocytes, thereby causing a cyclical infection which can have a periodicity of 48 to 72 hours, depending on the *Plasmodium* species. This phase of infection is defined as blood stage. Clinical symptoms of the disease can appear at this time. Some of the merozoites, under the right conditions, can differentiate into gametocytes. If a mosquito takes a blood meal at this stage, male and female gametocytes can generate extracellular gametes, and fuse in the mosquito gut to form a motile zygote, which then develops into ookinete, oocyst, and finally, sporozoites that migrate to the salivary gland.

### 1.3.3 The disease

Clinical features of malaria infection are determined by the *Plasmodium* species, as well as the host's age and immune system. Clinical symptoms are due to the replication of the parasite during the blood stage: synchronized rupture of infected erythrocytes is accompanied by release of pyrogenic mediators, which are responsible for the repeated episodes of fever, chills, headache and sweats [124]. The periodicity of these symptoms depends on the growth rate and synchronization of each species. For example, *P. vivax*, *P. ovale* and *P. falciparum* cause febrile episodes every 48 hours, whereas *P. malariae* every 72 hours. Several complications are associated with malaria infection, such as splenomegaly, hepatomegaly, renal and lung failure, acidosis, severe anemia, hypoglycemia, and cerebral malaria. Among these, hypoglycemia, severe anemia and cerebral malaria are the main causes of death. Malaria complications are related to the presence and activity of the parasite, as well as to the host immune responses. However, a clear understanding of the pathogenic mechanism involved is yet to be determined.
1.4 Rodent models of malaria

As a more detailed knowledge about malaria infection is needed, and as for obvious ethical reasons, studies in infected humans are limited, various rodent *Plasmodium* species and different inbred mouse strains have been used to model human malaria. Even though none of the mouse models exactly replicates the features of human infections, they represent valuable tools to investigate immunity and pathology in malaria. Four main rodent parasite species are commonly used as experimental models, *P. chabaudi*, *P. berghei*, *P. yoelii* and *P. vinckei*, with different strains available for each species (Table1.1).

**Table 1.1. Rodent models of malaria.** (Adapted from [125, 126]).

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Common use</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chabaudi</em></td>
<td>Mechanisms of immunity; factors that lead to immunopathology; vaccine studies; differences between resistant/susceptible mouse strains.</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>Pathogenesis of cerebral malaria; mechanisms of immunity; factors that lead to immunopathology.</td>
</tr>
<tr>
<td><em>P. yoelii</em></td>
<td>Mechanisms of immunity; factors that lead to immunopathology; vaccine studies.</td>
</tr>
<tr>
<td><em>P. vinckei</em></td>
<td>Pathogenicity and vaccine studies.</td>
</tr>
</tbody>
</table>

Based on their similarity in biology and pathogenicity to human malaria parasites, each is used to investigate different aspects of malaria infection (reviewed in [125-127]). In particular, the *P. yoelii* model is widely used to investigate mechanisms of immunity to malaria, as well as vaccine candidates. Non lethal strains of this parasite cause a self-clearing infection, characterized by a peak of parasitemia rarely exceeding 30% around day 8-12 post infection, and complete clearance of the parasite by day 20-25 post infection. On the other end, lethal strains of *P. yoelii* cause a lethal infection that leads to death around day 8-10 post infection due to high parasitemia. *P. berghei* has been widely utilized to study the pathogenesis of cerebral...
malaria, and will be described in detail in a dedicated section (see Section 1.6.1).

Many murine studies are performed by direct inoculation of iRBCs, and thus focus on the blood stage of the infection. However, sporozoites and mosquito models are also available, which allow to study the liver stage of malaria infection. Because animal models have been instrumental in understanding many aspects of malaria immunity and pathology, in the following paragraphs findings from human studies will be described in parallel with data obtained from animal models.

### 1.5 Immunity to malaria

Immunity to malaria is complex and still poorly understood. On a first exposure to malaria infection most humans develop a typical febrile syndrome and, in some cases, severe malaria-associated complications that can lead to death. However, following repeated exposure, partial immunity to malaria infection and essentially complete protection from malaria complications can develop. Nevertheless, immunity to malaria is short-lived and requires continuous exposure to parasite antigens to be maintained.

Innate immune responses are necessary to limit the parasite replication and to allow generation of specific adaptive responses, which are required for parasite clearance, but typically fail to completely eradicate the infection, thus resulting in chronic low-grade parasitemia. In both humans and mice, during the blood stage both cell-mediated and antibody-dependent responses are necessary to control the parasitemia. In particular, in the *P. chabaudi* model it has been shown that Th1 responses dominate early during infection, whereas induction of Th2 responses is required later after the peak of parasitemia (Figure 1.9) [128-130], even though more recent findings suggest that Th1 responses are important during the entire course of infection [131].
Figure 1.9. Immunity to malaria infection. Innate immune responses are necessary to limit the first wave of parasitemia and to allow generation of specific adaptive immunity. Both Th1 and Th2 responses are required to control the parasitemia (Adapted from NIMR and Langhorne group, 2012).

1.5.1 The role of spleen in malaria infection

Because, after the liver stage, malaria is essentially a blood infection, and because a large amount of blood flows through the spleen at any time, the spleen is the main organ where innate and adaptive immune responses to malaria parasite are generated. In particular, during malaria infection the spleen is involved in removal of infected red blood cells (iRBCs), uptake and presentation of parasite antigens and activation of specific adaptive immune responses, erythropoiesis and hematopoiesis. The fundamental role of spleen in malaria immunity is supported by studies in humans and rodents, where absence of spleen results in decreased clearance of iRBCs, prolonged blood parasitemia and slower generation of immune responses [132-137]. Nevertheless, in mouse models splenectomy seems to protect from severe malaria complications, such as cerebral malaria [138, 139], thus indicating that the spleen is the site where not only immune responses to malaria are generated, but also where pathogenic mechanisms are initiated.
The different specialized functions of the spleen reside in its intrinsic structure: (i) a white pulp containing immune effectors cells, (ii) a red pulp where senescent erythrocytes are removed, (iii) marginal zones separating the white from the red pulp, where bacteria, viruses and particles are removed. The blood enters the spleen through a central artery that gives rise to smaller arterioles and capillaries. Majority of them by-pass filtration by directly going to the marginal sinus and draining veins (closed circulation); the others empty into the red pulp filtrating beds, where blood flows slowly and makes contact with resident macrophages (open circulation) (Figure 1.10).

![Figure 1.10. Mouse spleen blood circulation.](image)

Parasite killing in the spleen occurs through production of free radicals, as well as through filtration and phagocytosis of iRBCs by the same mechanisms and phagocytic cells that are responsible for clearance of senescent erythrocytes [141, 142]. Two regions of the spleen are characterized by high phagocytic potential: the marginal zone and the red pulp. The marginal sinus is lined by reticular cells, marginal metallophilic macrophages (MMMs), located at the inner border adjacent to the white pulp, which are characterized by expression of the lectin Siglec-1 and have
been shown to phagocytose iRBCs [143], and ER-TR9+ MZMs, situated at the outer border closer to the red pulp, and which are known to be involved in elimination of bacteria and particles as the blood flows through the marginal sinus. The red pulp is formed by a network of reticular cells and fibers, and highly phagocytic F4/80+ macrophages, together forming the filtration system of the spleen, and continually surveilling particulate matter and cells present in the blood [144]. As senescent erythrocytes, iRBCs are characterized by decreased deformability and are easily trapped in the red pulp reticular network, where they are phagocytosed and cleared by resident macrophages [145, 146]. Moreover, squeezing of iRBCs through the red pulp mesh can cause the expulsion of rigid cytoplasmatic particles, a process known as 'pitting', which can result in removal of parasites, leaving the erythrocytes intact [147].

DCs located in the marginal zone are ideally placed to capture malaria antigens and present them to T cells in the central area of the white pulp, which following priming, will become activated and exert their specific effector responses. B cells are organized in follicles in the outer B cell zone of the white pulp, and upon interaction with follicular DCs, DCs and helper T cells, undergo clonal expansion and immunoglobulin isotype switching.

Figure 1.11. The role of the spleen during malaria. Follicular DCs present parasite antigens to B cells, which once activated, generate antibody responses. T cell responses are primed in periarteriolar lymphoid sheaths following antigen presentation by DCs. Blood iRBCs are removed by red pulp macrophages (Adapted from [151]).
Figure 1.11 summarizes the main functions of spleen during malaria infection. The architecture of the spleen has been shown to change during malaria infection, and typical features are splenomegaly, white pulp hyperplasia, and migration of macrophages to the white pulp. In particular, the red pulp expands and, in rodents, becomes a site of significant hematopoiesis [148-150]; F4/80⁺ macrophages are redistributed through the all spleen; MMMs become more diffused and MZMs are completely lost; [143]; B and T cells zones can merge and become more indistinct [148]. These changes are transient and usually do not prevent the development of immune responses to the parasite.

In non lethal murine malaria infections, another major change in the spleen is the temporary transition of the open circulation to closed circulation, due to the formation of layers of fibroblasts in the red pulp, thus creating physical barriers which channel the blood directly from the arterioles to the veins [143, 150, 152]. Moreover, iRBCs have been shown to adhere to such physical barriers, thus sequestrating in the spleen [153]. Spleen closure has several consequences, some of which favor the parasite, whereas other might be protective for the host. The major advantage for the parasite is that iRBCs are prevented from transiting through the red pulp, thus escaping macrophage-mediated clearance. On the other side, spleen closure limits the release of toxic and inflammatory mediators which are usually produced by activated macrophages when clearing iRBCs, and leaves developing adaptive responses unperturbed, as well as protects newly formed reticulocytes from infection. However, during peak parasitemia spleen closure may limit effective parasite clearance.

Even though information about spleen remodeling during human malaria is limited, post mortem analysis of spleens from children infected with *P. falciparum* showed extensive disorganization with some similarities to what observed in murine models [154].

**1.5.2 Innate immune responses to malaria infection**

Innate immune responses are the first line of defence during malaria infection. Several players of the innate immune system are important for malaria immunity, not only for their ability to directly remove infected red
blood cells, but also for bridging innate and adaptive immunity by activating those effector cells which are ultimately necessary for parasite clearance.

### 1.5.2.1 Macrophages

The importance of macrophages in iRBC clearance and control of parasitemia has been shown in experiments with lethal and non-lethal strains of *P. yoelii*, where depletion of monocytes/macrophages exacerbated parasite growth and anemia [155].

Macrophages can phagocytose iRBCs using two different mechanisms. One mechanisms does not require opsonizing antibodies: upon activation mediated by inflammatory cytokines such as TNF and IFN-γ, macrophages can bind to parasite antigens expressed on iRBCs via receptors on their surface [156]. In particular, in human malaria binding of the scavenger receptor CD36 to the *P. falciparum* erythrocytes membrane protein-1 (PfEMP-1) seems to be involved in this mechanism [157]. In the *P. chabaudi* murine model, CD36 is also believed to mediate non-opsonin dependent phagocytosis [158]. This mechanism may be important during early infection, however macrophages are also important for parasite clearance during adaptive immunity, when the second mechanism, the antibody-dependent phagocytosis, becomes prevalent. Indeed, in rodent models immune animals showed a more efficient clearance of iRBCs compared to non immune animals [159].

Beside spleen resident macrophages, in the *P. chabaudi* infection model a population of CD11b<sup>high</sup>Ly6C<sup>+</sup> monocytes arising from the BM has been shown to appear in the spleen and to be actively involved in control of acute parasitemia [160].

### 1.5.2.2 Dendritic cells

Due to their presence in sites of pathogen entry, their unique ability to sample, uptake, process and present antigens, as well as their capacity to integrate and respond to microbial and other immune cells signals, DCs have a central role during infection in activating and orchestrating both innate and adaptive immune responses. The presence of a large variety of pattern recognition receptors on DC surface, like TLRs, which allow them to
sense and interact with various conserved microbial molecules, is central in DC functions. The evidence that different DC subsets are equipped with different sensing receptors suggests specialized functions during various types of infection. These characteristics of DCs make them an inviting target for malaria immunotherapy. However, as of today, a clear understanding of DC functions, and in particular, of the role of different specific DC subsets in malaria immunity, as well as of the identity of the receptors mediating the interaction with parasite antigens and the mechanisms involved, is yet to be achieved. Some reasons for this can be found on the complexity of the parasite and DC system, the limited number of human studies, and the diversity of *Plasmodium* strains and genetic backgrounds of mice used in experimental models.

Protective immunity to blood stage malaria requires high titres of neutralizing antibodies, as well as malaria specific CD4+ T cells to effectively contain and clear the parasite [161]. During blood stage malaria, DCs in the marginal zone of the spleen are ideally placed to sample the blood flowing through the marginal sinus and, upon activation, they can migrate to the white pulp were they can initiate acquired immune responses. Even though DCs have been shown to interact with malaria parasites, and it is widely accepted that they have a crucial role in priming T cells responses, early studies aimed at investigating DC activation status and immunostimulatory capacity during malaria infection produced controversial results.

In humans, studies by Urban and colleagues showed that monocyte-derived DCs could interact with *P. falciparum* iRBCs by binding of CD36 to PfEMP-1. However, this interaction resulted in inhibition of DC maturation, thus affecting their ability to stimulate T cells [162, 163]. Nevertheless, a subsequent study showed that this inhibition is dose dependent and does not require interaction between CD36 and PfEMP-1 [164].

In mice, GM-CSF-conditioned BM-derived DCs co-cultured with *P. chabaudi* schizonts were shown to secret TNF-α, IL-6 and IL-12 [165]. *In vivo* studies in mice demonstrated that 6 days after infection with *P. chabaudi* DCs were fully functional [166], upregulated co-stimulatory molecules important for activating T cells such as CD40, CD86 and ICAM-1, and migrated from the marginal zone to the T cell area in the spleen within 5 days of infection.
However, other studies found that upon infection DCs exhibited an impaired immunostimulatory activity [168-170]. In studies aimed at understanding the effect of iRBCs on DCs, splenic CD11c⁺ cDCs were shown to be more efficient in uptaking iRBCs than RBCs and, in vivo, iRBCs induced DC maturation, production of IL-12 and IFN-γ, and CD4⁺ T cell maturation [171]. In particular, during *P. chabaudi* infection both CD8⁺ and CD8⁻ cDCs could present parasite antigens, but only CD8⁻ cDCs isolated during acute infection could activate antigen-specific CD4⁺ T cell responses [172]. Despite the contrasting results obtained by different groups, what has become evident is that the functional capacity of splenic DC subsets changes during infection, and that the antigen dose plays a part in such a modulation. Indeed, the amount of antigen is known to affect whether the generated immune responses are cell-mediated or antibody-mediated [173].

During early malaria infection, low parasite levels activate DCs to produce TNF-α and IL-12, which stimulate IFN-γ production by NK and naïve CD4⁺ T cells, and IL-12-associated protection has been observed as early as 6 days post infection [166, 172]. As the infection progresses and parasitemia increases, DCs produce less IL-12 and, instead, begin to produce IL-10, but they are still able to activate naïve CD4⁺ T cells [174]. During the later phase of infection, the induced, widespread systemic activation of DCs renders them refractory to TLR stimulation, thus dampening their ability to phagocytose antigens and priming T cells [174]. Apoptosis of CD8⁺ cDCs is observed at this phase in *P. chabaudi* infection, whereas the number of CD8⁻ cDCs increases in the spleen [172], and IL-4 and IL-10 production by proliferating CD4⁺ T cells prevails, which corresponds to a switch from Th1 to Th2 immune responses. At this stage, protection is essentially antibody-mediated [175] (Figure 1.11). As high doses of *P. falciparum* iRBCs have been shown to induce apoptosis in monocyte-derived human DCs, while low doses activate them to stimulate CD4⁺ T cell proliferation [164], it has been proposed that CD8⁺ cDCs, which are the major producers of IL-12 [176], might be important in early infection, when parasitemia is low, to activate Th1 responses, whereas CD8⁻ cDCs could have a major role during the acute phase to promote the switch from Th1 to Th2 immune responses [177].
Although CD8⁺ T cells are known to be protective during the liver stage of malaria infection [178], given that RBCs lack both MHC I and antigen processing machinery, cytotoxic T cells are generally considered to be ineffective during the blood stage. Nevertheless, CD8⁺ T cells are now known to be directly involved in cerebral malaria pathogenesis (reviewed in [179] and see Section 1.6.2.2). Moreover, recent studies have shown that splenic CD8⁺ cDCs efficiently present *P. berghei* antigens *in vitro* [180], and can cross-present antigens expressed during the blood stage *in vivo* to prime CD8⁺ T cells, which acquire cytotoxic effector functions [181, 182]. (Figure 1.12).

![Diagram of DCs in the spleen](image)

**Figure 1.12.** DCs in the spleen can prime T cell responses during blood stage malaria. (Adapted from [177]).

Interestingly, differences in DC functional capacity have been observed in non lethal versus lethal murine malaria models. In particular, in non lethal *P. yoelii* and *P. chabaudi* infections, DCs are fully functional, can secrete high levels of IL-12 [168, 183], and can induce protection when transferred in naive mice infected with a lethal strain [168]. By contrast, in mice infected with the lethal parasite strains *P. yoelii* YM, *P. berghei* and *P. vinckei*, DCs are unable to secrete IL-12 and prime T cells [168, 183]. These observations support the idea that high parasite load resulting in widespread DC activation causes a rapid down-regulation of their function. Distinct from splenic cDCs, pDCs are known to have poor antigen presentation properties, and have proved incapable of priming T cell responses during blood stage malaria. In fact, even though pDC numbers
have been shown to increase during infection with non lethal *P. chabaudi* and *P. yoelii* strains [168, 184], in *P. berghei* infection pDCs failed to process and present parasite antigens [180, 185]. Moreover, pDCs were unable to stimulate parasite-specific CD4⁺ T cells in *P. chabaudi* infected mice, due to their poor phagocytic properties and lack of adequate co-stimulatory signals [186]. pDCs are known for their ability to produce IFN-α, and in both humans and mice, IFN-α production seems to be associated with protection from cerebral malaria [187, 188]. However, pDCs ablation in *P. berghei* infected mice did not result in protection from cerebral pathology [182], suggesting that cells other than pDCs might be the main producers of IFN-α during malaria infection. Instead, pDCs are believed to have regulatory functions in reducing inflammation during acute infection. Indeed, a population of CD11c<sup>low</sup>CD45R<sup>high</sup> DCs, generally identified as pDCs, have been found to accumulate 7 to 10 days after a *P. yoelii* infection, and induce IL-10 expression by CD4⁺ T cells [184]. Moreover, recent studies suggest a role for pDCs as reservoirs of infectious parasites [189]. Indeed, some blood stage merozoites of *P. berghei*, *P. yoelii* and *P. chabaudi* were shown to have a tropism for pDCs in the spleen. Transfer of pDCs from *P. chabaudi* infected mice, after apparent clearance of blood infection, to naïve mice initiated more vigorous infections than transfer of RBCs, by releasing parasites that were infectious for RBCs[189].

Efficient resolution of malaria infection requires efficient uptake of parasite antigens by DCs for presentation, as well as regulated pro- to anti-inflammatory responses. However, the nature of the receptor-ligand interactions involved and necessary for parasite clearance is still poorly characterized. During blood stage infection, when iRBCs rupture to release merozoites, several products are released into the blood, which can activate TLRs and induce the production of pro-inflammatory cytokines. For example, glycosylphosphatidylinositol (GPI) has been shown to bind to TLR2 and mediate signal transduction [190, 191]. Hemozoin, a waste product deriving from the digestion of hemoglobin by the *Plasmodium* parasite, was initially suggested to bind to TLR9 [192, 193]. However a more recent study showed that a parasite protein-DNA complex is responsible for the engagement of the TLR9-MyD88 pathway and induction
of inflammatory cytokine responses in DCs [194]. MyD88 is an important adaptor molecule involved in most TLR signaling, and it has been shown to have a role in mediating symptoms, but not to be crucial for blood stage immunological control during *P. chabaudi* infection [195]. Early studies on TLR deficient mice showed that TLR2−/−, TLR4−/−, TLR6−/− and TLR9−/− mice infected with *P. chabaudi* showed no difference in parasitemia, body weight and temperature compared to wt mice. Consistently, deficiency of TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR9 and MyD88 could not protect from development of cerebral malaria [196]. However, a more recent study in *P. yoelii* infected mice reports that TLR9 and MyD88 are crucial for development of protective immunity to malaria as deficient mice produce lower pro-inflammatory and higher anti-inflammatory cytokines levels, have compromised ability to control parasitemia and are susceptible to death [197]. Similarly, it has recently been shown that treatment of mice immediately before or shortly after infection with *P. berghei* with chloroquine, a drug known to inhibit TLR9, protected mice from ECM [198].

At present, the exact role of TLR9 signaling on malaria infection outcome remains unclear, and even though DC TLR signaling appears to be involved in the modulation of malaria immune responses, it might not be necessary for direct parasite clearance.

In both humans and mice, DC and macrophage derived cytokines increase in the serum as soon as iRBCs start to be detectable in the blood, and are considered necessary for protection [199-201]. In humans, IL-12 and IL-18 have been associated with protection from severe malaria pathology [202-206]. In mice, one study showed that IL-12p40 deficient mice infected with *P. chabaudi* experienced higher parasitemia levels in comparison to wt mice, and could not easily resolve primary infection, as well as control challenge infection. Moreover, IL-12p40 deficient mice produced lower levels of Th1-dependent IgG2a and IgG3 antibodies, but higher levels of Th2-dependent IgG1, therefore the authors concluded that these results are consistent with a protective role of Th1 immune responses during blood stage malaria, and that IL-12 is required for efficient antibody-mediated immunity [131]. IL-12 is known to mediate production of IFN-γ, and one study from the same group on *P. chabaudi* infected IFN-γ deficient mice
showed similar results as mice developed higher parasitemia, suffered severe mortality and produced less IgG2a and IgG3 and more IgG1 [207]. However, in *P. yoelii* infection, even though a burst in IFN-γ was observed as early as 1 day post infection in wt mice, mainly produced by NK cells, the parasite load and course of infection did not differ in IFN-γ deficient mice compared to wt mice [155], thus suggesting that IFN-γ is not required for effective control of *P. yoelii* infection.

Studies using different *Plasmodium* strains have produced controversial results. Moreover, the receptors and mechanisms used by DCs to interact with iRBCs, capture parasite antigens and process them, as well as how they help to modulate innate immune responses and prime specific T cell responses, are not yet well understood. Therefore, further characterization is needed to gain more insights on how DCs work in different experimental models, which could help to understand the role of DCs in human malaria and how they could be exploited in immunotherapy.

**1.5.2.3 Monocytes and neutrophils**

In *P. chabaudi* infection, a population of monocytes defined as CD11b$^{\text{high}}$Ly6C$^+$ has been shown to rapidly generate in the BM and migrate to the spleen in a CCR2 dependent manner. These cells have been proposed to be important for controlling blood stage malaria parasites, as CCR2 deficient mice showed higher parasitemia levels, and CD11b$^{\text{high}}$Ly6C$^+$ monocytes from *P. chabaudi* infected wt mice could reduce parasitemia in CCR2 deficient mice [160].

In both mice and humans, neutrophils dysfunction due to an impairing of their oxidative burst has been observed during malaria infection. In particular, such acquired dysfunction appears to be a consequence of induction of a heme degrading enzyme in neutrophil progenitors in the BM [208].

**1.5.2.4 Natural killer T (NKT) cells**

NKT cells are a heterogeneous population of cells which share some functional properties of both NK cells and T cells. In the mouse model, NKT cells have been shown to contribute to innate immunity to malaria,
particularly during the liver stage [209]. Moreover, in mice infected with *P. yoelii* sporozoites an increase in the number of activated NKT cells was observed in the liver, and these cells could inhibit the growth of parasite in cultures of infected hepatocytes in an IFN-γ-dependent manner [209]. NKT cells recognize lipid-containing antigens in the context of the non classical MHC I molecule CD1d. It has been reported that, in *P. berghei* infection, CD1d restricted NKT cells from different mice backgrounds which have been activated by α-Galactosylceramide can influence T cells polarization towards Th1, as well as cytokine production and cerebral malaria pathogenesis [210]. However, CD1d deficient mice infected with *P. berghei* sporozoites showed immune responses similar to wt mice, thus suggesting that NKT cells might not be essential for resistance to pre-erythrocytic infection [209].

### 1.5.2.5 Gamma delta (γδ) T cells

γδ T cells are a small subset of T cells which posses a distinct T cell receptor (TCR) formed by one γ and one δ chain, and which appear not to be MHC restricted. In humans, in acute *P. falciparum* and *P. vivax* infection, polyclonal expansion of γδ T cells has been observed [211, 212], and *P. falciparum* activated γδ T cells produce high levels of IFN-γ [212, 213]. To become responsive to malaria, γδ T cells have been shown to require stimulation through their TCR. This occurs via recognition of soluble schizont-associated phosphorylated non-peptide antigens [214, 215], as well as exogenous cytokines signaling [216], suggesting that γδ T cells are not the first cell type responding to malaria infection.

In mice, γδ T cells have been shown to contribute to extra erythrocytic immunity in *P. yoelii* infection [209], and expansion of these cells has been observed during acute blood stage *P. chabaudi* infection [211]. As NK cells, γδ T cells seem to contribute to the production of IFN-γ, which is required for the activation of parasite specific alpha beta (αβ) T cells [217]. However, they do not seem to be essential in *P. yoelii* [217] and *P. chabaudi* blood stage immunity [218-221]. Nevertheless, γδ T cells have been shown to contribute to cerebral pathology in the *P. berghei* mouse model of cerebral malaria [222], even though their role is controversial. In fact, γδ T cells
deficient mice still develop ECM, but when anti γδ antibodies are used, which are activating rather than depleting, they can affect susceptibility to ECM.

1.5.2.6 NK cells
Because of their presence in blood and spleen, NK cells can easily encounter iRBCs and, in vitro, they have been shown to be the first cells to respond after exposure to *P. falciparum* infected erythrocytes [223]. In particular, NK cells responded by producing IFN-γ, with a peak production around 12 to 15 hours after exposure, whereas NKT cells and γδ T cells started to secrete IFN-γ only 24 to 48 hours after NK cell peak production, thus suggesting that IFN-γ secreted by NK cells might be the signal required to initiate the cascade of innate immune responses [223]. In *P. falciparum* studies, both IL-12 and IL-18, as well as direct contact between NK cells and iRBCs, have been observed to be required for NK cells activation and IFN-γ production. Moreover, NK cells from subjects exposed to *P. falciparum* were able to lyse iRBCs, indicating specific recognition of infected erythrocytes [224]. However, the ligands and receptors involved in the interaction between NK cells and iRBCs are still unknown.

In mice, infection with *P. chabaudi*, *P. berghei* and *P. yoelii* induce both IFN-γ production and NK cell-mediated cytotoxicity [225-227]. IL-12 has been shown to be required to activate NK cells, and their production of IFN-γ is necessary for development of protective immunity. In fact, ablation of this cell population results in higher parasitemia and less efficient parasite control in *P. chabaudi* infection, as well as higher mortality in *P. yoelii* infected SCID mice [217, 225, 228].

1.5.3 Adaptive immune responses to blood stage malaria infection
Protective immunity to blood stage malaria requires development of specific acquired immune response to eradicate the infection. In particular, both antibody-dependent and cell-mediated responses are necessary to control parasitemia.
1.5.3.1 Antibody-mediated immune responses

In both humans and mice, humoral responses are considered essential for eliminating parasites. In humans, this is supported by experiments of passive immunoglobulin (Ig) transfer [229]. In mice, transfer of serum from animals repeatedly infected with non lethal *P. yoelii* (hyperimmune serum) has been shown to improve the course of *P. yoelii* infection [230, 231]. In particular, when Ig isotypes in the hyperimmune sera were analyzed, the highest titre of antimalaria antibodies was found in the IgG2a subclass, with slightly lower titres in IgG1, IgG2b and IgG3. However, only passive transfer of antibodies of the IgG2a subtype could modulate parasitemia levels, thus leading the authors to conclude that IgG2a are the Ig predominantly responsible for parasite clearance in *P. yoelii* infection [232].

Malaria protective antibodies can mediate their effect either by blocking molecules important for RBC invasion on the merozoite surface, or by enhancing Fc-mediated phagocytosis by promoting merozoite agglutination after egress [233]. Because, in mice, activated macrophages have increased expression of Fc receptors for IgG2a antibodies, it has been proposed that they might preferentially phagocytose merozoites or iRBCs coated by the IgG2a subtype [232]. In humans, monocytes express only receptors for IgG1 and IgG3, and indeed only these antibody subtypes were able to mediate opsonization of *P. falciparum* iRBCs [234].

In *P. chabaudi* infection experiments, it has been shown that both IL-12 and IFN-γ are required for protective antibody-mediated immunity. In fact, both IFN-γ and IL-12p40 deficient mice had significantly lower levels of the Th1-dependent IgG2a antibodies, and higher levels of the Th2-dependent IgG1 antibodies, as well as higher parasitemia, higher mortality and could not resolve primary infection efficiently [131, 207]. Even though Th1 responses have been proposed to dominate early during *P. chabaudi* infection, whereas induction of Th2 responses would be required later after the peak of parasitemia [128-130], transfer of serum depleted of IgG2a antibodies resulted in reduction of its protective properties, thus suggesting that Th1 immune responses are important during the whole course of infection [131]. Several studies have tried to identify potentially protective antigens expressed on iRBCs, or on merozoites, but malaria antibody-mediated
immunity appears to be particularly complex, and responses to many antigens seem to be involved in protection [235]. One particular antigen expressed on merozoite surface, the merozoite surface protein (MSP-1), has been shown to elicit protective responses against blood stage in both human and mice malaria infection [236-238], and is a leading malaria vaccine candidate.

In humans, immunity to malaria develops slowly and continuous exposure to malaria antigens is thought to be necessary to generate and maintain immune memory to malaria. A rapid boosting of antibody responses after reinfection has been observed, suggesting the generation of memory B cells. However, two different studies have reported contrasting results regarding the presence of malaria-specific memory B cells [239, 240].

1.5.3.2 CD4⁺ T cell-mediated immune responses

In both human and rodent malaria, CD4⁺ T cells are necessary for clearance of *Plasmodium* parasites. In particular, CD4⁺ T cells contribute to parasite control and elimination in two different ways: (i) production of cytokines, which seems to be important for control, but does not eliminate completely the parasite, and (ii) helper function for antibody production by B cells, which is thought to be necessary for total clearance [129, 241-245]. The first evidence for the importance of T cells in malaria infection came from studies in Nu/Nu mice, which lack all T cells, where it was shown that the thymus plays a crucial role in providing help to B cells for the generation of protective antibodies [246]. Subsequent studies in B cell deficient mice and adoptive T cell transfer gave further evidence of the ability of T cells to directly control blood stage infection [246-249]. The particular importance of CD4⁺ T cells became evident in an experiment with T cell depleted mice, where repletion of CD4⁺ T cells, but not CD8⁺ T cells, conferred protection only in non splenectomized mice [250]. More recent studies have demonstrated that antigen presentation via MHC II and T cell co-stimulation are essential for protective blood stage immunity in both *P. yoelii* and *P. chabaudi* infection models [251].

The role of CD4⁺ T cells in blood stage immunity has been most studied in *P. chabaudi* infection, where they have been shown to be firstly important in
controlling the primary peak of parasitemia, and later to be required for initiating the switch from Th1 to Th2 responses, which are necessary for antibody production and complete parasite clearance [252, 253]. In particular, the switch occurs when the production of the Th1 IL-12, TNF and IFN-γ cytokines during early infection is later replaced by secretion of the Th2 cytokines IL-4 and IL-5 [254, 255]. Whereas control of peak parasitemia has been shown to be CD4+ T cell-dependent [243, 256], parasite eradication requires both CD4+ T cells and B cell antibody production [242]. Even though studies on memory CD4+ T cells are limited, recall responses of CD4+ T cells to several blood stage malaria antigens have been extensively documented in humans, and these cells are thought to be essential not only for induction, but also for maintenance of protective antibodies [257].

1.5.3.3 CD8+ T cell-mediated immune responses

Although CD8+ T cells are known to be important in immunity to the liver stage of malaria infection (reviewed in [258]), adoptive transfer and cell depletion studies in P. yoelii infected mice showed that CD8+ T cells were not sufficient to control parasitemia and were dispensable for blood stage parasite clearance [243, 259]. Nevertheless, a few studies provided some evidence for a CD8+ T cell role in blood stage immunity. One study found that CD8+ T cells specific for an epitope shared by both P. falciparum and P. yoelii could reduce parasitemia levels of about 50% [260]. Moreover, in the P. chabaudi model depletion of CD8+ T cells late in infection resulted in inability to clear the parasite [261]. However, in a subsequent study by the same group using CD8+ T cell deficient mice, the authors excluded a role for CD8+ T cells in blood stage immunity [262]. More recently, a robust parasite-specific CD8+ T cell response was shown to arise upon P. yoelii infection, which presented the classical signs of effector and memory CD8+ T cells [263].

If, on one side, the role of CD8+ T cells in protective immunity during blood stage malaria remains a matter of controversy, on the other side, their involvement in mediating cerebral malaria pathogenesis has become more and more evident (reviewed in [179] and see Section 1.6.2.2).
1.5.3.4 $T_{\text{regs}}$ and regulation of T cell responses

In malaria, as in other infections, a potent pro-inflammatory immune response is produced by the organism in the attempt to control the infection. However, such a strong response can result in pathology. A balance between pro-inflammatory and anti-inflammatory responses may be the key for an efficient parasite eradication without inducing host pathology.

A few cell types and mechanisms of the immune system are dedicated to regulating the overall immune response. CD4$^+$CD25$^+$Foxp3$^+$ $T_{\text{regs}}$ are known to play a crucial role in several infectious diseases. These regulatory cells can down-modulate inflammatory immune responses and prevent immune-mediated tissue damage by production of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β). However, an excessive $T_{\text{reg}}$ response can result in suppression of cell-mediated immunity, and thus can be an obstacle for an efficient clearance of the infection and lead to chronic disease.

In malaria infection, only a few studies have tried to address the role of $T_{\text{regs}}$. In the lethal $P.\,yoelii$ murine model, ablation of $T_{\text{regs}}$ before infection resulted in enhanced T cell responses and increased survival [264]. Similarly, $P.\,berghei$ infected mice showed delayed parasitemia when $T_{\text{regs}}$ were depleted [265]. In non-lethal $P.\,yoelii$ infection, $T_{\text{regs}}$ ablation led to an increase in T cell activation and a significant decrease in parasitemia [266]. Consistent with these results, TGF-β and IL-10 neutralization decreased lethality and promoted complete resolution of the infection in about 40% of lethal $P.\,yoelii$ infected mice [267], even though in the non-lethal model IL-10 was suggested to have only a limited effect on parasite clearance [266]. On the other side, in $P.\,chabaudi$ infection the importance of IL-10 for limiting severe disease has been shown, as deficient mice experienced enhanced pro-inflammatory cytokine responses and more severe pathology than wt mice [268, 269]. Therefore, $T_{\text{regs}}$, as well as IL-10 and TGF-β cytokines, are likely to be important in affecting the outcome of malaria infection. In particular, the timing of these anti-inflammatory responses might be crucial in determining whether host responses are suppressed before development of an effective protective immunity, as appears to
happen in lethal *P. yoelii* infection, or whether they are suppressed when pathology has already been caused.

### 1.5.4 Summary on immune responses to blood stage malaria

Based on the evidence obtained so far in human and murine studies, immunity to blood stage malaria can be summarized as follows (Figure 1.13). Immature DCs are able to recognize iRBCs or parasite products and uptake them. Antigen uptake leads to DC activation and maturation, which results in upregulation of MHCII and co-stimulatory molecules, as well as migration of DCs to the spleen, the primary site where immunity to blood stage malaria occurs.

**Figure 1.13. The players of immunity to blood stage malaria.** (Adapted from [270]).

In the spleen, activated DCs produce pro-inflammatory cytokines such as IL-12, responsible for activating NK cells to produce IFN-γ and for inducing the differentiation of naïve CD4⁺ T cells to Th1 cells, required for helping B cells to produce protective antibodies. IFN-γ production by NK cells also enhances DC maturation, and activates macrophage phagocytic and killing functions, thus further amplifying immune responses. As the infection progresses, antigen presentation declines, as well as IL-12 and IFN-γ production, whereas IL-10 and IL-4 production increases, which corresponds to a switch from Th1 to Th2 immune responses. At this stage
protection is essentially antibody-mediated, and regulatory cytokines such as IL-10 and TGF-β help to modulate excessive responses.

1.6 Cerebral malaria (CM)
CM is the most severe complication caused by *P. falciparum*. It affects mainly children under the age of five, and can cause death as soon as 14 days after infection. It is a neurological syndrome characterized by diffuse encephalopathy, loss of conscience and muscular tone, convulsions and coma, ultimately leading to death in 20-30% of the cases, even if patients are under treatment with anti-malaria drugs [271]. Supportive treatments such as exchange transfusion, ventilation aid, treatment of kidney failure and anti convulsants are necessary for patients suffering from CM, but not always available in many under-developed malaria endemic countries. Thus far, all efforts to improve CM treatment have proved unsuccessful, leaving not many options for treatment of suffering patients.

A deep understanding of the pathogenic mechanisms involved in CM is necessary in order to develop new effective therapeutic strategies. However, even though it has been extensively studied since the 19th century, the exact mechanisms leading to CM pathogenesis are still not fully understood, the complexity of the pathology standing as the main cause.

1.6.1 Experimental cerebral malaria (ECM)
Because of obvious ethical reasons human CM studies are basically limited to *post-mortem* examination, and the use of rodent parasites capable of inducing ECM has proven to be an invaluable tool and has become the model of choice for studying CM pathogenesis. Among all parasite species, *P. berghei* is the only one able to induce CM in rodents [272-275], and although several strains of mice are susceptible to CM development, *P. berghei* ANKA (*PbA*) infection in C57BL/6 mice is the best characterized model of ECM. Typically, susceptible C57BL/6 mice develop a neurological syndrome between 6 to 12 days after infection with *PbA*, characterized by paralysis, ataxia, deviation of the head, convulsions and coma. About 60-
100% of the mice succumb to the syndrome and die with relatively low parasitemia, whereas those mice which survive the CM phase die later on, during the second or third week post infection, due to high parasitemia and severe anemia. Despite the many similarities shared between the neurological syndrome induced in ECM and the human pathology, such as brain petechial hemorrhages, edema, increase of pro-inflammatory cytokines and local endothelial cell activation [276, 277], the relevance of the murine model to study human CM continues to be a matter of debate [278, 279]. The main reason for the controversy lies in the fact that, whereas iRBC sequestration in the human brain is considered to be a hallmark of CM, accumulation of iRBCs in ECM is less clear, with some studies confirming the presence of iRBCs in the brain of affected mice [280-283], and other reporting that ECM is more often associated with sequestration of immune cells rather than iRBCs [179, 284-288]. Nevertheless, it is evident that studies aimed at uncovering the mechanisms involved in ECM pathogenesis might provide new insights on understanding the human pathology.

1.6.2 CM pathogenesis
At present, three main events are thought to be involved in CM pathogenesis, acting in an intricate inter-play, and thus contributing to the pathology multifactoriality (reviewed in [286, 289]).

1.6.2.1 Sequestration of iRBCs
Sequestration of iRBCs in the brain microvasculature of patients who died of CM has been described as early as 1890, by Marchiafava and Bignami, and it has been a persistent histological finding, observed also in other tissues such as liver, lung, kidney, adipose tissue and placenta [290-298]. Based on these repeated observations, it has been proposed that cytoadherence of iRBCs to endothelial cells of capillaries and post-capillary venules compromises the local blood flow, as well as the integrity of the endothelium, thus causing petechial hemorrhages, hypoxia and deficient removal of waste products [299, 300]. As sequestration appears to be a signature of severe disease, several studies have been done to identify the
molecules involved in the cytoadherence. On one side it has been shown that the *P. falciparum* protein PfEMP1 is expressed on the surface of iRBCs, and interacts with endothelial cells [301, 302]. On the other side, molecules expressed on the endothelium, such as CD36, ICAM-1, VCAM-1, PECAM-1, E-selectin, P-selectin, thrombospondin and chondroitin sulfate, are up-regulated during infection, and can bind to iRBCs (reviewed in [303, 304]). Another factor proposed to be involved in the capillary occlusion is the decreased deformability of the iRBCs, due to the expression of parasite proteins on the cell surface. The decreased deformability promotes vessels plugging, which has been shown to occur independently of adhesion molecules, and to be an indicator of poor prognosis in animal models [305-307]. Uninfected RBCs are also believed to be less deformable due to oxidant stress, and to contribute to the capillary plugging [305]. One last mechanism likely to be involved is the ability of iRBCs to interact and form complexes with uninfected RBCs (rosettes), or with platelets (auto-agglutinates) [308, 309].

Cytoadherence has been proposed to offer a possibility to the parasite to escape passage through the spleen, where immunity to malaria usually occurs, and where less deformable RBCs are readily removed [310]. However, sequestration alone can not explain CM pathogenesis, because *P. falciparum* accumulation has been observed in the brain of infected patients who died from causes other than CM [311].

**iRBC sequestration in ECM**

The lack of parasite sequestration in ECM has been the main reason for the debate on the relevance of rodent models. In contrast to human CM, where sequestration of iRBCs in brain microvasculature is considered to be a major feature, ECM has been mainly associated to brain accumulation and activation of leukocytes and platelets. However, sequestration studies in the mouse model have produced controversial results.

The adhesion properties of *P. berghei* versus *P. falciparum* iRBCs has been reviewed in 2010 by Franke-Fayard and colleagues [312]. In *P. falciparum* infection, mature trophozoites, schizonts and developing gametocytes sequestrate in different tissues and, as a result, are not found in the
circulating blood. However, because infection in mice with \textit{P. berghei} generally results in an asynchronous parasite development, detection of \textit{P. berghei} sequestration is more complicated, and has led to the common misconception that \textit{P. berghei} cytoadherence to the host microvasculature is not as pronounced as it is in \textit{P. falciparum}. Indeed, when experimentally synchronized \textit{P. berghei} schizonts were used for mice infection, they all disappeared from the blood circulation, thus showing a sequestration phenotype similar to \textit{P. falciparum} [284, 313, 314]. More recently, the development of a technique which allows real time \textit{in vivo} imaging, by using a transgenic luciferase-expressing \textit{PbA}, has allowed to visualize that parasite sequestration occurs. The first observations using this system described iRBCs to accumulate mainly in lungs, adipose tissue, and spleen, but negligibly in the brain [283, 284, 315]. Moreover, it was demonstrated that the sequestration in this organs is CD36-dependent, but it is not necessary for CM development as CD36$^{-/-}$ mice are still susceptible [284]. However, later studies using optimized imaging parameters showed that \textit{P. berghei} iRBCs clearly accumulate in the brain [282, 283, 316, 317], thus indicating that additional factors other than CD36 might play a role. Two very recent works have finally proved that a striking interdependent relationship exists between parasite accumulation and cytotoxic T cell activity in the brain, such as one factor affects the other, and both are necessary for CM to develop [318, 319].

If on one side, there seems to be still no consensus on the involvement of iRBCs sequestration in ECM, on the other, some studies in humans show that not all patients who die from clinically diagnosed CM have sequestration of \textit{P. falciparum} iRBCs [320, 321], and that sequestration can occur in infected patients who died from causes other than CM [311]. Furthermore, as described in the mouse model, accumulation of leukocytes and platelets in brain microvasculature has also been observed in human CM [322-325]. These considerations suggest that, probably, human and experimental pathologies are not so far apart, and that other factors might be involved in CM development.
1.6.2.2 Inflammation

Malaria infection elicits a strong inflammatory response, aimed to containing and clearing the parasite load. However, excessive inflammation can promote pathology by increasing the expression of adhesion molecules on endothelial cells, thus enhancing iRBCs cytoadherence, and by recruiting leukocytes, which can accumulate and further contribute to the inflammation. Several players are involved in the inflammation cascade however, the identity and role of each of them, as well as the sequence of events leading to CM, are still far from being completely characterized.

Pro-inflammatory cytokines

During *P. falciparum* infection, the rupture of iRBCs causes the release of parasite molecules, like GPI. Mononuclear cells can recognize such molecules, become activated, secrete pro-inflammatory molecules including IL-1β, IL-6, TNF, and produce superoxide and nitric oxide (NO) [326]. Among these, the role of TNF has been the most studied. TNF, produced by macrophages, B and T cells and mast cells, is a potent pro-inflammatory cytokine which can bind to two different receptors: TNF receptor 1 (TNFR1) and TNFR2. High levels of TNF are found in the circulation of patients with CM, and serum TNF levels are believed to correlate with the severity of the pathology [327, 328]. TNF may act by increasing the expression of adhesion molecules on the endothelium. Indeed, TNF and its mRNA, together with TNFR2, have been detected in the brain of CM patients, suggesting that action of TNF through TNFR2 may be particularly important in the pathogenesis onset [285, 329, 330]. However, treatment with anti TNF antibody showed no survival amelioration [331], suggesting a limited role for this cytokine. In ECM, whereas TNF neutralization and TNFR2 deficiency prevented CM development in *P. berghei* infected CBA/Ca and C57BL/6 mice, respectively [332, 333], TNF-α deficient C57BL/6 mice showed the same susceptibility as control mice [334], suggesting that the role of TNF may vary in different mice genetic backgrounds. Another molecule belonging to the TNF family, Lymphotoxin α (LT α), seems to have a major role in ECM, as LT α deficient C57BL/6 mice are resistant to CM development [334].
Elevated levels of serum IFN-γ, another pro-inflammatory cytokine, have also been found in *P. falciparum* infected CM patients [335, 336], and in the mouse model, IFN-γ-regulated gene transcripts were found to be the most abundant in brain, spleen and BM of *P. berghei* infected C57BL/6 mice [337]. IFN-γ might act by enhancing production of TNF, and thus promoting up-regulation of endothelial cell adhesion molecules.

Even though pro-inflammatory cytokines seem to be involved, they cannot be considered the only players in CM pathogenesis, because elevated levels of these cytokines are found also in humans infected with the non lethal *P. vivax*.

**Endothelial cells and adhesion molecules**

Brain endothelial cells contribute to the formation of the blood-brain barrier (BBB). They respond to signaling by different cytokines by becoming activated, modulating adhesion molecules expressed on their surface, and producing cytokines themselves [338]. The vascular endothelium responds to TNF activation by up-regulating expression of ICAM-1 [339], which in turn, can bind to molecules expressed on leukocytes and platelets, such as LFA-1 [340]. Indeed, blockade of LFA-1 induces ECM resistance in mice [340-342], and ICAM-1 deficient mice do not develop ECM [343]. The action of activated leukocytes in the brain microvasculature likely promotes damage of the endothelium, thus compromising the integrity of the BBB [338, 344, 345].

**Leukocytes**

Even though brain leukocytes accumulation has been described in both humans and mice with CM, this event appears to be more prominent in the experimental model, with monocytes/macrophages, neutrophils, T lymphocytes and NK cells being the main cell types found in the cerebral vessels [280, 346-348].

Monocytes/macrophages and neutrophils are thought to be involved in the pathology onset, rather than on the acute disease. These mononuclear cells can secrete cytokines and chemokines, thus contributing to the recruitment of other leukocytes and promoting inflammation. Indeed, mice treated
before infection, but not after, with clodronate liposomes to deplete monocytes/macrophages, or with a monoclonal antibody to ablate peripheral neutrophils, showed resistance to ECM [348-350].

The first evidence of the involvement of T cells in CM pathogenesis came from experiments where neonatal thymectomy or treatment with anti thymocyte serum could prevent ECM in rat and hamsters infected with *P. berghei* [351, 352]. Later on, the use of depleting antibodies and T cell-deficient mice further confirmed the role of CD4+ and CD8+ T cells [139, 345, 348, 353-355].

CD4+ T cells have been proposed to be involved in the induction phase of ECM because *P. berghei* infected CD4 deficient mice, or mice injected with CD4 depleting antibodies before or during early infection, do not develop CM. However, later depletion cannot prevent the pathology [345, 348, 354]. Among all sequestered leukocytes, CD8+ T cells have proved to be the most directly involved in causing ECM. The first evidence of their role came from experiments where depletion of CD8+ T at the time of onset of the disease, on day 6 post infection, prevented the manifestation of the pathology [348, 356]. Moreover, cytotoxic T cells adoptively transferred to RAG-2 deficient mice were found to migrate to the brain where they induced ECM [356], even though this has never been reproduced by other groups. Because CD8+ T cells are cytotoxic effectors aimed at killing infected or malignant cells, the following experiments focused on their mechanism of causing pathology. Activated CD8+ T cells produce cytokines such as perforin and granzyme B (GzmB), through which they exert their cytotoxic function. Indeed, perforin deficient mice were resistant to ECM, even if iRBCs and leukocytes still accumulated in the brain [356]. Similarly, GzmB deficient mice were protected from ECM [318]. These results suggest that brain sequestered activated CD8+ T cells induce ECM by production of cytokines which damage the endothelium, thus compromising the integrity of the BBB and causing hemorrhage and edema. Even though studies with transgenic parasites expressing an epitope from chicken ovalbumin have confirmed that brain sequestered CD8+ T cells are parasite-specific [180, 181], only very recently CD8+ T cells specific for a native malaria *Plasmodium* epitope were shown to be induced during *P. berghei* infection, and to migrate to the
brain just before the manifestation of neurological signs. Moreover, the authors showed that activated brain endothelial cells acquire the ability to uptake and cross-present parasite antigens, thus becoming a target for specific cytotoxic T cells, and that this cross-presentation is associated with iRBCs accumulation in the brain [357]. Indeed, it was recently demonstrated that CD8⁺ T cells can induce ECM only when a critical parasite load is present in the brain and; vice versa, they play a critical role in maintaining parasite tissue sequestration during ECM [318, 319]. Therefore, iRBCs and cytotoxic T cells are not sufficient per se, and sequestration of both is necessary to induce pathology.

Interestingly, an increase in circulating CD8⁺ T cells has also been observed in humans with malaria [358, 359].

Chemokines and NK cells

Due to their role in leukocyte recruitment and inflammation, chemokines have been considered as potential mediators of CM. Several studies indicate a role for the chemokine receptors CCR5 and CXCR3. Indeed, mice deficient for CCR5 or CXCR3 are both significantly protected from ECM [356, 360]. However, when expression of these chemokines in brain sequestered T lymphocytes during CM was analyzed, only a small fraction of T cells expressed CCR5, whereas more than 90% of them was found to express CXCR3 [361]. In the same work it was also suggested that IFN-γ produced by NK cells is responsible for up-regulating the CXCR3 chemotaxis pathway and promote recruitment of T cells in the brain of mice with CM. Consistently, NK depletion affects recruitment of both CD4⁺ and CD8⁺ T cells, and CXCR3 deficient mice show an impairment of CD8⁺ T cells migration to the brain [361-364].

Interestingly, the chemokine IP-10, an IFN-γ-induced chemokine, has been implicated in human CM [365, 366].

Taken together these results suggest a role for chemotactic factors and their receptors in both human and experimental CM.
1.6.2.3 Hemostasis dysregulation

Dysregulation of the coagulation system has been often observed in humans infected with *P. falciparum*. In particular, these patients exhibit prolonged bleeding and blood coagulation times, platelet hyperaggregability, thrombocytopenia, brain microthrombi and petechial hemorrhage [367, 368]. Platelets, major players of the hemostasis system, were found to form emo-agglutinates with iRBCs in the brain microvasculature of infected subjects [309, 324, 369], thus promoting sequestration and vascular obstruction. Moreover, they express adhesion molecules such as LFA-1 and P-selectin, and they may promote adhesion by bridging the iRBCs to the endothelium [370]. It has been proposed that the observed thrombocytopenia in CM patients could be a response of the host to prevent platelet-mediated clumping of iRBCs and adhesion [371]. On the other hand, platelets can also release chemokines and contribute to inflammation [372, 373].

The role of platelets in CM pathogenesis is supported in the mouse model, as treatment with anti platelet antibodies before or at the time of infection prevents ECM [340].

Brain homeostasis is critical for normal brain functions, and therefore it needs to be tightly regulated. Endothelial cell, pericytes, astrocytes and glial cells contribute to the formation of the BBB, which main function is to ensure that no large molecules and pathogens in the blood have access to the brain tissue.

According to the current hypothesis, the combined action of sequestration, inflammation and hemostasis dysregulation results in capillary obstruction, reduced perfusion, local hypertension and, eventually, damage of the BBB and breaking of the brain homeostasis [286], [374]. Indeed, local rupture of the BBB and hemorrhage have been observed in both human and experimental CM.
1.7 Aims and objectives

DCs are a heterogeneous family of cells ideally located in strategic sites of the body, where they can detect invading pathogens and initiate immune responses.

To gain a more comprehensive understanding of the role of various DC subsets in immune protection and disease pathogenesis, we used the DTR-DT system to generate independent mouse strains, where \textit{in vivo} ablation of distinct DC subsets can be obtained. In particular, we chose three DC specific markers, Siglec-H, Clec9A and Clec4a4, exclusively expressed only on particular DC subsets, to drive the DTR expression in our transgenic mice. Siglec-H-, Clec9A- and Clec4a4-DTR mice allow specific ablation of pDCs, CD8$^+$ and CD103$^+$ cDCs, and CD8$^-$$CD11b^+$ cDCs, respectively, and have been valuable immunological tools, during this project, to address outstanding questions in the field of malaria, a parasitic infection that still remains an important global health problem. In particular, our specific aims were:

- to assess the relative contribution of distinct DC subsets in shaping the innate and acquired immune responses to the blood stage of malaria \textit{in vivo};
- to assess the involvement of distinct DC subsets in immune-mediated pathology in malaria infection.

To pursue these aims, we used the murine \textit{P. yoelii} and \textit{P. berghei ANKA} experimental models to infect our DTR-transgenic mice, and studied the consequences of the absence of the target myeloid cell subtypes.
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals
C57B6/J, BALB/c or ICR mice were purchased from the Center for Animal Resources of National University of Singapore, or obtained from our animal facility. Siglec-H-DTR, Clec9A-DTR and Clec4a4–DTR transgenic lines were generated in a BALB/c genetic background as described in Sections 2.2.1 to 2.2.9. To obtain ECM-susceptible mice, F1 mice were generated by crossing C57B6/J mice with BALB/c mice, or with homozygous Siglec-H-, Clec9A-, and Clec4a4–DTR BALB/c mice. All mice were maintained under specific pathogen-free conditions in our animal facility. All experiments were approved by the Institutional Animal Care and Use Committee.

2.1.2 Rodent malaria parasites
Green fluorescence protein (GFP) expressing Plasmodium yoelii 17X clone 1.1 non lethal strain (Py1.1-GFP) [375], Plasmodium yoelii 17X clone YM lethal strain (YM-GFP) (generous gift of Prof. Peter Preiser, School of Biological Science, Nanyang Technological University, Singapore), and Plasmodium berghei ANKA clone 15Cy1(PbA-GFP) [376] were used for malaria experiments.

2.1.3 Antibodies
Plasmodium yoelii (Py)-specific monoclonal antibodies were generated as described in Section 2.2.22. Anti mouse commercial antibodies are listed in Appendix 6.2, Table 6.1.

2.1.4 Media, buffers and solutions
Media, buffers and solutions are described in Appendix 6.1.
2.1.5 Reagents, chemicals and kits
All reagents, chemicals and kits used are listed in Appendix 6.2.

2.1.6 Equipment
Instruments used in this study are listed in Appendix 6.3

2.1.7 Computer software
Flow cytometry data were analyzed using FlowJo 7.6.1 software (TreeStar Inc, Ashland, OR).
Live stream imaging data were analyzed with IDEAS 3.0 image analysis software (Amnis, Seattle, WA, USA).
Graphs and statistical analysis were generated with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

2.2 Methods

2.2.1 Bacterial artificial chromosome (BAC) preparation
Siglec-H- and Clec9A-DTR transgenic mice were generated using a BAC approach. BAC constructs were designed and prepared by Wee Nih Fam (School of Biological Science, Nanyang Technological University, Singapore). Briefly, the BAC vectors contain a core expression cassette targeted precisely at the initiation codon of each locus in the corresponding BAC. The core expression cassette contains the human DTR cDNA, followed by 2A, a 18 amino acids linker which can efficiently direct cotranslation cleavage, tdTomato, a photostable red fluorescent protein (RFP) variant in which two genetically modified RFP domains are covalently fused, such that protein-protein interactions are restricted to intramolecular interactions within the same polypeptide chain, pA, a strong synthetic polyadenylation site, and the neomycin resistance gene for selection purpose (Figure 2.1).
Figure 2.1. Structure of the BAC vector. The core expression cassette contains the sequences of human DTR; cotranslation cleavage mediating linker 2A; tdTomato; synthetic polyadenilation site pA. After the Red/ET-mediated recombination, the core expression cassette is placed at the initiation codon of the chosen marker gene.

2.2.2 Gene targeting construct preparation

Clec4a4-DTR knock-in mice were generated using a gene targeting approach. The gene targeting construct was designed and prepared by Dr. Piotr Tetlak (School of Biological Science, Nanyang Technological University, Singapore).

Figure 2.2. Structure of the targeting vector. The core expression cassette contains the human DTR cDNA followed by pA and the neomycin resistance gene. After the knock-in, the core expression cassette is placed after the stop codon of the last coding exon in the Clec4a4.

Briefly, the targeting vector containing a long (10 Kb) and a short (2 Kb) homology arm for the selected loci, and the core expression cassette with
the human DTR cDNA followed by pA and the neomicine resistance gene, was targeted directly after the stop codon of the last coding exon in the Clec4a4 gene (Figure 2.2).

2.2.3 Embryonic stem (ES) cells and Mouse Embryonic Fibroblasts (MEFs) maintenance and passaging

Reagents:

ES cells culture medium
MEFs culture medium
0.5% Trypsin EDTA
1X Phosphate Buffer Saline (PBS)
ES cells and MEFs freezing medium

BALB/c male ES cell line (generous gift of Dr. Birgit Lederman, Novartis Pharma AG, Basel, Switzerland) was maintained in ES cell culture medium, onto a feeder layer of 30 Gy irradiated MEFs. Fresh medium was provided every day to ES cell cultures, and cells were passaged or frozen at a 1:8-10 ratio on the third day of culture, when they were about 70% confluent.

MEFs extracted from mouse embryos were maintained in MEF culture medium, providing fresh medium every second day. When confluent, MEFs were passaged or frozen at a 1:4 ratio, or 30 Gy irradiated for ES cells seeding.

Harvesting and passaging of ES cells and MEFs were performed using Trypsin EDTA after washing the cells with PBS and centrifugation at 1200 rpm for 5 minutes. Cells were frozen in 1mL of freezing medium.

2.2.4 ES cells electroporation and selection

Reagents:

0.5% Trypsin EDTA
1X PBS
300μg/mL G418

After thawing, ES cells were passaged at least once before electroporation and cultured into a 10 cm-plate with irradiated MEFs. For electroporation, 70% confluent ES cells were harvested, washed twice with PBS, gently mixed with 30-80 μg linearized BAC vector and resuspended in a final
volume of 800 μL of PBS. Cells-BAC suspension was placed into a 0.4 cm gap cuvette and electroporated at 250 Volts, 500 uFD. Only electroporations with a time constant of about 10 ms were considered successful. Electroporated ES cells were incubated for 10 minutes at 37 °C to let them recover, and then distributed into 4 new 10 cm-plates with freshly irradiated MEFs. 24 hours after electroporation, G418 antibiotic was added to ES cells medium to efficiently select the successfully electroporated ES cells colonies. After 7-9 days of selection, survived, nicely shaped, undifferentiated ES cell colonies were picked and split into two wells of a 96-well-plate. When confluent, one of the two wells was used for ES cell expansion; the replica well was used for screening.

2.2.5 ES cells screening

Reagents:

Blood and Tissue Kit
GoTaq Flexi DNA Polymerase
1% agarose gel
Gene ruler 1kb DNA ladder

The replica well was subjected to genomic DNA extraction according to the Blood and tissue Kit protocol, and the extracted DNA was used as template for Polymerase Chain Reaction (PCR) screening. ES cell clones electroporated with Siglec-H and Clec9A BAC constructs were tested for the presence of tdTomato sequence, as well as both 5’ and 3’ BAC ends, to confirm that the entire BAC vector was integrated in the genome. For ES cell clones electroporated with the Clec4a4 targeting construct, the presence of an amplification product spanning the 3’ of the core expression cassette and the region following the short homology arm was used for screening. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) house keeping gene was used for normalization. Primers used for PCR screening (Table 2.1) were purchased from 1st BASE Pte Ltd, Singapore. Standard PCR conditions were applied according to polymerase Kit protocol, specific suggested primers’ melting temperature and amplification product length. Amplification products were visualized by gel electrophoresis. Individual ES
cell clones fulfilling the screening criteria were expanded and frozen for blastocyst microinjection.

Table 2.1. Sequence of primers used for PCR screening.

<table>
<thead>
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2.2.6 Preparation of blastocyst donors and pseudopregnant foster mice

Reagents:

Pregnant Marel Serum (PMS)

Human Chorionic Gonadotropin (HCG)

For each session, 10 C57BL6/J 4 weeks old female mice were superovulated via intra peritoneal (i.p.) injection of 10 IU of PMS and, 46-48 hours later, 10 IU of HCG. Superovulation was used to reduce the total number of mice required to provide blastocysts for microinjection, to minimize variability in their quality, and to synchronize the production of a sufficient number of embryos at a given time. The age at which superovulation can most effectively be induced varies from strain to strain, but traditionally prepubescent female mice between 3-5 weeks of age have been used. The superovulated females were then mated to 10 C57BL6/J
male mice and used as blastocyst donors 3.5 days later, before the
embryos hatched from the zona pellucida.
2.5 days before the microinjection session, 10 ICR females were mated to
10 sterile (vasectomized) ICR males. The morning after, females were
checked for vaginal plugs and only plugged females were kept to be used
as foster mothers for blastocysts transfer on the injection day.

2.2.7 Preparation of ES cells for blastocyst microinjection

*Reagents:*

* ES cells culture medium
* 0.5% Trypsin EDTA
* 1X Phosphate Buffer Saline (PBS)

Selected ES cell clones were thawed four days prior to microinjection and
grown into a 6-well-plate well. Cells were passaged at least once before
microinjection, but they were always used at the lowest passage number
possible. ES cells from a 70% confluent 6-well-plate well were usually used
for each microinjection session.

About 15 minutes before required for injection, ES cells were washed with
PBS, harvested, centrifuged at 300g for 5 minutes and gently resuspended
in 5 ml of fresh ES culture medium to generate single cell suspension. Cells
were then incubated for 1 minute at room temperature to let MEFs settle
down; afterwards only the MEF-free upper fraction was collected and kept
on ice to be used for microinjection.

2.2.8 Blastocyst microinjection

*Materials:*

* M2
* M16

Blastocyst microinjection was performed by Dr. Piotr Tetlak. 3.5 days after
mating, donor females were sacrificed and blastocysts were collected from
each uterus. Embryos were washed in M2 medium and stored in M16
medium in a CO₂ incubator until microinjection.
A number of 8 to 10 ES cells were microinjected into a host blastocyst, and 9 to 12 injected blastocysts were transferred into one pseudopregnant foster mother.

2.2.9 Establishment of DTR-transgenic mice strains
Chimeric males generated via blastocyst microinjection were weaned on week 3 after birth, and mated with BALB/c females when 5 weeks old (the BALB/c ES cell line used is a male line, therefore only chimeric males are likely to succeed in germ line transmission). The resulting progeny was tested for transgenesis via PCR on genomic DNA extracted from the mice tail tip. Genomic DNA from tail tip was obtained as described in Wang et al. [377]. The same PCR conditions used for ES cells screening were applied. Positive heterozygous DTR male and female mice were crossed to obtain homozygous DTR mice, which were then mated with wt BALB/c or C57BL6/J mice to generate heterozygous DTR-transgenic mice to be used for experiments.

2.2.10 DT-mediated ablation
Reagents:
DT
For systemic ablation of target DC subsets, DTR-transgenic mice were injected i.p. twice in 24 hours with 4 (Siglec-H-DTR) or 10 (Clec9A-DTR, Clec4a4-DTR) ng/gram body weight (gbw) DT in 1X PBS added with 1% FCS. Depletion was analyzed 48 hours after the first injection. The DT dosage chosen for each of the DTR-transgenic lines was determined during characterization experiments as the lowest dose capable of obtaining an efficient ablation.

2.2.11 Cryopreservation of iRBC stock
Reagents:
$10^6$ iRBCs per mouse
1X PBS
1 to 3 mice were injected i.p. with $10^6$ RBCs infected with the parasite strain/species to amplify, obtained from a previous stock. 6 days post
infection (p.i.) for PbA-GFP and YM-GFP, and 10 to 12 day p.i. for Py1.1-GFP, the presence of the parasite was verified. In particular for parasitemia 1 μL of blood from the tail tip was resuspended in 500 μL of 1X PBS and the percentage of GFP+ iRBCs was evaluated by flow cytometry using a BD FACS Calibur. For PbA-GFP, which can induce ECM, the stock was prepared when ECM neurological signs were evident. At the time of stock preparation, blood obtained via retro orbital bleeding was collected in 30 mL of 1X PBS. The PBS-blood mix was then centrifuged at 400 g for 5 minutes, resuspended in 9.5 mL of fresh 1X PBS, and used to count the total number of RBCs, as described in Section 2.2.16, as well as to test the parasitemia by flow cytometry, in order to determine the total amount of iRBCs. After one more centrifugation, the stock was prepared by resuspending the RBCs in Alsever’s solution at a concentration of $10^7$ iRBCs/ml, distributed in 1.2 mL aliquots, and frozen at -80 °C in dedicated Styrofoam boxes for 24 hours, before being transferred in liquid nitrogen.

When iRBCs stock was needed for infection, frozen vials were removed from liquid nitrogen and thawed at 37 °C.

2.2.12 Malaria infection experiments

Reagents:

DT

100 μL of $10^7$ iRBCs/ml stock per mouse

Groups of 6 to 8 weeks old wt and DTR mice were used for malaria infection experiments. DTR mice were treated twice with DT before infection, and every 3 to 4 days during the infection period for a constant ablation of the target DC subsets. For infection, 100 μL of $10^7$ iRBCs/ml stock, corresponding to $10^6$ iRBCs, were injected i.p. to each mouse of both wt and DTR groups, and parasitemia (measured as described in Section 2.2.11) and survival were monitored starting from day 3 p.i. Serum samples were obtained on day 6 and on day 14 p.i. to test cytokines and parasite-specific antibodies production, respectively. ECM development was monitored every day from day 5 to day 12 p.i. using the rapid murine coma behaviour scale (RMCBS) score as previously described [378].
2.2.13 Preparation of serum samples for ELISA
100 to 300 μL of blood obtained by retro orbital bleeding was allowed to clot for 30 minutes at room temperature, and centrifuged at 13000 g for 3 minutes. Serum was then transferred to a new 1.5 mL tube and stored at -20°C.

2.2.14 Preparation of peripheral blood mononuclear cells (PBMCs) for flow cytometry

Reagents:
- 3% sodium citrate solution
- Ficoll-Paque™ PLUS
- 0.89% ammonium chloride

Approximately 200 μL of blood obtained by retro orbital bleeding was collected in 500 μL of 3% Sodium Citrate solution to prevent clotting, mixed, layered on top of 1 mL Ficoll-Paque™ PLUS and centrifuged at 900 g for 30 minutes. After centrifugation, the ring of PBMCs at the interface was collected and transferred to a clean tube with 2 mL FACS buffer, and spun again at 300 g for 10 minutes. After removing the supernatant, remaining contaminating erythrocytes were depleted via addition of 0.89% ammonium chloride and incubation for 10 minutes at room temperature. PBMCs were then washed again and labeled for flow cytometry.

2.2.15 Preparation of tissue mononuclear cells and RBCs for flow cytometry

Reagents:
- Collagenase D
- Digestion medium
- 0.89% ammonium chloride
- FACS buffer
- 1X PBS
- DNase I
- 33% Percoll solution

Spleen single cell suspension was obtained as described previously [379], by digesting the minced organs with 1mg/mL Collagenase D in digestion
medium for 1 hour at 37 °C, and meshing through a 100 μm cell strainer. For preparation of cells for Foxp3 staining, spleens were directly minced and meshed without collagenase digestion. Cells were then centrifuged at 300 g for 7 minutes, resuspended in 0.89% ammonium chloride and incubated for 10 minutes at room temperature to lyse erythrocytes, centrifuged again and washed with FACS buffer before being labeled for flow cytometry.

For analysis of brain leukocytes, mice were perfused intracardially with 15 ml 1X PBS to remove circulating leukocytes, brains were removed, minced and digested in 1 mg/mL collagenase D and 2 U/mL DNase I for 30 min at 37 °C, as described in Haque et al. [318]. Single cell suspension was obtained by homogenization through an 18 G needle, meshing through a 100 μm cell strainer and debris elimination via a 33% Percoll gradient. Cells were then washed, lysed in 0.89% ammonium chloride for 10 minutes to remove erythrocytes, and counted before staining for flow cytometry. For quantification of iRBCs brain accumulation, brains were minced, homogenized and meshed immediately after perfusion and collection. Following Percoll centrifugation and washing in FACS buffer, cells were counted and labeled for flow cytometry.

### 2.2.16 Cell counting

**Reagents:**

0.4% **Trypan blue**

For viable cell counting, 10 μL of cell suspension were mixed with 10 μL of Trypan blue and loaded on a Neubauer chamber. The 4 outer squares of the chamber were counted, and the concentration of viable cells was calculated using the following formula:

\[
\text{Total cells in 4 squares / } 4 \times 2 \times 10^4 = \text{cells/mL}
\]

For counting of iRBCs for stock preparation, 10 μL of the iRBCs suspension was loaded directly in a Neubauer chamber, the 4 outer squares of the chamber were counted, and the concentration of RBCs was calculated using the following formula:

\[
\text{Total RBCs in 4 squares / } 4 \times 10^4 = \text{RBCs/mL}
\]
2.2.17 Enrichment of spleen DCs

*Reagents:*

*OptiPrep<sup>TM</sup>*  
*Solution B*  
*Solution C*

Enrichment of spleen DCs was obtained by flotation through a low-density barrier [380]. Spleen mononuclear cells obtained as described in Section 2.2.15 were centrifuged at 300 g for 7 minutes, resuspended in 3 ml of solution B added with 1 ml OptiPrep<sup>TM</sup>, and overlayed with 4 ml of a 1:4.2 v/v OptiPrep<sup>TM</sup>/Solution C mix, followed by 3 ml of solution B. The gradient was then centrifuged at 600 g for 15 minutes without brake, and the ring of cells formed at the interface between the 1:4.2 v/v OptiPrep<sup>TM</sup>/Solution C mix and the solution B was collected and transferred to a clean tube. The enriched cDCs were then added with 10 ml solution B and centrifuged at 500 g for 10 minutes for washing, before being labelled for flow cytometry.

2.2.18 Cell labeling for flow cytometry

*Reagents:*

*FACS buffer*  
*Antibodies (listed in Appendix 6.2, Table 6.1)*  
*Brefeldin A*  
*Cell fixation buffer*  
*Permeabilization buffer*  
*Foxp3 staining kit*  
*Hoechst*

For staining of cell surface antigens, cells were added with fluorocrome-conjugated antibodies diluted in FACS buffer, incubated at 4 °C for 20 minutes, washed, and resuspended in FACS buffer for analysis. For intracellular cytokine staining, cells were incubated in 10 μg/ml brefeldin A for 3 hours at 37 °C/5% CO<sub>2</sub> before proceeding with cell surface staining, fixation, permeabilization and intracellular staining with fluorocrome-conjugated antibodies diluted in permeabilization buffer. Cells were then washed and resuspended in FACS buffer for analysis. For intracellular staining of Foxp3, cells were treated and stained according to the Foxp3 kit.
protocol. For quantification of iRBCs accumulation in the brain, cells were stained with anti CD45.2 antibody to discriminate RBCs from mononuclear cells, and with Hoechst which labels the parasite DNA.

2.2.19 Antigen uptake assay

**Reagents:**
- FACS buffer
- Antibodies (listed in Appendix 6.2, Table 6.1)
- Cell fixation buffer

Freshly isolated spleen DCs enriched as described in Section 2.2.17 were co-cultured for 2 hours with PbA-GFP iRBC at a 1:3 ratio, washed, stained, fixed and analyzed in live stream imaging.

2.2.20 Cytokines Enzyme Linked Immunosorbent Assay (ELISA)

**Reagents:**
- 1X PBS
- Assay diluent
- Wash buffer
- Mouse IFNγ ELISA MAX Kit (detection limit 10 pg/ml)
- 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate reagent A
- TMB substrate reagent B

For coating, 100 μL of capture antibody diluted in 1X PBS, as suggested in the kit protocol, were added to each well of a 96-well Nunc MaxiSorp plate, and incubated at 4°C over night. The plate was then washed 4 times with wash buffer and blocked with 100 μL of assay diluent per well for 1 hour to avoid non-specific binding. After washing 4 times, 100 μL per well of serum sample diluted 1:1 with assay diluent, as well as 100 μL per well of standard dilutions prepared as suggested in the kit protocol were added to the plate and incubated at room temperature for 2 hours. The plate was then washed 4 times, incubated at room temperature for 30 minutes with 100 μL per well of detection antibody prepared as suggested in the kit protocol, washed again for 4 times, and incubated at room temperature for 30 minutes with 100 μL per well of Avidin-HRP diluted in assay diluent as suggested in the kit protocol. The plate was finally washed 5 times and
added with 100 μL per well of a 1:1 mix of TMB substrate reagent A and B. Optical Density (OD) at 370 nm was measured every 5 minutes for 30-40 minutes.

2.2.21 Cell-based ELISA for parasite-specific antibodies

Reagents:
1X PBS
Blood from an infected mouse having about 20% parasitemia
Methanol
Blocking buffer
HRP-labeled anti-immunoglobulin antibodies (listed in Appendix 6.2, Table 6.1)
TMB substrate reagent A
TMB substrate reagent B

For coating with iRBCs, 100 μL of blood from an infected mouse having about 20% parasitemia were resuspended in 10 mL of 1X PBS and 100 μL 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 μL of methanol for 10 minutes. After washing 3 times with 1X PBS, 100 μL 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 as described in Section 2.2.13 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 μL of a 1:1 mix of TMB substrate reagent A and B were added to each well. OD at 370 nm was measured every 5 minutes for 30-40 minutes.

2.2.22 Generation of *P. yoelii*-specific monoclonal antibodies

*P. yoelii*-specific monoclonal antibodies from mice lacking Clec9A+ DCs were generated following standard protocols [381]. Briefly, splenocytes collected from DT treated Clec9A-DTR mice 14 days post infection with
Py1.1-GFP were fused with myeloma cells to generate hybridomas, which were plated in flat bottom 96-well plates. 10 μL of supernatant from all wells containing hybridoma clones were screened for *P. yoelii*-specific IgG1 and IgG2a using the cell-based ELISA for parasite-specific antibodies described in Section 2.2.21. Positive clones were subcloned by limiting dilutions, and wells containing only one large single colony were re-screened. Positive subclones were then frozen, as well as expanded for antibody purification. For purification of monoclonal antibodies, supernatants from expanded clones were applied to a GammaBind sepharose column, and antibodies were then eluted and dialyzed.

### 2.2.23 Passive immunization

Serum passive immunization was performed by injecting *P. yoelii* infected wt mice with a mix of sera obtained from *P. yoelii* infected DT treated Clec9A-DTR mice, or wt mice on day 14 p.i. In particular, 500 μL of the sera mix were injected i.p. on days 8 and 10 p.i. Antibody passive immunization was performed by injecting *P. yoelii* infected wt mice with a mix of 8 *P. yoelii*-specific monoclonal IgG1 antibodies generated in Clec9A ablazed mice. In particular, a mix containing 100 μg of each of the 8 IgG1 antibodies was injected i.p. on days 8, 10 and 12 p.i. in *Py1.1*-GFP experiments. In the YM-GFP experiment, 200 μg of each of the 8 IgG1 antibodies was injected i.p. 1 day prior and 1 day after infection.

### 2.2.24 Tetramer assay

**Reagents:**

- FACS buffer
- Live/Dead violet stain
- Antibodies (*listed in Appendix 6.2, Table 6.1*)
  - SQLLNALKY-H2D\(^b\) tetramer
- Cell fixation buffer

Brain single cell suspensions from uninfected or infected mice on day 6 p.i., prepared as described in Section 2.2.15, were firstly incubated for 30 minutes with Live/Dead violet stain, and subsequently with PE-labeled SQLLNALKY-H2D\(^b\) tetramer (generous gift of Prof. Laurent Renia,
Singapore Immunology Network, Agency for Science, Technology and Research, Singapore) at 4°C for 20 minutes. After washing, cells were stained, incubated for another 30 min at 4°C, washed again, fixed and analyzed in flow cytometry.

2.2.25 Brain histology

Reagents:

Organ fixation buffer

1X PBS

Ethanol

Xylene

Paraffin

Distilled water

Hematoxilin and Eosin (H&E) solutions

1% acid ethanol solution

Scott’s tap water substitute

DPX Mountant for histology

Brains dissected from infected and non-infected mice were fixed in organ fixation buffer for 24-48 h, washed twice with 1X PBS and submitted to the following dehydration and clearing steps: 80% ethanol for 1 hour, 90% ethanol for 1 hour, 100% ethanol for two hours, 1:1 ethanol:xylene for 30 minutes, xylene for 1 hour. Brains were then transferred in liquefied paraffin, incubated at 60 °C over night and embedded in paraffin. 5 μm sections were placed on glass microscope slides, deparaffinized 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 hematoxilin 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 xylene-based mounting medium and analyzed by light microscopy using a 40X magnification for image capture.

2.2.26 Spleen cryosection immunofluorescence

Reagents:
Optimal Cutting Temperature (OCT) compound
Acetone
Blocking buffer
1X PBS
Antibodies (listed in Appendix 6.2, Table 6.1)
Fc block
Fluorescence mounting medium

For preparation of cryosections, spleens were placed on dedicated molds containing the OCT compound on dry ice immediately after collection. After the OCT media had frozen, the organs were transferred to -80 °C until sectioning. 6 µm sections were placed on glass microscope slides, which were then air-dried and stored at -20 °C until staining for immunofluorescence.

For staining of cryosections, after removal from -20 °C slides were allowed to air-dry before and after fixation in cold acetone for 10 min. Blocking of non-specific binding was performed by incubation with Fc block for 15 minutes, followed by washing with 1X PBS. Sections were then stained with antibodies diluted in FACS buffer for 45 minutes and washed with 1X PBS. For biotinylated antibodies which required a subsequent fluorochrome-conjugated streptavidin step, a slide stained only with the fluorochrome-conjugated streptavidin was used as control for the streptavidin background signal. Slides were finally mounted using a fluorescence mounting medium and analyzed by fluorescence microscopy using a 20X magnification for image capture.

2.2.27 Statistical analysis

Comparison between 2 groups was performed using the Student t test or Mann-Whitney test, depending on whether data were parametric or non-parametric. Survival curves were analyzed using the Mantel-Cox long-rank
test. Statistical significance was accepted at \( p \text{ value} < 0.05 \). Data were analyzed with GraphPad Prism 5.0 software.
3. RESULTS

3.1 Generation and characterization of DTR transgenic mice

To generate our DTR-transgenic mice, we chose three DC specific markers exclusively expressed only on particular DC subsets, Siglec-H, Clec9A and Clec4a4, to drive the DTR expression, and obtain specific ablation of pDCs, CD8$^+$ and CD103$^+$ cDCs, and CD8$^-$CD11b$^+$ cDCs, respectively.

For Siglec-H-DTR and Clec9A-DTR mice, we used a BAC recombineering strategy to express the human DTR under the control of the chosen specific promoters. Recombination-mediated genetic engineering, or recombineering, is a technique based on homologous recombination systems, as opposed to the use of restriction enzymes and ligases, to combine DNA sequences in a specified order. It is particularly useful when big DNA molecules, such as BACs, need to be manipulated. We chose BAC transgenesis because it obviates the need to have fully characterized the promoter of choice. Via this strategy, we generated genetically modified ES cells, which were used in blastocyst microinjection, and allowed us to obtain Siglec-H-DTR and Clec9A-DTR mouse lines where the target DC subsets could be efficiently ablated. In the case of Clec4a4-DTR mice, using the same strategy we obtained 3 independent transgenic lines, but unfortunately, none of them ablated the target cell population. We hypothesized that such a result could be due to the lack of some important regulatory elements for the expression of Clec4a4 gene in the chosen BAC, therefore we decided to apply a gene targeting approach. Using this strategy, we obtained two independent lines, and this time, both efficiently ablated the target DC subset upon DT treatment.

The following paragraphs describe experiments performed to test efficiency and specificity of the DTR-DT ablation system in our DTR-transgenic mice. Given that the spleen is the organ of major interest in malaria immunity, only spleen results will be shown here.

Table 3.1 summarizes antibody combinations used in our FACS analysis to identify the target DC subsets and other immune effector cells of interest.
For CD8\textsuperscript{+}Cle9A\textsuperscript{+} CD8\textsuperscript{−}CD11b\textsuperscript{+} cDC analysis different combinations can be used, and are listed in the table.

**Table 3.1. Antibody combinations used for FACS analysis of spleen cell subsets.**

<table>
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<tr>
<th>Cell subset</th>
<th>Antibody combinations</th>
</tr>
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<tbody>
<tr>
<td>pDCs</td>
<td>CD11c\textsuperscript{int}Siglec-H\textsuperscript{+}</td>
</tr>
<tr>
<td>CD8\textsuperscript{+}Clec9A\textsuperscript{+} cDCs</td>
<td>CD8\textsuperscript{+}Clec9A\textsuperscript{+}</td>
</tr>
<tr>
<td></td>
<td>CD11c\textsuperscript{hi}CD8\textsuperscript{+}</td>
</tr>
<tr>
<td></td>
<td>CD8\textsuperscript{+}CD11b\textsuperscript{−} (gated on CD11c\textsuperscript{hi} cells)</td>
</tr>
<tr>
<td>CD8\textsuperscript{−}CD11b\textsuperscript{+} cDCs</td>
<td>CD11c\textsuperscript{hi}33D1\textsuperscript{+}</td>
</tr>
<tr>
<td></td>
<td>CD11b\textsuperscript{+}33D1\textsuperscript{+} (gated on CD11c\textsuperscript{hi} cells)</td>
</tr>
<tr>
<td></td>
<td>CD8\textsuperscript{−}CD11b\textsuperscript{+} (gated on CD11c\textsuperscript{hi} cells)</td>
</tr>
<tr>
<td>Red pulp macrophages</td>
<td>F4/80\textsuperscript{−}CD11b\textsuperscript{−lo} (gated on non CD11c\textsuperscript{hi} cells)</td>
</tr>
<tr>
<td>Other macrophages</td>
<td>F4/80\textsuperscript{−}CD11b\textsuperscript{+} (gated on non CD11c\textsuperscript{hi} cells)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>F4/80\textsuperscript{−}CD11b\textsuperscript{+} (gated on non CD11c\textsuperscript{hi} cells)</td>
</tr>
</tbody>
</table>

\textsuperscript{int} = intermediate
\textsuperscript{+} = positive
\textsuperscript{hi} = high
\textsuperscript{−} = negative
\textsuperscript{lo} = low

### 3.1.1 Characterization of Siglec-H-DTR mice

Siglec-H is a specific marker expressed exclusively on pDCs in BM, spleen and blood. To investigate whether Siglec-H\textsuperscript{+} pDCs could be efficiently ablated \textit{in vivo}, Siglec-H-DTR mice were injected i.p. twice in 24 hours with 4 ng/gbw DT (we chose this dose based on work of Jung and colleagues with the CD11c-DTR mouse [99]). 48 hours after the first injection, blood samples and spleens were collected, processed, and ablation efficiency was analyzed by flow cytometry. Results showed that Siglec-H\textsuperscript{+} pDCs were ablated with an 80 to 95% efficiency in both blood and spleen, compared to control treated wt mice or untreated Siglec-H-DTR mice. Moreover, the ablation was specific for Siglec-H\textsuperscript{+} DC subset because total CD11c\textsuperscript{+} DCs were not affected by DT treatment (Figure 3.1(a)). These results suggested that DT treatment of Siglec-H-DTR mice can mediate efficient pDC ablation, and that the effect is specific for this subset.
A subpopulation of macrophages in the marginal zone of the spleen, distinct from the siaoloadhesin Siglec-1* MMs, has been shown to express Siglec-H, together with SIGN-R1, a molecule expressed by MZMs, recognized by the ER-TR9 antibody [10]. To confirm depletion of these cells in our Siglec-H-DTR mice, spleen cryosections were prepared and stained with Siglec-1 and ER-TR9 antibodies. Indeed, ER-TR9* cells in the marginal zone appeared partially ablated in Siglec-H-DTR mice compared to wt mice (Figure 3.1(b)). Depletion of these MZMs should be considered when interpreting results obtained in Siglec-H-DTR mice.

**Figure 3.1. In vivo ablation of Siglec-H* cells.** (a) Representative dot plots showing the profile of pDCs in the blood, as well as pDCs and CD11c^{high} cDCs in the spleen of wt and DT-treated wt and Siglec-H-DTR mice (n = 3 per group). (b) Representative spleen cryosections of wt and DT treated Siglec-H-DTR mice stained with Siglec-1 and ER-TR9 antibodies.

Previous studies on CD11c-DTR mice reported that repetitive systemic DT application results in lethality [99]. To investigate whether long term pDC ablation could be obtained in our Siglec-H-DTR mice without causing lethality, and to verify if injection of higher doses of DT could further
increase the depletion rate, we performed a long-term experiment where three different groups of Siglec-H-DTR mice were treated with either 4, 8 or 16 ng/gbw DT every 7 to 8 days for 32 days.

Figure 3.2. Long-term in vivo ablation of Siglec-H⁺ pDCs. (a) Long-term ablation experimental strategy. (b) Graphs summarizing flow cytometry results of blood analysis in wt and Siglec-H-DTR mice treated with 4, 8 or 16 ng up to day 25 after the first DT injection (n = 3 mice per group per time point). (c) Representative dot plots showing pDC and CD11c<sup>high</sup> subsets in spleen of wt and Siglec-H-DTR mice treated with 4, 8 or 16 ng/gbw up on day 32 after the first DT injection (n = 3 mice per group).
To monitor Siglec-H⁺ pDC ablation, blood samples were collected, processed and analyzed via flow cytometry every 2 to 4 days for 25 days. On day 32, mice were sacrificed and pDC ablation in the spleen was analyzed (Figure 3.2(a)). Blood analysis showed that repeated DT injections could be performed in Siglec-H-DTR mice to obtain efficient prolonged pDC depletion without inducing lethality, even when higher DT doses were used. Moreover, the lower 4 ng/gbw DT dose was sufficient to obtain the same ablation efficiency as the higher 8 and 16 ng/gbw doses (Figure 3.2(b)). Siglec-H⁺ pDCs remained ablated for about 6 days after a single DT injection, and started to recover on day 7. This result is consistent with pDC turnover rate, which is known to be about 7 days.

Analysis of the spleen 32 days after the first DT injection showed that more than 90% of Siglec-H⁺ pDCs were still ablated in mice repeatedly treated with 4 or 8 ng/gbw DT. However, ablation efficiency decreased to about 67% in mice injected with the higher 16 ng/gbw dose, possibly due to the formation of neutralizing antibodies against DT, as reported in previous studies using DTR-transgenic mice [382]. Ablation was again confirmed to be specific, as total CD11c⁺ DCs were not affected, even after prolonged DT treatment (Figure 3.2(c)).

Taken together these results suggested that, in Siglec-H-DTR mice, a prolonged ablation of Siglec-H⁺ pDCs can be achieved via repeated DT injections for more than one month, without resulting in mice death. Moreover, injection of the lower 4 ng/gbw DT dose is sufficient to obtain an efficient ablation for all the period, without the risk of DT neutralizing antibody formation.

3.1.2 Characterization of Clec9A-DTR mice
Clec9A is a C-type lectin known to be expressed exclusively on CD8⁺ cDCs in spleen, LNs, and thymus, and on CD103⁺ cDCs in peripheral tissues. To investigate whether these DC subsets could be efficiently ablated in vivo, Clec9A-DTR mice were injected i.p. twice in 24 hours with 10 ng/gbw DT (the 10 ng/gbw dosage was chosen because it proved to be the lowest dose necessary to obtain an efficient ablation - data not shown). 48 hours after the first injection, spleens were collected, processed, and ablation efficiency
Figure 3.3. In vivo ablation of Clec9A+ cDC subset. (a) Representative dot plots showing expression of Clec9A on CD8+ cDCs in the spleen, and their efficient ablation upon DT treatment in Clec9A-DTR mice. (b) Representative dot plots showing the profile of DC subsets in spleens of wt and DT treated Clec9A-DTR and Siglec-H-DTR mice. For a better resolution spleen DCs were enriched before staining. (c) Absolute number of CD11c<sup>high</sup>CD8<sup>+</sup> DC, CD11c<sup>high</sup>CD8<sup>-</sup> and CD11c<sup>int</sup>Siglec-H<sup>+</sup> pDC subsets in spleens of wt and DT treated Clec9A-DTR and Siglec-H-DTR mice (n = 5 per group). Absolute numbers are expressed as mean ± SD. Student t test, **p < 0.01, ***p < 0.001.

was analyzed by flow cytometry. Because pDCs are know to express low levels of Clec9A [115], Siglec-H-DTR mice were included in the experiment and pDC ablation was also analyzed. Results confirmed that Clec9A is expressed on spleen CD11c<sup>high</sup>CD8<sup>+</sup> cDCs, and DT injection in Clec9A-DTR mice led to 80 to 90% ablation of this cDC subset, but not CD8<sup>-</sup> cDCs.
Moreover, about 50% of pDCs were depleted, whereas DT injection in Siglec-H-DTR mice resulted in complete pDC ablation (Figure 3.3(b), lower panel and Figure 3.3(c), right panel).

To investigate whether prolonged ablation of spleen CD8⁺Clec9A⁺ cDCs could be obtained in our Clec9A-DTR mice, we performed a repeated DT administration experiment.

In particular, as DTR-transgenic mice were to be subsequently used in malaria infection studies, we designed the prolonged ablation experiment according to the DT injection scheme to be used in malaria experiments, which consists of two doses of DT on two subsequent days, followed by DT injection every 3 days, for a total of 13 days (because CD8⁺ cDCs are known to have a turnover of about 3 to 4 days in the spleen [47], this scheme was chosen to ensure efficient and continuous ablation during the
early phase of malaria infection, when innate immunity is most relevant). Spleens were collected on days 9 and 16 after the first DT injection and pDC, total cDC, as well as CD8⁺ and CD8⁻CD11b⁺ subsets were analyzed by flow cytometry and compared with wt controls (Figure 3.4(a) and (b)). The analysis revealed that CD8⁺ cDCs were efficiently ablated in the spleen on day 9, as well as on day 16 (Figure 3.4(b), lower panel, left). pDCs were also ablated, but less efficiently than CD8⁺ cDCs, consistently with their lower Clec9A expression (Figure 3.4(b), upper panel, right). Because the fraction of CD8⁺ cDCs decreased in DT treated Clec9A-DTR mice, the proportion of CD8⁻CD11b⁺ cells reciprocally increased (Figure 3.4(b), lower panel, right).

Taken together these results suggested that, in Clec9A-DTR mice, CD8⁺Clec9A⁺ cDC subset can be efficiently ablated after 2 doses of DT, and continuous efficient ablation can be achieved by repeated DT administration, for a period long enough to study their role in malaria innate immunity. Because DT administration in Clec9A-DTR mice results also in partial ablation of pDCs, Siglec-H-DTR mice can serve as controls when interpreting results obtained with Clec9A-DTR mice.

### 3.1.3 Characterization of Clec4a4-DTR mice

Clec4a4 is a C-type lectin specifically expressed on CD8⁻CD11b⁺ cDCs in spleen, LNs and PP, but not on CD8⁺ DC subsets, and which is recognized by the 33D1 antibody. To investigate whether spleen CD8⁻CD11b⁺ cDCs could be efficiently ablated in vivo, Clec4a4-DTR mice were injected i.p. twice in 24 hours with 10 ng/gbw DT (the 10 ng/gbw dosage was chosen since it was the optimal dose necessary to obtain an efficient ablation - data not shown). 48 hours after the first injection, spleens were collected, processed, and ablation efficiency was analyzed by flow cytometry. Results confirmed that 33D1 is expressed on CD8⁻CD11b⁺ cDCs in the spleen, and showed that more than 90% of this cDC subset was efficiently ablated in DT treated Clec4a4-DTR mice, compared to wt mice (Figure 3.5(a), upper and middle panel and (b)), whereas the CD8⁺ cDC subset (Figure 3.5(a), middle panel and (b)), as well as pDCs were not affected (Figure 3.5(a), lower panel and (b)).
Figure 3.5. In vivo ablation of Clec4a4+ cDC subset. (a) Representative dot plots showing the profile of DC subsets in spleens of wt and DT treated Clec4a4-DTR mice. (For a better resolution spleen DCs from middle panel were enriched before staining). (b) Absolute number of CD11c\text{high}CD8\text{−} cDCs, CD11c\text{high}CD11b\text{+} cDCs and pDCs in spleens of wt and DT treated Clec4a4-DTR mice (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, **p ,< 0.01.

Because Clec4a4 is expressed on CD11b\text{+} cDC subsets, to test for the specificity of ablation in Clec4a4-DTR mice, we also analyzed the effect of DT treatment on other CD11b\text{+} populations, such as spleen macrophages. Surprisingly, about 70% of a population of CD11b\text{−}F4/80\text{+} macrophages was affected by DT treatment (Figure 3.6(a), upper panel and Figure(b)), even though flow cytometry results showed no evident 33D1 antibody recognition on these cells (Figure 3.6(a), lower panel). Moreover, as in Clec9A ablated mice (data not shown) we observed an increase in neutrophils
(CD11b+ F4/80− cells) in spleens of DT treated Clec4a4-DTR mice compared to wt mice (Figure 3.6(a), upper panel and Figure(b)).

**Figure 3.6. DT treatment in Clec4a4-DTR mice results in ablation of CD11b− F4/80+ macrophage and increased neutrophils in the spleen.** (a) Representative dot plots showing CD11b and F4/80 staining profile of spleen macrophages and neutrophils in wt and DT treated Clec4a4-DTR mice. (b) Absolute number of spleen macrophage and neutrophil populations based on results in (a) (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, *p, < 0.05.

Neutrophilia has been observed in other DTR-transgenic mice upon DT treatment [383, 384]. Even though neutrophils do not seem to have a major role in malaria immunity, they are thought to be involved in CM pathology onset [350]. Moreover, splenic red pulp F4/80+ macrophages are considered to be important for phagocytosis and clearance of iRBCs during malaria infection [145, 146]. Therefore, neutrophilia, as well as ablation of splenic CD11b− F4/80+ macrophages, should be carefully considered when interpreting results obtained from malaria infection experiments with Clec4a4-DTR mice.
Taken together these results suggested that DT administration in Cleca4a4-DTR results in an efficient ablation of the splenic CD8\(^{-}\)CD11b\(^{+}\) cDC subset, but also causes depletion of CD11b\(^{-}\)F4/80\(^{-}\) macrophages and neutrophilia.

3.1.4 Characterization of ablation efficiency in F1 DTR-transgenic mice

DTR-transgenic mice were originally generated in a BALB/c background. Different from C57B6/J mice, BALB/c mice are resistant to ECM development upon infection with *P. berghei* ANKA.

![Figure 3.7. In vivo ablation of target cell subsets in F1 DTR-transgenic mice.](image)

(a) Representative dot plots showing the profile of DC subsets in spleen of F1 wt and DT treated F1 Cleca4a4-DTR and Siglec-H-DTR mice. For a better resolution spleen DCs were enriched before staining. (b) Representative dot plots showing the CD11c and 33D1 staining DC profile in spleens of F1 wt and DT treated F1 Cleca4a4-DTR mice.
To obtain ECM-susceptible DTR-transgenic mice to be used in *P. berghei* ANKA infection experiments, C57B6/J mice were crossed with homozygous Siglec-H-, Clec9A-, and Clec4a4-DTR mice to generate DTR-heterozygous F1 mice, which will next be referred to as F1 Siglec-H-, F1 Clec9A-, and F1 Clec4a4-DTR mice, respectively. F1 mice from C57B6/J and BALB/c crossings were also obtained to be used as experimental controls, and will be referred to as F1 wt.

To monitor the ablation efficiency in F1 DTR-transgenic mice, flow cytometry was performed on spleens of DT treated F1 Siglec-H, F1 Clec9A-, and F1 Clec4a4-DTR mice, as well as F1 wt mice as control. Results showed that, as in original BALB/c mice, about 90% of all target DC subsets could be depleted in the respective F1 DTR-transgenic mice (Figure 3.7(a) and (b)).

### 3.1.5 Discussion

DCs are crucially important cells of the innate immune system. They are ideally located in strategic sites of the body such as skin, lung, spleen and gut, where they can detect invading pathogens and initiate immune responses. Their ability to bridge innate and adaptive immunity and to induce two complementary immunological functions, such as tolerance and immunity, makes them key coordinators of the immune system, and a possible target for immunotherapy. However, exploiting their functions depends on a precise understanding of the role that DCs play *in vivo*. The expression of a large variety of pattern recognition receptors on DC surface, such as TLRs, which allow them to sense and interact with various conserved microbial molecules, is central in DC functions. Also, in malaria infection DCs have been shown to interact with the parasite and to have a crucial role in activating immune responses. However, currently a clear understanding of the identity of the receptors mediating the interaction with parasite antigens, as well as of the role of different specific DC subsets in malaria immunity, is yet to be achieved. For many years, studies on DCs during malaria infection have treated them as a single population. In the meantime, DCs have been recognized as a heterogeneous compartment of cells with specialized functions, and therefore, should be dissected at the single subset level. To begin to understand the role and contribution of
specific DC subsets to immunity during malaria infection, we aimed at generating transgenic mice where such specific DC subsets could be selectively depleted. Currently, the most efficient method to ablate cells in vivo is the DTR-DT system, a genetic cell ablation approach in which the human DT receptor is expressed in transgenic mice under the control of a tissue-specific promoter. Using the DTR-DT strategy, Jung and colleagues generated in 2002 the first DTR-transgenic mouse for DC depletion: the CD11c-DTR mouse [99]. In this mouse model, the common cDC marker CD11c is used as a promoter to drive the DTR expression, allowing a systemic depletion of cDCs upon DT injection. Several studies using the CD11c-DTR mouse have already been performed to define essential in vivo roles of cDCs in different disease and infection models. For example, in malaria studies the CD11c-DTR mouse was used by deWalick and colleagues to show that cDCs, but not pDCs, are required for the induction of ECM in P. berghei infection [182]. However, a major limitation of this mouse is that CD11c is unspecifically expressed in all cDCs, therefore upon DT injection all cDC subsets are ablated. Moreover, CD11c is also expressed on MZMs in the spleen, and on alveolar macrophages. Thus, DT treatment of CD11c-DTR mice causes ablation of these cell populations also.

To investigate the role of different DC subsets in malaria infection, we chose three DC specific markers, Siglec-H, Clec9A and Clec4a4, exclusively expressed only on particular DC subsets, to drive the DTR expression in our transgenic mice for the specific ablation of pDCs, CD8+ and CD103+ cDCs, and CD8−CD11b+ cDCs, respectively.

Using a BAC recombineering strategy to express the human DTR under the control of the chosen specific promoters, we generated Siglec-H-DTR and Clec9A-DTR mouse lines where the target DC subsets could be efficiently ablated. In the case of Clec4a4-DTR mice, this approach was not successful, maybe due to the lack of some important regulatory elements for Clec4a4 gene expression in the chosen BAC. Therefore, we decided to apply a gene targeting strategy, and obtained two independent lines, which both efficiently ablated the target DC subset upon DT treatment. Since Clec4a4-DTR mice became available for experiments much later than
Siglec-H-DTR and Clec9A-DTR mice, fewer studies could be performed with these mice for this thesis.

Before proceeding with malaria infection experiments, all generated DTR-transgenic mice were tested for their efficiency and specificity in ablating the specific target DC subsets upon DT injection. Siglec-H is a specific marker expressed exclusively on pDCs in BM, spleen and blood. Experiments to characterize the efficiency of pDC depletion in Siglec-H-DTR mice showed that, upon i.p. injection of as little as 4 ng/gbw DT, 80 to 95% of pDCs could be efficiently ablated in vivo in all tissues and organs tested, including blood, spleen, gut and lung. Moreover, the ablation was specific for the Siglec-H+ DC subset, since total CD11c+ cDCs were not affected by DT treatment. The only non DC cells affected by DT treatment were spleen MZMs, which were reduced of about 50%. In fact, a subpopulation of ER-TR9+ MZMs in the spleen has been shown to express Siglec-H [10].

In the CD11c-DTR mouse generated by Jung and colleagues in 2002, repeated DT treatment was shown to cause lethality [99]. However, efficient long term ablation of pDCs could be obtained in our Siglec-H-DTR mice, for at least 32 days, by repeated DT treatment every 7 days. Moreover, we observed that the 4 ng/gbw DT dose was sufficient to obtain an efficient ablation, whereas the use of higher doses, such as 16 ng/gbw, did not significantly improve the ablation efficiency, and could instead lead to the generation of neutralizing antibodies when applied in long term ablation experiments, as previously reported [382].

Clec9A is a C-type lectin expressed exclusively on CD8+ cDCs in spleen, LNs, thymus, on CD103+ cDCs in peripheral tissues and, at lower levels, on pDCs [115]. In characterization experiments of the Clec9A-DTR mice, the dose of 10 ng/gbw DT showed to be the lowest amount of DT necessary to obtain an efficient 80 to 90% ablation of CD8+ cDCs in the spleen, as well as of the correspondent CD103+ cDC subset in peripheral tissues such as lung, gut, skin, heart and muscle (data not shown). Moreover, about 50% of pDCs were depleted, due to their lower Clec9A expression. In contrast, the CD8−CD11b+ and CD103−CD11b+ cDC subsets where not affected by DT injection in Clec9A-DTR mice, thus showing the specificity of ablation.
Because DT administration in Clec9A-DTR mice resulted also in partial ablation of pDCs, Siglec-H-DTR mice can serve as controls when interpreting results obtained with Clec9A-DTR mice.

DTR-transgenic mice were to be subsequently used in malaria infection studies, and in particular, we aimed at studying the role of different DC subsets during the early phase of malaria infection, when innate immunity is most relevant. For this purpose we needed to obtain a constant ablation of the target cell subsets for a long enough period. In Siglec-H-DTR mice we showed that an efficient pDCs depletion could be obtained for up to 32 days by injecting DT every 7 days. CD8$^+$ cDCs are known to have a turnover of about 3 to 4 days in the spleen [47]. In Clec9A-DTR mice, an efficient prolonged ablation of the target cell subset could be obtained by injecting DT every 3-4 days for at least 16 days. This period of time is long enough to study their role in malaria innate immunity, and a similar ablation scheme was subsequently applied to all DTR-transgenic mice in malaria infection experiments.

Clec4a4 is a C-type lectin known to be specifically expressed on CD8$^-$CD11b$^+$ cDCs in spleen, LNs and PP, but not on CD8$^+$ DC subsets. As in Clec9A-DTR mice, in Cle4a4-DTR mice the 10 ng/gbw DT dose proved to be the optimal amount of DT necessary to obtain an efficient ablation of CD8$^-$CD11b$^+$ cDCs in the spleen, without affecting the CD8$^+$ cDC and pDC subsets. However, surprisingly a population of CD11b$^-$F4/80$^+$ macrophages, which are known to be present in the spleen red pulp, were affected by DT treatment, even if no Clec4a4 expression could be observed in this population when analyzed by flow cytometry. Even though we could not reveal the mechanism behind the depletion of this macrophage population, one possible explanation is that CD11b$^-$F4/80$^+$ macrophages might express very low levels of Clec4a4, which are not detectable in flow cytometry. If this is the case, quantitative PCR could help to reveal the presence of Clec4a4 mRNA. Another possibility is that CD8$^-$CD11b$^+$ cDCs might have a role in the homeostasis of CD11b$^-$F4/80$^+$ macrophages, and ablation of CD8$^-$CD11b$^+$ cDCs might result in a perturbation of such homeostasis. Since splenic red pulp F4/80$^+$ macrophages are known to be important for phagocytosis and clearance of iRBCs during malaria infection [145, 146].
Ablation of these cells was taken into account when interpreting results obtained from the following malaria infection experiments with Clec4a4-DTR mice. Moreover, as in Clec9A-DTR mice (data not shown) an increase in neutrophils could be observed upon injection of DT in Clec4a4-DTR mice. Neutrophilia has been observed in other DTR-transgenic mice upon DT treatment [383, 384]. Even though neutrophils do not seem to have a major role in malaria immunity, they are thought to be involved in CM pathology onset [350]. Therefore neutrophilia was carefully considered in CM experiments with Clec4a4-DTR mice.

Since our original DTR mice were generated in a BALB/c background, we back-crossed them with C57B6/J mice to obtain ECM-susceptible F1 Siglec-H-, F1 Clec9A-, and F1 Clec4a4-DTR lines. Before proceeding with P. berghei ANKA infection experiments, we confirmed by flow cytometry that all target DC subsets could be depleted in F1 DTR-transgenic mice as efficiently as in original BALB/c DTR-transgenic mice.

In conclusion, all generated DTR-transgenic lines proved to be efficient in ablating the target DC populations upon DT treatment. Distinct from the already existing CD11c-DTR mice, our DTR-transgenic mice allow ablation of specific DC subsets. Based on the relevance of the DC compartment heterogeneity, recently several transgenic mice deficient for a particular DC subset have been generated, like the Batf3−/− mouse [24] and the human langerin-DTA mouse, which expresses the A subunit of DT under the human langerin promoter [383]. However, constitutive deficiency of a particular cell population may result in compensatory mechanisms which may confound investigation results, whereas conditional ablation allows avoidance of this effect. Transgenic mice for the conditional ablation of pDCs have been generated also by other groups [385, 386]. On the other hand, Clec9A- and Clec4a4-DTR mice represent a worldwide unique set of mice, and together with Siglec-H-DTR mice, were for us invaluable tools to investigate the role of different DC subsets in malaria immunity and pathology.
3.2 Importance of distinct DC subsets in malaria immunity

The *P. yoelii* infection model is widely used to investigate mechanisms of immunity to malaria. Non lethal strains of this parasite cause a self-clearing infection, characterized by a peak of parasitemia rarely exceeding 30% around day 8-12 post infection, and complete clearance of the parasite by day 20-25 post infection. To study the role of distinct DC subsets in malaria immunity, a GFP-expressing non lethal *P. yoelii* strain, defined as *Py*1.1-GFP, was used to infect mice that were conditionally ablated of different DC subsets. The infection outcome was compared with wt controls.

Figure 3.8 shows a schematic representation of the experimental protocol applied. Briefly, DTR-transgenic mice were injected twice with DT before infection with $10^6$ *Py*1.1-GFP iRBCs, administered i.p. Subsequently, DT was injected every 3 to 4 days up to day 13 p.i. to maintain a constant ablation of the target cell subsets. Parasitemia levels were monitored by flow cytometry starting from day 3 p.i. and until the parasite was cleared, usually around day 20 p.i. On day 6 and 14 p.i. blood samples were collected to test IFN-γ and parasite-specific antibodies by ELISA, respectively.

![Schematic representation of a typical *P. yoelii* infection experimental strategy.](image)

**Figure 3.8.** Schematic representation of a typical *P. yoelii* infection experimental strategy.

3.2.1 Characterization of myeloid cell subsets during *P. yoelii* infection

The spleen is known to undergo major transient changes during malaria infection, including splenomegalia, white pulp hyperplasia, red pulp expansion and macrophages redistribution. In *P. chabaudi* infection, apoptosis of CD8– cDCs has been observed in the spleen at the acute phase, when the number of CD8+ cDCs increases instead [172]. Moreover, a population of CD11b<sup>high</sup>Ly6C<sup>+</sup> monocytes arising from the BM in a CCR2
dependent manner has been shown to appear in the spleen and peak on day 14 p.i., and to be actively involved in control of acute parasitemia [160]. In *P. yoelii* infection, a population of CD11c<sup>low</sup>CD45R<sup>high</sup> DCs have been found to accumulate 7 to 10 days p.i., which induces IL-10 expression by CD4<sup>+</sup> T cells, and is believed to have regulatory functions in reducing inflammation during acute infection [184].

![Graphs showing percentage of parasitemia and cell counts over post-infection days](image)
To analyze changes in DC, macrophage, monocyte and neutrophil populations in the spleen during infection in our *P. yoelii* model, spleens from infected mice were collected on days 0 (prior to infection), 5, 9, 14 and 20 p.i., processed and analyzed in flow cytometry. Parasitemia was also measured during the infection time. Figure 3.9 shows parasitemia (Figure 3.9(a)), as well as absolute numbers (Figure 3.9(b)) and relative abundance (Figure 3.9(c)) of these populations of cells, at the different time points. As expected, the spleen cellularity increased, reaching up to 10 times the size of a normal mouse spleen in the steady state by day 14 p.i., and reverting back to about 5 times the size by day 20 p.i., when parasitemia was cleared. Even though the percentage of pDCs appeared to decrease during the infection, the total number of these cells increased substantially from day 9
to day 14 p.i. Also, percentage of total CD11c\textsuperscript{high} cDCs decreased during infection, even though they overall rose in absolute numbers, due to the increased cellularity of the spleen. Distinct to what was observed in the \textit{P. chabaudi} model, the CD8\textsuperscript{+} cDC subset experienced a decrease in both percentage and absolute numbers early during infection, but rose by day 14 p.i., only to decrease again by day 20. On the other hand, CD11b\textsuperscript{+} cDCs experienced a fluctuating trend in relative abundance, with the lowest values observed just before the peak parasitemia. However, overall these cells increased in absolute numbers, reaching the highest values on day 14 p.i., and had already partially decreased by day 20. As described in \textit{P. chabaudi} infection, we observed an increase in Ly6C\textsuperscript{high} inflammatory monocytes, which had a peak in percentage on day 5 p.i., but a maximum in absolute numbers on day 14 p.i., when the spleen reached the biggest size. F4/80\textsuperscript{+} macrophages experienced a drastic decrease in percentage, but a substantial increase in absolute numbers during the first 14 days of infection. Even though the percentage of neutrophils decreased during the first part of infection, both percentage and absolute numbers increased during the second phase, reaching a maximum on day 14 p.i., and decreasing afterwards.

These results showed that, during infection with non lethal \textit{P. yoelii}, even though the percentage of some cell populations decreases in the spleen, especially during the earlier time points, or experience a fluctuating trend, absolute numbers generally increase, and peak on day 14 p.i., due to the substantial rise in total spleen cellularity.

### 3.2.2 DT administration does not affect the course of \textit{P. yoelii} infection

To exclude a possible effect of DT on parasite cycle and growth, or on the course of infection, two groups of wt mice were infected with \textit{Py1.1-GFP}, one of them was injected with DT, whereas the other was administered PBS. For this experiment, a DT dose of 40 ng/gbw was used, which is four times the highest dose usually administered for ablation of the target cell populations. The comparison of parasitemia levels during the infection period showed that DT administration does not affect the parasite behavior (Figure 3.10).
3.2.3 Target DC subsets are efficiently ablated in Siglec-H- and Clec9A-DTR mice during early *P. yoelii* infection

As a next step, we wanted to confirm that target DC subsets could be efficiently ablated in DTR-transgenic mice during a typical *P. yoelii* experiment, and especially during the first phase of infection, when antigen uptake and presentation by DCs is considered to be most relevant. To this purpose, wt and DT treated Siglec-H- and Clec9A-DTR mice were infected with *Py1.1-GFP*, and spleens were collected from euthanized mice on days 5 and 9 p.i., processed and analyzed in flow cytometry (Clec4a4-DTR mice were generated at a later time point than Siglec-H- and Clec9A-DTR mice, and therefore were not included in this experiment).

![Figure 3.11. Siglec-H⁺ pDCs and CD8⁺Clec9A⁺ cDCs are efficiently ablated in the correspondent DTRtransgenic mice during early *P. yoelii* infection. Representative dot plots showing the profile of DC subsets in spleens of wt and DT treated Siglec-H-DTR and Clec9A-DTR mice (n = 3) infected with *P. yoelii*, compared to wt uninfected mice. For a better resolution spleen DCs were enriched before staining.](image)

Figure 3.10. DT administration does not affect the course of *P. yoelii* infection. Parasitemia of *P. yoelii* infected wt mice administered with 40ng/gbw DT or PBS (n = 6). Parasitemia is expressed as mean ± SD.
Results showed that both Siglec-H+ pDCs and CD8+ cDCs were ablated in the correspondent DTR-transgenic mice on both time points (Figure 3.11). Reduction of CD8+ cDCs was less dramatic than pDC ablation. A possible explanation is that mice received the last DT injection 3 to 4 days before the analysis on days 5 and 9 (see Figure 3.8), and CD8+ cDCs have a shorter turnover rate, compared to pDCs. Nevertheless, a relevant portion of CD8+ cDCs was still ablated at these time points.

3.2.4 Impact of ablation of distinct DC subsets in *P. yoelii* infection outcome

To begin to understand the role of the DC subsets targeted by our DTR-transgenic mice in immunity to *P. yoelii*, infection experiments were performed with Siglec-H-, Clec9A- and Clec4a4-DTR mice as described in Section 3.2, Figure 3.8.

3.2.4.1 Siglec-H-DTR mice

Figure 3.12 shows representative results obtained with Siglec-H-DTR mice. Ablation of Siglec-H+ pDCs consistently resulted in an earlier parasite clearance, compared to wt mice. In particular, after peak parasitemia on day

![Graphs showing impact of Siglec-H+ pDCs ablation on *P. yoelii* infection outcome](image)

*Figure 3.12. Impact of Siglec-H+ pDCs ablation on *P. yoelii* infection outcome. (a) Parasitemia (*n* = 13) (b) day 6 p.i. serum IFN-γ (c) day 14 p.i. serum parasite-specific IgG antibodies of wt and DT treated Siglec-H-DTR mice. Results are representative of 4 independent experiments. Parasitemia, IFN-γ and IgG antibodies are expressed as mean ± SD. Mann-Whitney test, *p*,< 0.05.*
11 p.i., Siglec-H depleted mice experienced a more than 50% decrease in blood parasite level in just one day, and cleared the infection completely by day 14, whereas wt mice managed to do so, as expected, around day 20 p.i. (Figure 3.12(a)).

Analysis of sera obtained on day 6 p.i. showed no difference in IFN-γ levels in Siglec-H ablated mice, compared to wt mice (Figure 3.12(b)). Such result is consistent with the fact that pDCs are not major IL-12 or IFN-γ producers. When serum levels of parasite-specific IgG antibodies were tested on day 14 p.i., no substantial difference was observed in Siglec-H ablated mice compared to wt mice, even though Siglec-H-DTR mice had slightly higher levels of the IgG1 subtype (Figure 3.12(b)). These results suggested that the more rapid parasite clearance observed in Siglec-H ablated mice, compared to wt mice, does not depend on altered IFN-γ production, or on major differences in parasite-specific protective antibody levels in the serum.

### 3.2.4.2 Clec9A-DTR mice

Figure 3.13 shows representative results obtained with Clec9A-DTR mice. As in Siglec-H depleted mice, ablation of Clec9A+ cDCs consistently resulted in a more rapid parasite clearance. In particular, Clec9A ablated

![Figure 3.13](image.png)

**Figure 3.13. Impact of Clec9A+ cDCs ablation on *P. yoelii* infection outcome.** (a) Parasitemia (*n = 10*) (b) day 6 p.i. serum IFN-γ (c) day 14 p.i. serum parasite-specific IgG antibodies of wt and DT treated Clec9A-DTR mice. Results are representative of 3 independent experiments. Parasitemia, IFN-γ and IgG antibodies are expressed as mean ± SD. Mann-Whitney test, *p < 0.05, **p < 0.01, ***p < 0.001.
mice began to clear the infection as early as 9 day p.i., thus avoiding the peak of parasitemia experienced by wt mice between days 10 and 12 p.i. Moreover, when Clec9A-DTR mice had completed parasite clearance on day 16 p.i., wt mice had only cleared about 50% of their peak parasitemia levels (Figure 3.13(a)). Analysis of sera obtained on day 6 p.i. showed significant lower IFN-γ levels in Clec9A ablated mice, compared to wt mice (Figure 3.13(b)). Such a result is consistent with the ability of CD8+ cDCs to produce high levels of the Th1 cytokine IL-12, which is known to mediate IFN-γ production.

Th1-dependent IgG2a antibodies, produced in response to increased IFN-γ levels, are considered to be the predominant Ig responsible for parasite clearance in both *P. yoelii* and *P. chabaudi* infection models [131, 232]. On the other hand, even though IgG1 antibody titres were shown to be only slightly lower than IgG2a titres in *P. yoelii* infected mice, passive transfer of this antibody subtype showed no effect on parasitemia [232]. When serum levels of parasite-specific IgG antibodies were tested on day 14 p.i., substantial differences were observed between wt and Clec9A ablated mice. In particular, total IgG parasite-specific antibodies were slightly increased in Clec9A ablated mice, compared to wt mice, and antibodies of the Th1-dependent IgG2a subtype were slightly decreased, consistently with the low IFN-γ levels experienced by these mice, compared to wt mice. On the contrary, and different from Siglec-H depleted mice, antibodies of the Th2-dependent IgG1 subtype were highly increased in DT treated Clec9A-DTR mice, suggesting a skewing towards a Th2 immune response (Figure 3.13(c)). Such result is consistent with what was observed in *P. chabaudi* experiments with IFN-γ and IL-12p40 deficient mice, which showed lower IgG2a and higher IgG1 antibody levels. However, in such experiments IFN-γ and IL-12p40 deficient mice experienced higher parasitemia, higher mortality and difficulty in resolving primary infection [131, 207], whereas in our experiments *P. yoelii* infected Clec9A ablated mice showed improved parasite clearance.

These results suggested that ablation of Clec9A+ cDCs results in a skewing to Th2, instead of Th1, immune responses in *P. yoelii* infected mice, which might be responsible for the observed improved infection clearance.
3.2.4.3 Clec4a4-DTR mice

Figure 3.14 shows representative results obtained with Clec4a4-DTR mice, which allow efficient ablation of CD8⁻CD11⁺ cDCs. DT treatment on *P. yoelii* infected Clec4a4-DTR mice caused a peak of parasitemia very early during infection, between day 6 and 8 p.i., when wt mice were still experiencing low parasite levels. After day 8, Clec4a4 ablated mice managed to contain their parasitemia to levels even lower than wt mice, which, on the contrary, had the expected peak of parasitemia between day 10 and 13 p.i. However, when wt mice started to clear the infection on day 14 p.i., parasitemia levels rose again in Clec4a4 ablated mice, and despite the *P. yoelii* used being a non lethal strain, by day 16 they all succumbed to the infection. In contrast, all wt mice survived and cleared the parasite by day 20 p.i. (Figure 3.14(a) and (b)).

![Figure 3.14](image_url)

*Figure 3.14. Impact of Clec4a4⁺ cDCs ablation on *P. yoelii* infection outcome. (a) Parasitemia (*n* = 7) (b) survival (*n* = 7) (c) day 6 p.i. serum IFN-γ (d) day 14 p.i. serum parasite-specific IgG antibodies of wt and DT treated Clec4a4-DTR mice. Results are representative of 3 independent experiments. Parasitemia, IFN-γ and IgG antibodies are expressed as mean ± SD. Mantel-Cox logrank test, ***p < 0.001; Mann-Whitney test, ***p < 0.001.*

Analysis of sera obtained on day 6 p.i. showed no significant differences in IFN-γ levels in Clec4a4 ablated mice, compared to wt mice (Figure 3.13(c)). Such result is not surprising since CD8⁻ cDCs are not major producers of IL-12.

When serum levels of parasite-specific IgG antibodies were tested on day 14 p.i., no substantial differences were observed in total IgG and IgG2a
subtype levels between wt and Clec4a4 ablated mice. However, ablation of Clec4a4⁺ cells caused a significant decrease in parasite-specific antibodies of the IgG1 subtype (Figure 3.14(d)). Because IgG1 antibodies are known to be Th2-dependent, such result is consistent with the ability of CD8⁻ cDCs to eliciting Th2 immune responses.

Red pulp F4/80⁺ macrophages are known to be important for containing parasite levels. As these macrophages are ablated in DT treated Clec4a4⁻ DTR mice, the very early peak of parasitemia observed in Clec4a4 ablated mice might be due to the absence of these cells. However, other mechanisms appear to come into place and prevent a further expansion of the parasite, since a rapid decrease in parasitemia was observed afterwards. Nonetheless, parasitemia levels rose again, and Clec4a4 ablated mice eventually succumbed to the infection. It is worth of notice that, even if parasitemia levels were on the increase, they were still relatively low at the moment of death.

**3.2.5 Absence of different DC subsets does not affect Tregs numbers during early P. yoelii infection**

Some evidence supports the idea that Tregs might be important in affecting the outcome of malaria infection. In particular, excessive Treg activity might suppress host responses before the development of an effective protective immunity. On the other hand, delayed or insufficient Treg suppressive activity might be one of the possible explanations for the excessive inflammatory responses and pathology observed in certain malaria patients. In a non-lethal P. yoelii infection, Abel and colleagues showed that the absolute number of Tregs increased in the spleen, and peaked during days 5 to 7 p.i., and that their ablation led to an increase in T cell activation and a significant decrease in parasitemia [266].

To investigate whether ablation of the target cell subsets in our P. yoelii infected DTR-transgenic mice caused changes in the Treg population, absolute numbers of spleen Tregs from wt mice and DT treated Siglec-H⁻, Clec9A⁻ and Clec4a4-DTR mice were analyzed by flow cytometry on day 5 p.i. As expected, spleen cellularity increased significantly in all infected mice, compared to the average 80 million cells normally counted on a wt
mouse spleen in steady state (Figure 3.15(a)). However, no differences in T\textsubscript{reg} percentage (Figure 3.15(b), graph on the left) or absolute numbers (Figure 3.15(b), graph on the right) were observed between wt mice and DT treated DTR-transgenic mice. Therefore, we concluded that ablation of the target cell subsets in \textit{P. yoelii} infected Siglec-H\textsuperscript{-}, Clec9A\textsuperscript{-} and Clec4a4-DTR mice does not affect the spleen T\textsubscript{reg} population.

![Figure 3.15](image)

\textbf{Figure 3.15.} DT treatment of Siglec-H\textsuperscript{-}, Clec9A\textsuperscript{-} and Clec4a4-DTR mice does not affect T\textsubscript{reg} percentage and absolute number in the spleen. (a) Absolute number of splenocytes. (b) Percentage (left) and absolute number (right) of spleen T\textsubscript{reg} \((n = 4)\). Absolute numbers and percentage are expressed as mean ± SD. Mann-Whitney test.

\textbf{3.2.6 The improved infection clearance observed in Clec9A ablated mice can be transferred by serum passive immunization}

In DT treated Clec9A-DTR mice, the improved parasite control and infection clearance was accompanied by a slight decrease in Th1-dependent parasite-specific IgG2a antibodies, and a conspicuous increase in Th2-dependent parasite-specific IgG1 antibodies (Figure 3.14(c)). To investigate whether this difference in antibody production was involved in the improved infection clearance observed, we first tested if such an enhanced parasite control could be transferred by serum passive immunization. In particular, a mix of sera collected on day 14 p.i. from wt or DT treated Clec9A-DTR mice was injected i.p. to \textit{P. yoelii} infected wt mice twice, just before and during the peak parasitemia on days 8 and 10 p.i. (Figure 3.16).
Similar to what we observed in *P. yoelii* infected Clec9A ablated mice, mice receiving Clec9A serum showed improved parasite control and clearance, compared to mice that did not receive the serum, and such effect was even more pronounced than in mice receiving wt serum. These results suggested that a factor present in the transferred serum was responsible for the phenotype observed in Clec9A ablated mice.

![Figure 3.16. Transfer of serum from Clec9A ablated mice results in improved parasite clearance.](image)

Parasitemia of *P. yoelii* infected wt mice receiving or non receiving a mix of sera collected on day 14 p.i. from infected wt or Clec9A ablated mice (*n* = 7). Red arrows indicate i.p. injection of 500μL of the sera mix. Parasitemia is expressed as mean + SD.

3.2.7 IgG1 antibodies from infected Clec9A ablated mice improve non lethal *P. yoelii* clearance, but do not protect from lethal *P. yoelii* infection

To test if the increase in IgG1 antibodies observed in *P. yoelii* infected DT treated Clec9A-DTR mice was the factor which helped to control the parasitemia, we generated *P. yoelii*-specific monoclonal antibodies from mice lacking Clec9A+ cDCs. Splenocytes collected from DT treated Clec9A-DTR mice 14 days post infection were used to generate a series of monoclonal antibodies using the standard hybridoma method [381]. Table 3.2 shows the number of IgG1 and IgG2a clones obtained. Consistent with the increase in IgG1 antibodies observed in the ELISA screening of sera from *P. yoelii* infected DT treated Clec9A-DTR mice on day 14 p.i. (Figure 3.13(b)), more IgG1 than IgG2a clones were found to be *P. yoelii*-specific during the hybridomas screening.
Table 3.2. Generated *P. yoelii*-specific monoclonal antibodies. Only clones scoring the highest values in the *P. yoelii*-recognition screening were further subcloned.

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<th>Clones</th>
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<td>IgG2a</td>
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To obtain monoclonal antibodies, only hybridoma clones with the best screening scores were subcloned, and among these, the 8 IgG1 subclones with the highest values were selected for further expansion and antibody purification.

To test the 8 purified *P. yoelii*-specific IgG1 monoclonal antibodies for their protective property, a mix containing 100 μg of each antibody was injected i.p. to *P. yoelii* infected wt mice on days 8, 10 and 12 p.i. Similarly to what observed in *P. yoelii* infected Clec9A ablated mice, as well as in the serum passive immunization experiment, mice receiving the IgG1 antibodies showed improved parasite control and clearance compared to mice that did not receive them (Figure 3.17(a)).

![Figure 3.17](image)

**Figure 3.17.** IgG1 antibodies from infected Cle9A ablated mice improve non lethal *P. yoelii* clearance, but do not protect from lethal *P. yoelii* infection. (a) Parasitemia of non lethal *P. yoelii* infected wt mice receiving or not receiving a mix containing 100 μg of each of the 8 parasite-specific IgG1 monoclonal antibodies generated from infected mice lacking Clec9A⁺ cDCs (n = 9). (b) Parasitemia and (c) survival of lethal *P. yoelii* infected wt mice receiving or non receiving a mix containing 200 μg of each of the 8 parasite-specific IgG1 monoclonal antibodies generated from infected mice lacking Clec9A⁺ cDCs (n = 5). Red arrows indicate i.p. injection of the mix containing the 8 purified IgG1 antibodies. Parasitemia is expressed as mean ± SD. Mantel-Cox logrank test.

To test if *P. yoelii*-specific monoclonal antibodies could also protect wt mice from infection with a lethal *P. yoelii* strain, a mix containing 200 μg of each of the 8 purified antibodies was injected i.p. to wt mice one day before and one day after infection with 10⁶ YM-GFP iRBCs. Lethal strains of *P. yoelii* cause a lethal infection that leads to death 8 to 10 days post infection, due
to high parasitemia. By injecting the antibodies just before and after infection, we hoped they could block the parasite amplification, and therefore prevent, or at least delay, the infection. However, injection of the IgG1 *P. yoelii*-specific monoclonal antibodies could not protect from infection with the lethal YM-GFP strain. In fact, all wt mice receiving the antibodies died with high parasitemia levels, even though 1-2 days later compared to wt mice not receiving the antibodies (Figure 3.17(b) and (c)). Therefore we concluded that the *P. yoelii*-specific monoclonal IgG1 antibodies generated from infected mice lacking Clec9A+ cDCs can improve non lethal *P. yoelii* clearance, but are not specific enough to protect from lethal *P. yoelii* infection.

**3.2.8 Discussion**

Despite years of research, a vaccine for malaria does not exist yet, and malaria still remains a devastating infectious disease and a major economic burden for many undeveloped countries. One of the main reasons for the difficulty in developing a valid vaccine is that an ideal one should effectively induce protective immunity. However, malaria immune responses are complex and still poorly understood. Therefore, to obtain the much needed knowledge which would allow a better basis for vaccine development, a deep understanding of immune mechanisms acting during malaria infection has to be achieved.

Innate immune responses are known to be necessary to limit the parasite replication and to allow generation of specific adaptive responses, which are required for parasite clearance. DCs have a central role during infection in bridging innate and adaptive immunity, due to their presence at sites of pathogen entry, the expression of a large variety of pattern recognition receptors, and their unique ability to sample, uptake, process and present antigens. Indeed, DCs have been shown to interact with malaria parasites and to have a crucial role in activating immune responses during malaria infection. However, DCs are a heterogeneous population of cells with specialized functions, and the specific role of each particular subset in malaria immunity is not yet completely understood. Moreover, it is still to be elucidated whether different subsets use different receptors to interact with
the parasite and parasite antigens, and the identity of such receptors. Obviously, identification of these molecules would provide a target for vaccine antigens delivery and other immunomodulatory strategies. Rodent models of malaria represent valuable tools to investigate immunity and pathology in malaria. In particular, the *P. yoelii* infection model is widely used to investigate mechanisms of immunity to malaria. By using the *P. yoelii* infection model in our DTR-transgenic mice, we wanted to investigate the consequences of ablation of the target DC subsets, in order to get more insights on their role in malaria immunity.

Before proceeding with *P. yoelii* infection experiments in our DTR-transgenic mice, some preliminary studies were performed. In particular, because the spleen is known to undergo major but transient changes during malaria infection, we analyzed variations in both relative abundance and absolute numbers of DC, macrophage, monocyte and neutrophil populations in the spleen during infection in our *P. yoelii* model. Consistent with what was reported previously in both humans and mice [148, 154], we observed splenomegalia and white pulp hyperplasia. In general, the analysis showed that, even though some populations of cells decreased in percentage in the spleen during infection, especially at the earlier time points, or experienced a fluctuating pattern, absolute numbers generally increased and peaked on day 14 p.i., due to the substantial rise in total spleen cellularity at this stage. Interestingly, whereas in *P. chabaudi* infection apoptosis of CD8+ cDCs has been observed in the spleen at the acute phase, when the number of CD8+ cDCs increases [172], in our *P. yoelii* infection model the CD8+ cDC subset experienced a decrease in absolute numbers early during infection, but rose by day 14 p.i.. On the other hand, the CD8−CD11b+ cDC subset experienced an overall increase in absolute numbers, which reached the highest values on day 14 p.i., and had already partially decreased by day 20. Such different results remind once again that different parasite species can interact with the host in diverse ways, and highlight the need to consider such variations when comparing results. On the other hand, as described in *P. chabaudi* infection [160], we observed an increase in Ly6C^{high} inflammatory monocytes in the spleen, which peaked on day 14 p.i., when the spleen reached the biggest size. However, in our model the relative
abundance of these cells reached the maximum on day 5 p.i. Ly6C<sup>high</sup> monocytes have been shown to be actively involved in control of acute parasitemia. Because the percentage of these cells reaches the maximum peak early in *P. yoelii* infection, their role in parasite clearance might be important earlier in this model, compared to *P. chabaudi* infection.

### 3.2.8.1 Siglec-H-DTR mice

Different from splenic cDCs, pDCs are known to have poor antigen presentation properties, and to be incapable of priming T cell responses during blood stage malaria. For example, in *P. chabaudi* infection, even though pDC numbers have been shown to increase in the spleen [168, 184], they could not stimulate parasite-specific CD4<sup>+</sup> T cells, due to their poor phagocytic properties and lack of appropriate co-stimulation [186]. Instead, cells expressing CD11c<sup>low</sup>CD45R<sup>high</sup>, which are usually considered pDC markers, have been found to accumulate 7 to 10 days after a *P. yoelii* infection, and are believed to have regulatory properties as they induced IL-10 expression by CD4<sup>+</sup> T cells [184]. Moreover, recent studies suggested a role for pDCs as reservoirs of infectious parasites. In fact, blood stage *P. berghei*, *P. yoelii* and *P. chabaudi* were shown to have a tropism for pDCs in the spleen, and transfer of pDCs from *P. chabaudi* infected mice after apparent clearance of blood infection, to naïve mice, initiated more vigorous infections than transfer of RBCs [189].

When pDCs were ablated in *P. yoelii* infected Siglec-H-DTR, we consistently observed a more efficient parasite clearance, compared to wt mice. Because infected ablated Siglec-H-DTR mice had no major differences in IFN-γ levels or in parasite-specific protective antibody levels in the serum, compared to wt mice, we concluded that the phenotype observed was not dependent on these factors. pDCs have been suggested to have immunomodulatory functions in different scenarios. Several lines of evidence have suggested that anti-inflammatory responses can dampen the strength of malaria protective immunity [264-267]. During our preliminary infection experiment in wt mice we observed that, even though the percentage of pDCs appeared to decrease, their total number increased from day 9 to day 14 p.i.. Moreover, Wong and colleagues previously
described a population of CD11c^{low}CD45R^{high} DCs with regulatory properties, which are usually considered to be pDCs, to accumulate 7 to 10 days after a *P. yoelii* infection in the spleen and induce IL-10 expression by CD4^{+} T cells [184]. Therefore, we reasoned that pDCs could be such a regulatory DC population, and that their ablation would remove the suppression on malaria protective immune responses, thus resulting in improved infection clearance. However, in their work Wong and colleagues showed that the regulatory CD11c^{low}CD45R^{high} DCs isolated from the spleen on day 10 p.i. did not express the pDC marker PDCA-1. Nevertheless, IL-10 intracellular staining of CD4^{+} T cells isolated after day 10 p.i. from the spleen of *P. yoelii* infected wt and pDC ablated Siglec-H-DTR mice could help to definitively exclude such a hypothesis. Moreover, because blood stage *P. berghei*, *P. yoelii* and *P. chabaudi* were shown to have a tropism for pDCs in the spleen and in *P. chabaudi* infection, pDCs have been suggested to act as reservoirs of infectious parasites [189]. Spleen pDCs from wt mice could be tested by flow cytometry at different time points during infection to verify if they harbour *Py1.1*-GFP. Clearly, in this scenario pDC ablation would result in a depletion of such a parasite reservoir, and could help to explain the improved parasite clearance.

### 3.2.8.2 Clec9A-DTR mice

Splenic CD11c^{high} cDCs have been shown to efficiently uptake iRBCs *in vivo*, upon which they mature and produce IL-12 [171]. Despite contrasting results obtained by different groups, and in different infection models, on the activation status of cDCs and their ability to efficiently uptake, process and present parasite antigens at different time points during infection, what has become evident is that the functional capacity of splenic cDC subsets changes during infection, and that the antigen dose plays a part in such a modulation. During early malaria infection, low parasite levels activate DCs to secrete IL-12, which stimulates NK and naïve CD4^{+} T cells to produce IFN-γ, necessary to activate malaria protective responses. As the infection progresses and parasitemia increases, DCs produce less IL-12, and instead, begin to produce IL-10 [174]. During the later phase of infection, the
induced, widespread systemic DC activation renders them refractory to TLR stimulation, thus impairing their ability to phagocytose antigens and to prime T cells [174]. In *P. chabaudi* infection, both CD8*+* and CD8*−* cDCs were shown to present parasite antigens, but only CD8*−* cDCs isolated during acute infection could activate antigen-specific CD4*+* T cell responses [172]. Moreover, in this infection model apoptosis of CD8*+* cDCs has been observed after acute infection in the spleen, when the number of CD8*−* cDCs increases instead [172]. Such an event has been correlated to an increase in IL-4 and IL-10 production by proliferating CD4*+* T cells, and to a switch from Th1 to Th2 immune responses [175]. In *P. yoelii* infection, the contribution of different cDC subsets to malaria immunity has not yet been investigated.

As in Siglec-H ablated mice, in Clec9A-DTR mice ablation of CD8*+*Clec9A*+* cDCs consistently resulted in an anticipated and improved parasite clearance upon *P. yoelii* infection, and Clec9A ablated mice began to clear the infection as early as 9 days p.i., when wt mice were just starting the acute parasitemia phase. However, different from Siglec-H ablated mice, and consistent with the particular ability of CD8*+*Clec9A*+* cDCs to produce high levels of IL-12, infected Clec9A ablated mice showed significant lower serum levels of the Th1 cytokine IFN-γ on day 6 p.i., compared to wt mice.

Since early production of IL-12 and IFN-γ is considered to be important to activate protective immune responses in malaria infection [166, 172], and Th1-dependent IgG2a antibodies produced in response to increased IFN-γ levels are believed to be the predominant Ig responsible for parasite clearance in both *P. yoelii* and *P. chabaudi* infection models [131, 232], we were surprised to observe an improved infection control in Clec9A ablated mice. In fact, IL-12p40 deficient mice infected with *P. chabaudi* have been shown to develop higher parasitemia levels compared to wt mice, and to fail in rapidly resolving primary infection, as well as in controlling challenge infection. Moreover, IL-12p40 deficient mice produced lower levels of Th1-dependent IgG2a and IgG3 antibodies, but higher levels of Th2-dependent IgG1, suggesting a protective role of Th1 immune response during blood stage malaria [131]. Similarly, it was shown that IFN-γ deficient mice infected with *P. chabaudi* develop higher parasitemia, suffer severe
mortality and produce less IgG2a and IgG3 and more IgG1 [207]. However, even though a burst in IFN-γ has been observed as early as 1 day p.i. in *P. yoelii* infection, parasite load and course of infection did not differ in IFN-γ deficient mice compared to wt mice [155], thus suggesting that IFN-γ might not be required for effective control of *P. yoelii* infection. Consistent with the dramatic reduction in serum IFN-γ levels in DT treated Clec9A-DTR mice, Th1-dependent IgG2a antibodies were slightly decreased in these mice, whereas Th2-dependent IgG1 antibodies were significantly increased, suggesting a skewing towards a Th2 immune response. Even though IgG1 antibodies are believed to play only a minor part in malaria immunity, we hypothesized that, in Clec9A ablated mice, the increase in IgG1 antibodies, and in particular, the generation of IgG1 antibodies specific for critical parasite antigens, could be responsible for the improved parasite clearance observed. If this would be the case, passive immunization of wt mice with serum obtained from *P. yoelii* infected Clec9A ablated mice, during the parasite clearance, should result in the same protection observed in Clec9A ablated mice. Indeed, when serum from DT treated Clec9A-DTR mice collected on day 14 p.i. was injected in infected wt mice on days 8 and 10 p.i., mice receiving the serum cleared the infection much more efficiently than mice that did not receive it, indicating that the factor responsible for the improved protection was present in the serum.

To test if the increase in IgG1 antibodies levels was the factor responsible for the improved protection observed in *P. yoelii* infected DT treated Clec9A-DTR mice, and to eventually characterize the repertoire of such antibodies, we generated *P. yoelii*-specific monoclonal antibodies from mice lacking Clec9A+ DCs. Consistent with the increase in IgG1 antibodies observed in the ELISA screening of sera from *P. yoelii* infected DT treated Clec9A-DTR mice on day 14 p.i, more IgG1 than IgG2a clones were found to be *P. yoelii*-specific during the screening of the hybridomas. Among the 41 IgG1 producing hybridoma clones obtained, only clones with the higher ELISA scores were subcloned, and, among these, the 8 IgG1 subclones with the highest values were selected for further expansion and antibody purification. When a mix containing 100 μg of each of the 8 antibodies was injected i.p. to *P. yoelii* infected wt mice on days 8, 10 and 12 p.i., mice experienced an
improved parasite control compared to mice that did not receive the antibodies, similar to what observed in Clec9A ablated mice. One particular merozoite antigen found in all malaria species, MSP-1, has been shown to elicit protective responses against blood stage in both human and mice malaria infection, and monoclonal antibodies directed towards this antigen have been shown to inhibit merozoite invasion [236-238]. Because lethal and non lethal P. yoelii strains are genetically close, we hypothesized that, if any of the 8 purified antibodies had particular protective functions, they would be able to protect also from infection with a lethal P. yoelii strain. In particular, by injecting a mix containing 200 μg of each of the 8 purified antibodies just before and after infection with $10^6$ YM-GFP iRBCs, we expected the antibodies to block the parasite expansion and prevent, or at least delay, the infection. However, all injected mice succumbed to the infection with high parasitemia levels, even though lethality was delayed of about one day compared to mice that did not receive the antibodies. Possible reasons for the failure of the antibodies to confer protection is that the antigens they recognize might not be shared by the lethal and non lethal strains, or they might be polymorphic. Characterization of the antigens recognized by the 8 antibodies would help to clarify this aspect.

On a subsequent experiment with the non lethal P. yoelii model, we divided the 8 antibodies in three groups, in the attempt to identify if one or a few of them were responsible for the improved protection observed (data not shown). However, in this case no difference was observed between mice receiving one of the three antibody mix and mice receiving no antibody. Considering these results, we reasoned that the improved infection control observed in Clec9A ablated mice could be due to factors other than the increase in parasite-specific IgG1 antibodies. The more efficient parasite clearance observed in mice infected with non lethal P. yoelii in serum and antibody passive immunization experiments might be simply due to the fact that mice receiving the passive immunization have an advantage compared to mice that do not receive it, rather than to the presence of specific protective IgG1 antibodies in the serum or in the antibodies mix administered. Indeed, also mice receiving serum from day 14 infected wt mice and Siglec-H-DTR mice (data not shown) showed an improved
infection outcome, even if less pronounced than mice receiving serum from Clec9A ablated mice. Moreover, beside parasite-specific antibodies, the serum contains other important factors, such as cytokines, which might be responsible for the observed phenotype. Nevertheless, to completely exclude a role for parasite-specific IgG1 antibodies in Clec9A ablated mice, other clones of the generated antibodies should be tested. These considerations bring forward the possibility that the improved infection clearance observed in both Siglec-H and Clec9A ablated mice might be the result of the same mechanism, and the fact that in Clec9A-DTR mice pDCs are also partially ablated supports this possibility.

A few lines of evidence support the idea that T\(_{\text{regs}}\) might be important in affecting the outcome of malaria infection. In particular, excessive T\(_{\text{reg}}\) activity might suppress host responses before an effective protective immunity has time to develop. In non lethal \(P.\) \(yoelii\) infection it was shown that the absolute number of T\(_{\text{regs}}\) increases in the spleen and peaks during days 5 to 7 p.i., and that their ablation leads to an increase in T cell activation and a significant decrease in parasitemia [266]. However, no difference in T\(_{\text{reg}}\) proportion or absolute numbers was observed between wt mice and DT treated Siglec-H- and Clec9A-DTR mice, thus excluding a role for these cells, at least during early infection. A profiling of cytokines production in the serum of both Siglec-H and Clec9A ablated during the whole infection period might provide more insights into the mechanism underlying the observed improved infection clearance.

### 3.2.8.3 Clec4a4-DTR mice

In \(P.\) \textit{chabaudi} infection, CD8\(^{-}\) cDCs have been shown to be important after peak parasitemia to promote the switch from Th1 to Th2 immune responses, necessary for an efficient antibody-mediated protection [175]. However, in \(P.\) \textit{yoelii} infection the contribution of this particular cDC subset to malaria immunity has not yet been elucidated.

When Clec4a4 ablated mice were infected with \(P.\) \textit{yoelii}, they experienced a first peak of parasitemia very early during infection, while wt mice had still low parasite levels in the blood, and a second peak later, after day 14 p.i., when wt mice were clearing the infection. Surprisingly, even though the
parasite used was a non lethal strain, all ablated mice died within day 16 p.i., with relatively low parasitemia levels. Analysis of serum IFN-γ levels on day 6 p.i., parasite-specific IgG antibodies on day 14 p.i., as well as T_{regs} on day 5 p.i., revealed that none of these factors were involved in the observed phenotype. Further experiments to test if Clec4a4 ablated mice develop a more severe anemia or a faster high reticulocytemia might help to explain the observed lethality.

Beside causing ablation of CD8^-CD11b^+ cDCs, DT treatment in Clec4a4-DTR also results in depletion of red pulp F4/80^+ macrophages. This particular subset of macrophages is known to be important, especially during early infection, for phagocytosing iRBCs and containing parasite levels. Therefore, we reasoned that the very early peak of parasitemia observed in Clec4a4 ablated mice might be due to the absence of these important phagocytes. Spleen histological sections prepared at this time point might help to understand if a greater amount of iRBCs is actually present in the spleen of Clec4a4 ablated mice, compared to wt mice. After the first peak, parasitemia levels rapidly decreased in Clec4a4 ablated mice, suggesting that other mechanisms might come into place and prevent a further expansion of the parasite. Other phagocytic cells, such as Siglec-1^+ MMMs, might eventually compensate the absence of the F4/80^+ macrophages. However, such a ‘rescue’ might occur too late in time, as all Clec4a4 ablated mice succumbed to the infection. Because mice died with relatively low parasitemia values, high parasitemia cannot be considered the cause of death, even though anemia cannot be excluded, since it can occur even at low parasitemia levels. iRBCs have been shown to adhere to endothelial cells of capillaries and post-capillary venules, not only in the brain, but also in other tissues such as liver, lung, kidney, adipose tissue and placenta, in both human and mice (reviewed in [312]). Even in P. yoelii infection, cytoadherence has been reported by Martin-Jauilar and collaborators [153]. In the absence of the F4/80^+ macrophages, after the first unusual peak of parasitemia, iRBCs might be able to sequester in different tissues, causing irreversible damage to vital organs. Indeed, we observed hematuria in Clec4a4 ablated mice during the few days preceding death, which suggests a severe damage of the kidney. Such hypothesis
would explain why these mice die with relatively low levels of parasitemia. Histological analysis of organs such as kidney, lung and liver, at this time point, might help to investigate this possibility.

Innate immune responses are known to be important to contain parasite load during early malaria infection, in order to give time to adequate adaptive immune responses to develop, and the amount of antigen is known to influence the nature of immune responses [173]. In Clec4a4 ablated mice, the absence of F4/80+ red pulp macrophages, resulting in high parasite load early in infection, might induce widespread, systemic activation of DCs, rendering them refractory and impairing their ability to prime T cells. Functional studies on the ability of DCs from Clec4a4 ablated mice to activate T cells, at this early stage of infection, might help to elucidate this possible scenario.

Taken together these results suggest that Siglec-H+ plasmacytoid DCs and Clec9A+ cDCs contribute to immune responses during infection with non-lethal *P. yoelii*, as ablation of both subsets results in an improved control and clearance of the parasite. Because, different from wt and Siglec-H ablated mice, DT treated Clec9A-DTR mice had significantly reduced serum levels of the Th1 cytokine IFN-γ, and generated a clear isotype profile of parasite-specific antibodies, with higher IgG1 than IgG2a levels, further investigations are required to elucidate the mechanism involved in the protection observed in these DTR-transgenic mice. Moreover, even though our results do not exclude a role for Clec4a4+ cDCs in malaria immune responses, the higher susceptibility observed in DT treated Clec4a4-DTR mice during non-lethal *P. yoelii* infection is most probably best explained by the concomitant ablation of F4/80+ red pulp macrophages in these mice, and further studies are needed to confirm this possibility.
3.3 Importance of distinct DC subsets in malaria pathology

*P. berghei*, the only rodent parasite able to induce ECM, has been extensively used to study the pathogenesis of CM. Infection of susceptible C57BL/6 mice with *P. berghei* results in development of a neurological syndrome, typically between 6 to 12 days p.i., which in about 60 to 100% of cases leads to death with relatively low parasitemia levels. Mice that manage to survive the syndrome, die later during infection, due to high parasitemia and severe anemia. To assess the involvement of distinct DC subsets in immunity-mediated pathology, a GFP-expressing *P. berghei* ANKA strain, defined as *PbA*-GFP, was used to infect the different DTR-transgenic mice.

Figure 3.18 shows a schematic representation of our typical monitoring of a *P. berghei* infection experiment. Briefly, DTR-transgenic mice were injected twice with DT before infection with $10^6$ *PbA*-GFP iRBCs administered i.p. DT was subsequently injected every 3 to 4 days up to day 13 p.i. to maintain a constant ablation of the target cell subsets. Parasitemia levels were monitored by flow cytometry from day 3 p.i., until all the mice succumbed to the infection, and compared with age-matched wt infected mice. On day 6 p.i., blood samples were collected to test IFN-γ levels in the serum. ECM development was monitored every day from day 5 to day 12 p.i. using the previously described rapid murine coma behaviour scale (RMCBS) score [378].

![Schematic representation of a typical *P. berghei* infection experimental strategy.](image)

Such scoring system consists of 10 parameters to be evaluated, each of which can be given a maximum score of 2, in the case of a healthy mouse, or a minimum score of 0, in the case of a sick mouse which fails to show
any response. Therefore, the total final score ranges from a maximum of 20 to a minimum of 0. Scores lower than 12 are considered as a clear sign of ECM development.

3.3.1 Both CD8$^+$ and CD8$^-$ splenic cDC subsets phagocytose *P. berghei* iRBCs

Malaria complications, such as CM, are known to be caused not only by the presence and activity of the parasite, but also by the host immune responses. In particular, CD8$^+$ cytotoxic T cells have been shown to be directly involved in ECM pathogenesis. cDCs are known to efficiently phagocytose pathogens and pathogen antigens, and present them to activate adaptive immune responses. In *P. chabaudi* infection both splenic CD8$^+$ and CD8$^-$ cDCs have been shown to present parasite antigens [172]. To investigate whether these cDC subsets were able to efficiently phagocytose iRBCs in our *P. berghei* infection model, which is a necessary requisite for antigen presentation, live stream imaging was performed on enriched spleen DCs 2 hours after co-culture with *PbA*-GFP iRBC at a 1:3 ratio. This technique combines quantitative image analysis and flow cytometry. Thus, cells stained with different antibodies and flowing in a stream, as in flow cytometry, can be live imaged, as in a fluorescent microscope. Because it is possible to collect multiple fluorescent images per cell, molecules phagocytosed by distinct subpopulations can be simultaneously determined. Results showed that both splenic CD8$^+$ and CD8$^-$ cDCs can phagocytose *P. berghei* iRBCs (Figure 3.19(a) and (b)).

![Figure 3.19](image.png)

**Figure 3.19.** Both splenic CD8$^+$ and CD8$^-$ cDC subsets can phagocytose *P. berghei* iRBCs. Representative live stream imaging of splenic enriched CD11c$^+$CD8$^+$ (a) and CD11c$^+$CD8$^-$ (b) cDCs after co-culture with *PbA*-GFP iRBC at a 1:3 ratio.
3.3.2 C57BL/6-BALB/c F1 mice are susceptible to ECM

C57BL/6 mice are susceptible to ECM development upon *P. berghei* infection, whereas BALB/c mice are resistant. Because our DTR-transgenic mice were originally generated in a BALB/c background, C57B6/J mice were crossed with BALB/c mice to obtain F1 wt mice (to be used as experimental controls), or with homozygous DTR-transgenic mice to obtain F1 Siglec-H-, F1 Clec9A-, and F1 Clec4a4-DTR mice. To test the susceptibility of F1 mice to ECM, F1 wt mice were infected with PbA-GFP and survival, RMCBS score and parasitemia were monitored. Results showed that more than 70% of mice died during the typical ECM phase, between day 6 and day 12 p.i., with severe ECM signs, low RMCBS scores, and with relatively low parasitemia levels. On the other hand, the few mice that survived the ECM phase died due to hyperparasitemia (Figure 3.20(a), (b) and (c)). Because such an infection outcome is similar to what is usually observed in C57BL/6 mice, we concluded that C57BL/6-BALB/c F1 mice are as susceptible to ECM development as C57BL/6 mice, upon *P. berghei* infection.

![Figure 3.20. C57BL/6-BALB/c F1 mice are susceptible to ECM upon infection with *P. berghei*. (a) Survival (b) RMCBS scores and (c) parasitemia of F1 wt mice infected with PbA-GFP (*n* = 11). RMCBS score and parasitemia are expressed as mean ± SD](image_url)

3.3.3 DT administration does not affect the course of *P. berghei* infection and ECM susceptibility

To exclude a possible effect of DT on parasite cycle and growth, or on ECM development and overall infection outcome, two groups of F1 wt mice were infected with PbA-GFP, and one of them was injected with 10 ng/gbw DT, whereas the other was administered with PBS. Survival, RMCBS scores and parasitemia were compared. Results showed no significant differences between DT or PBS injected mice, as all mice from both groups died during
the ECM phase, with low RMCBS scores and relatively low parasitemia levels (Figure 3.21(a), (b) and (c)). Therefore we concluded that DT injection in F1 mice does not affect the course of *P. Berghei* infection, or ECM susceptibility.

Figure 3.21. DT administration does not affect the course of *P. berghei* infection and ECM development in F1 mice. (a) Survival (b) RMCBS scores and (c) parasitemia of F1 wt mice infected with PbA-GFP and injected with 10ng/gbw DT or PBS (*n* = 7). RMCBS score and parasitemia are expressed as mean ± SD. Mantel-Cox logrank test.

### 3.3.4 Ablation of Clec9A⁺ cDCs induces resistance to ECM development

Using C57BL/6 CD11c-DTR mice and pDC depleting antibodies, deWalick and colleagues previously showed that cDCs, but not pDCs, are required for the induction of ECM in *P. berghei* infection [182]. However, it is still not known whether both cDC subsets, CD8⁺Clec9A⁺ and CD8⁻CD11b⁺ cDCs, are equally responsible, or whether only one of them is mainly involved in ECM pathogenesis. Our co-culture experiment with *PbA*-GFP iRBC showed that both splenic CD8⁺ and CD8⁻ cDCs can phagocytose *P. berghei* iRBCs (Figure 3.19(a) and (b)). However, since CD8⁺ T cells have been shown to be directly involved in ECM pathogenesis, and because CD8⁺Clec9A⁺ cDCs are known to be specialized in cross-presentation to CD8⁺ T cells, we hypothesized that CD8⁺Clec9A⁺ cDCs might have a more relevant role in ECM. To test this hypothesis, we infected F1 wt and DT treated F1 Clec9A⁻DTR mice with *P. Berghei*, and monitored the infection as described in Section 3.3, Figure 3.18. Because Clec9A is weakly expressed also on pDCs, F1 Siglec-H-DTR mice were included in the experiment to exclude any role for pDCs.

As expected, most F1 wt mice and DT treated F1 Siglec-H-DTR mice (about 75% and 90%, respectively) developed clear ECM signs around day 7-8 p.i.
and died during the ECM phase with low parasitemia levels (Figure 3.22(a), (b) and (c)). On the contrary, DT treated F1 Clec9A-DTR mice showed no visible signs of ECM and none of them died during this period. Instead, these mice died later, during the second phase of infection, as did the few F1 wt mice and Siglec-H ablated mice which survived the ECM phase (Figure 3.22(a) and (b)). Such phenotype is similar to what observed in P. berghei infected resistant BALB/c mice. The absence of a neurological syndrome in Clec9A ablated mice was also confirmed by the lack of the typical ECM histological signs in brain sections prepared on day 7 p.i. In fact, none of the sections from DT treated F1 Clec9A-DTR mice showed intravascular accumulation of iRBCs and leukocytes, or extensive areas of hemorrhage, as observed in F1 wt mice (Figure 3.22(d)).

![Figure 3.22. Ablation of Clec9A+ cDCs induces resistance to ECM development. (a) Survival (b) RMCBS scores and (c) parasitemia of F1 wt mice and DT treated F1 Clec9A-DTR and Siglec-H-DTR mice infected with PbA-GFP \(n = 12\). (d) Representative histological H&E stained sections from brains of F1 wt mice and DT treated F1 Clec9A-DTR mice on day 7 p.i. Pial and parenchymal vessels containing iRBCs and leukocytes, as well are areas of hemorrhage, can be detected only in the brain of F1 wt mice (indicated with black arrows). (e) Serum IFN-γ levels of F1 wt and DT treated F1 Clec9A-DTR mice on day 6 p.i. Results are representative of 3 independent experiments. RMCBS score, parasitemia and IFN-γ are expressed as mean ± SD. Mantel-Cox logrank test, *\(p < 0.05\); Mann-Whitney test, ***\(p < 0.001\).](image)

Elevated levels of the pro-inflammatory cytokine IFN-γ have been found in P. falciparum infected CM patients [335, 336], as well as in P. berghei infected C57BL/6 mice [337], and IFN-γ have been proposed to enhance
TNF production, thus promoting up-regulation of adhesion molecules on endothelial cells. Indeed, IFN-γ levels in sera collected from F1 wt mice on day 6 p.i. were significantly higher than those present in sera from ECM resistant Clec9A ablated mice (Figure 3.22(e)).

Taken together, these results showed that Clec9A ablated mice are resistant to ECM development, and that such protection is solely due to ablation of Clec9A+ cDCs, and not to pDCs depletion, as Siglec-H ablated mice had an infection outcome similar to F1 wt mice. Therefore, F1 Siglec-H-DTR mice were omitted in the following investigations.

3.3.5 Ablation of Clec9A+ cDCs results in reduction of splenic T cell activation during P. berghei infection

During ECM, parasite-specific T cells are primed by cDCs in the spleen, and are found in the brain at the onset of the neurologic symptoms [182, 348]. In particular, T cells expressing the activation marker CD69 have been shown to increase and peak on day 4 p.i. in the spleen [361].

![Figure 3.23](image)

**Figure 3.23. Reduced splenic T cell activation in absence of Clec9A+ cDCs during P. berghei infection.** (a) Representative dot plots showing the expression profile of CD69 on splenic CD4+ and CD8+ T cells in uninfected F1 wt mice (left panel) and 4 days p.i. in F1 wt mice (middle panel) and DT treated F1 Clec9A-DTR mice (right panel). (b) Graphs summarizing absolute numbers of data showed in (a) (n = 5). Mann-Whitney test, *p < 0.05.

To investigate whether ablation of Clec9A+ cDCs affected spleen T cell priming during P. berghei infection, expression of CD69 on splenic CD4+ and CD8+ T cells isolated from F1 wt and DT treated F1 Clec9A-DTR mice,
on day 4 p.i., was analyzed. Consistent with what shown previously, the fraction of spleen CD69+CD8+ and CD69+CD4+ T cells increased in infected F1 wt mice (Figure 3.23(a), middle panel and (b)). However, in the absence of Clec9A+ cDCs, the amount of splenic CD8+ and CD4+ T cells expressing CD69+ was similar to the amount in uninfected F1 wt mice (Figure 3.23(a), left and right panel, and (b)). This result suggested a direct involvement of this particular cDC subset in activating splenic T cell responses during *P. berghei* infection.

### 3.3.6 Ablation of Clec9A+ cDCs decreases dramatically the accumulation of activated CD8+ T cells and iRBCs in the brain during *P. berghei* infection

ECM is characterized by an accumulation of leukocytes in the brain. Among all, activated CD8+ T cells producing cytokines such as IFN-γ, perforin and GzmB, through which they exert their cytotoxic function, have been proved to be the most directly involved in causing ECM [318, 356]. Moreover, it was recently demonstrated that such CD8+ T cells are parasite specific [357], and that they can induce ECM only when a critical parasite load is present in the brain, and vice versa, such as a strong interdependence exist between cytotoxic T cells and iRBCs in the brain, and accumulation of both is necessary to induce the pathology [318, 319]. Because F1 Clec9A ablated mice showed to be resistant to ECM, we hypothesized that they would experience a lower degree of leukocyte and iRBC accumulation in the brain during the ECM phase. To test this hypothesis, we first monitored cerebral accumulation of different leukocytes such as NK cells, NKT cells, CD8+, and CD4+ T cells in perfused brains of F1 wt mice and DT treated F1 Clec9A-DTR mice on day 7 p.i. Interestingly, mice lacking Clec9A+ cDCs showed a significant decrease in cerebral leukocyte accumulation, especially CD8+ T cells, but also CD4+ T cells and NKT cells, whereas the absolute number of NK cells was comparable to that of F1 wt mice (Figure 3.24(a)). Moreover, whereas in F1 wt mice a large portion of brain CD8+ T cells secreted IFN-γ and strongly expressed GzmB, the few CD8+ T cells detectable in Clec9A ablated mice showed significantly lower IFN-γ and GzmB levels (Figure 3.24(b)). Consistently, compared to wt
mice, in DT treated Clec9A-DTR mice a significant lower fraction of brain CD8\(^+\) T cells isolated on day 6 p.i. were stained by the SQLLNAYL-H-2D\(^b\) tetramer, a very recently developed tetramer which carries a malaria *Plasmodium* peptide [357] (Figure 3.24 (c) and (d)).

![Graphs and dot plots](image)

**Figure 3.24.** Clec9A\(^+\) cDCs control cerebral parasite-specific CD8\(^+\) T cells accumulation and parasite load during ECM. (a) Absolute numbers of CD8\(^+\), CD4\(^+\), NKT, and NK cells in the brain of F1 wt and DT treated F1 Clec9A-DTR mice on day 7 p.i. (n = 10). (b) Absolute numbers of IFN-\(\gamma\) and GzmB-expressing CD8\(^+\) T cells in the brain of F1 wt and DT treated F1 Clec9A-DTR mice on day 7 p.i. (n = 10). (c) Representative dot plots showing the percentage of SQLLNAYL-H-2D\(^b\) tetramer "CD8\(^+\)" T cells in the brain of F1 wt and DT treated F1 Clec9A-DTR mice on day 6 p.i. (d) Graph summarizing percentages of data showed in (c) (n = 5). (e) Representative dot plots showing the percentage of Hoechst\(^{low}\)GFP\(^+\) and Hoechst\(^{high}\)GFP\(^{high}\) iRBCs in the brain of F1 wt and DT treated F1 Clec9A-DTR mice on day 7 p.i. CD45.2\(^+\) leukocytes were excluded from the analysis. (f) Graph summarizing absolute numbers of data showed in (e) (n = 6). Absolute numbers and percentages are expressed as mean ± SD. Mann-Whitney test, *\(p < 0.05\), **\(p < 0.01\).

These results suggested that parasite antigen presentation to CD8\(^+\) T cells is hampered by ablation of the Clec9A\(^+\) cDC subset, and the almost total absence of cerebral CD8\(^+\) T cells, as well as the poor activation status of the few present in Clec9A ablated mice, contributed to their resistance to ECM.
Next, because CD8⁺ T cells are necessary to induce ECM pathology, but are not sufficient per se, we measured cerebral parasite burden in perfused brains of F1 wt mice and DT treated F1 Clec9A-DTR mice on day 7 p.i. Interestingly, flow cytometry analysis showed that mice lacking Clec9A⁺ cDCs had a significant lower amount of mature (Hoechst^high^GFP^high^), but not young (Hoechst^low^GFP⁺), iRBCs in the brain, compared to F1 wt mice (Figure 3.24 (e) and (f)). Together with the substantial decrease in activated and parasite-specific CD8⁺ T cells, the reduction in brain parasite load explains the lack of neurological syndrome in *P. berghei* infected Clec9A ablated mice.

3.3.7 Clec4a4⁺ cDCs are not involved in ECM pathogenesis during *P. berghei* infection

Results obtained with Clec9A ablated mice strongly suggested that Clec9A⁺ cDCs are the cDC subset responsible for priming pathology-inducing cytotoxic T cells, and mediating ECM. However, to exclude a possible role of Clec4a4⁺ cDCs, we performed a similar analysis with DT treated Clec4a4⁻ DTR mice. In particular, we first compared ECM susceptibility, survival and parasitemia with F1 wt mice. The obtained results clearly showed that mice ablated of Clec4a4⁺ cDCs were even more susceptible than wt mice, as all of them died during the ECM phase, with low RMCBS scores and low parasitemia, whereas some of the F1 wt mice survived the ECM period and died later with high parasitemia levels (Figure 3.25 (a) to (c)).

![Figure 3.25. Clec4a4 ablated mice are more susceptible to ECM than wt mice. (a) Survival (b) RMCBS scores and (c) parasitemia of F1 wt mice and DT treated F1 Clec4a4-DTR infected with *PbA-GFP* (*n* = 9). RMCBS scores and parasitemia are expressed as mean ± SD. Mantel-Cox logrank test, *p* < 0.05.](image)
To further confirm that Clec4a4+ cDCs are not involved in inducing ECM pathology, we monitored the accumulation of activated CD8+ T cells and iRBCs in perfused brains of F1 wt mice and DT treated F1 Clec4a4-DTR mice on day 7 p.i. The analysis showed that ablation of Clec4a4+ cDCs did not affect any of these events, as both were comparable to what observed in F1 wt mice (Figure 3.26(a) to (c)). Therefore, we concluded that Clec4a4+ cDCs are not involved in controlling accumulation of cytotoxic T cells and iRBCs during ECM in *P. berghei* infected mice.

![Figure 3.26. Clec4a4+ DCs do not control cerebral CD8+ T cells accumulation and parasite load during ECM.](image)

**3.3.8 Discussion**

CM is the most severe complication caused by *P. falciparum*. Even though it has been extensively studied since the 19th century, the exact mechanisms leading to CM pathogenesis are still not fully understood, one of the main reasons being the complexity and multifactoriality of the pathology. Even though the *P. berghei* mouse model of CM does not exactly replicate all the features of human CM, it has proven to be an invaluable tool to study its pathogenesis, and it has been extensively used for this purpose. Indeed, it is evident that studies aimed at uncovering the mechanisms involved in murine ECM pathogenesis might provide new insights for a better understanding of the human pathology. As other malaria complications, CM pathogenesis is caused not only by the presence and activity of the parasite, but also by the host immune responses. A precise understanding
of the involvement of immune responses in CM pathogenic would help to develop new effective therapeutic strategies. Among other factors, CD8$^+$ cytotoxic T cells have been shown to be directly involved in ECM pathogenesis. Because of their efficiency in processing and presenting antigens, cDCs are the best candidates for the activation of such cytotoxic T cells. Indeed, it has been shown that cDCs are the APC required for the induction of ECM in *P. berghei* infection [182]. However, it is still not known whether CD8$^+$Clec9A$^+$ and CD8$^-$CD11b$^+$ cDC subsets are equally responsible, or whether only one of them is mainly involved in activating the pathology-inducing CD8$^+$ T cells. Since CD8$^+$Clec9A$^+$ cDCs are known to be specialized in cross-presentation to CD8$^+$ T cells, we hypothesized that CD8$^+$Clec9A$^+$ cDCs might have a more relevant role in ECM.

In our co-culture experiment, we showed that both splenic CD8$^+$ and CD8$^-$ cDCs can efficiently phagocytose *P. berghei* iRBCs. After excluding any possible effect of DT on ECM development and infection outcome, and after confirming the ECM susceptibility of our F1 wt and F1 DTR-transgenic mice, we proceeded to investigate the involvement of these two cDC subsets, as well as pDCs, in ECM pathogenesis.

pDCs have previously been shown to be dispensable for ECM development [182]. Indeed, infection of Siglec-H ablated F1 mice with *P. berghei* resulted in ECM development and death in about 90% of the cases, similar to F1 wt mice. On the contrary, interestingly ablation of CD8$^+$Clec9A$^+$ cDCs during *P. berghei* infection induced resistance to ECM, and none of the typical ECM histological signs observed in brains of F1 wt mice, such as plugged vessels and hemorrhage, was present in Clec9A ablated F1 mice. Moreover, in these mice levels of IFN-γ, which has been proposed to favor CM by promoting upregulation of adhesion molecules on endothelial cells, were significantly lower than in F1 wt mice.

During ECM, parasite-specific T cells are primed by cDCs in the spleen, and are found in the brain at the onset of the neurologic symptoms [182, 348]. In particular, T cells expressing the activation marker CD69 have been shown to increase and peak on day 4 p.i. in the spleen [361]. However, in the absence of Clec9A$^+$ cDCs, the amount of splenic CD69$^+$CD8$^+$ and
CD69^+CD4^+ T cells was similar to the amount detected in uninfected F1 wt mice, suggesting a direct involvement of this particular cDC subset in activating splenic T cell responses during *P. berghei* infection.

ECM is characterized by an accumulation of leukocytes in the brain. Among all, activated CD8^+ T cells producing cytokines such as IFN-γ, perforin and GzmB, through which they exert their cytotoxic function, have proved to be the most directly involved in causing ECM [318, 356]. Studies with transgenic parasites expressing an epitope from chicken ovalbumin have shown that brain sequestered CD8^+ T cells are parasite-specific [180, 181]. Very recently, CD8^+ T cells specific for a native malaria *Plasmodium* epitope were identified during *P. berghei* infection, and showed to migrate to the brain just before the manifestation of neurological signs. However, activated, parasite-specific CD8^+ T cells are not *per se* sufficient to induce ECM, but they can do so only when a critical parasite load is present in the brain, and vice versa. Therefore, a strong interdependence appears to exist between cytotoxic T cells and iRBCs in the brain, and accumulation of both is necessary to induce the pathology [318, 319].

Consistent with their resistance to ECM, DT treated F1 Clec9A-DTR mice experienced a significantly lower degree of cerebral leukocyte accumulation, compared to F1 wt mice, during the ECM phase, especially of CD8^+ T cells, but also CD4^+ T cells and NKT cells. Moreover, the few CD8^+ T cells detectable in Clec9A ablated mice showed significantly lower IFN-γ and GzmB levels, and only a small fraction of them was specific for the SQLLNAKYL-H-2D^b tetramer. Furthermore, Clec9A^+ ablated mice had a significantly lower amount of mature, but not immature, iRBCs in the brain, compared to F1 wt mice. Such a result is particularly interesting as in *P. falciparum* infection sequestration of mature stages of the parasite has been associated with severe disease [312]. Together with the substantial decrease in activated CD8^+ T cells, the reduction in brain parasite load explains the lack of neurological syndrome in *P. berghei* infected Clec9A ablated mice. These results suggest that Clec9A^+ cDCs are the cDC subset responsible for priming pathology-inducing cytotoxic T cells, and mediating ECM.
To evaluate a possible involvement of the Clec4a4+ cDC subset in ECM pathogenesis, we performed a similar analysis with Clec4a4 ablated F1 mice. However, these mice appeared to be even more susceptible to ECM development that F1 wt mice. In fact, analysis of CD8+ T cells activation and accumulation, as well as iRBCs sequestration, in the brain, revealed no significant differences with F1 wt mice, thus excluding a role for Clec4a4+ cDCs in controlling accumulation of cytotoxic T cells and iRBCs during ECM, in *P. berghei* infected mice. Such a result is consistent with the poor cross-presentation properties of CD8−CD11b+ cDCs. Because DT treatment in Clec4a4-DTR mice results also in ablation of red pulp F4/80+ macrophages, which are known to be important for parasite clearance, the absence of these important phagocytes might be a possible explanation for the exacerbated susceptibility of Clec4a4 mice to ECM development.

Distinct from CD8−CD11b+ cDCs, CD8+Clec9A+ cDCs are particularly equipped and efficient in cross-presentation. Moreover, they are anatomically optimally located, together with MZMs, in the marginal zone of the spleen [13], a location that allows an efficient uptake of parasitized erythrocytes from the blood flowing through the marginal sinus. However, different from macrophages, which are specialized in phagocytosis and clearance of iRBCs, CD8+Clec9A+ cDCs possess unique antigen presentation properties. Our results suggest that such specific properties are directly responsible for the priming and activation of CD8+ T cell responses, which can lead to severe cerebral pathology during *P. berghei* infection.

A population of human cDCs, defined as CD141+CD1c− or BDCA3+ DCs, has been proposed to be the counterpart of mouse CD8+Clec9A+ cDCs, and has been shown to have similar cross-priming properties [80, 82]. This particular human cDC subset was recently suggested as an attractive clinical target to enhance cross-priming of cytotoxic T cells in tumor therapy [387]. Future experiments aimed at uncovering the nature of the interaction between these cDCs and iRBCs might provide possible targets for the development of immunotherapies to prevent severe malaria.
4. GENERAL CONCLUSION

Despite years of research, malaria still remains a devastating infectious disease, and a better understanding of malaria infection is desperately needed. Thanks to the generation of a unique set of mice, the Siglec-H-, Clec9A-, and Clec4a4-DTR mice, where specific DC subsets can be efficiently ablated, we have contributed to the understanding of the role of these key regulator cells of the immune system in malaria immunity and pathology. Even though some of the obtained results are not definitive, they open the way for new investigations, which might help to uncovering important mechanisms involved in malaria immunity, and might provide new insights for a better understanding of human malaria infection. Indeed, a better knowledge of the molecular basis of the host-parasite interaction is a prerequisite for the development of therapies to improve malaria infection outcome, and vaccines targeting appropriate DC subsets might be one of such possible therapies.
5. REFERENCES


6. APPENDIX

6.1 Media, buffers and solutions

ES cells culture medium:
Dulbecco’s Modified Eagle Medium (DMEM)
1 mM sodium pyruvate
0.1 mM non essential amino acids
100 U/mL penicillin/streptomycin
2 mM L-glutamine
0.05 mM 2-mercaptoethanol
15% inactivated Fetal Calf serum (FCS)
1 μg/L Leukemia Inhibitory Factor (LIF)

MEFs culture medium:
Dulbecco’s Modified Eagle Medium (DMEM)
100 U/mL penicillin/streptomycin
2 mM L-glutamine
0.05 mM 2-mercaptoethanol
10% inactivated FCS

ES cells and MEFs freezing medium:
Inactivated FCS
10% dimethyl sulfoxide

1% agarose gel
1X TAE buffer
1% agarose
0.005% ethidium bromide
50X TAE buffer (Tris/acetate/EDTA)
242 g Tris base
57.1 ml glacial acetic acid
37.2 g Na₂EDTA·2H₂O
Distilled H₂O to 1 L

Diphtheria Toxin (DT)
1X PBS
1% mouse serum
0.4 ng/μL or 1 ng/μL DT (depending on the DTR-transgenic line)

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

3% sodium citrate solution
3% sodium citrate
Distilled H₂O

0.89% ammonium chloride solution
Ammonium chloride
Distilled H₂O

Fluorescence Activated Cell Sorting (FACS) buffer
1X PBS
2% FCS
Digestion medium
Iscove’s Modified Dulbecco’s Medium (IMDM)
2% FCS

Solution B: Hank’s Balanced Salt Solution (HBSS)
100 mM Hepes
100 mM EDTA
Distilled H₂O
pH adjusted to 7.4

Solution C: Diluent
0.88% NaCl
1 mM EDTA
0.5% Bovine Serum Albumin (BSA)
10 mM Hepes
pH adjusted to 7.4

Cell fixation buffer
1X PBS
2% paraformaldehyde
pH adjusted to 7.4

Blocking buffer
1X PBS
10% FCS

Permeabilization buffer
1X PBS
0.5% saponin

Assay diluent
1X PBS
1% BSA
Wash buffer
1X PBS
0.05% Tween 20

Organ fixation buffer
1X PBS
4% paraformaldehyde
pH adjusted to 7.4

1% acid ethanol solution
Ethanol
1% HCl

Scott’s tap water substitute
For 500 mL:
1 g sodium bicarbonate
10 g magnesium sulphate
Distilled water
6.2 Reagents, chemicals and kits

Ammonium chloride  Sigma, St. Louis, MO, USA
2-mercaptoethanol  Sigma, St. Louis, MO, USA
Acetone  Fisher Scientific, Longhorough, UK
Agarose  1st BASE Pte Ltd, Singapore
Blood and Tissue Kit  Qiagen, Valencia, CA, USA
Brefeldin A  Sigma, St. Louis, MO, USA
BSA  Sigma, St. Louis, MO, USA
Clec9A BAC: RP23-394L7  BACPAC Resources Center, CHORI, Oakland, CA, USA
Collagenase D  Roche, Basel, Switzerland
D-(-)-Glucose  Sigma, St. Louis, MO, USA
Dimethyl sulfoxide  Sigma, St. Louis, MO, USA
DMEM  Gibco, Grand Island, NY, USA
DNase I  Fermentas, Waltham, MA, USA
DPX Mountant for histology  Sigma, St. Louis, MO, USA
DT  Sigma, St. Louis, MO, USA
EDTA  USB, Cleveland, OH, USA
Ethanol  Merck KGaA, Darmstadt, Germany
Ethidium bromide  Bio-Rad, Hercules, CA, USA
FCS  Chemicon International, Temecula, CA, USA
Ficoll-Paque™ PLUS  GE Healthcare, Uppsala, Sweden
Fluorescence mounting medium  Dako, Golstrup, Denmark
Foxp3 Fixation/Permeabilization Concentrate and Diluent  eBioscience, San Diego, CA, USA
G418  PAA, Velizy-Villacoublay, France
GammaBind™ PLUS Sepharase  GE Healthcare, Uppsala, Sweden
Generuler 1kb DNA ladder  Fermentas, Waltham, MA, USA
Glycerine  Affymetrix, Santa Clara, CA, USA
GoTaq Flexi DNA Polymerase  Promega, Fitchburg, WI, USA
H&E  Merck KGaA, Darmstadt, Germany
HCG  Sigma, St. Louis, MO, USA
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abcam, Cambridge, UK  
AbD Serotec, Oxford, UK  
BioLegend, San Diego, CA, USA  
eBioscience, San Diego, CA, USA  
Pharmigen, San Diego, CA, USA  
SouthernBiotech, Birmingham, AL, USA
### 6.3 Equipment

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<td>ImageStreamX</td>
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<td>Leica EG1150 H - Embedding</td>
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<td>Microscope Nikon Eclipse 80i</td>
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<td>Multiskan Spectrum</td>
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<tr>
<td>Thermocycler T3000</td>
<td>Biometra GmbH, Goettingen, Germany</td>
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7. AUTHOR’S PUBLICATIONS

8. POSTERS, INVITED TALKS

4th International Singapore Symposium of Immunology, Singapore, February 2011.
Poster presentation: “Specific ablation of dendritic cell subsets in vivo”. **Piva, L.**, Aminah, S., Fam, W., Tetlak, P., Kumar, A., Ruedl, C.

Singapore Malaria Network Meeting 2012, Singapore, February 2012.
Oral presentation: “Importance of distinct myeloid cell subsets in protection and pathology to malaria parasite”. **Piva, L.**, Tetlak, P., Claser, C., Karjalainen, K., Renia, L., Ruedl, C.

3rd Advanced Singaporean Immunology PhD student Retreat, August 2012.
Oral and poster presentation: “Role of distinct DC subsets in blood-stage malaria immunity”. **Piva, L.**, Renia, L., Ruedl, C.

12th International Symposium on Dendritic Cells (DC2012), Daegu, Korea, October 2012.
Poster presentation: “Clec9A⁺ dendritic cells mediate the development of experimental cerebral malaria”. **Piva, L.**, Renia, L., Karjalainen, K., Ruedl, C.