Modulation of adaptive and innate immune responses by myeloid cells during malaria

Lai, Si Min

2019

Lai, S. M. (2019). Modulation of adaptive and innate immune responses by myeloid cells during malaria. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106032>

<https://doi.org/10.32657/10220/48099>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MODULATION OF ADAPTIVE AND INNATE
IMMUNE RESPONSES BY MYELOID CELLS
DURING MALARIA**

LAI SI MIN

SCHOOL OF BIOLOGICAL SCIENCES

2018

**MODULATION OF ADAPTIVE AND INNATE
IMMUNE RESPONSES BY MYELOID CELLS
DURING MALARIA**

LAI SI MIN

School of Biological Sciences

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

2018

Statement of Originality

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

12/4/2019

.....
Date

Lai Si Min

.....

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

12.04.2019

.....
Date

Christiane Ruedl

.....

Authorship Attribution Statement

This thesis contains material from 1 paper(s) published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

Chapter 3 and 4 are published as Lai, S. M., et al. (2018). "Organ-Specific Fate, Recruitment, and Refilling Dynamics of Tissue-Resident Macrophages during Blood-Stage Malaria." *Cell Reports* 25(11): 3099-3109.e3093.

DOI: <https://doi.org/10.1016/j.celrep.2018.11.059>

The contributions of the co-authors are as follows:

- Prof Christiane Ruedl initiated and conceptualized the study.
- Prof Christiane Ruedl prepared and edited the manuscript drafts. The manuscript was revised by Prof Christiane Ruedl and Prof Klaus Karjalainen.
- I, Dr Pravesh Gupta and Dr Sheng Jian Peng performed all the laboratory work and investigations at the School of Biological Sciences. I and Prof Christiane Ruedl also formally analyzed and interpreted the data.
- Dr Francesca Zolezzi and her core facility at Singapore Immunology Network conducted the RNA sequencing experiment. Dr Duan Kaibo carried out the bioinformatics analysis of RNA sequencing data.
- Dr Evan Newell at Singapore Immunology Network carried out the high-dimensional UMAP analyses and assisted in interpretation of UMAP data.
- Dr Laurent Renia at Singapore Immunology Network co-supervised the PhD project and provided guidance.

12/4/2019
.....
Date

Lai Si Min
.....

Acknowledgements

I would like to take this opportunity to sincerely thank my supervisor, Associate Professor Christiane Ruedl, for her constant guidance, motivation and encouragement these four years. Her mentorship and trust in me have been instrumental for my PhD progression, for allowing me to have the freedom to explore scientific angles, her patience to my bouts of bullheadedness and helpful advice in tackling the subtler nuances of academia and professional life. I would also like to thank my co-supervisor Professor Laurent Renia, for his generosity, expertise, valuable advice and suggestions for my PhD, and always encouraging me to look deeper into interesting phenomenon.

To the members of the CR/KK lab;

Professor Klaus Karjalainen for his knowledge and thought-provoking questions and fine appreciation of beer,

Dr Pravesh Gupta, my mentor-in-crime for imparting his knowledge, skills and engaging discussions about our projects, science and life,

Dr Sheng Jian Peng, for valuable discussions and great experimental ideas/suggestions,

Irene, Dr Ng See Liang, Dr Teo Yi Juan, colleagues whom I could always count on for discussions, technical help and everyday conversations,

Yolanda and Monika, for exemplary technical help and responsibility of daily tasks,

Dr Piotr Tetlak, Dr Abdul Rashid, Chen Qi, Mei Ling and Arun.

Thank you and my greatest appreciation for my time in the lab.

I would also like to thank our collaborator Dr Evan Newell from SlgN, for his indispensable help in UMAP analyses of our voluminous data for the manuscript on a tight schedule, and Dr Carla Claser for her generosity and patience in my many queries about malaria.

My SIgN and NTU PhD comrades-in-arms: Wei Li, Victor, Daniel, Hiroko, Jelin, Ania, Adam, Jerzy, Militsa, Ain, Wint, Eric, Meshal and CresPion RMs; always helpful, humourous, encouraging and vivacious, living life largely at and outside of work.

My close friends from university and elsewhere: Andre, Wei Lun, Xiangyi, Heidi, Alisa, Natalie, Bao Qi, Bryan, Leah, Joe, Bijil, Helen, Yvonne, Ashley, Viktor, Sunny and the rest of my great friends and teammates; thank you for encouraging, cheering me on and all the laughter when I am uninspired, and to do more sports instead of resigning to the sedentary life.

Lastly but not least, I thank my family for always supporting me in my endeavours, to pursue my interests and never pressuring me to conform. My sisters Wei Min for her fresh new perspectives and independence, and Yue Min for her free spirit. I am eternally grateful to my parents, Lai Ming and Esther Chew, for always wishing the best for me and sacrificing so much on the behalf of my sisters' and my education, even in arduous times.

<u>Table of Contents</u>	Page
Statement of Originality	
Supervisor Declaration Statement	
Authorship Attribution Statement	
Acknowledgements	1
Table of Contents	3
Abbreviations	9
List of Figures	13
List of Tables	16
Abstract	17
1. Introduction	19
1.1. The myeloid cell lineage and mononuclear phagocyte system	19
1.2. Dendritic cells	19
1.2.1. Conventional DCs	20
1.2.2. Inflammatory DCs	21
1.3. Macrophages	21
1.3.1. Tissue-resident macrophages	22
1.3.2. Origins of tissue-resident macrophages	25
1.3.3. Fate-mapping models for macrophage ontogeny	26
1.3.4. Transcriptional factors and cytokines in TRMØs development and differentiation	26
1.4. Monocytes	28
1.5. Malaria: A global crisis	29
1.5.1. Life cycle of <i>Plasmodium</i> in mammalian hosts	30
1.5.2. Animal models of malarial disease	32
1.5.3. Human models and studies of malaria	35
1.5.4. Clinical manifestations of malaria	36
1.6. Structure of the spleen	37
1.6.1. Importance of the spleen in malaria	39

1.7. Immunity to malaria	41
1.7.1. Liver-stage anti- <i>Plasmodium</i> immunity	41
1.7.2. Blood-stage anti- <i>Plasmodium</i> immunity	41
1.7.3. Immunopathology in malaria	46
1.7.4. Immunoregulatory mechanisms in malaria	47
1.8. Cell ablation systems	48
1.8.1. DT-DTR system	48
1.8.2. DT-DTR models for DC depletion	49
1.8.3. Clec9A DT-DTR model for ablation of Clec9A ⁺ DCs	50
1.9. Objectives and aims of the study	51
2. Materials and Methods	52
2.1. Materials	52
2.1.1. Mice and ethics statement	52
2.1.2. Parasites	52
2.1.3. List of commercial antibodies and cell viability dyes	52
2.1.4. Buffers, media and solutions	55
2.1.5. Reagents, chemicals and kits	56
2.1.6. Equipment and instruments	58
2.1.7. Data analysis and computational software	59
2.2. Methods	59
2.2.1. Mice and ethics statement	59
2.2.2. Parasite cryopreservation and maintenance	59
2.2.3. Tamoxifen (TAM) administration, DT injection and <i>Plasmodium</i> infection	60
2.2.4. Blood parasitemia monitoring	60
2.2.5. Serum collection and processing	61
2.2.6. Sample processing and leukocyte isolation from organs	62
2.2.7. Antibody staining for flow cytometry	62
2.2.8. ELISA for serum cytokines	64
2.2.9. ELISA for <i>Plasmodium</i> -specific Ig	64
2.2.10. Serum passive transfer	65
2.2.11. Cell sorting for RNA-sequencing analyses	66
2.2.12. RNA isolation for QPCR	66

2.2.13. Cytospin for morphological examination	67
2.2.14. Data analyses and statistical significance	67
3. Results	69
3.1. Characterization of specific depletion in Clec9A-DTR mouse model	69
3.1.1. Specific and efficient ablation of Clec9A ⁺ CD8 ⁺ and CD103 ⁺ DCs in organs of Clec9A-DTR mice	69
3.1.2. Non-DC myeloid populations were intact following Clec9A ⁺ DC ablation in Clec9A-DTR mice in spleen, lung and liver	70
3.1.3. Summary of the efficiency and effectiveness of Clec9A-DTR mouse model	72
3.2. Elucidating the role of Clec9A⁺ CD8⁺ and CD103⁺ cDC1s in <i>Plasmodium chabaudi chabaudi</i> AS infection and immunity	72
3.2.1. IL-12 production is primarily from CD8 ⁺ cDC1s and not CD11b ⁺ cDC2s during steady-state and <i>PccAS</i> infection	72
3.2.2. Clec9A ⁺ DC depletion results in greater magnitude of prolonged parasitemia and elevated mortality in <i>PccAS</i> -infected mice	73
3.2.3. Clec9A ⁺ DCs modulate cellular proliferation and activation during acute phase of <i>PccAS</i> infection	74
3.2.4. Ablation of Clec9A ⁺ DCs results in impaired T _{EFF} polyfunctionality and proinflammatory cytokine responses during acute <i>PccAS</i> infection	75
3.2.5. Systemic cytokine milieu was skewed towards anti-inflammatory rather than pro-inflammatory in Clec9A ⁺ DC-depleted mic	84
3.3. Roles of Clec9A⁺ DCs in controlling <i>Plasmodium chabaudi chabaudi</i> AS chronic malaria	85
3.3.1. Absence of Clec9A ⁺ DCs do not impair germinal center reactions and total <i>Plasmodium</i> -specific IgG production, but skews Ig towards T _{h2} -polarized IgG1	85

3.3.2.	Serum passive transfer of Clec9A-DTR	
	<i>Plasmodium</i> -specific IgG1 confers less partial protection	
	than WT T _{H1} -polarized <i>Plasmodium</i> -specific IgG2c	88
3.3.3.	Clec9A ⁺ DCs influence <i>Plasmodium</i> -specific Ig	
	class-switch recombination by modulating IFN γ	
	production in effector follicular T helper (T _{FH}) cells	91
3.3.4.	Absence of Clec9A ⁺ DCs results in persistent impaired	
	CD4 and CD8 T _{EFF} during recrudescence episodes	94
3.4.	Macrophage Ontogeny Tracking in Acute Malaria	99
3.4.1.	Infection kinetics in WT Balb/c and Kit ^{MerCreMer/R26}	
	mice during <i>Plasmodium yoelii</i> acute malaria	99
3.4.2.	Myeloid kinetics in WT Balb/c and Kit ^{MerCreMer/R26}	
	mice during <i>Plasmodium yoelii</i> acute malaria	100
3.4.2.1	<i>Tissue-resident macrophages are differentially</i>	
	<i>repopulated but infiltrating monocytes have similar</i>	
	<i>kinetics in the organs of Py1.1GFP-infected mice</i>	101
3.4.2.2	<i>Py1.1GFP infection induces transient activated</i>	
	<i>tissue-resident macrophages</i>	105
3.4.2.3	<i>Distinct organ-specific myeloid subset clusters</i>	
	<i>are distinguished by high-dimensional analyses</i>	106
3.4.3.	Myeloid ontogeny tracking during Py1.1GFP infection	111
3.4.3.1	<i>Splenic RPMϕs and liver KCs, but not lung AMϕs,</i>	
	<i>are majorly replenished by BM-derived progenitors</i>	
	<i>during Py1.1GFP infection</i>	111
3.4.3.2	<i>Self-renewing non-FcγR Mϕs are evident in spleen</i>	
	<i>and lung, but not liver during Py1.1GFP infection</i>	112
3.4.3.3	<i>BM-derived, niche-refilling monocytes are stable in the</i>	
	<i>long-term and exhibit similar turnover kinetics as their</i>	
	<i>embryonic-derived counterparts</i>	117

3.4.4.	Post-entry monocytes gain molecular and gene expression characteristics of embryonically-seeded tissue-resident MØs	119
4.	Discussion	123
4.1.	Clec9A-DTR mouse model is a robust system to study roles of Clec9A⁺ DCs in <i>Plasmodium</i> infection	123
4.2.	Clec9A⁺ DCs are key drivers influencing downstream immune responses against <i>Plasmodium chabaudi chabaudi</i> AS infection	123
4.2.1.	Absence of Clec9A ⁺ DCs dysregulates induction of T _{H1} proinflammatory responses and T effector polyfunctionality in <i>PccAS</i> infection	123
4.2.2.	An intact innate immune compartment compensates for polyfunctional proinflammatory responses in the absence of Clec9A ⁺ DCs	125
4.2.3.	Germinal center reactions, B-cell responses and Ig production are not abrogated in absence of Clec9A ⁺ DCs	126
4.2.4.	Absence of Clec9A ⁺ DCs diminishes T _{H1} -like effector T _{FH} and proinflammatory signals required for class switch recombination to more protective T _{H1} IgG2 and IgG3	128
4.2.5.	The T _{H1} - T _{FH} relationship conundrum	129
4.2.6.	Clec9A ⁺ DC deficiency results in persistent T effector and memory precursor impairment during recrudescence <i>PccAS</i> infection	131
4.3.	Caveats and limitations in the Clec9A-DTR <i>PccAS</i> infection model	133
4.4.	Organ-specific tissue-resident macrophages are replenished with different kinetics during acute <i>Plasmodium yoelii</i> infection	135
4.4.1.	Emptied embryonically-seeded TRMØ niches in spleen and liver are partially replenished by BM-derived monocytic progenitors that differentiate into stable, bona fide TRMØs	135
4.4.2.	Depleted lung alveolar MØs almost exclusively self-renew	

with negligible input from BM-derived circulatory monocytes	
during acute <i>Py1.1GFP</i> infection	139
4.5. Rapidly-responding Ly6C^{hi} inflammatory monocytes	
and monocyte-derived MØs massively infiltrate afflicted	
tissues and contribute to <i>Py1.1GFP</i> clearance	139
4.5.1. Ly6C ^{hi} inflammatory monocytes rapidly and massively infiltrate	
organs upon <i>Py1.1GFP</i> infection	140
4.5.2. Concerted efforts of FrII inflammatory monocytes and	
monocyte-derived / other tissue-resident MØs in resolution	
of <i>Py1.1GFP</i> infection	141
4.6. BM monocyte-derived MØs are phenotypically and	
transcriptionally similar to <i>bona fide</i> embryonic	
tissue-resident MØs	142
4.7. <i>Plasmodium</i> infection as a new precedent for the	
general mechanism of beneficial cell suicide	144
5. Summary	146
6. Significance of studies on current malaria immunological	
research	150
6.1. Clec9A ⁺ DCs and their roles in anti- <i>Plasmodium</i>	
immunity	150
6.2. Tissue-resident MØ repopulation kinetics during	
<i>Plasmodium</i> infection	151
6.3. Concluding remarks	152
7. Appendix	153
7.1. Cell phenotype profile	153
7.2. List of antibody panels	155
7.3. Supplementary figures	159
8. References	163
9. Author's publication(s)	182
10. Oral and poster presentations	183

Abbreviations

ACT	Artemisinin-based combination therapy
ADCI	Antibody-dependent cellular inhibition
APC	Antigen presenting cells
AGM	Aorta-gonad-mesonephros
AMØ	Alveolar macrophage
Baf8	Basic leucine zipper ATF-like transcription factor 3
BBB	Blood-brain barrier
BM	Bone marrow
BMEM	Memory B-cells
CD	Cluster of differentiation
cDC	Conventional dendritic cells
cKit	Tyrosine-protein kinase Kit
CSF-1/MCF-1	Colony stimulating factor 1
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CDP	Common dendritic progenitor
CLEC9A	C-type lectin receptor 9A
CMP	Common myeloid progenitor
CMoP	Common monocyte progenitor
CTL	Cytotoxic T lymphocytes
CX3CR1	CX3C chemokine receptor 1
CXCR5	C-X-C chemokine receptor type 5
DNA	Deoxyribonucleic acid
DNGR1	Dendritic cell NK lectin group receptor-1
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Experimental cerebral malaria
ELISA	Enzyme-linked immunoabsorbent assay
EMP	Erythroid myeloid precursor
ESAM	Endothelial cell-specific adhesion molecule
FACS	Fluorescence activated cell sorting

FcR	Fragment of crystallization receptor
FL	Fetal liver
FLMo	Fetal liver monocyte
FrI	Fraction I (TRMØs)
FrII	Fraction II (monocytes)
FrIII	Fraction III (neutrophils)
FVS	Fixable viability stain
gbw	Gram per body weight
GC	Germinal center
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol
GzA	Granzyme A
GzB	Granzyme B
HB-EGF	Heparin-binding epidermal growth factor receptor
HSC	Haematopoietic stem cell
i.p.	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
Id2	Inhibitor of DNA binding 2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMØs	Interstitial macrophage
IRF8	Interferon regulatory factor
KC	Kupffer cell
KLRG1	Killer lectin receptor group 1
LCs	Langerhans cells
LN	Lymph node
LXR	Liver X receptor
MA-ALI/ARDS	Malaria associated acute liver injury/acute respiratory distress syndrome
MARCO	Macrophage receptor with collagenous structure
MERTK	MER proto-oncogene, tyrosine kinase

MDP	Monocyte dendritic cell precursor
MFG-E8	Milk fat globule-EGF factor 8 protein
mg	Milligram
MHC	Major histocompatibility complex
mL	Milliliter
MMM	Marginal metallophilic macrophages
MØ	Macrophage
MPEC	Memory precursor effector cells
MPS	Mononuclear phagocyte system
MZ	Marginal zone
MZM	Marginal zone macrophages
NACLAR	National Advisory Committee for Laboratory Animal Research
NHP	Non-human primate
NK	Natural killer
NKT/iNKT	Natural killer T / induced
NO	Nitric oxide
OD	Optical density
p.i.	Post-infection
PAMPs	Pathogen associated molecular patterns
PB/PC	Plasmablast / plasma cell
PbA	Plasmodium berghei ANKA
PBS	Phosphate-buffered saline
PCA	Principle component analysis
PccAS	<i>Plasmodium chabaudi chabaudi</i> AS
PD1	Programmed cell-death 1
pDC	Plasmacytoid DC
PRR	Pattern recognition receptors
P-Sp	Para-aortic splanchnopleura
PV	Parasitophorous vacuole
Py1.1GFP	<i>Plasmodium yoelii</i> 1.1 GFP
qPCR	Quantitative polymerase chain reaction

RBC/iRBC	Red blood cell / infected red blood cell
Relb	RELB proto-oncogene, NF-KB subunit
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RP	Red pulp
RPMØ	Red pulp macrophage
SIGN-R1	Specific ICAM-3 grabbing nonintegrin-related 1
SPICE	Simplified presentation of incredibly complex evaluations
SLEC	Short lived effector cells
SMA	Severe malarial anemia
SS	Steady state
TAM	Tamoxifen
T _{CM}	T central memory cell
TCR	T cell receptor
T _{EFF}	T effector cell
T _{EM}	T effector memory cell
TF	Transcription factor
T _{FH}	Follicular T helper cell
Th	T helper cell
T _{REG}	Regulatory T cell
TIM	T-cell immunoglobulin mucin
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRMØ	Tissue resident macrophages
UI	Uninfected
UMAP	Uniform manifold approximation and projection
WHO	World Health Organization
WT	Wildtype
YFP	Yellow fluorescent protein
YS	Yolk sac
γδT	Gamma delta T-cells
µL	Microliter

List of figures

Figure 1.1 Phenotype and functional specialization of distinct tissue-resident macrophages.	22
Figure 1.2 Three proposed models of tissue resident macrophage ontogeny.	24
Figure 1.3 Countries endemic for malaria and estimated malaria cases in 2016 by WHO regions.	30
Figure 1.4 Life cycle of the <i>Plasmodium</i> parasite.	32
Figure 1.5 Distinct microarchitectural regions in the spleen and their unique immune cell subsets.	37
Figure 1.6 Differences in splenic microarchitecture between human and mice	39
Figure 1.7 Updated CD4 ⁺ T _h anti- <i>Plasmodium</i> immunity model	44
Figure 1.8 Innate and adaptive immunity to <i>Plasmodium</i> infection	46
Figure 1.9 DT-DTR conditional system of in vivo depletion for cell subsets of interest	49
Figure 2.1 Gating strategy for tri-colour flow cytometry of blood parasitaemia	61
Figure 3.1 Clec9A ⁺ DCs are efficiently specifically depleted for both short and long-term in the spleen, liver and lungs of DT-injected Clec9A-DTR mice during steady-state conditions.	70
Figure 3.2 DT administration induces monocytosis but not unspecific depletion of CD11b ⁺ macrophage and monocyte subsets in the spleen, liver and lungs of DT-injected Clec9A-DTR steady-state mice following short or long-term depletion.	71
Figure 3.3 IL-12 is produced majorly by CD8 ⁺ DCs during <i>Plasmodium chabaudi</i> AS infection.	74
Figure 3.4 Absence of Clec9A ⁺ DCs resulted in overall lowered CD45 ⁺ leukocyte and drastically reduced CD3 ⁺ T-cell subsets in the spleen, lung and liver of <i>PccAS</i> -infected Clec9A-DTR mice	76

Figure 3.5 Depletion of Clec9A ⁺ DCs leads to drastically reduced activation of CD4 ⁺ and CD8 ⁺ effector T-cells in malaria-afflicted organs during acute <i>PccAS</i> infection	78
Figure 3.6 Absence of Clec9A ⁺ DCs results in dramatically diminished functionality of CD4 ⁺ and CD8 ⁺ effector T-cells in malaria-afflicted organs during acute <i>PccAS</i> infection	80
Figure 3.7 Polyfunctionality of effector T and NK-cells during acute <i>PccAS</i> infection is severely impacted in the absence of Clec9A ⁺ DCs	83
Figure 3.8 Clec9A ⁺ DC depletion leads to increased systemic anti-inflammatory IL-10 levels	84
Figure 3.9 Clec9A ⁺ DC depletion does not impair germinal center B-cell reactions but influences polarization of <i>Plasmodium</i> -specific Ig isotypes produced	87
Figure 3.10 IgG1-predominant Clec9A-DTR immune serum confers less partial protection against <i>PccAS</i> challenge	90
Figure 3.11 Impaired T _{H1} effector T follicular helper (T _{FH}) cell functionality is evident in <i>PccAS</i> -infected Clec9A-DTR mice	93
Figure 3.12 CD4 ⁺ effector T-cells are less T _{H1} -polarized and adopt a IFN γ *IL-10 ⁺ phenotype during recrudescence <i>PccAS</i> infection	95
Figure 3.13 Clec9A ⁺ DC-deficient mice have severely impaired terminally-differentiated effector and memory precursor CD4 T-cell numbers during recrudescence <i>PccAS</i> infection	98
Figure 3.14 <i>P. yoelii</i> blood-stage infection in WT Balb/c and Kit ^{MerCreMer/R26} mice	100
Figure 3.15 Gating strategy for tissue-resident macrophages and monocyte subsets	101
Figure 3.16 Tissue-resident macrophages are differentially repopulated in organs of Kit ^{MerCreMer/R26} mice during <i>Py1.1GFP</i> infection	104
Figure 3.17 Repopulated tissue-resident macrophages are stable	

long-term in organs of <i>Kit^{MerCreMer/R26}</i> after <i>Py1.1GFP</i> infection	105
Figure 3.18 Activated transient tissue resident macrophages in <i>Py1.1GFP</i> -infected mice are laden with ingested haemozoin and highly vacuolated	106
Figure 3.19 High dimensional analysis of myeloid dynamics shows distinct clusters in spleen, liver and lung	109
Figure 3.20 Myeloid subset expression plots show distinct time-course kinetics during <i>Py1.1GFP</i> infection	110
Figure 3.21 Spleen tissue resident MØ and liver Kupffer cells are partially replenished by bone marrow progenitors, while lung alveolar macrophages are self-renewed during <i>Py1.1GFP</i> infection	114
Figure 3.22 Turnover kinetics of monocyte-refilled MØs do not differ from the “original” embryonic-derived counterparts	118
Figure 3.23 Repopulating BM emigrants have similar gene signatures as originally seeded tissue-resident macrophages	121
Figure 3.24 Tissue-resident macrophages retain a more activated genetic profile after recovery from <i>Py1.1GFP</i> infection	122
Figure 5.1 Replenished tissue-resident MØs, except the lung, have dual origins during <i>Py1.1GFP</i> infection	148

Supplementary Figures

Supplementary Figure 1 Clec9A ⁺ DC depletion results in significant reduction of CD3 ⁺ T-cell subsets, particularly effector T-cells in spleen, lung and liver	159
Supplementary Figure 2 Clec9A ⁺ DC depletion results in severely impaired splenic CD4 T _{EFF} polyfunctionality	159
Supplementary Figure 3 Depletion of Clec9A ⁺ DCs does not impair B-cell cellularity but results in differential polarization of <i>Plasmodium</i> -specific Ig isotypes in <i>PccAS</i> -infected mice	160
Supplementary Figure 4 CD3 ⁺ T-cell subsets remain severely depleted in the absence of Clec9A ⁺ DCs during recrudescence <i>PccAS</i> infection	161
Supplementary Figure 5 KLRG1 ⁺ CD4 T _{EFF} are present during Recrudescence <i>PccAS</i> infection and reduced in Clec9A ⁺ DC-deficient mice	161
Supplementary Figure 6 Tissue-resident macrophages are differentially repopulated in organs of WT Balb/c mice during <i>Py1.1GFP</i> infection	162

List of tables

Table 1.1 Fate mapping mouse models and their labelled cell subsets	26
Table 2.1 List of antibody reagents	52
Table 2.2 List of chemicals and reagents	56
Table 2.3 List of equipment and instruments	58
Table 2.4 List of computational software	59
Table 2.5 Serum serial dilutions for <i>Plasmodium</i> -specific Ig isotypes	65
Table 3.1 UMAP analyses parameters	106

Abstract

Dendritic cells and macrophages are highly heterogeneous members of the mononuclear phagocyte system, that share a common lineage precursor in their differentiation progression. They are found in virtually all tissues and have broadly unifying roles in immunity with overlapping functions such as antigen presentation, cytokine production and regulation of both the innate and adaptive arms of immunity. Dendritic cells are dedicated immune cells unrivalled for antigen presentation, T-cell activation and bridging the dual arms of immunity, with little other non-immune roles. Macrophages have a multitude of indispensable roles in embryo development and adult tissue homeostasis, in concert with their immune functions, largely imparted by potent phagocytosis. Their complex heterogeneity and functional specialization extend to numerous subpopulations and requires investigation as separate entities rather as a collective whole in steady-state, disease or infection models. Here we focused on the specific conventional DC subset, Clec9A⁺ CD8⁺ lymphoid or CD103⁺ non-lymphoid DCs; specialized in cross-presentation, sensors of apoptotic/dying cells and potent inducers of IL-12-mediated proinflammatory responses, in the context of chronic malaria. From the macrophage axis in acute malaria, we were interested in embryonically-seeded tissue-resident macrophages, including the CD169⁺ subset, found in the splenic red pulp and marginal zone, liver hepatic sinusoids, lung alveolar spaces and other tissues with tissue-specific functional specializations.

To study CD8⁺/CD103⁺ DCs, we utilized the Clec9A-DTR mouse model that allowed for specific and efficient ablation of Clec9A⁺ DCs. Depletion of Clec9A⁺ DCs resulted in a striking phenotype in the *Plasmodium chabaudi chabaudi* AS model of chronic malaria, characterized by higher acute blood parasitemia, multiple severe recrudescence episodes and some associated mortality. Acute-phase T_{H1} proinflammatory responses, T effector cytokine-producing polyfunctionality and follicular T helper effector functionality were drastically impaired in Clec9A-DTR mice. During the chronic phase, Clec9A-deficient animals showed striking anti-*Plasmodium* Ig polarization to the less cytophilic IgG1, with little class-switching to other Ig isotypes; as well as persistently impaired T effector and T memory precursor generation.

Hence, the absence of Clec9A⁺ DCs had far-reaching impacts on downstream immune effector responses to *PccAS* infection, indicating the key roles and indirect influences this DC subset has in shaping anti-*Plasmodium* immunity.

To study the tissue-resident macrophage turnover during acute malaria infection, we utilized the Kit^{MerCreMer/R26} inducible fate-mapping mouse model which labelled cKit⁺ bone marrow progenitors and their subsequent progeny as YFP⁺ in adult mice, after tamoxifen pulsing. Tissue-resident macrophages mostly self-renewed with little monocytic input in unperturbed conditions. *Plasmodium yoelii* 1.1GFP infection resulted in macrophage disappearance during the pre-crisis period, followed by massive influx of bone marrow-derived inflammatory monocytes that developed into inflammatory macrophages as well as differentiated to tissue-resident macrophages; and in conjunction with local proliferation of surviving embryonic-seeded macrophages, replenished the emptied splenic and liver niches. In contrast, the lung alveolar macrophages were exclusively self-renewing. Transcriptional profiling in all organs showed high degree of similarity between BM-originated replenished macrophages and *bona fide* tissue-resident macrophages, as well as longevity beyond resolution of infection. Our results demonstrated that *Plasmodium* infection resulted in efficient monocytic refilling of tissue-resident macrophage compartments with a stable dual ontogeny upon recovery, which were also able to adopt tissue-specific genetic expression profiles of *bona fide* embryonic-origin tissue-resident macrophages.

1. Introduction

1.1 The myeloid cell lineage and mononuclear phagocyte system

Phagocytes are first termed by one of the recognized forefathers of immunology – Ilya Metchnikoff, who observed and investigated macrophages and ‘microphages’ (polymorphonuclear leukocytes), in a variety of organisms, and their roles in immunity and tissue homeostasis, over a century ago [1]. Since then it has evolved through some iterations, including Aschoff’s reticuloendothelial system; to our current knowledge and classifications. The mononuclear phagocyte system (MPS), a broadly encompassing classification termed four decades ago by van Furth and colleagues, describes three types of highly phagocytic cells: monocytes, macrophages and dendritic cells [2]. MPS are later found to be differentiated from a common bone marrow (BM) progenitor with no granulocyte potential, the clonogenic macrophage dendritic cell precursor (MDP) [3-5]. MDPs can further differentiate to more restricted common DC progenitors (CDPs) [6] and common monocyte progenitors (CMOPs) [7], the precursors for cDCs and monocytes/macrophages respectively. Functionally, they are collectively involved in innate and adaptive immunity, maintenance of tissue homeostasis and recovery from tissue injury or insult [8, 9].

1.2 Dendritic cells

Dendritic cells (DCs) are a highly heterogeneous myeloid lineage found in all tissues in an immature state. They possess an arsenal of PRRs to recognize and capture foreign antigens, with classical migratory DCs undergoing maturation *en route* to the lymph nodes (LN), losing their dendrite morphology, upregulating activation and costimulatory molecules in the process; where they are primed in LNs to activate naïve T-cells through MHC-I and MHC-II pathways. Resident DCs that do not migrate to LNs but remain in their seeded tissues for effector functions also exist. Their primary function is as a professional antigen-presentation cell (APC), resulting in the activation of naïve T-cells and initiating the start of adaptive immunity [10, 11]. Hence, they are commonly known as the bridge between innate and adaptive immunity.

DCs can be broadly classified into conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [12]. Phenotypically, all cDCs express CD24 and high levels of CD11c and MHC-II, while pDCs conversely express low levels of these markers, and are B220⁺ Ly6C⁺ Siglec-H⁺. pDCs also do not possess the dendrite morphology of cDCs, are rounded in shape like lymphocytes and have limited effector T-cell priming ability even after activation [13]. They mainly produce Type I interferons in response to viral pathogens [12, 14-16], and their development is controlled by the transcription factor E2-2 and Flt3 ligand [6, 17]. In humans, the pDC counterparts are CD123⁺ DCs, with similar functional specialization [18].

1.2.1 Conventional DCs

cDCs originate from the BM haematopoietic stem cells (HSC) and are crucially dependent on PU.1 [19] and Flt3 ligand signalling [6, 20] for their differentiation program. cDCs arise from the common myeloid progenitor (CMP) → MDP (still has macrophage/monocyte potential) → CDP and subsequently to pre-DCs [5-7, 21]. cDCs are heterogenous and can be broadly classified as cDC1 and cDC2, and further delineated into different subsets in different tissues.

cDC1s consist of CD8⁺ lymphoid or CD103⁺ non-lymphoid DCs which have superior cross-presentation abilities [22], where exogenous antigens can be presented on MHC-I pathway to CD8⁺ cytotoxic T-lymphocytes (CTL) [13]. Clec9A is a cDC1-specific marker that functions as a necrotic cell sensor [23], and aids in cross-presentation of dead cell antigens to CD8⁺ CTLs [24]. The cDC1 counterpart subset in humans, CD141⁺ DCs, have similar functions and also express Clec9A [18]. Recently, CD8⁺ cDC1s have also been reported to be able to present glycolipid antigens to iNKT cells [25]. Functionally, they produce large amounts of IL-12, which triggers expansion and IFN γ production from activated immune effector cells, particularly T-cells [13]. cDC1 development and differentiation are controlled by the master regulator IRF8 [26], and also requires other transcription factors (TF) like Id2 [27] and Batf3 [28] in a sequential manner, where their deletion or blockade leads to defects in cDC1 development [29].

cDC2 or CD11b⁺ DCs, and their human counterpart CD1c⁺ DCs [18], are the more abundant DC subset in lymphoid (except thymus) and non-lymphoid organs. Their differentiation progression are not as well-characterized as cDC1 and they appear to be more heterogenous [13]. Transcription factors important for CD11b⁺ DC development include Relb [30], Notch2 [31] and IRF4 [32], with the latter influencing functions like MHC-II expression [33] and migration [34]. Phenotypically, some CD11b⁺ DCs also express CD4 or ESAM [31], but these subsets are not distinctly delineated phenotypically like cDC1s. There is also considerably different functional specialization from cDC1s. cDC2s are neither excellent cross-presenters nor potent producers of IL-12, but rather produce IL-6 or IL-23 [13, 35]. They are regarded as the superior subset for CD4⁺ T-cell activation due to high surface expression of MHC-II molecules, compared to cDC1s [31, 33].

1.2.2 Inflammatory DCs

Inflammatory DCs, or monocyte-derived DCs, are a non-conventional DC subset differentiated from BM inflammatory monocytes upon infection, inflammation or injury [36]. They are also commonly known as Tip-DCs due to their production of TNF α and nitric oxide [37], and phenotypically express markers from both lineages, including DC markers like CD11c, MHC-II, CD11b but also the macrophage marker CD64 [13].

1.3 Macrophages

Macrophages are professional phagocytes that are found in virtually all tissues in the body. They comprise of dual origins, embryonically-seeded tissue resident macrophages (TRM ϕ) [38] and those derived from BM inflammatory monocytes – a population that is minor in steady-state but expand massively during infection or injury [39, 40]. Although well-known for their potent immune effector functions, most macrophage subsets, unlike DCs, are dually involved in a multitude of tissue-specific functions; and are crucial to the maintenance of homeostasis and tissue integrity [9]. Some examples include liver Kupffer cells (KCs) and splenic red pulp M ϕ (RPMs) which are involved in homeostatic iron recycling through clearance of senescent or damaged RBCs and scavenging of heme [38, 41, 42], as well

as lung alveolar MØ (AMs) that clear pulmonary surfactant to prevent airway obstruction and congestion [43]. A detailed phenotypical profile and functional specialization of some distinct TRMØs from different organs and tissues are summarized in Figure 1.1:

Tissue Origin	Macrophage subset	Functions	Phenotypic markers
Adipose	Adipose MØs	Control of insulin sensitivity and thermoregulation	F4/80 ⁺⁺ , CD45 ⁺⁺ , PPAR γ ⁺
Blood	Ly6C ^{lo} monocytes	Intravascular patrolling and housekeeping	CX3CR1 ⁺⁺ , Ly6C ⁺ , F480 ⁺ , CD115 ⁺
Bone	Osteoclasts	Bone resorption	Calcitonin receptor ⁺
	Bone marrow MØs	Erythropoiesis, haematopoiesis, self-renewing stem cell niches	F4/80 ⁺⁺ , CD169 ⁺⁺
Central nervous system	Microglia	Immune surveillance, neuron remodelling and survival	F4/80 ⁺⁺ , CD45 ⁺ , CD11b ⁺⁺
	Perivascular MØs	Immune surveillance	F4/80 ⁺⁺ , CD45 ⁺⁺⁺ , CD11b ⁺⁺ , CD163 ⁺⁺
Gastrointestinal tract	Intestinal MØs	Intestinal homeostasis and immune regulation to gut commensals	F4/80 ⁺⁺ , CD64 ⁺⁺ , CD11b ⁺⁺ , CD11c ⁺⁺ , CX3CR1 ⁺⁺⁺
Liver	Kupffer cells	Iron recycling, clearance of senescent RBCs, circulatory system immune surveillance	F4/80 ⁺⁺⁺ , CD169 ⁺⁺⁺ , CD11b ⁺ , CD68 ⁺
Spleen	Red pulp MØs		F4/80 ⁺⁺⁺ , Spi-C ⁺⁺ , CD206 ⁺⁺
Spleen	Marginal zone MØs	Immune surveillance	Tim-4 ⁺⁺ , CD68 ⁺⁺ , CD209b ⁺⁺ , MARCO ⁺⁺
	Marginal metallophilic MØs		CD68 ⁺⁺ , CD169 ⁺⁺ , F4/80 ⁺
	Tingible body MØs	Germinal center reaction housekeeping	CD68 ⁺⁺
Skin	Langerhans cells	DC-like, Immune surveillance and T lymphocyte interactions	F4/80 ⁺⁺ , CD11b ⁺⁺ , CD11c ⁺⁺ , Langerin ⁺⁺⁺
	Dermal MØs	Immune surveillance	F4/80 ⁺⁺ , CD11b ⁺⁺ , CD11c ⁺⁺ , CD206 ⁺⁺ , CD169 ⁺⁺
Lung	Alveolar MØs	Immune surveillance, pulmonary surfactant clearance	F4/80 ⁺ , CD68 ⁺ , CD11b ⁺ , CD11c ⁺⁺⁺ , CD206 ⁺⁺ , Siglec F ⁺⁺
	Interstitial MØs	DC maturation and activation	F4/80 ⁺⁺ , CD68 ⁺⁺ , MHC-II ⁺⁺

Figure 1.1 Phenotype characteristics and functional specialization of distinct tissue-resident macrophages. Surface marker expression levels are denoted by low (*), positive (++) and high (+++). Adapted from [38].

1.3.1. Tissue-resident macrophages

Tissue-resident macrophages (TRMØs) seed tissue-specific microanatomical niches in various organs. They are long-lived relative to other leukocytes and are generally sessile in their tissue niches. There is a high degree of heterogeneity associated with TRMØs, attributed by

ontogeny, functional specialization and organ-specific microenvironments [9, 38, 44, 45]. TRMØs are well-equipped as first-line responders and are commonly referred to as 'immune sentinels'; however unlike DCs, they also perform a multitude of crucial organ-specific homeostatic roles such as, but not limited to, neuronal pruning, iron recycling and bone formation [9, 38, 46]. Within these roles itself, further subset and functional heterogeneity or diversity is present, even in the same organ or tissue. For the past few decades, the accepted dogma proposed by van Furth, was that TRMØs are derived from BM haematopoiesis and refilled throughout adulthood via circulatory monocytes [47], despite existence of early contradictory studies [48, 49]. A series of recent ground-breaking studies have ascertained now that most tissue-specific TRMØs, during steady-state conditions, are derived from embryonic yolk sac (YS) or foetal liver (FL) origins, and maintain locally through self-renewal with minimal contribution from adult circulatory BM-derived monocytes [38, 50-55]. Notable exceptions are the gut [56] and dermis [57], which do not self-renew and are constantly replaced by BM monocytes. Additionally, the heart and pancreas also exhibit mixed ontogeny, with BM-derived replenishment occurring at a slow rate, but eventually diluting out embryonically-seeded TRMØs [54, 58, 59] over the lifespan of the organism. Recently, Sheng *et al* has reported foetal haematopoietic stem cells (HSCs) emerging from the developing foetus' structure, the aorta-gonad-mesonephros (AGM) as the source of embryonic-seeded TRMØs, instead of primitive YS erythroid-myeloid progenitors (EMPs); in most tissues aside from brain microglia and minorly in skin Langerhans cells (LCs) [60]. Three models of foetal macrophage ontogeny have been proposed according to the current schools of thought put forth by Gomez Perdiguero *et al*, Hoeffel *et al* and Sheng *et al*, as reviewed in Figure 1.2 [58]. There are two unifying agreements from these three models - firstly that the brain microglia are derived from yolk sac primitive haematopoiesis, and not from other foetal precursors nor adult BM monocytes, the result of physical circulatory separation due to the blood-brain barrier. Secondly, regardless of primitive yolk sac, yolk sac-derived foetal monocyte or foetal HSCs origins, the foetal liver is the overall convergent site for foetal haematopoiesis that will form the basis for TRMØ seeding and adult haematopoiesis.

Figure 1.2 Three proposed models of tissue resident macrophage ontogeny.

Model 1 - Gomez Perdiguero *et al* [52] - Early YS EMPs give rise to microglia and YS MØs that seed embryonic tissues and self-proliferate *in situ* to give rise to majority of TRMØs. Some early EMPs and foetal HSCs seed the foetal liver as foetal monocytes and contribute minorly to TRMØ pool in tissues.

Model 2 - Hoeffel *et al* [51] - Early YS EMPs give rise to microglia. Late YS EMPs can give rise to some YS MØs and majority of foetal monocytes that seed foetal liver and proceed to populate tissues to establish TRMØ. Some TRMØ may derive from foetal HSCs arising from AGM or early YS-derived MØs.

Model 3 - Sheng *et al* [60] - Early YS EMPs give rise to microglia. Late YS EMPs can give rise to some YS MØs, and may also seed foetal liver, whose foetal monocytes migrate to tissues to establish TRMØ together with early YS-derived MØs. AGM-derived foetal HSCs migrate to foetal liver and predominately seed embryonic tissues to establish majority of TRMØs. Red arrows represent the proposed primary differentiation pathways, while black arrows represent possible alternative pathways in the respective models. YS – yolk sac, HE – hemogenic endothelium, AGM - aorta-gonad-mesonephros, EMP - erythroid-myeloid progenitors, HSC - haematopoietic stem cells, MO – monocytes. Adapted from [58].

1.3.2 Origins of tissue-resident macrophages

In foetal haematopoiesis, there are several spatially, temporally-separated and functionally distinct waves that give rise to myeloid precursors of different ontogeny and lineage potential [58, 61, 62]. The first ‘primitive’ wave occurs from extra-embryonic yolk sac origins at E7.5 and generates early EMPs that differentiate to YS MØs without monocytic intermediates, and strong evidence from multiple groups suggest; is the origin of brain microglia. A second ‘transient definitive’ YS wave on E8.5 then generates late EMPs that are c-Myb-dependent and migrate via circulatory system to foetal liver (FL) at E9.5. These FL precursors have an expanded differentiation repertoire, including MPS and granulocytes, and initiate intra-embryonic haematopoiesis [46, 51, 52, 55, 58, 61, 63]. Foetal HSCs from the para-aortic splanchnopleura (P-Sp) and aorta-gonad-mesonephros (AGM) regions lastly arrive in FL at E10.5, complementing c-Myb⁺ late EMPs and heralding the next wave of definitive haematopoiesis, with multi-lineage and long-term reconstitution potentials [46, 55, 58, 60, 61]. It is currently not certain if early and late EMPs are differently-originated progenitor populations or distinct maturation/transitions of YS-derived progenitors, which are eventually programmed by the FL tissue microenvironment. All these myeloid, lymphoid and erythroid progenitors of differing origins converge in FL to collectively contribute to foetal tissue-resident MØ differentiation and seeding of organs between E12.5 to E14.5, before transition to adult haematopoiesis beyond E16.5 in BM and spleen [51, 54, 55, 58, 61]. The seminal studies involved in elucidating these very precise temporal waves have enabled the development of valuable experimental tools to further study developmental ontogeny and haematopoietic processes in embryo and adult mice, such as fate-mapping models.

1.3.3 Fate-mapping models for macrophage ontogeny

Fate-mapping models to track MØ ontogeny mostly utilize the tamoxifen-inducible (TAM) Cre-recombinase system, under the control of endogenous cell or lineage-specific promoter of interest, coupled to a floxed reporter gene controlled by ubiquitous reporters like Rosa26 [55, 64, 65]. Some fate-mapping models are shown below in Table 1.1:

Fate-mapping model	Ontogeny labelled
<i>Runx1</i> ^{MerCreMer} [63]	Early YS progenitors Late YS EMPs HSCs
<i>Csf1r</i> ^{MerCreMer}	YS EMPs YS MØs
<i>Tie2</i> ^{MerCreMer}	YS EMPs Foetal HSCs
<i>Flt3</i> ^{Cre} [52]	FL HSCs
<i>Myb</i> ^{-/-} [46, 53]	Late YS EMPs
<i>Kit</i> ^{MerCreMer/R26} [52, 54, 60]	Late EMPs HSCs
<i>S100a4</i> ^{Cre} [51]	Fetal monocytes

Table 1.1: Fate mapping mouse models and their labelled cell subsets.

Fate mapping allows studies to be performed in precise temporal windows after TAM administration, a powerful advantage for ontogeny tracking in embryonic tissues where differentiation and developmental events are specifically timed. However, a disadvantage would be incomplete labelling - whilst never reaching 100%, in some models even remain particularly low (<20%), which could result in underreporting or misinterpretation of results.

1.3.4. Transcriptional factors and cytokines in TRMØs development and differentiation

TRMØs generally have a common surface phenotype - CD11c^{lo} CD11b⁺ F480^{hi} MHCII⁺, with some exceptions (i.e. CD11c^{hi} CD11b^{lo} lung AMØ) and additional marker variations (i.e. CD169^{hi} liver KCs) between different tissues and organs. The cytokine macrophage-colony stimulating factor or

colony-stimulating factor 1 (M-CSF/CSF1) is critical in the differentiation, self-renewal capability and survival of TRMØs as mice deficient in M-CSF have a multitude of myeloid deficiencies and dysregulated homeostatic functions [38, 66, 67]. Other tissue-specific cytokines such as GM-CSF and IL-34 are critical for lung AMØ and microglia/skin LC development [46, 68-70]. IL-4 and a T_h2-inclined microenvironment also promotes global TRMØ local proliferation and expansion without involvement of inflammatory monocytes [46, 71, 72].

PU.1 is a transcription factor (TF) well-known as the master regulator of myeloid and B-cell differentiation [73]. It is regulated by *RUNX1* and high concentrations favour myeloid progenitor fate towards downstream macrophage and DC progression by acting on *Csf1r* and *Flt3* respectively [19, 74, 75]. *Csf1r* encodes the receptor for *Csf1* and IL-34, indispensable cytokines for MPS cell survival, proliferation and maintenance, with the latter exerting pro-survival effects specifically on microglia and LCs [70, 76]. Yolk sac precursors and MØs that seed microglia/LCs, and the source of primary waves of progenitors migrating to foetal liver, an integral event in foetal haematopoiesis, are dependent on PU.1 and *Csf1r* for development [63, 77]. Additional TFs then act on and through PU.1, to shape the myeloid lineage differentiation pathways – but the core macrophage lineage program deduced as part of the Immunological Genome (ImmGen) Project, generally consists of CD14, CD64, CX₃CR₁, PRRs, Fcγ receptors, Sirpα, AIF1, MerTK and F4/80 [78-80]. From here, niche-specific signals and epigenetic landscaping further act on TFs in different tissues and organs to determine TRMØ phenotype, function and transcriptional profiles; wherein it is also possible to have more than one distinct niche within an organ [45, 81].

Spi-C, a PU.1-related transcription factor is indispensable for splenic RPM development, absence of which renders mice devoid of RPM and hence, defects in erythrocyte clearance and iron recycling [42, 77, 82]. The transcription factor liver X receptor, LXRα is crucial for marginal zone (MZM and MMM) MØs development, differentiation and renewal in prenatal and adult environments [77, 83]. LXRα-deficient mice have altered splenic MZ

microarchitecture, displaced MZ B-cells and increased susceptibility to certain bloodborne pathogens [84]. In the absence of MZ MØs, Ly6C^{hi} and Ly6C^{lo} inflammatory and resident monocytes can refill the empty niche, however the possibility of replenishment from embryonic precursors should not be excluded [84]. Marginal metallophilic MØs also require lymphotoxin $\alpha\beta$ and follicular B-cells for their presence at the marginal zone [41, 85].

Liver KCs also require LXR α and Spi-C for functional specialization, as they have overlapping functions with splenic RPMs in RBC clearance and iron recycling [83, 84]. Recently, Id3 has also been found to be necessary for KC development and its functions can be compensated to some degree by Id1 [78]. Lung alveolar MØs are dependent on GM-CSF for differentiation and development from foetal monocytes upon birth [68, 78, 86]. GM-CSF also induces expression of PPAR γ which in tandem, are required for proper acquisition of AMØ function [87].

1.4. Monocytes

Monocytes are perhaps the most versatile and mobile cells of the MPS, with the capacity to rapidly generate heterogenous subsets of macrophages and dendritic cells, depending on the specific insult or inflammation. Monocyte development, like TRMØs, is critically dependent on M-CSF [50]. Two major subsets have been identified in the blood with morphological, phenotypically and functional differences, the Ly6C^{hi} and Ly6C^{lo} monocytes. Ly6C⁺ inflammatory monocytes require expression of CCR2, and its ligands CCL2 and CCL7, for egress from the bone marrow, and respond rapidly in large quantities to infection or injury [50, 88, 89]. Inflammatory monocytes can also be quickly dispatched from the spleen which serves as a reservoir for monocytic precursors [90]. Ly6C^{lo} CXCR3⁺ 'resident' monocytes, which are normally differentiated from Ly6C^{hi} blood monocytes in steady-state but can also arise directly in the BM; patrol the endothelium and do not display the same propensity for homing to inflamed tissues. An intermediate monocyte population with characteristics of both subsets have also been identified, although it is still uncertain if they are a separate subset or a transitional stage. [50, 91, 92]. Human counterparts to murine monocytes are CD14⁺ classical and CD14^{lo} CD16⁺ non-classical monocytes, and the intermediate

population has also been reported [50, 93]. Signals that govern monocytic mobility, extravasation and differentiation into either DC or MØ are largely dependent on local tissue and inflammatory milieu [3, 4, 88, 89].

1.5. Malaria: A Global Crisis

Malaria is a devastating disease and global crisis that has afflicted mankind for thousands of years [94]. The 2017 WHO Malaria Report detailed an estimated 216 million cases and 445000 deaths, with 91% of mortality from the African region and the remaining from Southeast Asia. Most deaths (70%) are attributed to children under the age of 5, with 285000 cases recorded in 2016 (WHO Report 2016, 2017). Geographically, it is still largely endemic in regions of Sub-Saharan Africa (90%) with rising prevalence in Southeast Asia (7%) and Eastern Mediterranean regions (2%) (Figure 1.3). Although the incidence of infections has declined 18% from the last decade due to preventive and ACT therapy, some regions have experienced a surge in cases (WHO Report 2017), and malaria remains a significant global burden due to severe pathology imparted by a multitude of malarial-associated syndromes and complications. Moreover, the recent emergence of multidrug-resistant *Plasmodium* strains to ACT, with Southeast Asia as a hotspot, is worrying (WHO Report 2017).

Longstanding global efforts at developing an effective malaria vaccine have been futile, largely due in part by the complexity of the causative organism and host immune responses [95]. Hence, uncovering deeper aspects of the correlates of protection in *Plasmodium* infection may reveal new insights into parasite-host relationship, and would be valuable in the ongoing quest to control and eradicate this disease.

Figure 1.3 Countries endemic for malaria and estimated malaria cases in 2016 by WHO regions. Map and schematics from WHO Malaria Reports 2016 and 2017 showing malaria distribution and prevalence. AFR – African, SEAR – Southeast Asia, EMR – Eastern Mediterranean, WPR – West Pacific, AMR – Americas regions.

1.5.1 Life cycle of *Plasmodium* in mammalian hosts

The causative agents of malaria are the intracellular, apicomplexan parasites of *Plasmodium spp*, transmitted by female *Anopheles* mosquitoes [94]. Six species are known to infect humans: *P.falciparum*, *P.vivax*, *P.knowlesi*, *P.malariae*, *P.ovale* and *P.cynomolgi* [96]. *Plasmodium spp* have a complex three-stage sexual and asexual life cycle involving two hosts, illustrated in Figure 1.4 [94, 97-100]. *P.falciparum* remains the most widespread infection, particularly in Sub-Saharan Africa and causes the most severe disease and complications. However, *P.vivax* is a rising epidemic with a larger geographical distribution and is the predominant infection outside of Africa (36%); imparted by higher parasite fitness in more temperate regions. More than 50% of *P.vivax* cases are found in Southeast Asia (WHO Report 2016).

The infection is initiated when a *Plasmodium*-infected female *Anopheles* mosquito takes a blood meal, injecting a small amount of *Plasmodium* sporozoites residing in its salivary glands, into the skin of the host (Figure 1.4, left). Some of these motile sporozoites successfully encounter a blood vessel and migrate to the liver through systemic circulation, a process that only takes a few minutes [101, 102]. In the liver, they initiate the pre-erythrocytic or liver-stage infection. Sporozoites attach to the endothelium via surface moieties like proteoglycans found on hepatocytes and cross the endothelium layer [102]. There, they transmigrate through Kupffer cells, liver sinusoidal endothelial cells and hepatocytes, seeming to choose their target host cell, before settling in one of them, inside a parasite-specific organelle known as the parasitophorous vacuole (PV) [101-105]. There, asexual replication commences to give rise to thousands of merozoites. Close to maturation, the PV membrane ruptures and merozoites await in the host cell for release via budding of host cell membrane-enclosed vesicles, known as merozoites (Figure 1.4, right), in a bid to avoid immune detection by Kupffer cells [101, 102, 105]. Some species like *P.vivax* and *P.ovale* enter an additional dormant hypnozoite stage which is linked to reoccurring infection [96, 106]. Merozoites are shuttled out of the liver into the circulation, reducing in size due to circulatory shear forces and arrive in the lung, where they arrest in blood capillaries and finally rupture, releasing free infectious merozoites into lung microvasculature [101, 105]. It was proposed that this mode of action allows merozoites to infect nearby erythrocytes accumulated due to packing of capillaries with large merozoites, facilitating the transition into symptomatic blood-stage infection quickly [105].

Merozoites infect erythrocytes and go through distinct stages during blood-stage infection; from rings to trophozoites to mature schizonts, which rupture to give rise to more merozoites. Multiple rounds of replication occur, and number of parasites in the blood increase exponentially [106, 107]. Some merozoites undergo sexual reproduction to produce gametocytes, which upon uptake by the next feeding mosquito, mature inside the midgut to give rise to ookinetes and oocytes. Sporozoites are then generated by meiosis and migrate into mosquito salivary glands, completing the *Plasmodium* life cycle for the next round of infection (Figure 1.4, left) [107, 108].

Figure 1.4 Life cycle of the *Plasmodium* parasite. Infection begins when a female *Anopheles* mosquito injects sporozoites (Spz) residing in its salivary glands, into the host whilst taking a blood meal. Some of the sporozoites encounter a blood vessel and migrate to liver via circulation. There they transmute through the endothelium layer to reach host hepatocytes, invade and multiply exponentially in a parasite-specific organelle known as parasitophorous vacuole (PV), beginning the liver stage infection. Upon PV rupture, merozoites are budded off, encapsulated in host hepatocyte membrane in merozoites, to avoid detection by immunosurveillance tissue resident Kupffer cells, enter the bloodstream and travel to the lung microvessels. Merozoites rupture to release thousands of merozoites that infect RBCs and initiate the blood stage infection. Multiple rounds of asexual replication in progression from early ring stage → trophozoites → schizonts results in lysis of RBCs and exponential increase of merozoites. Some of the ring stages deviate from the cycle to undergo sexual reproduction that give rise to gametocytes, which are taken up by another feeding mosquito. There the parasite gametocyte matures in the *Anopheles* midgut into sporozoites and restarts the life cycle in a new host. Diagrams are adapted from [101] and UC Riverside (<https://phys.org/news/2013-12-scientists-chemical-modification-human-malaria.html>).

1.5.2 Animal models of malarial disease

Animal models have historically been indispensable to human understanding of disease, physiological processes and pathological disorders. In malaria, human studies are traditionally limited to postmortem or clinical samples that may be subject to individual variation or restricted to certain tissue types or blood only. Hence, murine and non-human primate (NHP) models have helped tremendously to further our understanding of this complex disease [109, 110].

Three murine *Plasmodium* species and their strains are commonly used to study malaria: *P.berghei*, *P.yoelii* and *P.chabaudi*. *P.berghei* ANKA (*PbA*) is the most commonly used strain for studying experimental cerebral malaria (ECM), with pathology similar to cerebral malaria (CM) in humans [111].

Unfortunately, due to high mortality rate of *PbA*-infected mice, this strain is generally not suitable for study of acute or humoral immunity, although a recent study has investigated humoral aspects of germinal center (GC) B-cell and antibody production in *PbA* [112]. *Plasmodium chabaudi chabaudi* (*Pcc*) and *Plasmodium yoelii* (*Py*), which cause chronic and acute malaria respectively, are both well-utilized murine models to study acute and humoral immune effector responses [110, 113]. A large body of literature about anti-*Plasmodium* immunity has been produced from *Pcc* spanning early innate recognition [114] to memory recall responses [113, 115]. Susceptibility or resistance to *Plasmodium* species is also dependent on genetic background: C57BL/6 mice are commonly used for *PbA* ECM studies due to high susceptibility to neurological symptoms, while with *Pcc* they are resistant and allow for insights into acute and chronic phase infection. Other pathogenic malaria syndromes can be studied with specific *Plasmodium* /host combinations, i.e. *PbA* and DBA/2 mice for acute lung injury model [98, 113].

In addition to the wealth of blood-stage infection studies, murine models have also contributed in elucidating liver-stage infection, once believed to be immunologically silent; but in actuality triggers potent innate [116], CD8⁺ cytotoxic T-lymphocyte (CTL) [117] and humoral immune responses [118]. The mosquito-borne sporozoites required to initiate liver-stage infection more closely mimics the natural route of infection, and attenuated sporozoites have also been utilized as potential malaria vaccine targets for long-term correlates of protection [119].

However, there are still definite caveats about mouse models in translation to human malaria, largely due to incomplete emulation of human disease, relevant extrapolation of immune responses and the divergent genetics of both human and murine *Plasmodium* spp., as well as host species [110, 120].

NHP models have the advantage of closer genetic similarity and disease progression to humans than mice. *P.cynomolgi* closely mimics human *P.vivax* infection in macaques [121], allowing studies into this emerging *Plasmodium* species that is currently unculturable, and *P.knowlesi* infects both primates and humans [109]. *P.coatneyi* infection in macaques result in

severe malaria that is similar to human *P.falciparum* infection, characterized by overlapping syndromes like cerebral malaria, deep vasculature sequestration, anemia, renal and metabolic dysregulations [122, 123]. Some NHPs of the *Aotus* and *Samiria* spp. can also be infected with adapted strains of the most prevalent human parasites *P.falciparum* and *P.vivax*, developing both liver and blood-stage infections [123, 124]; allowing for perhaps the closest comparisons and extrapolations to *bona fide* human malaria. NHP models are also valuable as human co-infections (e.g. HIV and malaria) are common in endemic regions, and their study can be mimicked with SIV / *Plasmodium* spp. co-infection [125]. Recent work in whole-genome sequencing [126], emerging research platforms [123] and availability of immune characterization tools [127, 128] of members of the *Macaca* family have also aided in NHP-based research. However, NHP models have greater technical, cost and logistics constraints compared to mice; and usually also require special ethical regulations, hence widespread NHP models remains to be unfeasible for many malarial research facilities.

To circumvent the technical, financial and ethical issues faced by NHP models, humanized mouse models; usually created in an immuno-deficient background, provide an alternative resource. Humanized mice with human hepatocytes [129], CD8⁺ CTLs [130] and rudimentary haematopoietic or immune systems [131], have been developed and utilized successfully in investigating both liver and blood-stage infections; as well as liver-to-blood stage transmission in *P.falciparum* and *P.vivax* [123, 132]. Vaccine candidate studies using attenuated sporozoites can also be performed in humanized mice [120], but with less of the translational issues faced by pure murine models for preclinical data. However, their greatest current limitation lies in the lack of stable, self-renewing haematopoiesis to generate human blood and immune cells in reconstituted humanized mice [133], rather than short-term solutions like repeated RBC perfusions [134]. Without these models, insights into long-term humoral immunity towards human parasites, or blood-stage infection studies of the unculturable, reticulocyte-trophic *P.vivax* still cannot be achieved.

1.5.3 Human models and studies of malaria

Although many of the experiments and procedures performed in animal models to dissect the intricacies and mechanisms of *Plasmodium*-specific immunity cannot be emulated in humans due to obvious ethical or technical issues, long-standing human studies have also provided indispensable data. Experimental malaria in humans, known as controlled human malaria infections (CHMI), have been carried out since the 1960 – 70s [135], and can be either liver or blood-stage depending on induction by sporozoites or infected RBCs [136, 137]. In more recent years, CHMI have been greatly focused on clinical trials for testing potential vaccine candidates and their efficacy, or live sporozoite inoculation under anti-malarial drug cover; with the ultimate goal of a commercially-available malaria vaccine.

Liver-stage sporozoite infection in humans, like mice, is also potentially immunogenic and allows for development of robust anti-*Plasmodium* immunity [138, 139]; while subduing the symptomatic blood-stage infection by prophylaxis. CHMI and naturally-acquired infections have also elucidated the presence and involvement of T_{H1} $CD4^+$ proinflammatory responses, $CD8^+$ CTLs, follicular T helper cells and *Plasmodium*-specific humoral immunity as core correlates of protection in humans [138, 140-142], correlating well with animal models. Sterile immunity, the optimal endpoint in vaccination, has been achieved using irradiated or attenuated *P.falciparum* sporozoites (e.g. Sanaria PfSpz) [143, 144] and specific liver-stage antigens (e.g. circumsporozoite protein, CSP) under CHMI conditions [145], but this still remains to be replicated in the field with long-term effectiveness in naturally endemic regions [146]. The RTS,S vaccine, comprising a *P.falciparum* CSP protein fragment coupled to hepatitis B antigen, is a prime example, where field clinical trials underperformed with only 30 – 50% efficacy compared to preclinical immunization data, and subsequent yearly decreases in durability of protection [137, 147, 148].

Nevertheless, it is still invaluable as RTS,S/AS01 is currently the only malaria vaccine candidate to enter Phase III clinical trials [149]. Very recently, PfSpz attenuated sporozoites have also begun Phase I field studies in Mali, with some promising results (30 – 40% efficacy) relative to other similar clinical trials [150]. Hence, the human data generated by CHMI

or field studies, with compliment from animal models; continue to be critical in the future advancement and optimization of RTS,S, as well as other potential malaria vaccines.

1.5.4 Clinical manifestations of malaria

Malaria is a major multifactorial systemic disease that presents with a variety of clinical manifestations, and most mortality is associated with severe complications. The liver-stage infection is known to be clinically silent and asymptomatic [97]. Clinical symptoms, manifestations and complications arising from the blood-stage infection are largely due to massive destruction of infected RBCs, sequestration of infected RBCs in organ or tissue microvasculature, inflammation arising from cyclic rupture of schizonts into bloodstream and release of toxic parasite products from lysis of host RBCs [103, 107, 120, 151].

P.falciparum causes the most severe disease in humans, with complications such as CM, which upon onset often leads to quick death. Clinical symptoms include multi-organ failure, anaemia, ataxia, seizures, cerebral haemorrhage and coma. Most victims are children aged five years or below, and it is known to be caused in part by immunopathogenic hyper-inflammation due to deep vascular sequestration of iRBCs [110, 152].

Aside from CM, other serious manifestations include severe malarial anaemia (SMA) and malaria-associated acute lung injury, or the more severe acute respiratory distress syndrome (MA-ALI/ARDS). Other complications include, but are not limited to, kidney/liver failure, placental malaria, metabolic acidosis, high fever and thrombocytopenia. With the exception of CM, these complications have also been documented in *P.vivax* and *P.ovale* malaria. [98, 110, 153, 154].

1.6 Structure of the spleen

The spleen has a unique trabecular architecture demarcated by distinct regions, divided into the red pulp (RP) and white pulp (WP), separated by the marginal zone (MZ) [155-157]. Each region has intrinsically distinct functions, the RP is involved in senescent RBC and iron recycling, the WP as the inductive site of adaptive immunity, and the MZ, a boundary between the circulation and red pulp, is a key region for blood filtration and phagocytic activity. Blood flows into the spleen via the central arterioles and branches out into smaller capillaries, flowing through the MZ and into the RP [155, 158, 159]. Not all blood will enter this region, in fact most of it directly drains into efferent veins and exits the spleen, a system known as closed circulation. The remainder is slowly filtered through the MZ-RP regions ('open circulation') and subjected to the scrutiny of splenic myeloid cells, which efficiently capture foreign antigens and pathogens [156, 159, 160].

The spleen, being a key lymphoid organ, contains a diverse repertoire of immune cells in all microarchitectural regions, including tissue resident macrophages, monocytes, DCs, T and B-cells. F4/80^{hi} red pulp macrophages (RPMØs) reside in the RP and are functionally specialized in homeostatic iron recycling and erythrophagocytosis of senescent or effete RBCs [38, 41, 42, 161]. Murine RP is also a site for erythropoiesis and is especially important during infections when anemia is induced [162]. The white pulp houses a plethora of DCs, macrophages, T-cells and B-cells. Distinct T and B-cell zones separate T and B lymphocytes [41, 156, 161, 163]. Follicular DCs are found with immature B-cells in B-cell follicles and aid in their development. During induction of immune responses, these B-cell follicles become sites of B-cell maturation, expansion and antibody production, known as germinal centers (GCs) [163, 164]. Tingible-body MØs, white pulp MØs and conventional CD8⁺ or CD4⁺ DCs are also found in the WP [165]. In the marginal zone, two subsets of tissue resident MØs are found, SIGNR1⁺ MARCO⁺ marginal zone MØs (MZM) found in the outer MZ and CD169⁺ marginal metallophilic MØs (MMM) in the MZ sinus, facing the WP [38, 41, 155, 161, 166]. Additionally, MZ DCs and T-independent, IgM-producing MZ B-cells are found together with MMM and MZMs [155, 167, 168]. All MZ immune subsets are strategically positioned to detect or

capture bloodborne foreign antigens (Figure 1.5). Overall, the diverse splenic resident immune cell populations, microanatomical structure and high volume of blood circulation makes the spleen the primary site for induction of immunity to, and its importance to bloodborne infections, including malaria [161, 169].

Figure 1.5 Distinct microarchitectural regions in the spleen and their unique immune cell subsets. The spleen is compartmentalized into red pulp, marginal zone and white pulp regions. Iron recycling, erythro-phagocytotic F480^{hi} red pulp (RP) macrophages are abundant in RP. The marginal zone separates white pulp (WP) from RP and contains MZ B-cells, MZ DCs and two subsets of macrophages – SIGNR1⁺ marginal zone macrophages (MZM) are found in the outer MZ ring and CD169⁺ marginal metallophilic macrophages (MMM) in the inner MZ sinus facing WP. WP contains B and T-cell regions, follicular DCs that maintain B-cell zones and MFG-E8⁺ tingible-body macrophages that engulf apoptotic cells during rapid germinal center expansion. Diagrams are adapted from [38].

The microanatomical structure of human spleen differs from the mouse, although immune cell subsets and the open/closed circulatory systems are similar (Figure 1.6). Human spleens are sinusoidal, do not have a defined MZ sinus, and have an additional perifollicular zone [170-172]. This additional layer of mechanical filtration removes damaged, abnormal or infected RBCs, whose altered deformability would prevent their squeezing through the narrow endothelial slits of RP venous sinuses to rejoin the circulating blood. No evidence so far has supported presence of erythropoiesis in human spleen, unlike in mice [170-173].

Figure 1.6 Differences in splenic microarchitecture between human and mice. Human spleen has perifollicular zone and an undefined marginal zone unlike mice. Venous sinusoids in red pulp (RP) allow additional layer of mechanical filtering for circulating blood. DC – dendritic cell, FDC – follicular DC, RBC – red blood cell, Ret – reticulocytes, MZM – marginal zone MØ, MMM – metallophilic MØ, PALS – periarteriolar lymphoid sheath. Diagrams are adapted from [172] .

1.6.1 Importance of the spleen in malaria

The distinct microanatomical structure of the spleen as discussed prior is designed to retain and filter blood, allowing ample opportunity for immune cells to contact with any foreign substances or pathogens to initiate appropriate immune responses. During malaria infection, the spleen undergoes massive and significant architectural changes such as disruption of well-demarcated marginal zone, diffusion of T-cell zones/B-cell follicles and extra-follicular plasmablast foci formation [169, 174-177].

Two very early studies have observed reduced trapping of *P.yoelii* parasites in the spleen during pre-crisis period, characterized by fibroblast barrier formation, splenomegaly and extramedullary haematopoiesis, and later massive destruction of iRBCs when blood parasitemia declines [160, 178]. This response has beneficial and detrimental effects on anti-*Plasmodium*

immunity – blockade of open circulation results in no blood flow to the phagocyte-rich RP, which decreases macrophage-mediated erythrophagocytic clearance of parasites and increased percentage blood parasitemia [179]. However, closed circulation allows for containment of *Plasmodium* parasites, proinflammatory cytokines, toxic metabolic or cytolytic byproducts and hence, protection from immunopathology. The barrier also physically separates parasites from the developing adaptive immune responses in WP, and RP extramedullary haematopoiesis which replenishes the RBC pool, preventing disruption of T/B-cell maturation and infection of developing RBCs that would exacerbate the disease [160, 178]. Later studies by Krucken *et al* also observed closed circulation in *P.chabaudi* infection, limiting the iRBCs accessible to RPMØs, but was still accompanied by efficient destruction of iRBCs [175].

In murine and human malaria, iRBCs containing parasites results in altered deformability. Mechanical trapping and pitting, a phenomenon where foreign material is expelled from iRBCs attempting to squeeze through RP, are also mechanisms of parasite control in the spleen [180, 181]. Highly phagocytic RPMØs, abundant in large numbers in RP, also contribute to clearance of iRBCs if open circulation permits their contact with iRBCs [161, 182]. Although they are less phagocytic than RPMØs, MZ MØs also ingest bloodborne particulates, and their close proximity with other immune cells could enhance antigen presentation either directly or indirectly [183-185]. cDCs are professional APCs with the advantage of high mobility, unlike rather sessile tissue resident macrophages, and migrate into lymph nodes or white pulp where they activate adaptive immunity players like T_{h1} proinflammatory CD4/CD8 effector T-cells and B-cells [186-188]. In the later stages of *Plasmodium* infection, parasite-specific antibodies generated in splenic WP germinal centers and extrafollicular foci are required for control and clearance of parasites to subpatent levels [189-191].

The importance of the spleen in murine and human malaria is emphasized by splenectomy studies or patients, whom displayed overall impairment of parasite clearance, higher blood parasitaemia and delayed induction of anti-*Plasmodium* immunity [192, 193]. In the *PccAS* murine model, although

splenectomized mice are able to eventually generate anti-*Plasmodium* Ig, they were still susceptible to recurrent infections [194].

1.7 Immunity to malaria

1.7.1 Liver-stage anti-*Plasmodium* immunity

While the blood-stage *Plasmodium* infection is symptomatic and immunogenic, the liver-stage infection in contrast is asymptomatic. Although sporozoite immunization have been shown to effectively induce immunity and protection against re-challenge [118, 195], liver-stage infection via the natural route of mosquito transmission was thought to be relatively immunologically silent. However, emerging evidence are revealing that active and robust immunological responses are generated during liver-stage infection. Innate immune responses characterized by Type I IFN and IFN γ are observed in the liver following *P.berghei* and *P.yoelii* sporozoite infection, which aided in reducing parasite load in infected mice [116, 196]. Interestingly, IFN γ -producing NKT cells were found to be the main cell subset responsible for early control of liver-stage parasites [196]. Type I IFN and IFN γ production also promotes monocyte / macrophage involvement, and liver Kupffer cells are known to clear liver-stage parasites [197, 198]. Sporozoite immunization studies have also largely contributed to elucidating the role of adaptive CD8⁺ CTLs [199-201] and tissue-resident memory T-cells [117] in killing and protection against liver-stage infection, wherein in some circumstances sterile immunity can be achieved [202]. As such, potent anti-*Plasmodium* immunity and vaccination strategies could be potentiated by targeting the liver stage infection.

1.7.2 Blood-stage anti-*Plasmodium* immunity

Immune responses to blood-stage *Plasmodium* are complex, and protection ultimately involves both cell-mediated and humoral immunity. Much of the work on elucidating immunological mechanisms in *Plasmodium* infection has been established by Langhorne, J, through the *P.chabaudi* model that allows for both acute and chronic phase studies.

In general, malaria is associated with strong T_{H1} proinflammatory responses that are crucial for initial parasite control. One of the key cytokines involved

is IL-12, which has stimulatory and proliferative effects on both innate and adaptive immune cells. IL-12 is crucial for the induction of both innate and adaptive immunity, acting on natural killer (NK) cells, macrophages and T-cells. IL-12 mediates its effects through IFN γ , the hallmark cytokine of T_{H1} responses. IL-12 and IFN γ operate in a feed-forward loop that will eventually have compounding influences on downstream effector responses [98, 191, 203-205]. IFN γ KO or IL-12KO mice have significantly higher parasitaemia and mortality rates during acute infection [191, 203]. Interestingly, Type I IFN, which is important for liver-stage immunity, has a converse immunosuppressing effect on blood-stage immunity by dampening CD4⁺ T_{H1} proinflammatory responses [206, 207] and promoting IL-10-producing, anti-inflammatory T_{H1} cell differentiation, a finding also reported in humans [208]; highlighting the complexity of anti- *Plasmodium* immunity.

Dendritic cells (DCs) have been extensively studied and known to be involved in various facets of anti-*Plasmodium* immunity for nearly the past two decades [188, 209, 210]. Splenic conventional DCs (cDCs) are the initial responders to bloodborne *Plasmodium* infection possessing a multifunctional repertoire, participating in early parasite clearance by phagocytosis [211] and later on as crucial activators of naïve T-cells and B-cells by initiating the adaptive immune response via antigen presentation [212]. It is well-established that IL-12 derived from cDC1s activates innate immune effector cells such as NK-cells and $\gamma\delta$ T-cells to produce IFN γ [213-216], perpetuating the IL-12/ IFN γ feed-forward loop [191, 217] and initiating IFN γ -dependent downstream cascades that eventually activate macrophages, T-cells and B-cells; whose concerted efforts are required for *Plasmodium* parasite killing, clearance and resolution of infection [203, 204, 218, 219]. cDC2s, which are significantly lesser producers of IL-12, and hence not as efficient at inducing proinflammatory responses; are potent at priming CD4⁺ T-cells instead [188, 220]. pDCs, which do not possess the same functionality of conventional DCs, also expand during *Plasmodium* infection but the extent of their contribution is unclear. However, production of IFN α , a potent inhibitor of IL-12; and IL-10 by pDC suggests anti-inflammatory roles [212, 221, 222].

IFN γ , regulated by IL-12 produced from cDC1s, is the central mediator molecule involved in anti-*Plasmodium* immunity, particularly in induction of CD4⁺ T_h effector responses crucial for controlling both acute and chronic infection [203, 218, 223]. During *PccAS* infection, immune responses exhibit distinct kinetics; a predominantly T_{h1} proinflammatory environment during acute phase infection for rapid clearance of parasites, which subsequently wanes and transits to humoral immunity and antibody production in the chronic phase, required for long-term protection [190, 224-229]. CD4⁺ T-cells are also crucial in the protection against *Plasmodium* infections, as key producers of IFN γ and activating phagocytes like macrophages. Later in the infection, they provide T-cell help to B-cells and direct *Plasmodium*-specific antibody production. Mice depleted of CD4⁺ T-cells in acute phase infection exhibit impaired responses for parasite clearance and higher parasitaemia [98, 205, 226, 227, 230]. Additionally, the cDC1-mediated IL-12 / IFN γ axis can also promote expansion of CD8⁺ CTLs that contribute to some extent in parasite killing, but are not as functionally indispensable as CD4⁺ T_h cells [231, 232], and have immunopathogenic implications in some animal models (e.g. ECM) [233].

The waning of the acute T_{h1} response and transition to humoral immunity is heralded by a decline in peak parasitaemia, decrease in proinflammatory cytokines, the shift to B-cell antibody production and induction of protective immune memory. Coinciding with this time-frame, proinflammatory IL-12-producing CD8⁺ cDCs start to undergo apoptosis, and CD8⁻ DCs that specialize in induction of humoral immunity become the major DC component [172, 225, 234].

The classical paradigm of anti-*Plasmodium* immunity proposed by Mosmann and Coffman [235], and supported with seminal work from Langhorne [227, 236, 237], describes biphasic T_{h1} to T_{h2} responses, heralded by its hallmark cytokines IFN γ and IL-4; as the infection transitions from acute to chronic phase [238, 239]. However, with advancing insights into T-cell plasticity and functional subsets, recent findings present irrefutable evidence about the critical involvement of other T_h subsets, such as follicular T helper cells (T_{FH}), in establishing effective *Plasmodium*-specific responses [229, 240, 241]. As such, the classic T_{h1} - T_{h2} paradigm

is an incomplete and overly simplified view of anti-*Plasmodium* immunity, and an updated model has been proposed by Perez-Mazliah and Langhorne [225] to encompass the multi-faceted T_h immune responses during *Plasmodium* infection (Figure 1.7).

$CD4^+$ T_{FH} have garnered a great amount of research interest recently due to their previously underestimated involvement in humoral immunity, in both infection and immunization studies [242]. A rare $CD4^+$ T_h subset, their signature cytokine, IL-21 is found to be crucial in germinal center B-cell reactions, leading to downstream class-switch recombination for high-affinity antibody production and subsequent long-lived plasma cell differentiation [243, 244]. Their remarkable effector plasticity also imparts various functions and outcomes [245]. In anti-*Plasmodium* immunity, $CD4^+$ T_{FH} are now believed to be the key drivers of the protective antibody response instead of $CD4^+$ T_{h2} , whose previously described contribution is currently questionable in the light of new findings (Figure 1.7) [240, 246].

Figure 1.7 Updated $CD4^+$ T_h anti-*Plasmodium* immunity model. Activated $CD4^+$ T_h differentiate into various subsets with differing effector cytokines and proposed effector functionality during *Plasmodium* infection. Inclusion of the updated T_h subsets and their effector function beyond the old T_{h1} - T_{h2} paradigm are shown in pink rectangle. DC – dendritic cells, iRBC- infected RBC, T_h – T helper cell, IL – interleukin, T_{r1} - FoxP3-regulatory T-cell, T_{FH} - follicular T helper cell, Ig – immunoglobulin, IFN γ – gamma interferon, MØ – macrophage. Adapted from [225].

In addition to $CD4^+$ T_h , the other myeloid cells - monocytes and macrophages, are also crucial to anti-*Plasmodium* immunity in murine and

human malaria. Likewise to CD4⁺ and CD8⁺ T-cells, they are activated by IFN γ , produced by the aforementioned cells as well as from NK/NKT and $\gamma\delta$ T-cell sources [197]. Early *Plasmodium* infection triggers a rapid, IFN γ -dependent monocytic egress from the BM followed by infiltration into the spleen, where they exert their effector functions as inflammatory monocytes, or differentiate into effector macrophages [219, 247, 248], aiding the existing pool of tissue-resident macrophages. Monocytes/macrophages present a diverse repertoire of parasite clearance and killing strategies, including non-opsonic and opsonic phagocytosis, reactive oxygen species (ROS) and production of proinflammatory cytokines like IL-12, IL-18, IL-6 and tumour necrosis factor (TNF α) [197, 249-251]. During late infection when humoral immunity has been established, antibody-dependent opsonization of iRBCs and antibody-dependent cellular inhibition of parasite growth by macrophages and monocytes further contribute to overall parasite clearance [197, 249, 252]. In addition to parasite killing, specific subsets of the heterogeneous macrophage population, such as CD169⁺ tissue-resident macrophages, also control systemic and local immunopathology through anti-inflammatory mechanisms like IL-10 production [253].

Finally, long-term protection against malaria, as with many other infections, is ultimately mediated by pathogen-specific antibodies. In malaria, antibodies and humoral immune responses are crucial for clearing blood-stage infections as well as providing protective immunity to future infections. Mice deficient of B-cells are shown to be unable to clear *P.yoelii* and *P.chabaudi* infections, despite a normal T_{h1} proinflammatory response during acute infection. Memory T and B-cells produced in primary infection maintain both short-lived and long-lived anti-*Plasmodium* responses. Upon re-infection, mice have accelerated and heightened immune responses, evidenced by delayed and lowered parasitaemia onset, as well as activation of T effector memory cells and secretion of high-affinity, high-avidity *Plasmodium*-specific antibodies. A summary of the multifaceted immune effector responses in malaria infection are illustrated in Figure 1.8.

Figure 1.8 Innate and adaptive immunity to *Plasmodium* infection. Infected RBCs are recognized by DCs via PRRs and phagocytosed. DCs process parasite antigens, present them to naïve T-cells, and produce IL-12 and IL-18. IL-12 activates NK cells and T-cells towards proinflammatory T_H1 CD4 T-cells and they produce IFN γ . $\gamma\delta T$ and NKT-cells also contribute to IFN γ production, which has autocrine effects and activates macrophages. MΦs increase phagocytosis of opsonin-mediated iRBC and mediate parasitocidal activity by ROS and NO and produce proinflammatory cytokines, leading to febrile symptoms. B-cells activated by CD4 T-cells in late infection produce *Plasmodium*-specific Ig that neutralize or opsonize iRBCs/*Plasmodium* parasites to increase phagocytic clearance by MΦs. Anti-inflammatory cytokines IL10 are secreted by T_H1 CD4, MΦs and T_{reg} to curtail immunopathology from excessive inflammation or as infection resolves. Adapted from [254].

Importantly, this also holds true for humans and *P.falciparum* infection, where repeated exposure and high titers of circulating anti-*Plasmodium* antibodies contribute to clinical immunity and protection. However, sterile immunity, although achieved in CHMI and murine models, remains rare or even non-existent amongst naturally acquired infections in humans, and is attributed to many factors, such as short-lived memory responses, parasite exposure and antigen variation [174, 190, 191, 224, 228, 255].

1.7.3 Immunopathology in Malaria

Cerebral malaria, as well as experimental cerebral malaria in *P.berghei* ANKA mouse models, is a classic example of deleterious inflammation and immunopathology. ECM is orchestrated mainly by increased parasite biomass, microvascular sequestration and leukocyte infiltration into cerebral blood vessels and tissues, as a result of chemokine-recruited trafficking. The leukocytes include CD4⁺ and CD8⁺ T-cells, macrophages, NK-cells and NKT cells. IFN γ and lymphotoxin α produced by T-cells, and TNF α from

macrophages exacerbates inflammation and recruits more leukocytes. The cytolytic destruction of cross-presenting microvessels and activated endothelial cells, by perforin and granzyme B from CD8 T-cells and NK-cells, and ROS from macrophages is postulated to destroy the integrity of the blood-brain barrier (BBB), resulting in the symptoms of ECM [197, 230, 233, 256, 257]. The anti-inflammatory cytokine IL-10 can alleviate the symptoms of and protect from ECM by reducing parasite sequestration and the subsequent downstream associated immunopathology [258].

In humans, iRBC sequestration to brain endothelium via CD36 and ICAM-1 also disrupts BBB integrity. In mice, it is still debatable if this phenomenon occurs and contributes to ECM [256].

1.7.4 Immunoregulatory mechanisms in malaria

Regulation and control of immunopathology in the context of malaria is maintained during infection in a few ways. Firstly, the apoptosis of CD8⁺ DCs after ascending peak parasitaemia, and handover of CD4⁺ T-cell activation by CD8⁺ DCs, is one of the measures of the host to control excessive inflammation together with inducing humoral immunity. Secondly, through the production of IL-10, an anti-inflammatory cytokine that potently suppresses IL-12, IFN γ and TNF α . The importance of inflammatory control measures during infection is highlighted in studies with IL-10KO mice, which present with elevated IL-12, IFN γ and TNF α levels, but high mortality and anaemia, not associated with increased parasitaemia. The crucial role of IL-10 as an immunoregulatory cytokine is also underscored by observations of Sanni *et al*, where IL-10KO mice display cerebral edema and haemorrhage during a *P.chabaudi* infection, which was never documented to have cerebral effects in wildtype mice [259-262].

Altogether, immune responses to malaria are complex and multi-faceted. There is a fine balance between proinflammatory responses to control infection and to cause immunopathology, which can lead to serious complications like death of the host. The concerted efforts and precise interactions of various immune effector cell subsets are required both spatially and temporally during the progression of *Plasmodium* infection, to ensure appropriate downstream immune responses are generated,

eventually resulting in elimination of parasites and long-term clinical protection.

1.8 Cell ablation systems

Depletion of specific cell populations of interest is the best theoretical way to study their function in an experimental setting. However, in practice, this is more challenging than theorized. Clodronate liposomes was an effective method for myeloid cell depletion but does not discriminate between DCs and MØs [263, 264]. Similarly, the MAFIA mouse model using suicide drug administration ablated both DCs and MØs non-specifically by Fas-mediated apoptosis [265]. Cre-recombinase techniques heavily utilized for generating fate-mapping models [65, 266] are dependent on turnover kinetics which differ on cell types, and not very feasible for slow turnover cells like tissue-resident macrophages [58]. Hence, the diphtheria toxin - diphtheria toxin receptor (DT – DTR) system developed by Saito *et al* (Figure 1.9), remains an elegant and highly specific method for ablating cell subsets of interest.

1.8.1 DT-DTR system

This system utilizes the high sensitivity (10^3 - 10^5 more than murine DTR) of the human DTR receptor, a membrane-anchored form of the heparin-binding epidermal growth factor receptor (HB-EGF), to DT produced by *Corynebacterium diphtheriae* [267, 268]. DT has two subunits A and B, B binds to DTR causing internalization of the endosome [268, 269]. Proteolysis occurs, resulting in linkage of subunit A and B in the acidic endosomal environment, inducing subunit B to form pores in the endosome and allow release of subunit A into the cytoplasm [268]. Subunit A inhibits the activity of Elongation factor 2 involved in protein synthesis, halting the process and ultimately leading to apoptosis of affected cell [268]. Taking advantage of this difference, the human DTR construct is engineered to be controlled by cell or tissue-specific promoters of the gene/subset of interest in the mouse. Upon administration of DT, it allows for quick and specific ablation of cells by apoptosis, hence limiting necrosis-induced inflammation which would complicate interpretations [267]. DT-DTR depletion however does induce some transient neutrophilia [270] and long-term depletion loses efficacy due to immune response mounted against foreign protein DT [271], and should be considered for experimental design.

Figure 1.9 DT-DTR conditional system of in vivo depletion for cell subsets of interest. Human DTR with 10^3 - 10^5 fold higher sensitivity to DT is expressed under tissue-specific promoter or enhancer in the mouse, and cell subsets of interest are specifically and efficiently depleted upon DT administration. Adapted from [267].

1.8.2 DT-DTR models for DC depletion

The CD11c-DTR transgenic mouse model has been instrumental in establishing the roles cDCs play in, but not limited to, *Plasmodium* infection, by allowing the ablation of DCs. However, simultaneous unspecific ablation of other CD11c-expressing immune cells, including macrophages, NK-cells and some T/B-cell subsets, exacerbates disease severity and limits this model [263, 272]. Moreover, the cDC population is heterogenous and can be further delineated into CD8⁺ (cDC1), CD8⁻ (cDC2) and CD4⁻CD8⁻ subsets in lymphoid tissues; as well as CD103⁺ cDC1 counterparts in the peripheral organs. Both cDC1 and cDC2 have been established as potent inducers of effector T-cell activity, with functional bias towards CD8⁺ and CD4⁺ T-cell help respectively, hence influencing CTL responses and humoral immunity [12, 273-278]. Although newer mouse models such as Langerin-DTR and zDC-DTR that deplete less accessory cells have been developed, there still exists a gap in specifically ablating cDC subsets of interest [272].

1.8.3 Clec9A DT-DTR model for ablation of Clec9A⁺ DCs

Clec9A (c-type lectin domain family 9, member a), also colloquially known as DNGR-1, is a c-type lectin-like molecule that is highly expressed on lymphoid and non-lymphoid cDC1s. Batf3-dependent cDC1s can be classified into CD8⁺ DCs, found predominantly in lymphoid tissues such the spleen and lymph nodes; and CD103⁺ DCs present in non-lymphoid peripheral organs [23]. To bridge the gap, we recently described the Clec9A-DTR transgenic mouse, which allows for specific ablation of CD8⁺/CD103⁺ cDC1s, but not CD11b⁺/CD8⁻ DCs *in vivo*, via targeting of the highly specific cDC1 marker, Clec9A [279]. Hence, the specificity of Clec9A on cDC1s was utilized by our lab to generate the Clec9A-DTR transgenic mouse model, and upon DT administration, specific systemic ablation of Clec9A⁺ DCs could be achieved [257].

1.9 Objectives and aims of the study

Dendritic cells and macrophages are phenotypically heterogeneous and functionally diverse cells of the myeloid lineage. Both types of cells are also crucial first-line immune effectors with overlapping and unique functions, positioned at strategic locations and niche microenvironments throughout tissues. Despite the wealth of knowledge available on anti-*Plasmodium* immunity, the complexity of malaria infection warrants continuous and more in-depth study of the associated immune cell subsets, as well as their infection and turnover kinetics. Previously, we have characterized the CD8⁺ CTL priming roles of Clec9A⁺ cDC1s and their exacerbation of ECM [257]; as well as important first-line immunity and control of immunopathology imparted by CD169⁺ tissue-resident macrophages during *Plasmodium berghei* ANKA infection [253]. Following up on these published findings, this project has two main arms of interest:

1. Investigate the roles and contributions of Clec9A⁺ DCs in the induction of multi-organ immune responses in a murine model of acute and chronic malaria, *Plasmodium chabaudi chabaudi* AS.
2. Elucidate the ontogeny and repopulation kinetics of returning tissue-resident macrophages after infection-induced necrosis in the early phase of non-lethal, self-resolving *Plasmodium yoelii* (Py1.1) infection; and compare their phenotypic and gene expression signatures to the original embryonic-derived residents.

We exploit two specific mouse models for the aims described above; first the Clec9A-DTR transgenic mouse that allows for specific, efficient but also reversible ablation of Clec9A⁺ DCs, and second the Kit^{MerCreMer/R26} reporter mouse which labels bone-marrow derived myeloid precursors and cells YFP⁺.

2. Materials and Methods

2.1 Materials

2.1.1. Mice and ethics statement

Clec9A-DTR and CD169-DTR transgenic mice were generated in Balb/c background as described in [257] and [280]. Clec9A-DTR mice were then backcrossed eight to ten generations into C57BL/6J background (The Jackson Laboratory, Bar Harbour, ME, USA). IFN γ KO C57BL/6J mice were bred in-house. Kit^{MerCreMer/R26} mice were generated as reported in [60], and maintained with either WT Balb/c or WT C57BL/6J mice in the animal facility of Nanyang Technological University of Singapore.

2.1.2 Parasites

Plasmodium chabaudi chabaudi AS (*PccAS*) strain were used for *PccAS* infections and transgenic GFP-expressing *Plasmodium yoelii* 17X non-lethal clone 1.1, *Py1.1GFP*, was used for *Py* infections.

2.1.3 List of commercial antibodies and cell viability dyes

Anti-mouse antibody	Fluorochrome	Clone	Company	Catalog No.
CD45.2	APCCy7 APC/Fire™ 750	30-F11	Biolegend	103116 103154
CD45	BUV737	104	BD Biosciences	564880
CD3	BUV737	17A2	BD Biosciences	564618
CD4	APC/Fire™ 750 Alexa Fluor® 488 FITC BUV395	GK1.5 GK1.5	Biolegend BD Biosciences	100460 100423 100406 563790
CD8	APC/Fire™ 750 PerCPCy5.5 APC BUV395	53-6.7	Biolegend BD Biosciences	100766 100734 100712 563786
CD69	PE	H1.2F3	eBioscience	104508
CD62L	APC PE	MEL-14	Biolegend	104412 104408
CD44	FITC BV510	IM7	Biolegend BD Biosciences	563114

NK1.1	BV605	PK136	BD Biosciences	563220
PD-1	Biotin PE	RMP1-30 J43	Biolegend eBioscience	109106 12-9985-81
Streptavidin	BUV737 BUV395 BV421 PE Cy7		BD Biosciences Biolegend	564293 564176 56325 405206
CD43	PE Cy7	S11	Biolegend	143210
IFN- γ	PE-CF594 FITC	XMG1.2	BD Biosciences Biolegend	562303 505806
IL-10	PECy7	JES5-16E3	Biolegend	505026
TNF- α	APC	MP6-XT22	Biolegend	506308
Granzyme B	Pacific Blue	GB11	Biolegend	515408
Granzyme A	PerCP-eFluor 710	GzA-3G8.5	eBioscience	46-5831-82
$\gamma\delta$ TCR	APC	GL3	Biolegend	118116
CD19	BUV395 FITC	1D3	BD Biosciences	563557 553785
B220	FITC BUV395	RA3-6B2	Biolegend BD Biosciences	103206 563793
CD38	BV421	90/CD38	BD Biosciences	562768
CD23	PerCPCy5.5	B3B4	Biolegend	101618
CD27	PerCPCy5.5	LG.3A10	BD Biosciences	563603
CD138	PE	281-2	Biolegend	142504
GL-7	Alexa Fluor® 647	GL7	Biolegend	144606
IgD	PE Cy7 FITC	11-26c.2a	Biolegend	405720 405704
IgM	APC Cy7	RMM-1	Biolegend	406516
IgG (minimal X- reactivity)	BV605	Poly4053	Biolegend	405327
Bcl-6	BV421	K112-91	BD Biosciences	563363
CXCR5	Biotin	L138D7	Biolegend	145510
IL-21	APC	FFA21	eBioscience	17-7211-80
KLRG-1	BV605	2F1	BD Biosciences	564013
CD127 (IL-7R)	APC	N.A	In-house CR Lab	N.A
CCR7 (CD197)	Biotin	4B12	Biolegend	120104
CD11c	PE Cy7 APC BV605	N418	Biolegend BD Biosciences	117318 117310 744179
I-A/I-E (MHC-II)	BV421 FITC	M5/114.15.2	Biolegend	107632 107606

CD11b	BUV395 BB515 PerCPCy5.5	M1/70	BD Biosciences Biolegend	563553 564454 101228
F4/80	PE	BM8	Biolegend	123110
Ly6C	BV605 PerCPCy5.5	HK1.4	Biolegend	128036 128012
Ly6G	FITC BUV395	1A8	Biolegend BD Biosciences	127606 563978
CD169	APC	REA197	Miltenyi Biotec	130-104- 954
CD169	eFluor 660	SER-4	eBioscience	50-5755-80
REA Isotype Control	APC	REA293	Miltenyi Biotec	130-113- 446
Tim-4	PE Cy7	RMT4-54	Biolegend	130010
Siglec-H	FITC	551	Biolegend	129604
CD103	PerCPCy5.5	2E7	Biolegend	121416
CD24	APC	M1/69	Biolegend	101814
CD64	APC	X54-5/7.1	Biolegend	139306
CCR2	APC	475301	R&D Systems	FAB5538A
CCR3	Alexa Fluor® 647	J073E5	Biolegend	144508
cKit	APC	2B8	Biolegend	105812
MerTK	APC	108928	R&D Systems	FAB5912A
CD209a	eFluor 660	MMD3	eBioscience	17-2092-80
Siglec F	APC	E50-2440	BD Biosciences	562680
Ki-67	APC	16A8	BD Biosciences	652406
CD34	eFluor 660	RAM34	eBioscience	50-0341-80
Fc Block	Purified	2.4G2	In-house	In-house
HRP-IgG (minimal X-reactivity)	From goat	N.A	Biolegend	405306
IgG1, Human ads- HRP	From goat	N.A	Southern Biotech	1070-05
IgG2a, Human ads-HRP	From goat	N.A	Southern Biotech	1080-05
IgG2b, Human ads-HRP	From goat	N.A	Southern Biotech	1090-05
IgG2c, Human ads-HRP	From goat	N.A	Southern Biotech	1079-05
IgG3, Human ads- HRP	From goat	N.A	Southern Biotech	1100-05
IgM, Human ads- HRP	From goat	N.A	Southern Biotech	1020-05
Viability dyes	Fluorochrome	Clone	Company	Catalog No.
BD Horizon™ Fixable Viability Stain 510	BV510	N.A	BD Biosciences	564406

LIVE/DEAD® Fixable Blue Dead Cell Stain	Blue – UV	N.A	Thermo Fisher Scientific	L34961
---	-----------	-----	--------------------------------	--------

Table 2.1. List of antibody reagents

2.1.4 Buffers, media and solutions

Alsever's solution for cryopreservation

For 500 mL:

10.25 g D-(+)-Glucose

3.95 g trisodium citrate · 2H₂O

2.1 g NaCl

50 mL glycerine

450 mL dH₂O

pH adjusted to 6.1

ELISA blocking and assay buffer 1

1X PBS

10% FCS

ELISA blocking and assay buffer 2

1X PBS

1% Bovine Serum Albumin (BSA)

ELISA wash buffer

1X PBS

0.05% Tween-20

Cell fixation buffer

1x PBS

4% paraformaldehyde

pH adjusted to 7.4

Diphtheria Toxin (DT)

1X PBS

1% mouse serum

2 ng/μl DT

IMDM 2%

Iscove's Modified Dulbecco's Medium (IMDM)

2% Fetal Calf serum (FCS)

PBS 2%

1X PBS

2% FCS

Permeabilization buffer

PBS 2%

0.5% saponin

70% Percoll solution

For 500ml:

10X PBS, 35 ml

Percoll, 315 ml

IMDM, 150 ml

40% Percoll solution

For 500ml:

10X PBS, 20 ml

Percoll, 180 ml

IMDM, 300 ml

35% Percoll solution

For 500ml:

10x PBS, 17.5ml

Percoll, 157.5ml

1x PBS, 325ml

Red blood cell (RBC) lysis buffer

0.89% ammonium chloride

dH₂O

3% Sodium citrate solution

70 µl of 20% sodium citrate solution in dH₂O

500 µl 1 X PBS

2.1.5 Reagents, chemicals and kits

Name	Company
Acrylic nail polish	Drugstores
Arcturus PicoPure™ RNA Isolation Kit	Thermo Fisher Scientific, Waltham, MA, USA
Ammonium chloride	Sigma, St. Louis, MO, USA
Agencourt RNACleanXP magnetic beads	Beckman Coulter, IN, USA

Brefeldin A	Sigma, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma Aldrich, St. Louis, MO, USA
Collagenase D	Roche, Basel, Switzerland
Collagenase D	SERVA Electrophoresis GmbH, Heidelberg, Germany
Corn oil	Sigma Aldrich, St. Louis, MO, USA
D-(+)-Glucose	Sigma Aldrich, St. Louis, MO, USA
Dihydroethidium	Sigma Aldrich, St. Louis, MO, USA
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich, St. Louis, MO, USA
DPX Mountant for histology	Sigma Aldrich, St. Louis, MO, USA
Diphtheria toxin (DT)	Sigma, St. Louis, MO, USA
DNA Gel Loading Dye (6X)	Thermo Fisher Scientific, Waltham, MA, USA
DNA High Sensitivity Reagent Kit	Perkin Elmer, Waltham, MA, USA
DNase I (digestion)	Roche, Basel, Switzerland
DNase I, RNase-free (RNA purification)	Thermo Fisher Scientific, Waltham, MA, USA
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher Scientific, Waltham, MA, USA
eBioscience™ Intracellular Fixation & Permeabilization Buffer Set	Thermo Fisher Scientific, Waltham, MA, USA
Ethanol (absolute)	Sigma Aldrich, St. Louis, MO, USA
ERCC RNA Spike-In Mix Ambion®	Thermo Fisher Scientific, Waltham, MA, USA
Fetal calf serum (FCS)	Biowest, Nuaille, France
Giemsa-Wright stain	Sigma Aldrich, St. Louis, MO, USA
Glycerine	Affymetrix, Santa Clara, CA, USA
Hoechst 33342	Sigma Aldrich, St. Louis, MO, USA
Illumina Nextera XT Kit	Illumina, San Diego, CA, USA
Ionomycin	Sigma Aldrich, St. Louis, MO, USA
IMDM	Gibco, Grand Island, NY, USA
Isoflurane	Piramal Healthcare, India
Magnesium sulfate	Sigma Aldrich, St. Louis, MO, USA
Methanol (absolute)	Sigma Aldrich, St. Louis, MO, USA
Mouse IFN-γ ELISA MAX	BioLegend, San Diego, CA, USA
Mouse IL-10 ELISA MAX	BioLegend, San Diego, CA, USA
Mouse TNF-α ELISA MAX	BioLegend, San Diego, CA, USA
Mouse IL-6 ELISA MAX	BioLegend, San Diego, CA, USA
Mouse IL-4 ELISA MAX	BioLegend, San Diego, CA, USA
eBioscience™ Mouse IFN-γ ELISA Ready-SET-Go!™ Kit	Thermo Fisher Scientific, Waltham, MA, USA
eBioscience™ Mouse IL-10 ELISA Ready-SET-Go!™ Kit	Thermo Fisher Scientific, Waltham, MA, USA
Nuclease-free Water	Thermo Fisher Scientific, Waltham, MA, USA
Ovation® Pico WTA System V2	NuGEN, San Carlos, CA, USA
Paraformaldehyde (PFA)	Sigma Aldrich, St. Louis, MO, USA
PBS (1x)	Gibco, Grand Island, NY, USA

PBS (10x) RNase-free	Thermo Fisher Scientific, Waltham, MA, USA
Percoll™	GE Healthcare, Uppsala, Sweden
Phorbol myristate acetate (PMA)	Sigma Aldrich, St. Louis, MO, USA
PrecisionFAST qPCR SYBR Master Mix	Integrated Sciences, NSW, Aus
Quant-iT™ RiboGreen™ RNA Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
Saponin	Fluka Chemie AG, Buchs, Switzerland
Sodium bicarbonate	Sigma Aldrich, St. Louis, MO, USA
Sodium Chloride	Merck KGaA, Darmstadt, Germany
Sodium citrate	Sigma Aldrich, St. Louis, MO, USA
Tamoxifen	Sigma Aldrich, St. Louis, MO, USA
TMB Substrate Reagent (1x)	BioLegend, San Diego, CA, USA
Trisodium citrate·2H ₂ O	Fluka Chemie AG, Buchs, Switzerland
Trypan blue	Sigma Aldrich, St. Louis, MO, USA
Tween 20	Sigma Aldrich, St. Louis, MO, USA

Table 2.2 List of chemicals and reagents

2.1.6 Equipment and instruments

Name	Company
Agilent 2100 Bioanalyzer	Agilent, Santa Clara, CA
BD LSR Fortessa X-20 3-laser	Becton Dickinson, San Jose, CA, USA
BD LSR Fortessa X-20 5-laser	Becton Dickinson, San Jose, CA, USA
BD FACSAria™ I	Becton Dickinson San Jose, CA, USA
BD FACSAria™ III	Becton Dickinson San Jose, CA, USA
Cytospin™ 4 Cytocentrifuge	Thermo Scientific, Waltham, MA, USA
DiaMag02 Magnetic Rack	Diagenode, Liege, Belgium
ENDURO™ Gel XL Electrophoresis System	Labnet, Edison, NJ, USA
Illumina ECO™ Real Time PCR System	Illumina, San Diego, CA, USA
Illumina HiSeq 2500 System	Illumina, San Diego, CA, USA
Microscope Nikon Eclipse 80i	Nikon Instruments, Melville, NY, USA
Multiskan Spectrum	Thermo Scientific, Waltham, MA, USA
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer with Wi-Fi	Thermo Scientific, Waltham, MA, USA
Innova 4200 Incubator-Shaker	New Brunswick Scientific, MD, USA

Eppendorf ThermoMixer® F2.0	Thermo Fisher Scientific, Waltham, MA, USA
Eppendorf™ 5424R Microcentrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Infinite® 200 PRO Plate reader	Tecan, Männedorf, Switzerland
Thermocycler T3000	Biometra GmbH, Goettingen, Germany

Table 2.3 List of equipment

2.1.7 Data analysis and computational software

Software	Company
Flowjo X	TreeStar Inc, Ashland, OR
Graphpad Prism 6.0	Graphpad Software Inc, La Jolla, CA
SPICE 5.1	NIAID, Maryland, USA
FeatureCount program	WEHI, Melbourne, Australia http://subread.sourceforge.net/
<i>R-3.1.2 custom scripts</i>	<i>R Project</i> http://www.R-project.org/

Table 2.4 List of computational software

2.2 Methods

2.2.1 Mice and ethics statement

Experimental animals as described in 2.1.1 were age and sex-matched, and referred to as WT, Clec9A-DTR, CD-169-DTR or cKit^{MerCreMer/R26} throughout the text. Diphtheria toxin was injected to DTR mice between 6-10 weeks, while tamoxifen was administered to cKit^{MerCreMer/R26} mice between 5-8 weeks of age. The following studies were carried out in strict accordance with NAACCR (National Advisory Committee for Laboratory Animal Research) recommended guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore. The Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University of Singapore approved the protocols (Authorization No: IACUC SBS/NIE A-0127 and A-0258).

2.2.2 Parasite cryopreservation and maintenance

PccAS and *Py* infection stocks were maintained by infecting Balb/c or C57BL/6J WT mice intraperitoneally (i.p.) with 10⁶ iRBCs. When blood parasitemia is between 5 – 15%, with majority of ring or early trophozoite-

stage parasites as monitored by Giemsa thin blood smears, mice were terminally bled. Whole blood with 20% sodium citrate solution was suspended in 40mL ice-cold PBS and periodically shaken to dissuade clotting. RBCs were spun down at 1400rpm for 5min and quickly resuspended in 1mL cold 1x PBS. A Giemsa blood smear and 1:200 dilution (in 1x PBS) of the resuspended RBC pellet were performed to determine % of iRBCs (see 2.2.4) and total cell count (via Neubauer haemocytometer chamber) respectively, before final resuspension in 10mL PBS. The number of iRBCs was calculated based on earlier % iRBC and total cell count to yield 10^7 iRBCs/mL per cryovial for infection stocks, or 10^8 iRBCs/mL per cryovial for ELISA iRBC plate coating. RBCs were spun down, resuspended in corresponding amount of Alsever's Buffer, and aliquoted into 1.2mL/cryovials. Cryovials were acclimatized at -80°C for 24h before transfer to liquid nitrogen for cryopreservation of infection stock.

2.2.3 Tamoxifen (TAM) administration, DT injection and *Plasmodium* infection

Tamoxifen tablets were dissolved in corn oil overnight with shaking. 300 μL of TAM emulsion (4mg/mouse) was administered by gavage to mice over five consecutive days. Kit^{MerCreMer/R26} mice were utilized experimentally 4-5 weeks after TAM administration to allow for YFP labelling of cKit⁺ bone marrow-derived myeloid progenitors.

To ablate relevant cell populations of interest, 10 – 20 ng/g per body weight (g.b.w) of diphtheria toxin (DT) was injected i.p. into DTR mice on day -1 and day 0 of infection. Ablation was maintained by DT injection every 3 to 4 days post-infection (d.p.i), up till 28 d.p.i. For heterozygous C57BL/6J-DTR strains, WT littermate controls were also administered DT to normalize effects of DT toxicity, if any.

For induction of rodent malaria, WT, Clec9A-DTR and Kit^{MerCreMer/R26} mice were infected i.p. with 10^6 PccAS or Py iRBCs immediately thawed from frozen stocks described in 2.2.2.

2.2.4 Blood parasitemia monitoring

Blood parasitemia of *Plasmodium*-infected mice were monitored over the course of infection, spanning from day 3 – day 45 (depending on purpose

of experiment) by a modified protocol of the tri-colour flow cytometry method developed by [281]. Briefly, 1µl of mouse tail vein blood was suspended in 200µl PBS in a 96-well plate and spun down to pellet cells. Cells were stained with 8µM Hoechst 33342, 5µg/mL dihydroethidium and 1:600 anti-mouse CD45-APCCy7 in PBS 2% for 20min at 4°C. Cells were washed 1x with 200µl PBS, spun down and resuspended in 300 - 500µl of FACS Buffer (1x PBS/PBS 2%) for acquisition on a BD LSR Fortessa X-20 3-laser instrument. The gating strategy for % iRBCs is illustrated in Figure 2.1 below. Thin Giemsa blood films were used to determine sub-patent parasitemia below the tri-colour flow cytometry detection limit. Thin blood smears were fixed in absolute methanol, heat-dried at 50°C, stained for 5min in 20% filtered Giemsa-wright (diluted in dH₂O) and visualized under 100x magnification with an oil objective. At least 8-10 fields (or 1000 RBCs) were enumerated for calculation of percentage blood parasitemia.

Figure 2.1: Gating strategy for tri-colour flow cytometry of percentage blood parasitemia. From FSC/SSC, doublets and triplets were excluded (P2), and then split into two subgates; (P3) for CD45⁺ leukocytes and (P4) for Hoechst⁺ HD⁺ (DNA/RNA⁺) and HD⁺ (RNA⁺) populations. Percentage parasitemia is calculated from (P4 – P3)_{infected} – baseline (P4 – P3)_{uninfected}.

2.2.5 Serum collection and processing

Mice were bled by retro-orbital sinus/plexus puncture by glass capillaries under isoflurane or anaesthesia (Ketamine/xylazine) influence. Terminally-bled mice were sacrificed by cervical dislocation. Serum was allowed to clot for at least 30min at room temperature (serum antibodies) or on ice (cytokines assay) before centrifuging at 16000g for 10min in a 4°C benchtop microcentrifuge. Serum was carefully pipetted into fresh Eppendorf tubes,

avoiding buffy coat layer contamination and spun down again for 5min to remove any residual RBCs. Serum was finally aliquoted into fresh Eppendorf tubes and stored immediately at -20°C (antibodies assay), -80°C (cytokines assay) or kept on ice till usage.

2.2.6 Sample processing and leukocyte isolation from organs

Mice were bled as in Section 2.2.5, and perfused intracardially with 10mL 1x PBS. Spleens, lungs and livers were collected either in 2ml Eppendorf tubes or 12-well plates with 2% IMDM on ice. Organs were minced, transferred to fresh tubes and topped up with 5-7ml of 2% IMDM. 1mg/ml collagenase D and 20µg/mL were added to organ suspensions, and incubated at 37°C in a shaking incubator for 45min (spleen), 60min (liver) and 90min (lung). After digestion, organs were passed through a 100µ wire mesh and gently mashed to obtain a single-cell suspension. For liver and lung samples, digested tissues were first homogenized by a 18G and 21G needle five times respectively, before mashing through the 100µ wire mesh. The single-cell suspensions were spun down at 350g for 5min, and then resuspended in 10mL 0.89% NH₄Cl lysis buffer directly (spleen) to lyse RBCs, for 10min at room temperature (RT). For leukocyte enrichment in non-lymphoid organs, cells were resuspended in 10mL of RT 35% Percoll/PBS solution and centrifuged at 600g for 10min, for removal of debris, lipids and non-leukocyte cell fractions. The enriched cell pellet was then subjected to RBC lysis as described above. Both splenic and non-lymphoid organ suspensions were spun down and washed 1x with 15mL PBS 2% to remove residual lysis buffer. Following which, cells are resuspended in 1 – 5mL of 2% IMDM, fine-filtered to remove debris, and left on ice till usage.

2.2.7 Antibody staining for flow cytometry

Total cell count of single-cell suspensions was performed using a Neubauer chamber haemocytometer.

i.) Extracellular staining

Approximately 10⁷ cells/well in a v-bottom plate were used for various staining panels: myeloid, activation markers, T-follicular helper cells and GC B-cell reactions. For myeloid and B-cell staining, cells were first incubated

with 10µg/ml FcR block and 0.1µg/ml fixable viability stain in 1x PBS for 20min at 4°C, washed with 200µl 1x PBS and centrifuged at 500g for 3min. Extracellular staining with the corresponding antibody panels (see 2.1.3 and Appendix 7.2 for commercial anti-mouse antibody clones) was performed for 20min at 4°C, followed by washing with 1x PBS and resuspension in 50-150µl PBS 2% for FACS acquisition.

ii.) Stimulation and intracellular cytokines/ transcription factor staining

Approximately 10^7 cells/well were aliquoted to a 96-well plate in duplicates, centrifuged to pellet cells, resuspended in 200µl 2% IMDM and transferred to sterile flat bottom 96-well plates.

For stimulated samples, 20µl PMA/ionomycin (final concentration 10ng/ml) was added to duplicate wells. A similar set of duplicate wells acting as non-stimulated internal controls were also aliquoted, and both sets of samples were incubated for 2.5h at 37°C. After PMA/ionomycin stimulation, 20µl Brefeldin A (final concentration 10µg/ml) was added to all wells to trap cytokine production, and further incubated at 37°C for 2.5h.

Cells were then transferred to 96-well V-bottom plates for maximum pellet recovery, washed 2x with 200µl PBS 2%, and subjected to fixable viability and extracellular staining with the corresponding antibody mastermixes (see Appendix 7.2) as described above in (i). After washing, cells were first resuspended in 100µl of PBS, and then 100µl of 4% paraformaldehyde (PFA) was added to fix the cells (final concentration = 2% PFA), for 30min at room temperature. For overnight fixing, cells were resuspended in 200µl of 1% PFA (diluted in 2% IMDM) and incubated at 4°C. Fixed cells were centrifuged at 600g, washed 1x with 200µl PBS and 1x with 200µl Permeabilization Buffer (0.5% saponin/PBS) and incubated with corresponding intracellular antibody mastermix (in Permeabilization Buffer) at RT for 20min. Cells were then washed 1x with 200µl Permeabilization Buffer and 1x with 200µl PBS, and resuspended in 100µl PBS 2% for acquisition.

For nuclear transcription factor staining, manufacturer's instructions for eBioscience™ FoxP3 transcription factor staining buffer kit (see 2.1.5 reagent list) were followed accordingly.

Stained cells were primarily analyzed on a BD LSR Fortessa X-20 5-laser instrument and data acquired on Flowjo X software. Cell sorting was performed on a BD FACS Aria™ Ilu 4-laser instrument under 4-way purity mode.

Note: Most commercial antibodies were used at an optimized dilution of 1:600 in PBS 2% unless stated otherwise (see Appendix 7.2 for detailed panels and dilutions).

2.2.8 ELISA for serum cytokines

Serum was obtained as described in Section 2.2.5, and quick-thawed just prior to use. 1% BSA was used as the blocking buffer and reagent diluent in this assay and 0.05% PBS-Tween 20 as the wash buffer.

Serum IFN γ , TNF α , IL-10, IL-6 and IL-4 were quantified using Mouse ELISA MAX™ Standard Kit or eBioscience™ Mouse ELISA Ready-SET-Go!™ Kit (as per manufacturer's instructions. OD_{370nm} was read on a Multiskan Spectrum ELISA plate reader every 5 min for 20 min (up to 60 mins for IL-10).

2.2.9 ELISA for *Plasmodium*-specific Ig

10% FCS/PBS was used as the blocking buffer and reagent diluent in this assay. 1x PBS was used as washing buffer. Serum was obtained as described in Section 2.5, and quick-thawed prior to use.

50 μ l of poly-lysine solution was added to uncoated ELISA plates (NUNC) and incubated at 4°C overnight. Plates were washed 1x and allowed to air-dry before use. *PccAS* or *Py* iRBC cryovials (10⁸/mL stock as described in 2.2.2) were thawed, centrifuged at 16000g for 3min to pellet iRBCs, and resuspended in 10mL 1x PBS. 50 μ l of iRBC suspension (~10⁶ iRBCs) was aliquoted to poly-lysine-coated ELISA plates and spun down at 10000rpm for 3min. Adhered iRBCs were fixed with 50 μ l absolute methanol for at least 20min at RT. Plates were washed 3x and blocked with 200 μ l blocking buffer for 2h at RT or 4°C overnight.

Serum samples were serially diluted at the respective concentrations summarized in Table 2.5. 50µl of serially diluted serum was added to wells and incubated for 2h at RT or 4°C overnight. Plates were washed 3x, and 50µl of HRP-conjugated goat anti-mouse Ig antibodies (see list in 2.1.3) was added to wells and incubated for 30min at RT. Plates were washed 5 – 7x, and 50µl fresh 1x TMB substrate was added to wells. OD_{370nm} was read on a Multiskan Spectrum ELISA plate reader at every 5 min interval, up to 25 min.

Ig Isotype (1:5000)	Serial Dilutions (3-fold) Day 9, 14 & 21	Serial Dilutions (3-fold) > Day 21
IgG	1:50, 1:150, 1:450, 1:1350	1:50, 1:150, 1:450, 1:1350
IgM	1:50, 1:150, 1:450, 1:1350	1:50, 1:150, 1:450, 1:1350
IgG1	1:10, 1:30, 1:90, 1:270	1:50, 1:150, 1:450, 1:1350
IgG2a/IgG2b	1:10, 1:30, 1:90, 1:270	1:50, 1:150, 1:450, 1:1350
IgG2c	1:10, 1:30, 1:90, 1:270	1:50, 1:150, 1:450, 1:1350
IgG3	1:10, 1:30, 1:90, 1:270	1:50, 1:150, 1:450, 1:1350

Table 2.5: Serum serial dilutions for *Plasmodium*-specific Ig isotypes.

2.2.10 Serum passive transfer

Serum samples were screened as described in Section 2.2.9 for *Plasmodium*-specific Ig and isotype polarization before being pooled for passive transfer. Outliers were removed from common pool. The serum passive transfer protocol is adapted from [191].

C57BL/6J wildtype male mice were designated as recipients (BRC, A*STAR or in-house), split into 4 groups receiving: (i) sham PBS, (ii) uninfected serum, (iii) wildtype *PccAS*-infected serum and (iv) Clec9a-DTR *PccAS*-infected serum. Recipient mice received 180 µl of undiluted serum at specific time points and were infected with *PccAS*. Percentage blood parasitemia was monitored from day 8 onwards as described in 2.2.4, for a period of 40 days, and expressed in either percentage % or log₂ blood parasitemia.

2.2.11 Cell sorting for RNA-sequencing analyses

Tissue-resident MØ obtained from the liver, lungs, and spleen of uninfected or *P. yoelii*-infected, recovered animals at 35 d.p.i. were sorted with 4-way purity using a BD FACSAria™ (see Appendix 7.2 for antibody panel). Total RNA was extracted using the Arcturus PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's protocol. All mouse RNAs were analyzed on an Agilent Bioanalyzer for quality assessment; the RNA Integrity Number (RIN) range was 6.5–10, median of 9.2. cDNA libraries were prepared with 2 ng of total RNA and 1 µl of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion) using the SMARTSeq v2 protocol [282] using 20 µM TSO and 250 pg of cDNA with 1/5 reaction of Illumina Nextera XT kit (FC-131-1-24). Length distribution of the cDNA libraries was monitored using a DNA High Sensitivity Reagent Kit (CLS760672) on the PerkinElmer Labchip. All eight samples were subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2500 at Rapid mode (17 samples/lane). The paired-end reads were mapped to the Mouse GRCm38/mm10 reference genome using the STAR alignment tool [283]. Mapped reads were summarized to gene level using featureCounts (V1.5.0-P1) software [284] and with GENCODE gene annotation, Release M10 [285]. Genes with less than 10 average number of reads per sample in all cell sub-populations, were omitted from further analysis. For differentially expressed gene (DEG) analysis, one of the best performing RNA-seq data analysis pipelines recommended by the MicroArray Quality Control (MAQC) project (Consortium, 2014); the limma/voom pipeline was used. Comparisons of pre- and post-infection MØs for each of the tissues were done using limma and DEGs were selected with Benjamini-Hochberg [286] adjusted p-values of < 0.05. Hierarchical clustering and principal component analysis were done with Log2-transformed value of Fragments Per Kilobase of transcript per Million mapped reads (FPKM). All RNA-Seq analyses were performed in R-3.1.2 (URL <http://www.R-project.org/>).

2.2.12 RNA isolation for qPCR

Splenic and liver tissue-resident MØ and monocytes were sorted using a BD FACSAria™ III (see Appendix 7.2 for antibody panel) into 1.5ml Eppendorf tubes in PBS 2%. Total RNA was extracted using the Arcturus

PicoPure™ RNA Isolation Kit and quantified using Quant-iT™ RiboGreen™ RNA Assay Kit (both Thermo Fisher Scientific) according to manufacturer's protocol. Appropriate amount of RNA (normalized to sample with lowest concentration, up to 5ng/ml) was used for downstream amplification, purification and conversion to cDNA with the Ovation® Pico WTA System V2 and Agencourt RNAClean kits as per manufacturer's protocol, while remaining unused RNA was kept at -80°C. Total converted cDNA was quantified with a Nanodrop and stored at -20°C until usage. Working aliquots diluted to 20ng/μl in dH₂O were used for RT-qPCR with PrecisionFAST SYBR qPCR kit (as per manufacturer's instructions), and triplicate reactions were run on an Illumina ECO™ Real Time PCR System for 40 cycles. Median values of triplicates were normalized internally to β-actin and presented as relative expression fold-change.

2.2.13 Cytospin for morphological examination

Cell fractions sorted as described in 2.2.12 and 2.2.13 were collected in PBS 2% in 5ml Falcon tubes and spun down at 350g. Cell pellet was transferred to 1.5ml Eppendorf tubes, washed 1x with 1ml PBS 2% and diluted to about 0.5 – 1 x10⁵ cells/200 μl if required. 200 μl cell suspension was loaded into the funnel of the cytopsin apparatus assembly coupled with positively-charged glass slides, and centrifuged at 300rpm in a Cytospin™ 4 Cytocentrifuge for 5min. Slides were carefully disassembled to avoid smearing of spun-down cells and air-dried. Cytospins could be stained immediately with Giemsa-wright stain as described in 2.2.3 for microscopy or fixed in absolute methanol (5min) for storage and later staining. Stained cytopsin slides were then mounted with glass coverslips using DPX Mountant for histology and sealed with acrylic nail polish for long-term storage.

2.2.14 Data analyses and statistical significance

Flow cytometry data was analysed with Flowjo X software. Graphpad Prism 6.0 was used for plotting graphs and calculating statistical significance with either unpaired Student's *t-test* or nonparametric Mann-Whitney U test. Survival curves were analyzed using the Mantel-Cox log-rank test.

Significance is determined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

For poly-functionality data, Boolean gating function in Flowjo X was utilized and the resulting data was analyzed by SPICE 5.1. Probability values and significance of SPICE pie charts were calculated in-program using Monte-Carlo-based group analysis.

For the high-dimensional flow cytometry data analyses - phenograph cell clustering and UMAP dimensionality reduction; custom R and Python scripts were used. After manually gating on cells of interest and exporting using FlowJo X, compensated .fcs files were opened in R using the flowCore (Bioconductor.org) package. Light scattering parameters “FSC-A” and “SSC-A” were normalized (minimum = 0, maximum = 4.5) on a linear scale. Fluorescent parameters were Log-transformed (using flowCore functions) with automatically determined “w” and “t” parameters, and “m” parameter set to 4.5. After transformation, PhenoGraph clustering was performed using the using the Rphenograph package (github.com/JinmiaoChenLab/Rphenograph), $k = 20$. For UMAP, transformed values were exported and used for input for a custom python script for implementing the “umap” Python package (github.com/lmcinnes/umap), using parameters: $n_neighbors = 15$, $min_dist = 0.2$, $metric = "euclidean"$, $n_components = 1$. Calculated values were then plotted in R as dot-plots coloured by relative marker intensities or as heat-plots (using the heat plot.2 function and default parameters for hierarchical clustering) of median marker intensities within each PhenoGraph cluster. Annotation of clusters was performed by reloading generated .fcs files into Flowjo X to validate marker expression.

3. Results - Role of Clec9A⁺ DCs in Acute and Chronic Malaria

3.1 Characterization of specific depletion in Clec9A-DTR mouse model

Clec9A (c-type lectin domain family 9, member a), also colloquially known as DNGR-1, is a c-type lectin-like molecule that is highly expressed on lymphoid and non-lymphoid cDC1s [23]. The specificity of Clec9A on cDC1s is utilized by our lab to generate the Clec9A-DTR transgenic mouse model which expresses the human DT receptor on the Clec9A gene promoter. Firstly, we aim to characterize the efficiency and specificity of Clec9A⁺ DC depletion in our mouse model.

3.1.1 Specific and efficient ablation of Clec9A⁺ CD8⁺ and CD103⁺ DCs in organs of Clec9A-DTR mice

Upon the administration of two doses of DT (10ng/gbw) at day -2 and day -1 before harvest (day 0), flow cytometric analysis of CD24⁺ DC subsets showed efficient and specific depletion in both percentage and absolute numbers of Clec9A⁺ CD8⁺ and CD103⁺ DCs, in the spleen, liver and lung (Figure 3.1). In the liver, an additional CD11b⁺ CD103⁺ double-negative (DN) DC subset, postulated to be Clec9A⁺ XCR1⁺ CD8⁺ DCs, also showed depletion. In contrast, the counterpart CD11b⁺ cDC2s were not ablated and present in higher numbers in the three organs, likely a compensatory mechanism in the wake of the ablation of CD8⁺/CD103⁺ DCs. The specific CD8⁺/CD103⁺ DC depletion was maintained long-term for 2 weeks (Figure 3.1, right panel) and beyond (not shown), although higher percentage and numbers of DCs were observed compared to short-term depletion, indicating the slow insensitivity of the mice to continuous DT administration.

Figure 3.1. Clec9A⁺ DCs are efficiently specifically depleted for both short and long-term in the spleens, livers and lungs of DT-injected Clec9A-DTR mice during steady-state conditions.

Flow cytometry analysis showing specific depletion of live CD45⁺CD11c^{hi}MHC-II^{hi}CD24⁺ CD8⁺/CD103⁺ DCs in Clec9A-DTR mice. For short-term depletion, mice were injected with 20ng/gbw DT at day -2 and day -1 and harvested on day 0. For long-term depletion, mice were subjected to the same baseline procedure and additionally injected with DT every 3-4 days until harvest at day 14. Representative short-term depletion dot plots (left) are shown from the spleen (top), lung (middle) and liver (bottom) of SS and DT-injected WT Clec9A-DTR mice. Bar charts show the absolute numbers of CD11b⁺, CD8⁺/CD103⁺ DCs and CD11b⁻CD103⁻CD8⁺ DCs (liver only) in SS (white) and DT-injected WT (black) Clec9A-DTR mice at short-term (middle) and long-term (right) timepoints. Data shown in SEM are representative of three independent experiments with n=3 mice/group, 6-10 weeks old. Statistical significance was determined with unpaired Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.1.2 Non-DC myeloid populations were intact following Clec9A⁺ DC ablation in Clec9A-DTR mice in spleen, lung and liver

The specificity of the Clec9A-DTR model was also supported by the lack of unspecific ablation of other CD11b⁺ myeloid subsets in the same mice. Tissue-resident macrophages (FrI), bone-marrow derived infiltrating monocytes (FrII) and neutrophil (FrIII) numbers were intact in Clec9A-DTR mice (Figure 3.2), though significant monocytosis was observed in spleen and liver, particularly following short-term depletion (Figure 3.2, middle panel). The significantly higher FrII splenic monocyte count in Clec9A-DTR mice could have attributed to the corresponding increase in FrI, as some of

the infiltrating FrII monocytes differentiated to monocyte-derived FrI macrophages. Likewise, day 14 CD11b⁺ myeloid subset profiles were similar to untreated steady-state mice, reaffirming no long-term unspecific depletion with continuous DT administration; and though monocytosis was still present in the spleen, overall the magnitude was lower than in short-term depletion (Figure 3.2, right panel).

Fig 3.2: DT administration induces monocytosis but not unspecific depletion of CD11b⁺ macrophage and monocyte subsets in the spleen, liver and lungs of DT-injected Clec9A-DTR steady-state mice following short or long-term depletion.

Flow cytometry analysis showing intact CD45⁺CD11c^{hi}MHC-II^{hi}CD11b⁺ myeloid populations and DT-induced monocytosis in Clec9A-DTR mice following DT administration. For short-term depletion, mice were injected with 20ng/gbw DT at day -2 and day -1 and harvested on day 0. For long-term depletion, mice were subjected to the same baseline procedure and additionally injected with DT every 3-4 days until harvest at day 14. Representative short-term depletion dot plots (left) show myeloid subsets characterized by CD11b and F4/80 expression – (1) FrI: CD11b^{int} F4/80⁺ CD169⁺ macrophages, (2) FrII: F4/80^{int} CD11b⁺ Ly6C⁺ monocytes and (3) FrIII - CD11b⁺ Ly6G⁺ neutrophils from the spleen (top), lung (middle) and liver (bottom) of SS and DT-injected WT Clec9A-DTR mice. Bar charts show the absolute numbers of FrI, FrII and FrIII myeloid subsets in SS (white) and DT-injected WT (black) Clec9A-DTR mice at short-term (middle) and long-term (right) timepoints. Data shown in SEM are representative of three independent experiments with n=3 mice/group, 6-10 weeks old. Statistical significance was determined with unpaired Student's t-test, ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.1.3 Summary of the efficiency and effectiveness of Clec9A-DTR mouse model

Here we present the Clec9A-DTR mouse model as an effective tool to allow for rapid, specific and efficient depletion of CD8⁺ and CD103⁺ DCs without unspecific effects on other myeloid populations or significant reduction of pDCs. It can be useful for both acute and long-term studies in tissue-specific immunity and kinetics of disease models such as during *Plasmodium* infection.

3.2 Elucidating the role of Clec9A⁺ CD8⁺ and CD103⁺ cDC1s in *Plasmodium chabaudi chabaudi* AS infection and immunity

Lymphoid CD8⁺ and non-lymphoid CD103⁺ cDC1s not only produce abundant IL-12, but also possess intrinsic capacities of cross-presentation to potently induce CD8⁺ cytotoxic T-lymphocyte (CTL) responses [11, 186, 287, 288]. IL-12 stimulates IFN γ production from activated T effector (T_{Eff}) as well as innate immune cells, perpetuating a highly T_{H1} proinflammatory environment important for induction of efficient immune responses in many infections, including malaria [210]. Despite the wide body of literature unanimously agreeing on the importance of cDC1s, IL-12 and IFN γ , the role of cDC1s has always been heavily implied in the context of *Plasmodium chabaudi chabaudi* AS infection, largely due to the lack of experimental tools that allow for specific cDC1-targeting, instead of broad DC depletion - for example CD11c-DTR mice. Hence, we first sought to characterize the kinetics of *Plasmodium chabaudi chabaudi* AS infection in our Clec9A-DTR mouse model.

3.2.1 IL-12 production is primarily from CD8⁺ cDC1s and not CD11b⁺ cDC2s during steady-state and *PccAS* infection

Firstly, we showed that during *Plasmodium chabaudi chabaudi* AS infection, splenic CD8⁺ cDC1s were indeed the major producers of IL-12, and not CD11b⁺ cDC2s (Figure 3.3A). Higher IL-12 expression was already present in steady-state CD8⁺ cDC1, which increases 2-fold during *PccAS* infection, whilst infected CD11b⁺ cDC2s barely upregulated IL-12 above the baseline.

This is in line with literature about the IL-12 production capabilities of cDC1s in response to infection and pathogens [287, 289].

3.2.2. Clec9A⁺ DC depletion results in greater magnitude of prolonged parasitemia and elevated mortality in *PccAS*-infected Clec9A-DTR mice

We first monitored the infection profile in *PccAS*-infected WT and Clec9A-DTR mice. Both WT and Clec9A-DTR mice presented with detectable blood parasitemia from 6 – 7 d.p.i. which steadily increased to the acute peak parasitemia at 10 – 11 d.p.i. Blood parasitemia of Clec9A-DTR mice surpassed their WT counterparts by 9 d.p.i., and reached significantly higher levels by the peak, following which both groups showed a rapid decline. Strikingly, at around 16 – 17 d.p.i., Clec9A-DTR mice began to experience significantly pronounced and prolonged recrudescence episodes that persisted well up to 4 weeks post-infection, while WT mice cleared blood parasites to sub-patent levels by 3 weeks post-infection (Figure 3.3B). There was also associated mortality observed in Clec9A-DTR mice (variable, up to 60 – 70%) that interestingly correlated with later recrudescence timepoints at lower magnitude of log blood parasitemia rather than the acute peak (Figure 3.3C). In contrast, none of the WT mice died, suggesting the absence of Clec9A⁺ cDC1s increased the susceptibility of C57BL/6J mice to *PccAS* infection.

Fig 3.3: IL-12 is produced majorly by CD8⁺ DCs during *Plasmodium chabaudi* *chabaudi* AS infection. Depletion of Clec9A⁺ DCs results in greater magnitude of acute blood parasitemia, prolonged significant recrudescence and elevated mortality in *PccAS*-infected mice

qPCR of IL-12 expression in sorted steady state (SS) and infected CD8⁺ and CD11b⁺ DCs. Mice were harvested on day 3 after *PccAS* infection. Bar graph represents the fold-change expression of IL-12 in splenic SS CD8⁺ cDC1 (black), infected CD11b⁺ cDC2 (blue) and infected CD8⁺ cDC1 (red), relative to SS CD11b⁺ cDC2 (white), and normalized to β -actin expression. Data shown is the sample triplicate mean value and representative of two independent experiments with n=3 mice/group, 6-10 weeks old. (B) Infection profile curve showing log blood parasitemia of WT (black circles) and Clec9A-DTR mice (blue circles) from 7 d.p.i. to 50 d.p.i. Mice were administered 2 doses of 10ng/gbw DT on day -1 and day 0 of infection and depletion was maintained by DT injection every 3-4 days up to 28 d.p.i. (C) Survival curve of WT (black) and Clec9A-DTR (blue) mice infected with *PccAS*. Data shown are representative of > 8 independent experiments with n=3-8 mice/group, 6-10 weeks old. Statistical significance of log blood parasitemia was determined using Mann-Whitney U test at *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3.2.3 Clec9A⁺ DCs modulate cellular proliferation and activation during acute phase of *PccAS* infection

During acute-phase *PccAS* infection, activation and proliferation of parasite-specific T_{Eff} cells crucial for control of parasitemia, are driven by cytokines IL-12 and IFN γ [191, 203]. The earlier results in Figure 3.3 confirmed that Clec9A⁺ DCs were indeed potent IL-12 producers; and they are well-

established in literature as mediators of antigen-dependent T-cell proliferation during infection and induction of immunity – including in malaria [210, 290, 291]. The striking phenotype we observed eluded to the importance of Clec9A⁺ DCs in *PccAS* infection, hence to further dissect their roles, we next evaluated the cellularity and activation of immune effector cells in the spleen, lung and liver at 9 d.p.i.

Gross macroscopic examination of organs harvested from infected mice at 9 d.p.i. revealed comparatively smaller spleen sizes in Clec9A-DTR mice than WT mice (data not shown), which was confirmed by significantly reduced numbers of CD45⁺ cells (Figure 3.4A). The overall CD45⁺ cellularity was also lower in lung and liver of Clec9A-DTR mice, albeit not significantly. Further delineation of CD45⁺ to the broad CD3⁺ and CD3⁻ fractions revealed drastically reduced numbers in the former in all three organs (Figure 3.4B). Absolute numbers of CD3⁻ cells were less affected and only showed significant reduction in the spleen, whereas the lung and liver did not appear to have discernible differences. An in-depth analysis was then performed on these CD3⁺ and CD3⁻ fractions to profile the cell subsets and offer a global glimpse into any differences present between WT and Clec9A-DTR mice.

CD3⁺ cells were delineated into CD4⁺, CD8⁺, NKT, $\gamma\delta$ and non-classical T-cell subsets; and CD3⁻ cells into B-cells, NK-cells, DCs and mixed myeloid populations as specified by the gating strategy in Figure 3.4C. Cell profiling pie charts revealed the greatest differences in CD4⁺ and CD8⁺ T-cells, in which Clec9A-DTR mice had visibly lower relative proportions than WT littermates in the spleen, liver and lung (Fig 3.4D). NKT, $\gamma\delta$ and CD4⁻CD8⁻NK1.1 $\gamma\delta$ ⁻ non-classical T-cell subsets that formed a minority of overall total CD45⁺ cells also had reduced relative proportions in Clec9A-DTR mice, particularly in the liver. This was in line with earlier observations of decreased CD3⁺ cells in Figure 3.4B. CD3⁻ NK-cells and mixed myeloid populations in lung and liver, and also B-cells in the case of the spleen, appeared to have higher relative proportions in Clec9A-DTR mice than WT littermates. Detailed cell counts indeed revealed significantly reduced numbers in most CD3⁺ T-cell subsets in all three organs of Clec9A-DTR mice, reiterating the greatest impact on CD4⁺ and CD8⁺ T-cells

(Supplementary Figure 1), consistent with above findings. In contrast, CD3⁻ subsets did not show an overall unifying trend in all three organs, except for the spleen that had consistently lowered numbers in all subsets assessed (Supplementary Figure 1).

Fig 3.4: Absence of Clec9A⁺ DCs resulted in overall lowered CD45⁺ leukocyte and drastically reduced CD3⁺ T-cell subsets in the spleen, lung and liver of *PccAS*-infected Clec9A-DTR mice

Total live CD45⁺ cellularity in SS and *PccAS*-infected WT and Clec9A-DTR mice at 9 d.p.i. Bar charts show absolute numbers of FVS⁻ CD45⁺ leukocytes in the spleen (top), lung (middle) and liver (bottom) of SS (white), WT (black) and Clec9A-DTR (blue) mice. (B) Absolute numbers of CD3⁺ and CD3⁻ cell subsets in SS and *PccAS*-infected WT and Clec9A-DTR mice at 9 d.p.i. Bar charts show absolute numbers of FVS⁻ CD45⁺ CD3⁺ (left) and FVS⁻ CD45⁺ CD3⁻ (right) leukocytes in the spleen (top), lung (middle) and liver (bottom) of SS (white), WT (black) and Clec9A-DTR (blue) mice. Data shown in (A) and (B) are SEM values representative of >3 experiments with n=3-4 mice/group, 6-10 weeks old. Statistical significance was determined using unpaired Student's t-test, ns = not significant, *p < 0.05, **p < 0.01 and ***p<0.001. (C) Gating strategy of detailed cell profiling in SS and *PccAS*-infected mice. Representative plots from the liver show the subsets profiled: CD3⁺ T-cells (1) CD4⁺, (2) CD4⁺CD8⁻, (3) CD8⁺, (4) NK1.1⁺ NKT, (5) NK1.1⁻ CD4⁺CD8⁻ non-classical, (6) $\gamma\delta$ ⁺ and CD3⁻ (7) CD19⁺ B-cells, (8) CD19⁻ NK1.1⁻ myeloid, (9) NK1.1⁺ NK-cells, (10) MHCII^{hi} CD11c^{hi} dendritic cells and (11) CD11c⁺ mixed myeloid populations. (D) Pie charts representative of the proportion of profiled leukocyte subsets in the spleen (top), lung (middle) and liver (bottom) of SS (left), *PccAS*-infected WT (middle) and Clec9A-DTR (right) mice at 9 d.p.i. Data shown in (C) and (D) are representative of 3 independent experiments with n=3-4 mice/group, 6-10 weeks old. Pie charts in (D) were calculated from SEM values of absolute numbers of each individual cell subset, as a proportion of total CD45⁺ population.

It was evident so far that Clec9A-DTR mice were deficient in the proliferation of CD4⁺ and CD8⁺ T-cells compared to WT counterparts, hence we next assessed the activation status of these T-cells using the cell surface markers CD62L, CD44 and KLRG-1.

Activated T-cells displaying a classical CD62L^{lo} and CD44^{hi} [292-294] phenotype were observed in both WT and Clec9A-DTR mice, indicating successful immune induction to *PccAS* infection (Figure 3.5A). Slightly elevated proportions of CD44⁺ CD4⁺ effector T-cells (T_{EFF}) (Figure 3.5A, top) were observed in all three organs of WT mice compared to Clec9A-DTR, but overall differences were not significant. However, Clec9A-DTR mice presented with dramatically lowered levels of CD44^{hi} CD62L^{lo} CD8⁺ T_{EFF} than WT, particularly in spleen and lung; and to a lesser extent in the liver (Figure 3.5A, bottom). Additionally, expression of the killer cell lectin-like receptor G1 (KLRG1), classically associated with terminally differentiated effector cells, and more recently described as an excellent correlative activation marker, particularly for T_{h1} responses [288, 295, 296]; was also significantly elevated on WT CD4⁺ and CD8⁺ T_{EFF} in the spleen, lung and liver, compared to Clec9A-DTR mice (Figure 3.5B).

Fig 3.5: Depletion of Clec9A⁺ DCs leads to drastically reduced activation of CD4⁺ and CD8⁺ effector T-cells in malaria-afflicted organs during acute *PccAS* infection (A) Clec9A-DTR mice have significantly reduced proportions of CD44⁺ CD8⁺ T-cells. Bar charts show percentages of CD62L and CD44 expression on live CD3⁺ CD4⁺ (top) and CD8⁺ (bottom) T-cells in the spleen (left), lung (middle) and liver (right) of UISS (white), *PccAS*-infected WT (black) and Clec9A-DTR (blue) mice. (B) KLRG-1⁺ effector T-cells are drastically reduced in Clec9A-DTR mice. Bar charts show percentages of KLRG-1 expression on live CD3⁺ CD4⁺ (top) and CD8⁺ (bottom) T-cells in the spleen, lung and liver of SS (white), *PccAS*-infected WT (black) and Clec9A-DTR (blue) mice. Data shown in (A) and (B) are SEM values representative of 3 independent experiments at 9 d.p.i. with n=3-4 mice/group, 6-10 weeks old. Statistical significance was determined using Mann-Whitney U test, ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Overall, Clec9A-DTR mice were not drastically impaired in CD4⁺ T_{EFF} activation but displayed significant defects in CD8⁺ T_{EFF} activation as well as an impairment in T_{H1}-inclined activation and effector T-cell differentiation during acute *PccAS* infection. These observations corresponded with the known functions of Clec9A⁺ DCs in T-cell proliferative capability, CD8⁺ T_{EFF} activation and induction of T_{H1} proinflammatory responses [22, 220, 278] to infection or foreign antigenic stimuli such as vaccinations.

3.2.4. Ablation of Clec9A⁺ DCs results in impaired T_{EFF} polyfunctionality and proinflammatory cytokine responses during acute *PccAS* infection

In *Plasmodium* infection, CD4⁺ T-cell-deficient mice fail to control the parasite and eventually die [227, 237], highlighting CD4⁺ T-helper (T_H) involvement as a crucial component to the induction and establishment of *Plasmodium*-specific adaptive immune responses [224, 226, 227, 230, 237]. During acute-phase infection, a highly proinflammatory environment, largely imparted by IFN γ -producing CD4⁺ T_{H1} is necessary to activate and stimulate proliferation of immune effector cells like macrophages and B-

cells, to clear the parasites and induce humoral immunity [204, 219, 297, 298]. Since different DC subsets prime distinct T-cell responses during inflammation and infection, we assessed cytokine production as a measure of CD4⁺ and CD8⁺ T_{EFF} function in the spleen, lung and liver of *PccAS*-infected WT and Clec9A-DTR mice, at 9 d.p.i.

Flow cytometric analysis revealed significantly reduced proportions and numbers of IFN γ ⁺ CD4⁺ T_{EFF} in all three organs, in the absence of Clec9A⁺ DCs (Figure 3.6A – C, left). Similarly, CD8⁺ T_{EFF} from Clec9A-DTR mice exhibited pronounced impairment in IFN γ production and cytotoxicity potential, evident by drastically reduced numbers of granzyme B⁺ (GzB⁺) and IFN γ ⁺GzB⁺ CD8⁺ T_{EFF} subsets (Figure 3.6A – C, middle). Additionally, we also observed a substantial IFN γ ⁺IL10⁺ population, postulated to be terminally-differentiated T_{EFF} cells capable of limiting self-induced immunopathology [299-301] in both CD4⁺ (Figure 3.6A – C, left) and CD8⁺ (Figure 3.6A – C, right) T-cells of infected WT and Clec9A-DTR mice. IFN γ ⁺IL10⁺ CD4⁺ and CD8⁺ T_{EFF} cells were significantly more prevalent in non-lymphoid lungs and livers (Figures 3.6B and 3.6C) than spleens (Figure 3.6A) of WT mice compared to Clec9A-DTR.

Fig 3.6: Absence of Clec9A⁺ DCs results in dramatically diminished functionality of CD4⁺ and CD8⁺ effector T-cells in malaria-afflicted organs during acute *PccAS* infection

Clec9A-DTR mice have significantly reduced numbers of cytokine-producing CD4⁺ and CD8⁺ effector T-cells at 9 d.p.i. (A-C) Representative dot plots show percentages of live CD3⁺ IFNγ⁺ IL10⁺ single or double –producing CD4⁺, CD8⁺, and IFNγ⁺ GzB⁺ single or double –producing CD8⁺ T-cells in the spleen (A), lung (B) and liver (C) of WT or Clec9A-DTR mice. Mice were harvested at 9 d.p.i. and isolated cells were stimulated with PMA/ionomycin to induce cytokine production. Bar charts show absolute numbers of IFNγ⁺ CD4⁺, IFNγ⁺ IL10⁺ CD4⁺, IFNγ⁺ CD8⁺, IFNγ⁺ GzB⁺ CD8⁺ and IFNγ⁺ IL10⁺ CD8⁺ T-cells in the spleen (A), lung (B) and liver (C) WT (black) and Clec9A-DTR (blue) mice. Baseline cytokine production of steady-state controls are denoted by the dashed red lines on bar graphs. Data shown in (A) and (B) are SEM values representative of >5 independent experiments at with n=3-4 mice/group, 6-10 weeks old. Statistical significance was

determined using Mann-Whitney U test, ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

In recent years, new evidence has emerged supporting the dynamic nature of immune effector cells, relating to the notion that they are not restricted to static single cytokine production but rather, a myriad of cytokines; a phenomenon termed polyfunctionality [225, 302, 303].

Here, we investigated polyfunctionality in terms of production of pro-inflammatory cytokines IFN γ , TNF α , cytolytic granzymes GzB and GzA, as well as the anti-inflammatory cytokine IL-10. Delineation of percentage live CD45 $^{+}$ lymphocytes into subsets of interest - CD3 $^{+}$ classical T-cells (CD4 $^{+}$ and CD8 $^{+}$ T-cells), CD3 $^{+}$ non-classical T-cells ($\gamma\delta$ and NKT-cells) and CD3 $^{-}$ NK1.1 $^{+}$ NK-cells were gated as shown in Supplementary Figure 2A. The broad CD3 $^{-}$ NK1.1 $^{-}$ fraction consisting of B-cells and myeloid cells was not further investigated in the following section. Steady-state data was also not shown as cytokine production was negligible.

Stimulated CD4 $^{+}$ and CD8 $^{+}$ T-cells in spleen, lung and liver of WT and Clec9A-DTR mice showed evident polyfunctionality and heterogeneity during *PccAS* infection. However, infected WT T_{EFF} displayed greater heterogeneity (pie slices) and polyfunctionality (overlapping coloured arcs) than Clec9A-DTR, in the context of single and multiple cytokine-producers, succinctly illustrated via visual disparity between SPICE pie charts. These differences were statistically significant across all three organs in both T-cell compartments (Figure 3.7A) except for splenic CD4 $^{+}$ T-cells. However, unstimulated splenic CD4 $^{+}$ T-cells did show significant differences ($p = 0.0307$) between WT and Clec9A-DTR mice (Supplementary Figure 2B), which was probably masked by unspecific PMA/ionomycin stimulation.

In-depth SPICE analysis (Figure 3.7A) also revealed that majority of polyfunctional CD4 $^{+}$ and CD8 $^{+}$ T_{EFF} in spleen, lung and liver were secreting IFN γ . Concurrently, a large proportion of WT CD8 $^{+}$ IFN γ $^{+}$ T_{EFF} also co-secreted cytolytic GzA and GzB (Figure 3.7A, bottom). Although CD4 $^{+}$ and CD8 $^{+}$ T_{EFF} from Clec9A-DTR mice were also capable of co-producing IFN γ and granzymes, they were significantly lesser in proportion compared to WT mice. TNF α , unlike IFN γ , was produced at a much lower magnitude and

without distinctly different proportions between WT and Clec9A-DTR mice. Prevalent IL-10 production was seen in CD4⁺ T_{EFF} (Figure 3.7A, top) and to a lesser extent in CD8⁺ T_{EFF} (Figure 3.7A, bottom), particularly in the liver. These IL-10⁺ T_{EFF} almost always exclusively aligned with IFN γ production, in conjunction with other cytokines/granzymes across all organs, recapitulating the self-limiting IFN γ ⁺ IL10⁺ CD4⁺ and CD8⁺ T_{EFF} subsets described in Figure 3.6.

Aside from adaptive T_{EFF}, we also investigated the polyfunctionality of innate immune cells, broadly categorized into CD3⁺ CD4-CD8⁻ non-classical T-cells (consisting of NKT and $\gamma\delta$ T) and CD3⁻ NK1.1⁺ NK-cells. Innate effector populations in infected organs of WT and Clec9A-DTR were also polyfunctional and showed less striking differences between the two groups than T_{EFF} (Figure 3.7B). In fact, Clec9A-DTR splenic NK-cells and liver CD4⁻ CD8⁻ NKT/ $\gamma\delta$ T and NK-cells were significantly more heterogenous than WT counterparts. Most innate polyfunctional effector cells were IFN γ ⁺ GzB⁺ GzA⁺, with little producers of TNF α .

IL-10 production was prominently observed in NK-cells (Figure 3.7B, bottom) and nearly undetectable in CD4-CD8⁻ non-classical T-cells (Figure 3.7B, top). Interestingly, splenic WT NK-cells produced hardly any IL-10, in direct contrast to lung and liver WT NK-cells. Likewise, IL-10⁺ NK-cells were also exclusively IFN γ ⁺, reminiscent of the IFN γ ⁺ IL-10⁺ CD4 and CD8⁺ T_{EFF} described in Figure 3.7A, and have been reported with self-limiting capabilities in other infection models [304, 305].

Fig 3.7: Polyfunctionality of effector T and NK-cells during acute *PccAS* infection is severely impacted in the absence of Clec9A⁺ DCs Clec9A-DTR T and NK-cells have overall less propensity for effector cytokine polyfunctionality at 9 d.p.i. (A) SPICE pie charts depicting polyfunctional CD4⁺ (top) and CD8⁺ (bottom) T_{Eff} in spleen (left), lung (middle) and liver (right) of WT and Clec9A-DTR mice. Cytokines and cytolytic granules Granzyme A, Granzyme B, IFN γ , IL-10 and TNF α are represented as coloured arcs as per figure legend(s) over SPICE pies. Overlapping arcs and pie slices correspond to multiple cytokine-producing and the proportional heterogeneity of cell subpopulations respectively. (B) SPICE pie charts depicting polyfunctional CD3⁺ CD4⁻ CD8⁻ NKT and $\gamma\delta$ T-cells (top) and CD3⁻ NK1.1⁺ NK-cells (bottom) in WT and Clec9A-DTR mice assayed for the same conditions as (A). p-values on top of SPICE pie charts denote significant differences between WT and Clec9A-DTR mice. Legend 1 defines colour-matched cytokines in all groups, except lung CD8⁺ and NK-cells (Legend 2) which do not produce detectable TNF α in both (A) and (B). SPICE pies in (A) and (B) are generated from cumulative data of stimulated samples from 2 independent experiments, with n=7-8 mice/group at 6-10 weeks of age. Statistical significance is defined by the heterogeneity between two SPICE pies, analyzed using in-built Monte Carlo method, with *p < 0.05, **p < 0.01 and ***p < 0.001.

Overall, polyfunctional adaptive T_{EFF} responses prominent in WT mice were significantly impaired in Clec9A-DTR; whereas in contrast innate immunity did not appear to be defective in these Clec9A⁺ DC-depleted animals during acute *PccAS* infection.

3.2.5. Systemic cytokine milieu was skewed towards anti-inflammatory rather than pro-inflammatory in Clec9A⁺ DC-depleted mice

The reduced local proinflammatory cytokine production attributed to impaired T_{EFF} polyfunctionality in afflicted organs of Clec9A-DTR mice was paralleled systemically through significantly lower levels of serum IFN γ (Figure 3.8, left). TNF α , which did not display locally distinct trends in earlier SPICE analysis, was also systemically reduced in Clec9A-DTR mice (Figure 3.8, middle). Conversely, serum IL-10 was also elevated, despite the lack of correlation with SPICE analysis (Figure 3.8, right). This suggested other cellular sources of TNF α and IL-10 that were not examined in this study, like B-cells and macrophages. Overall, the systemic cytokine milieu in Clec9A-DTR mice was skewed towards anti-inflammatory IL-10, instead of pro-inflammatory IFN γ and TNF α like in WT counterparts.

Fig 3.8: Clec9A⁺ DC depletion leads to increased systemic anti-inflammatory IL-10 levels.

Quantification of systemic IFN γ , TNF α and IL-10 levels in *PccAS*-infected WT (black) and Clec9A-DTR (blue) mice. Serum cytokines were measured at 9 d.p.i in pg/mL, and steady-state baseline is denoted by red dashed lines on individual graphs. Data shown in SEM is cumulative of three independent experiments, n = 3-4/group, 6-10 weeks old. Statistical significance was determined using Mann-Whitney U test, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3.3 Roles of Clec9A⁺ DCs in controlling *Plasmodium chabaudi* *chabaudi* AS chronic malaria

Following acute-phase infection, *Plasmodium*-specific antibody and immune memory responses are indispensable for the resolution of, and subsequent protection against chronic *PccAS* infection [115, 174, 224, 228, 237, 255, 306, 307].

To recapitulate, Clec9A-DTR mice developed multiple dramatic recrudescence as opposed to WT littermates (Fig 3.3B). CD4⁺ T_h cells are crucial in establishing antibody-mediated immunity via antigen-dependent activation and proliferation of B-cells, germinal center (GC) formation, maturation and production of antibodies [308, 309]. Supported by the evident impaired T_{EFF} functionality observed so far, we hypothesized involvement of Clec9A⁺ DCs in downstream modulation of the T-B-cell axis, which subsequently influences the aptitude of infected mice to resolve *PccAS* malaria.

3.3.1 Absence of Clec9A⁺ DCs does not impair germinal center reactions and total *Plasmodium*-specific IgG production, but skews Ig towards T_{h2}-polarized IgG1

We evaluated the impact of Clec9A⁺ DCs on splenic B220⁺ CD19⁺ B-cells and *Plasmodium*-specific antibody production at 16 -18 d.p.i., a time interval coinciding with the interface between acute and recrudescence infection. We broadly assessed non-antigen-specific GC B-cell, plasmablast/plasma cell (PB/PC) and B memory (B_{MEM}) precursor subsets with surface markers as referenced in [255] and [310] (summarized in Appendix 7.1 Table 3).

Firstly, despite the stark disparity in T-cell cellularity between WT and Clec9A-DTR mice, it was not paralleled in absolute numbers of B-cells (Supplementary Figure 3A). Both B220⁺ and sub-gated B220⁺CD19⁺ fractions were not significantly decreased in Clec9A-DTR mice.

Comparable percentages of CD19⁺ CD38^{lo} GL-7⁺ B-cells were observed in the spleens of infected WT and Clec9A-DTR (Figure 3.9A, top row), indicating ongoing GC formation and reactions. Surface-bound IgG and IgM on B-cells could be used as an indicator for B-cell activation and functionality, as they downregulate IgD upon maturation. Surface-bound

IgM and IgG (sIgG and sIgM) staining of IgD⁻ GC B-cells, revealed no stark differences (Figure 3.9B, top row) between both groups, although percentage of IgG⁺ GC B-cells was slightly higher in WT mice. These trends were recapitulated in splenic B220⁺ IgD⁻ CD38^{lo} CD138⁺ plasmablasts (PB) (Figure 3.9A and B, middle row). IgD⁻ CD23⁻ CD38^{hi} B_{MEM} precursors comprising a small fraction (about 2-3%) of total B220⁺CD19⁺ B-cells, were significantly higher in proportion and surface IgM/IgG in WT than Clec9A-DTR mice (Figure 3.9A and B, bottom row). Taken together however, these results suggest Clec9A-DTR mice were not defective in initiating GC reactions, B-cell responses or antibody class-switching (IgM → IgG), despite severe impairment in CD4 T_{EFF} polyfunctionality.

Lastly, serum *Plasmodium*-specific immunoglobulins (Ig), a direct readout of antibody production was assessed. OD_{370nm} of total IgG, although lower in Clec9A-DTR mice, were overall comparable between WT and Clec9A-DTR mice (Figure 3.9C - D), corroborating well with B-cell observations. Conversely, *Plasmodium*-specific IgM was significantly higher in WT than Clec9A-DTR mice, their increased production could be attributed to other B-cell subsets, such as IgM⁺ marginal zone B-cells, that were not investigated [167].

Upon further assessment of *Plasmodium*-specific IgG isotypes as a proxy for B-cell class-switching recombination (CSR), distinct differences in Ig polarization were observed between WT and Clec9A-DTR mice. WT serum contained a diverse mixture of IgG2b, IgG2c and IgG3, with T_{h1}-polarized IgG2c as the dominant isotype and low levels of IgG1. In stark contrast, Clec9A-DTR serum had a reverse phenotype containing predominantly T_{h2}-inclined IgG1, with diminished levels of IgG2 isotypes and nearly undetectable IgG3 (Figure 3.9C - D).

A

Gated on live CD3⁻ B220⁺ CD19⁺ cells

B

C

D

Fig 3.9: Clec9A⁺ DC depletion does not impair germinal center B-cell reactions but influences polarization of *Plasmodium*-specific Ig isotypes produced

Flow cytometry analysis showing induction of live B220⁺ CD19⁺ B-cell responses in *PccAS*-infected WT and Clec9A-DTR spleens during recrudescence at 16-18 d.p.i. (A) Representative plots and bar charts show percentage of CD38^{lo} GL-7⁺ germinal center (GC) B-cells (top row), CD38^{lo} CD138⁺ plasmablasts (middle row) and IgD⁻ CD23⁻ CD38^{hi} B_{MEM} precursors (bottom row) in WT and Clec9A-DTR mice. (B) Bar charts depict percentage of surface IgG (sIgG⁺) and IgM (sIgM⁺) on GL-7⁺ GC B-cells (top), CD138⁺ plasmablasts (middle) and CD38^{hi} B_{MEM} (bottom) in SS (white), WT (black) and Clec9A-DTR (blue) mice. Data shown in SEM per subset is calculated relative to root % B220 fraction, and cumulative of three independent experiments, n = 3/group, 6-10 weeks old. (C-D) *Plasmodium*-specific immunoglobulin (Ig) isotypes in the serum of WT and Clec9A-DTR mice during *PccAS* infection. (C) 3-fold serial dilution curves and (D) dot plots show levels of IgG and IgM (left), IgG1 and IgG2c (middle), IgG2b and IgG3 (right) isotypes in SS (white), WT (black) and Clec9A-DTR (blue) mice at 14-17 d.p.i. Ig levels are obtained from OD_{370nm} values (– blank OD_{370nm}). Baseline steady-state Ig levels in (D) are depicted as red dashed lines on individual plots. IgG and IgM values are derived from 1:150 dilution and isotype IgG1, IgG2b, IgG2c, and IgG3 values from 1:30 dilution. Data shown in SEM are cumulative of five independent experiments, n = 3-7/group, 6-10 weeks old. Statistical significance for (B) and (D) was determined using Mann-Whitney U test, and (C) by 2-way ANOVA with Bonferroni's post-test: ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3.3.2 Serum passive transfer of Clec9A-DTR *Plasmodium*-specific IgG1 confers less partial protection than WT T_{h1}-polarized *Plasmodium*-specific IgG2c

The apparent disparity in *Plasmodium*-specific IgG1 and IgG2b/IgG2c polarization in Clec9A-DTR and WT mice respectively led us to deduce whether Ig isotypes imparted differential protective effects during recrudescence episodes. A serum passive transfer experiment was designed to investigate this hypothesis using *PccAS*-infected C57BL/6J WT recipients administered different groups of pooled donor serum (pre-screened to fit expected Ig polarization profiles – Supplementary Figure 3B), following the schedule described in Figure 3.10 (top), and monitoring their infection kinetics.

Infected C57BL/6J WT male mice receiving either steady-state control serum and sham PBS presented course of infection similar to WT mice described in Figure 3.3A; with peak parasitemia observed at 10 d.p.i., before rapid resolution to low levels with sub-patent recrudescence (Figure 3.10, bottom). C57BL/6J WT recipients administered three doses of IgG1-predominant Clec9A-DTR immune serum showed similar onset and

magnitude of acute infection, as well as recovery kinetics (red curve) as control mice (clear and black curves), indicating negligible protection. In a second serum passive transfer performed with four doses of immune serum - including one pre-infection dose (Figure 3.10B), recipients of Clec9A-DTR immune serum presented with delayed onset and two-fold reduced percentage blood parasitemia as compared to controls (Figure 3.10B, red and black curves), suggesting some partial protection conferred by Clec9A-DTR serum, that was dose-dependent.

Conversely, both C57BL/6J WT recipient groups of IgG2c-predominant WT immune serum presented with delayed onset (3 – 4 days later), and two to four-fold lower magnitude percentage blood parasitemia; relative to WT controls (Figure 3.10A - B, blue curves). IgG2c-predominant WT immune serum recipients also showed greater delayed onset and roughly two-fold lower magnitude of acute percentage blood parasitemia compared to recipients of IgG1-predominant infected Clec9A-DTR serum and controls (Figure 3.10A - B). Mice which received four doses of WT immune serum had the lowest magnitude and latest onset of infection. This suggests that IgG2c-polarized serum could indeed confer greater degree of partial protection against acute *PccAS* infection than IgG1-polarized serum in a dose-dependent manner.

Interestingly, despite the heightened boost in controlling acute parasitemia, WT immune serum recipients additionally experienced a prolonged period of low-grade recrudescence peaking around 1 – 1.5%. This phenomenon typically persisted in the week following acute infection, before reduction to sub-patent levels, and was not observed in other recipient groups (Figure 3.10).

Figure 3.10: IgG1-predominant Clec9A-DTR immune serum confers less partial protection against *PccAS* challenge

IgG1-predominant Clec9A-DTR immune serum does not ameliorate *PccAS* challenge in WT recipients when passively transferred. (A – B) Experimental timeline shows schedule of passive transfers after *PccAS* infection (top). Dot graph (bottom) shows percentage blood parasitemia of *PccAS*-infected C57BL/6J WT recipients after receiving either (1) sham PBS (diamonds), (2) steady-state control serum (clear circles), (3) IgG2b/2c-skewed WT immune serum (blue circles) or (4) IgG1-skewed Clec9A-DTR immune serum (red squares) in (A) three doses (at +2h p.i., 1 d.p.i and 2 d.p.i.) or (B) 4 doses (at -2h p.i., +2h p.i., 1 d.p.i and 2 d.p.i.). Sera for passive transfer was pre-tested for Ig polarization and

pooled from (n = 10) mice. Data shown is representative of two individual experiments with n=4-5 mice/group, 6 – 8 weeks old.

Taken together, findings so far implicated that differential Ig isotype polarization played a role in the phenotype manifestation of Clec9A-DTR mice and their inability to control *PccAS* infection beyond the acute phase. Although these early *Plasmodium*-specific Ig were not capable of complete protection by serum passive transfer, the T_{h1}-polarized IgG2/IgG3 isotypes were able to control acute infection more effectively; and we had earlier demonstrated that upstream T_{h1} proinflammatory and T_{EFF} responses were severely compromised consequently due to absence of Clec9A⁺ DCs. To unify these two concepts in our infection model, we revisited upstream T_{h1} T_{EFF} influences on antibody production, as well early memory T (T_{MEM}) precursors or T_{EFF} involvement during recrudescence.

3.3.3 Clec9A⁺ DCs influence *Plasmodium*-specific Ig class-switch recombination by modulating IFN γ production in effector follicular T-helper (T_{FH}) cells

Antibody affinity maturation and class-switch recombination (CSR) in the splenic GCs is a complex process orchestrating concerted efforts from the triumvirate of B, T-cells and follicular DCs - to ultimately produce protective antibodies. A specialized CD4⁺ T_h subset – T follicular helper cells (T_{FH}), are indispensable for GC survival and maintenance by direct interaction with GC residents and influencing local cytokine milieu with their diverse effector cytokines [308, 309, 311-314].

The recrudescence phenotype and immune responses we observed led us to revisit T_{FH} functionality during acute *PccAS* infection to determine if upstream events were responsible for downstream preferential Ig skewing. CD4⁺ T_{FH} were elucidated by expression of the T_{FH} lineage master transcription factor B-cell lymphoma 6 (Bcl6), along with surface markers of activated T_{FH}, PD-1 and CXCR5 (see Appendix 7.1 Table 2); and assessed for production of effector cytokines [315-318].

Stimulated WT and Clec9a-DTR mice were nearly equivalent in proportion of Bcl6⁺ CD4⁺ T-cells, however in absolute numbers WT had significant higher levels of T_{FH} than Clec9A-DTR mice. Both CXCR5⁺ PD-1⁺ and

CXCR5⁺ T_{FH} [308, 319] subsets were lower in proportion but not significantly so in Clec9A-DTR mice, compared to WT mice (data not shown). However, taking into account splenic cellularity, WT mice had nearly two-fold greater numbers of activated Bcl6⁺ CXCR5⁺ PD-1⁺ T_{FH} than Clec9A-DTR mice (Figure 3.11A).

IL-21 is the classical effector cytokine associated with CD4⁺ T_{FH} functionality and it exerts both autocrine and downstream effects on T_{FH} themselves and B-cells. However, owing to their phenotype plasticity, T_{FH} can secrete also a variety of cytokines for different effector functions, such as, but not limited to, IL-4, IFN γ or IL-10 [242, 245]. An IFN γ -polarized tissue microenvironment has been reported to be involved in influencing CSR of IgG2a antibodies produced inside GCs or extrafollicular B-cell responses [310, 320]. Hence, IFN γ production of T_{FH} was subsequently assessed in *PccAS*-infected mice.

In the absence of Clec9A⁺ DCs, Bcl6⁺ CXCR5⁺ T_{FH} showed significantly lower proportion and absolute numbers of IFN γ ⁺ effector cells in both PD-1⁺ and PD-1⁻ fractions (Figure 3.11B) compared to WT mice. This reiterated an overall less T_{H1}-inclined systemic and local tissue proinflammatory environment, which could synergistically attribute to impairments in downstream CSR of *Plasmodium*-specific Ig isotypes such as IgG2b/IgG2c, necessary for greater and sustained protection against *PccAS* chronic malaria.

Fig 3.11: Impaired T_{H1} effector T follicular helper (T_{FH}) cell functionality is evident in *PccAS*-infected Clec9A-DTR mice

(A) Reduced absolute numbers of CD4⁺ T_{FH} in *PccAS*-infected Clec9A-DTR mice at 9 d.p.i. Representative dot plots show proportions of live splenic CD3⁺ CD44⁺ Bcl6⁺ CD4⁺ T_{FH} (top) and Bcl6⁺ CXCR5⁺ or CXCR5⁺ PD-1⁺ activated CD4⁺ T_{FH} (bottom) in infected WT (left) and Clec9A-DTR (right) mice. Bar charts show absolute numbers of Bcl6⁺ T_{FH} (top) and Bcl6⁺ CXCR5⁺ PD-1⁺ (bottom) T_{FH} in WT (black) and Clec9A-DTR (blue) mice. (B) Decreased proportions and absolute numbers of Bcl6⁺ CXCR5⁺ PD-1⁺ activated effector T_{FH} producing IFN γ in the absence of Clec9A⁺ DCs. Representative dot plots show proportions of IFN γ ⁺ Bcl6⁺ CXCR5⁺ PD-1⁺ or PD-1⁻ T_{FH} in WT (left) or Clec9A-DTR (right) sub-gated from (A). Bar charts represent absolute numbers of IFN γ ⁺ Bcl6⁺ CXCR5⁺ PD-1⁺ (left) and CXCR5⁺ PD-1⁻ (right) effector T_{FH} in WT (black) and Clec9A-DTR (blue) mice. Steady-state baseline controls for both (A) and (B) are denoted with red dashed lines across individual graphs. Data shown in SEM are cumulative of three independent experiments with n=11/group, 6 – 10 weeks old. Statistical significance was analyzed using Mann-Whitney test, *p < 0.05, **p < 0.01 and ***p < 0.001.

3.3.4 Absence of Clec9A⁺ DCs results in persistent impaired CD4 and CD8 T_{EFF} during recrudescence episodes

Given the impairment in the ability of Clec9A⁺ DC-deficient mice to mount sufficient T_{H1} proinflammatory responses during acute *PccAS* infection, we sought to investigate their aptitude during recrudescence. *PccAS* chronic malaria is a biphasic infection, and the immune response correspondingly shifts from T_{H1}-dominant towards humoral and antibody-mediated immunity. Although B-cell response broadly evaluated via the proxy of serum antibodies did not show defects in overall *Plasmodium*-specific Ig, only distinct skewing of isotypes; the establishment of immune memory is another crucial arm of humoral immune response. We lastly investigated the effector T-cells and memory T-cell precursors during the first recrudescence episode. As our timepoint was during ongoing infection and not re-challenge or recall, we referred to T_{MEM} subsets profiled as precursors instead of true T_{MEM}, to be more technically stringent. Utilizing cell surface CD44, a marker for antigen-experienced T-cells [321], CD127 (IL-7R α), an important marker of T-cells destined for memory differentiation lineage [322], and KLRG-1, which is highly expressed on activated and terminally-differentiated cells [323] (Appendix 7.1 Table 4), we broadly analysed the global differences in polyclonal CD4⁺ and CD8⁺ T-cell subsets between WT and Clec9A-DTR mice.

Firstly, total CD3⁺ cellularity strikingly remained compromised in Clec9A-DTR mice during recrudescence in spleen, lung and liver (Supplementary Figure 4, left). This was translated further into CD8⁺, and particularly CD4⁺ overall, as well as CD44⁺ activated T-cell numbers in all three organs (Supplementary Figure 4, middle columns). Interestingly in WT mice, the CD4-CD8⁻ double-negative T-cell compartment consisting of mainly $\gamma\delta$ T-cells, together with NKT cells (Supplementary Figure 4, right); expanded exponentially nearly 6-fold and was largely accountable for the massive increase in splenic cellularity. Likewise, WT lung and liver CD4-CD8⁻ T-cells also showed significantly increased numbers, but at more modest magnitudes compared to the spleen.

During recrudescence, unstimulated CD4⁺ T_{EFF} produce little *ex vivo* IFN γ , and the propensity for IFN γ production even after PMA/ionomycin

stimulation was greatly reduced compared to acute malaria (Figure 3.12). In fact, differences between WT and Clec9A-DTR polyfunctionality were less discernible. Higher proportions of single IL-10⁺ CD4⁺ T_{EFF} were seen in Clec9A-DTR mice, and although IFN γ ⁺ cells were still elevated in WT mice; a significant proportion were IFN γ ⁺IL10⁺ CD4⁺ T_{EFF} (Figure 3.12). These observations were in line with the waning of T_{h1} proinflammatory responses and a transition to cytokine environments that support the development of humoral and cell-mediated immunity during chronic malaria [225]. Hence, we do not further emphasize on T_{EFF} polyfunctionality in the following analyses.

Figure 3.12: CD4⁺ effector T-cells are less T_{h1}-polarized and adopt a IFN γ ⁺IL-10⁺ phenotype during recrudescence *PccAS* infection.

Flow cytometry analysis of CD4⁺ T-cells polyfunctionality during *PccAS* recrudescence infections. Representative dot plots show IL-10⁺ / IFN γ ⁺ single (1, 3) and double-producing (2) CD4⁺ T-cells in WT (left) and Clec9A-DTR (right) mice during recrudescence. Bar charts show percentages of PMA/ionomycin stimulated spleen (row 2), lung (row 3) and liver (row 4) CD4⁺ T-cells in SS (white bars), *PccAS*-infected WT (black bars) and Clec9A-DTR mice (blue bars). Unstimulated control (top row). Data shown in SEM are representative of 3

independent experiments with n=3-5 mice/group, 6-10 weeks old. Statistical significance was determined using Student t-test, ns = not significant, *p < 0.05, **p < 0.01 and ***p<0.001.

To analyse polyclonal T memory precursors and effector T-cell subsets in infected and control mice, we adapted a gating strategy as described by Stephens and Langhorne to profile T central memory (T_{CM}) precursors, T effector memory (T_{EM}) precursors and effector T-cells (T_{EFF}) [115, 224, 324]. We focussed on $CD4^+$ T-cells only as the roles and contributions of $CD8^+$ T memory cells in malaria are still contended. Briefly, after excluding $CD62L^{hi}CD44^-$ naïve T-cells, activated $CD44^+$ T-cells were broadly gated into $CD62L^+CD127^-$ T-cells, $CD62L^+CD127^+$ precursor T_{CM} , $CD62L^-CD127^+$ precursor T_{EM} and $CD62L^-CD127^-$ T_{EFF} . $CD27$, the costimulatory receptor important for activated T-cell survival, and $KLRG1$, correlative marker of terminal differentiation, were then used in concert to further delineate T_{EM} and T_{EFF} subsets (Figure 3.13A and Appendix 7.1 Table 4).

WT mice presented with significantly higher absolute numbers of $CD4^+$ T_{CM} , T_{EM} and $CD44^+ CD62L^- CD127^- T_{EFF}$ than Clec9A-DTR mice (Figure 3.13B and C, left). $CD4^+$ T_{EM} could be further sub-gated using expression of $CD27$ into early $CD27^+$ and late $CD27^-$ T_{EM} from $KLRG1^-$ cells, showing the progressive differentiation pathway from early to late precursors. T_{CM} were also $CD27^+$ (not shown). WT mice did not show significant differences in $CD27^+$ early T_{EM} (T_{EME}) cells but had up to 3-fold higher $CD27^-$ late T_{EM} (T_{EML}) numbers in the spleen, lung and liver than Clec9A-DTR mice (Figure 3.13B, right). This suggested that a greater proportion of T_{EM} precursors in WT mice were differentiated to late T_{EML} instead of remaining in early T_{EME} state, unlike Clec9A-DTR mice.

The $CD127^-$ effector (T_{EFF}) fraction undergoes massive expansion, and all subsequent-derived T_{EFF} subsets showed drastically higher numbers in WT mice than Clec9A-DTR mice in all organs (Figure 3.13C). Similar to T_{EM} , $CD4^+$ T_{EFF} could be further delineated by $CD27$ expression into $CD62L^+ CD27^+$ early T_{EFFE} (Figure 3.13A insert, Figure 3.13C left), $CD62L^-CD27^+$ transitioning T_{EFFINT} and $CD62L^-CD27^-KLRG-1^+$ late T_{EFFL} . The significantly increased numbers of T_{EFF} in WT mice were also concentrated in the later differentiation stages, T_{EFFINT} and particularly T_{EFFL} , rather than

T_{EFFE} which was only modestly higher than in Clec9A-DTR mice (Figure 3.13C, right). Interestingly, we also observed distinct KLRG1⁺ CD4⁺ T_{EFF} populations (both CD27⁺ and CD27⁻), especially in the lung and liver, which were proportionately and numerically higher in WT mice than Clec9A-DTR mice (Supplementary Figure 5). KLRG-1 is a well-described marker of terminally-differentiated CD8⁺ T_{EFF} and highest levels of expression are often correlated with CD8⁺ short-lived effector cells (SLEC); but is not known to be strongly linked in CD4⁺ T_{EFF} [323]. Some studies in *Toxoplasma gondii* models have also reported the appearance of KLRG-1⁺ CD4⁺ T_{EFF} following acute infection, however no unique function nor certainty of terminal differentiation has yet to be attributed to them [325]. Nonetheless, it could represent a distinct differentiated subset within polyclonal CD4⁺ T_{EFFL}, which was likewise impaired in Clec9A-DTR mice. Altogether, these results suggest there might be a defect, or a delay in the differentiation pathway to late-stage CD4⁺ T_{MEM} precursors and effector cells in Clec9A-DTR mice.

In conclusion, the recrudescence phenotype in Clec9A⁺ DC-deficient mice was characterized by intact B-cell responses and antibody production, T_{H2}-polarized IgG1 isotype predominance and prolonged defects in T-cell cellularity, which resulted in impaired expansion of non-classical CD4⁺CD8⁻ T-cells as well as CD4⁺ T_{EFF} and T_{MEM} precursor subsets. Taken together, these compounding factors could contribute to an overall reduced capacity and capability of Clec9A-DTR mice to control chronic *PccAS* infection compared to WT counterparts.

A

Gated from live CD45⁺ CD3⁺ CD44⁺ CD4 T-cells:

B

C

Figure 3.13: Clec9A⁺ DC-deficient mice have severely impaired terminally-differentiated effector and effector memory CD4 T-cell numbers during recrudescent *PccAS* infection.

(A) Flow cytometry analysis of CD3⁺ CD4⁺ T-cell subsets during *PccAS* recrudescent infection (16 – 18 d.p.i). Representative dot plots of CD44⁺ CD4⁺ T-cell subsets in the spleen (left), lung (middle) and liver (right) of WT and Clec9A-DTR mice. Dot plots in row 2 correspond to CD127⁺ T_{EM} and row 3 to CD127⁻ T_{EFF} subsets. (1) denotes. Numbers on dot plots show percentages of respective fractions. Coloured numbers denote subsets of interest - T_{CM}, T_{EM}, T_{EME}, T_{EML}, T_{EFF}, T_{EFFE}, T_{EFFINT} and T_{EFFL}. Insert plot at bottom: further sub-gating of T_{EFFE} from parent plot. (B - C) Bar charts show total numbers of CD127⁺ T_{EM}/T_{EFF} (B) and CD127⁻ T_{EFF} (C) cell subsets profiled in spleen, lung and liver of SS (white bars), *PccAS*-infected WT (black bars) and Clec9A-DTR mice (blue bars). Coloured numbers denote corresponding subsets of interest in (A). Data shown are SEM values cumulative of 3 independent experiments with n=3-4 mice/group, 6-10 weeks old.

Statistical significance was determined using Mann Whitney's test, ns = not significant, *p < 0.05, **p < 0.01, ***p<0.001 and ****p<0.0001.

3.4 Results - Macrophage Ontogeny Tracking in Acute Malaria

During *Plasmodium* infection, the large amounts of parasite, and resultant inflammation and immune responses results in striking changes in local organ microarchitecture. Particularly in the spleen, the distinct steady-state organizations of splenic resident macrophages, such as marginal metallophilic and marginal zone macrophages (MMM, MZMs), are disrupted and become disperse [174, 253]. Unpublished data from our laboratory also supported these findings. Given the importance of macrophages in the clearance of *Plasmodium* parasites both in early and late infection, we sought to understand their refilling kinetics in malaria afflicted organs, by using the Kit^{MerCreMer/R26} reporter mouse, that labels precursors / cells of BM origin YFP⁺ after administration of tamoxifen.

3.4.1. Infection kinetics in wildtype Balb/c and Kit^{MerCreMer/R26} mice during *Plasmodium yoelii* acute malaria

In this series of investigations, we utilized *Plasmodium yoelii* (Py1.1GFP strain) as it is an acute model of malaria, which allowed us to evaluate the infection kinetics without prolonged periods of natural recovery, as would chronic *Plasmodium chabaudi chabaudi* AS.

Py1.1GFP infection caused a steady increase in percentage blood parasitemia detectable from 3 d.p.i. to a peak around 9 – 11 d.p.i, with average 20% iRBCs in WT Balb/c mice (Figure 3.14A). A small recrudescence was observed around 13 – 15 d.p.i, and the infection self-resolves by 30 d.p.i. In Kit^{MerCreMer/R26} mice a similar trend was also observed, though ascension was more modest and peak parasitemia (9 – 10 d.p.i.) was overall lower at 12 – 15% iRBCs than in the Balb/c background (Figure 3.14B). The infection was likewise resolved by 30 d.p.i. and no mortalities were observed for both groups of mice.

Figure 3.14: *P. yoelii* blood-stage infection in WT Balb/c and *Kit^{MerCreMer/R26}* mice
 Infection kinetics of *P. yoelii* (*Py1.1GFP*) from 3 – 33 d.p.i. Curve shows percentage of infected red blood cells (iRBCs) in (A) WT and (B) *Kit^{MerCreMer/R26}* mice. Data shown are in SEM and representative of > 5 independent experiments with n=4-6 mice/group, 6-10 weeks old.

3.4.2 Myeloid kinetics in WT Balb/C and *Kit^{MerCreMer/R26}* mice during *Plasmodium yoelii* acute malaria

For myeloid analysis, we derived CD45⁺ CD11c^{lo}/CD11c^{int} myeloid populations using CD11b, F480, Ly6G and CD169 (Figure 3.16 and Appendix 7.1 Table 1). Total CD45⁺ cells were gated as initial attempts at traditional forward/side scatter plots overstated the duration of MØ disappearance (> 35 d.p.i.) as they were located very high up on the side scatter scale. FrI denoted the tissue-resident macrophages (TRMØ) which were also CD169⁺ (not shown), in line with recently published work by Gupta *et al* [253] and others. FrII monocytes and macrophages were subgated further using Ly6C and MHC-II to delineate differentiating and transitioning populations as previously described by Sheng *et al* [60]. Although FrIII Ly6G⁺ neutrophils, Ly6C^{int} MHCII⁻ FrIIai monocytes/neutrophils and Ly6C⁻ MHCII⁻ eosinophils were illustrated by this gating strategy, we did not emphasize heavily on these populations. Hence, in the following sections, only Ly6C⁺ FrIIai monocytes would be predominantly discussed.

Figure 3.15: Gating strategy for tissue-resident macrophages and monocyte subsets

Gating strategy of flow cytometry analysis of myeloid populations during *Py1.1GFP* infection in spleen, lung and liver. From CD45⁺ CD11b⁺ population, F480^{hi} FrI (tissue-resident macrophages), F480^{int} FrII (monocytes/macrophages) and F480^{lo} FrIII (neutrophils) are gated. Further subgating from FrII yields Ly6C^{hi} FrIIai and Ly6C^{int} FrIIai monocytes, Ly6C⁺ MHCII⁺ FrIIb monocytes, FrIIc macrophages and Ly6C⁻ MHCII⁻ eosinophils (Eo).

3.4.2.1 Tissue-resident macrophages are differentially repopulated but infiltrating monocytes have similar kinetics in the organs of *Py1.1GFP*-infected mice

Figure 3.15A shows representative plots of timepoints assessed in Balb/C WT and Kit^{MerCreMer/R26} mice and the general trend of myeloid kinetics throughout the course of *Py1.1GFP* infection. Since Balb/C and Kit^{MerCreMer/R26} mice had slightly different infection kinetics (Figure 3.14), we profiled myeloid kinetics in both to determine if there were any disparities that might affect interpretation of results.

A detailed profiling of FrI and FrII subsets in Kit^{MerCreMer/R26} mice illustrated the stark disappearance of spleen and liver FrI early from 3 - 4 d.p.i. FrI remained severely depleted from 4 – 7 d.p.i. in spleen and progressively recovered from 10 – 14 d.p.i, although proportion was lower relative to steady-state (Figure 3.16A and C). Liver FrI rapidly began to recover following day 3 disappearance, though proportions remained lower than

steady-state till 7 d.p.i, and greatly increased from 10 - 14 d.p.i. (Figure 3.16A and E) Overall for spleen and liver, day 35 end-point Frl were not significantly different from steady-state. The lung however, undergoes a more gradual decline and AMØs never fully disappeared at any timepoint unlike spleen MØs and liver KCs. Proportion of Frl was lowest in lung between 10 – 14 d.p.i., but upon recovery was present at significantly higher levels than steady-state (Figure 3.16A and D).

Frl subsets, which follow the differentiating pathway from Frla → Frlb → Frlc [60], expanded greatly in proportion following Py1.1GFP infection. A massive influx of Frlai monocytes from 3 - 4 d.p.i. was observed in all three organs, followed by concomitant increase firstly in Frlb (3 – 5 d.p.i) and sequentially Frlc (7 – 14 d.p.i) a few days later, when Frlai subsides. (Figure 3.16B-E). Interestingly, Frlc MØs also remained elevated in proportion after recovery in all three organs.

Similarly, in Balb/C WT mice the overall trend recapitulates that observed in *Kit^{MerCreMer/R26}* mice, but with a more prolonged period of disappearance in Frl MØs (Supplementary Figure 6A – C). Particularly in the lung, the kinetics were distinct in that a decrease was first observed from 3 d.p.i., followed by steady decline and although never fully disappearing, remained significantly reduced till 14 d.p.i. (Supplementary Figure 6B). Aside from the spleen, both lung and liver Frl are significantly higher in proportion at 35 d.p.i. than steady-state.

Frlai and Frlb inflammatory monocytes also followed relatively similar kinetics as *Kit^{MerCreMer/R26}* mice in terms of influx during early infection, with Frlb increasing as Frlai decreases (Supplementary Figure 6A – C). Lastly, Frlc differentiated MØs, remained at low proportions until 5 (spleen) or 6 d.p.i. (lung and liver), when they progressively increased to peak at 10 d.p.i. as Frla and Frlb subset % began to wane (Supplementary Figure 6B – C). At 15 d.p.i., spleen and lung continued to show high proportion of Frlc while liver has begun to decline. High % Frlc persisted in the spleen till 21 d.p.i., which could be due to the small Frla influx observed at 15 d.p.i. (Supplementary Figure 6A), and subsequent continued differentiation. By

36 d.p.i., all three organs had declined substantially, but still showed significantly higher proportions of Frlc MØs relative to steady-state (Supplementary Figure 6B – C).

Long-term repopulation kinetics of recovered day 35 Frl in spleen, liver and lung were similar or slightly higher in proportion than steady-state mice. Frl analysed on 60 or 90 d.p.i were not significantly different in proportion from day 35 Frl (Figure 3.17), indicating that the system had returned close to steady-state, and remained stable after recovery without quick turnover.

A

B

C

D

E

Figure 3.16: Tissue-resident macrophages are differentially repopulated in organs of *Kit^{MerCreMer/R26}* mice during *Py1.1GFP* infection

Myeloid population kinetics in *Kit^{MerCreMer/R26}* mice during *Py1.1GFP* infection. Representative plots of (A) Frl TRM, FrlI monocytes, FrlII neutrophils and (B) FrlI subsets: FrlIai, FrlIb monocytes, FrlIc macrophages and Eo eosinophils in spleen, lung and liver from d.p.i. 3 - 35. (C-E) % kinetics of Frl (red, top row) TRM, FrlIai (blue, 2nd row) monocytes, FrlIb (green, 3rd row) and FrlIc (purple, bottom row) in (C) spleen, (D) lung and (E) liver from 3 - 35 d.p.i. Data shown are SEM values calculated relative to total % CD11b⁺ myeloid cells; and cumulative of 2 independent experiments with n=3-4 mice/group, 6-10 weeks old upon TAM treatment. Statistical significance was determined using Mann Whitney's test against steady-state baseline (D0): *p < 0.05, **p < 0.01, ***p<0.001 and ****p<0.0001.

Figure 3.17: Repopulated tissue-resident macrophages are stable long-term in organs of *Kit^{MerCreMer/R26}* after *Py1.1GFP* infection

Myeloid population kinetics in *Kit^{MerCreMer/R26}* mice after recovery from *Py1.1GFP* infection. Representative plots of Frl TRM, FrlI monocytes and FrlII neutrophils in spleen (left), lung (middle) and liver (right). Dot curves show % kinetics of Frl from d.p.i. 35 – 90 in spleen (red), lung (blue) and liver (green). Data shown are SEM values calculated relative to total % CD11b⁺ myeloid cells; and cumulative of 2 independent experiments with n=3-4 mice/group, 6-10 weeks old upon TAM treatment. Statistical significance was determined using Mann Whitney's test against recovery endpoint (D35): ns = not significant.

3.4.2.2 *Py1.1GFP* infection induces transient activated tissue-resident macrophages

As mentioned earlier, Frl MØs in recovered mice had very high side scatter characteristics, indicating increased granularity and volume. Indeed, haemozoin deposits, an inflammatory metabolite of *Plasmodium* parasites [153], became visually apparent as acute infection progresses, and even after recovery and clearance of parasite, organs were still laden with haemozoin. Macrophages and myeloid cells which had phagocytosed haemozoin particles could match the criteria for high side scatter. Cytospins were performed on sorted Frl to determine cell morphology. Figure 3.18A

(1-6) showed copious amounts of haemozoin from sorted Frl MØs during day 10 peak infection in the liver. In addition, many of these Frl MØs were also highly vacuolated (images 2, 3, 4) and cell volume was large (images 5, 6) compared to steady-state MØs.

Frl TRMØs also appeared to display a positional shift during *Py1.1GFP* infection (Figure 3.18B). An overlay of CD11b⁺ and F480⁺ plots at 10 d.p.i. revealed infected TRMØs in all three organs were shifted towards the right (CD11b^{hi}) compared to steady-state TRMØs and had higher count of CD11c and CD169 expression (Figure 3.18B). We termed this as an ‘transient activated’ MØ phenotype that was most prevalent from 7- 10 d.p.i around peak infection, but subsided and was not seen at 35 d.p.i or later timepoints in recovered mice.

Figure 3.18: Activated transient tissue resident macrophages in *Py1.1GFP*-infected mice are laden with ingested haemozoin and highly vacuolated

Frl macrophages at 10 d.p.i in *Py1.1GFP*-infected *Kit^{MerCreMer/R26}* mice. (A) Representative microscopy images (1-6) of sorted liver Frl KCs showing haemozoin deposits and vacuoles. Fixed cytopsin slides are stained with Giemsa-wright stain and images are acquired on 100x magnification. (B) Dot plots show overlay of SS and infected CD11b⁺ / F480⁺ Frl macrophages (top) and their CD11b, CD11c and CD169 expression counts in spleen (left), lung (middle) and liver (right). Red dots and histograms denote infected WT MØ and blue denotes steady-state (SS) baseline.

3.4.2.3 Distinct organ-specific myeloid subset clusters are distinguished by high dimensional analyses during Py1.1GFP infection

Lastly, to present with a global overview and unbiased data-driven analyses of myeloid subsets during *Py1.1GFP* infection, we applied the high-dimensional methods of PhenoGraph cell clustering [326] and Uniform Manifold Approximation and Projection (UMAP) analysis; a novel, fast, and reliable non-linear dimensionality reduction technique [327] to our timepoint data. Triplicates of each timepoint from steady-state to 35 d.p.i, in spleen, liver and lung were analysed with a multi-parameter array shown in Table 3.1.

FSC	SSC
CD45	FVS AF
CD11c	MHCII
CD11b	F480
Ly6C	CD169

Table 3.1. UMAP analyses parameters

Spleen and liver samples were analysed as a unified data set as their myeloid kinetics and phenotypes were very similar, but separately from lung samples which exhibited an overall different expression profile and kinetics.

UMAP projections for spleen/liver (Figure 3.19A) and lung (Figure 3.19B) generated 32 and 31 distinct clusters respectively, which represented different activated, non-activated and non-myeloid subsets. Utilizing the corresponding phenograph clustering heat maps (Figure 3.19C and D), together with back-gating on Flowjo X software allowed for annotation of numbered clusters according to Table 4.1 parameters. Cluster delineation in the spleen/liver revealed FrI F480^{hi} TRMØs in clusters {1, 2, 8, 21, 31}, FrIIai Ly6C^{hi} monocytes in {12, 25}, FrIIb Ly6C⁺ MHCII⁺ transitional/inflammatory monocytes in clusters {9, 10, 28} and FrIIc MHCII⁺ differentiated MØs in {3, 16, 18, 22}, while FrIII neutrophils and eosinophils were respectively found in clusters 32 and 20 (Figure 3.19A).

In the lungs (Figure 3.19B), FrI alveolar MØs were distinctly in cluster {15, 25} due to their unique CD11c^{hi} expression profile. FrII subsets were found

in cluster 6 (Frllai), cluster {8, 18} (Frllb), cluster {10, 16, 20, 30} (Frllc) and cluster 19 (eosinophils), as well as neutrophils in cluster 2.

A time-course series of expression plots were also generated for UMAP analysed spleen, liver and lung samples. Like Figure 3.19, the lung was separated from spleen and liver due to its distinct marker expression profile. This allowed for visualization of expression kinetics of particular markers of interest temporally, graded from low (blue) to high (red) expression. We followed the kinetics of F480 (Figure 3.20A and D), Ly6C (Figure 3.20B and D) and CD11c (Figure 3.20C and D), and observed increased expression and the subsequent waning at timepoints corresponding to higher parasite loads and recovery respectively. In spleen/liver (Figure 3.20A - C), Frl TRMØs were expectedly F480^{hi} in steady-state, and upon their brief disappearance, the refilling F480⁺ clusters spatially corresponded to the Frllai monocytes (clusters 12, 25 - F480^{int}) and Frllb inflammatory/transitional monocytes (clusters 9, 10, 28 - F480^{hi}) from 5 d.p.i onwards (Figure 3.19A).

Concurrently, increased CD11c expression which marked the activated transient macrophages in Figure 3.18 could also be observed in the refilling Frl and monocytic fractions (Figure 3.20C). Interestingly, after resolution of infection, Frllc MØs remain high in CD11c expression, which had subsided in Frl TRMØs. Ly6C expression (Figure 3.20B) was highest during peak monocytic influx (5 – 7 d.p.i, Frllai and Frllb clusters), and remain elevated relative to steady-state throughout days 10 – 14 p.i., corresponding well with previous myeloid kinetics results in Figure 3.19 and Supplementary Figure 6. In the lung, likewise high F480 (Figure 3.20D, top) and CD11c (Figure 3.20D, bottom) expression was seen on Frl AMs (cluster 15, 25), and various Frl subsets (clusters 6, 8, 18, 10, 20, 16, 30), which was also correspondingly Ly6C^{hi} at monocytic influx timepoints (Figure 3.20D, middle); as infection progresses.

By 35 d.p.i, the overall myeloid profile and expression had re-localized to roughly the original positioning as steady-state baseline. Interestingly, recovered spleen/liver F480^{hi} Frl TRMØs, though sitting in the same

positional area; had a visually different profile (condensed blob) from steady-state controls (sharp diagonal). Back-gating of UMAP data on Flowjo X software revealed recovered Frl TRMØs were high in SSC, corroborating well with haemozoin deposition and engulfment as supported by cytospin data (Figure 3.18A), hence their disparity from steady-state samples.

Figure 3.19: High dimensional analysis of myeloid dynamics shows distinct clusters in spleen, liver and lung

Phenograph clustering and UMAP analyses of myeloid subsets in SS and *Py1.1GFP*-infected spleen, liver and lung. (A - B) UMAP projections of (A) spleen/liver and (B) lung based on FSC/SSC and eight surface expression markers – FVS, CD45, CD11c, MHC-II, CD11b, F4/80, Ly6C and CD169. Circled myeloid subsets on UMAP plots corresponds to colour legends. (C - D) Heat maps (intensity scale, 0 – 4) of median parameter (columns) expression values (post-transformation values, see methods) for each phenograph cluster (rows) for the concatenated data obtained from (C) spleen/liver and (D) lung samples.

Figure 3.20: Myeloid subset expression plots show distinct time-course kinetics during *Py1.1GFP* infection

Expression plots of UMAP analyses parameters from D0 – D35 in *Py1.1GFP*-infected mice. Time course expression of (A) F480, (B) Ly6C and (C) CD11c are shown in spleen (top), liver (bottom) and the same markers in (D) lung. Colour-mapped plots indicate low to high expression of parameters: blue (low) → green → yellow → orange → red (high). X and y-axis scale: 0.0 – 1.0, 0.2 interval.

3.4.3 Myeloid ontogeny tracking during *Py1.1GFP* infection

Following the detailed myeloid kinetics and dynamics profiling in *Py1.1GFP*-infected mice, we next tracked the ontogeny and turn-over of these subsets. *Kit^{MerCreMer/R26}* reporter mice developed by our lab [60] is a valuable tool to track myeloid ontogeny, as tamoxifen (TAM) administration in foetal or adult mice allows for labelling of cKit⁺ HSC or BM-derived progenitors and their progeny, via yellow fluorescent protein (YFP) expression. Relative YFP labelling of subsets presented were internally normalized using FrIII neutrophils as 100%, due to their quick turn-over and pure BM origin.

Tissue resident macrophages were mostly foetal-derived; only a miniscule percentage of them are BM-derived and YFP⁺. Typically, our steady-state data shows FrI TRMØs had baseline YFP⁺ of less than 15% and 10%, in the spleen/liver and lung respectively. Steady-state FrIIai and FrIIb monocytes had normalized YFP labelling close to 100%, indicating majority of BM origin. Meanwhile FrIIc MØ are about 75 – 80% labelled in spleen and liver, and about 60 – 70% in the lung, indicating this fraction was a mixed population with either slower turnover rates, some non-BM-derived, self-renewing MØ, or both scenarios.

3.4.3.1. Splenic RPMØs and liver KCs, but not lung alveolar MØs, are majorly replenished by BM-derived progenitors during *Py1.1GFP* infection

During early *Py1.1GFP* infection, YFP⁺ FrI remained at low baseline levels in spleen (Figure 3.21A and B) and liver (Figure 3.21C and D) as they began to disappear. From 7 d.p.i. onwards, YFP labelling was significantly higher in spleen (Figure 3.21A and B, red curves) and liver FrI (Figure 3.21C and D, red curves) and progressively increased to 50 – 70% as more of the depleted fraction was replenished. This clearly suggested refilling of the emptied niche from emigrant BM-derived progenitors during *Py1.1GFP* infection, coinciding well with previous data showing the influx of FrIIai/FrIIb inflammatory monocytes (Figure 3.16B, D, F) and their differentiation into F480^{hi}-expressing MØ (Figure 3.20A). Although YFP labelling did not reach 100%, indicating partial refilling and persistence of self-renewing tissue-resident MØ, BM-derived progenitors contributed a larger role in refilling the niche.

In the lung, despite decline in alveolar MØ during *Py1.1GFP* infection, YFP⁺ labelling remained at low levels close to steady-state and did not dramatically increase unlike spleen and liver (Figure 3.21E and F, red curve). This reiterated the sufficient self-renewing properties of AMØs and little contribution from circulatory BM-derived progenitors despite observed massive monocytic infiltration (Figure 3.16B and E, Figure 3.20D); in replenishing lung tissue-resident AMØs.

Frllai and Frllb monocytic subsets in all three organs (Figures 3.21A - F, blue and orange curves) consistently had high YFP labelling throughout course of infection, indicating definite BM origin; although during the influx period YFP labelling of Frllb inflammatory monocytes (Fig 3.21A, C, E, column 3 and Fig 3.21B, D, F, orange curves) commonly exceeded that of baseline neutrophils (>100%).

3.4.3.2. Self-renewing non-FrI MØs are evident in spleen and lung, but not liver during *Py1.1GFP* infection

Interestingly, mixed population Frllc MØ presented with varying kinetics in the afflicted organs. There was a unifying phenotype previously observed in spleen, lung and liver between 3 – 5 d.p.i. where proportion of Frllc MØ also declined relative to steady state (Figure 3.16A and B), particularly in the liver; before the beginning of a sharp ascension from 7 d.p.i. onwards. In spleen, Frllc ontogeny tracking showed decreased YFP labelling from 70-80% to about 50% at 7 d.p.i and was subsequently maintained around 60% throughout the infection and recovery (Figure 3.21B, green curve). This suggests the increased Frllc MØ fraction had a slower turnover rate or were not purely differentiated from Frllb inflammatory monocytes, but also contained a self-renewing MØ population. Intriguingly, it did not appear to solely give rise to F480^{hi} FrI TRMØ as time-course F480 expression was lower than Frllai and Frllb fractions (Figure 3.20A, row 1), and could indicate other self-renewing MØ populations in the splenic myeloid landscape that were depleted during *Py1.1GFP* infection.

In the liver, despite similar early infection profile as spleen, Frllc MØ YFP labelling conversely maintained close to steady-state proportions at 4 - 7 d.p.i. when Frllai / Frllb monocytic influx was highest and subsequently increases to 90 – 100% (or beyond) after recovery (Figure 3.21C, green curve). This indicated that, unlike spleen, the incoming expansion of Frllc MØ were mostly of BM origin, and as a larger proportion were visually F480^{hi} (Figure 3.20A, row 2); also suggest BM emigrants at least partially progressed through a Frllai → Frllb → Frllc differentiation pathway to replenish FrI liver KCs.

Lung Frllc presented labelling kinetics resembling a combination of both spleen self-renewal and liver BM-derived refilling. From 10 – 14 d.p.i., there was a sharp increase (50 - 60% → 90 - 100%) in YFP labelling of Frllc MØ (Figure 3.21F, green curve) which coincided with massive infiltration (Figure 3.16B, middle column) of Frllb and Frllc, indicating BM origin. However, by 35 d.p.i. %YFP⁺ Frllc were at steady-state levels. This suggested that lung FrII infiltrating monocytes and differentiated MØ did not persist after resolution of infection (either short-lived or do not retain in tissues), nor did they contribute to FrI AMØ refilling as evidenced by negligible %YFP⁺ throughout the entire course of infection.

A

Spleen CD11b⁺ myeloid cells

B

C

Liver CD11b⁺ myeloid cells

D

E

Lung CD11b⁺ myeloid cells

F

Figure 3.21: Spleen tissue resident MØ and liver Kupffer cells are partially replenished by bone marrow progenitors, while lung alveolar MØs are self-renewed during *Py1.1GFP* infection

YFP labelling of refilling bone marrow progenitors in spleen (A, B), liver (C, D) and lung (E, F) of Kit^{MerCreMer/R26} mice during the course of *Py1.1GFP* infection. (A, C, E) Representative plots show Frl tissue-resident MØ (left), FrlIai / FrlIb monocytes (2nd and 3rd) and FrlIc differentiated MØ (right) from 3 – 90 d.p.i. (B, D, F) Dot curves show % time-course kinetics of YFP labelling in Frl, FrlIai, FrlIb and FrlIc populations from (A, C, E), normalized to baseline YFP% of FrlII neutrophils (not shown). Data shown are SEM values cumulative of 2 independent experiments with n=3-4 mice/group, 6-10 weeks old upon TAM treatment. Statistical significance (for Frl only) was determined using Mann Whitney's test against steady-state baseline (D0): *p < 0.05, **p < 0.01, ***p<0.001 and ****p<0.0001.

3.4.3.3 BM-derived, niche-refilling monocytes are stable in the long-term and exhibit similar turnover kinetics as their embryonic-derived counterparts

We had earlier shown that repopulated Frl TRMØ proportions remained stable at 60 and 90 d.p.i. following resolution of *Py1.1GFP* infection (Figure 3.17). Myeloid ontogeny kinetics in recovered mice reiterated these results as virtually all subsets, and importantly the replenished Frl MØs; maintained equivocal proportion of YFP⁺ cells from 35 d.p.i. till 90 d.p.i., suggesting stability and longevity of BM-derived monocytes (and self-renewing tissue-resident precursors, in the case of the lung) refilling the local tissue niches (Figures 3.21B, D, F, right x-axis). The stable turnover kinetics of repopulated myeloid subsets was also emphasized in a reverse experiment where Kit^{MerCreMer/R26} were first infected with *Py1.1GFP* and allowed to resolve the infection naturally, before TAM administration and analyses of myeloid YFP-labelling after 2 – 3 months (experimental schedule - Figure 3.22A). Clearly, YFP labelling did not defer greatly from steady-state controls in most myeloid subsets of all organs (Figure 3.22B), indicating long-term stability.

The only exception with significant differences would be splenic FrlIc MØs, however this was in line with YFP labelling results in Figure 3.21A and B, wherein initial percentage of steady-state YFP⁺ FrlIc MØs was high but drops at 7 d.p.i, and was thereafter maintained consistently at lower percentage of YFP labelling until the experimental endpoint. Importantly, replenished Frl MØs compartments in spleen and liver also exhibited nearly identical YFP labelling to the 'original' embryonic-derived counterparts,

indicating they regained similar turnover kinetics upon resolution of *Py1.1GFP* infection (Figure 3.22B, column 1).

Figure 3.22: Turnover kinetics of monocyte-refilled MØs do not differ from the “original” embryonic-derived counterparts

Refilled myeloid subsets have similar turnover kinetics as original embryonic-derived counterparts. (A) Schematic representation of adult fate-mapping protocol using pre-infected *Kit^{MerCreMer/R26}* mice. Mice (8–10 weeks old) were infected with *P. yoelii* and allowed to recover. Tamoxifen was injected i.p. on five consecutive days from 35 d.p.i., and mice were sacrificed 65 and 100 days later, and analyzed for the presence of YFP⁺ cells. (B) Bar graphs show normalized % YFP labelling of tissue-resident MØ and monocytes in spleen (top - blue), liver (middle - red) and lung (bottom - green) of SS (white bars) and recovered infected WT (rWT) mice. Data shown in SEM are cumulative of two independent experiments, *n* = 6/grp. Statistical significance was determined using Mann Whitney’s test against steady-state baseline (D0): ns = non-significant, **p* < 0.05 and ***p* < 0.01.

3.4.4. Post-entry monocytes gain molecular and gene expression characteristics of embryonically-seeded tissue-resident macrophages

Our results so far suggested that original embryonic seeded tissue-resident macrophages and bone marrow-derived refilling inflammatory monocytes during *Py1.1GFP* infection were phenotypically very similar. We lastly investigated the molecular relationships between steady-state embryonic-seeded progenitors and BM-derived emigrants from recovered infected mice using RNAseq.

Hierarchical clustering generated dendrograms (Figure 3.23A) which showed close clustering of steady-state and recovered Frl MØs within each organ group. Between organs, spleen and liver clustered closer together than did the lung. These results were recapitulated in a Principal Component Analysis (PCA) which likewise showed close clustering of steady-state and recovered Frl MØs (Figure 3.23B), indicating a high degree of similarity between original and refilled compartments. The total number of differentially expressed genes (DEGs) between steady-state and recovered Frl MØs was overall minimal; with the highest similarity in lung (1.7% DEGs, Figure 3.23C), which correlated well with fate-mapping data supporting the self-renewal capabilities of alveolar MØs and negligible contribution of BM-derived progenitors, despite the massive monocytic influx.

The spleen and liver recovered Frl MØs, despite largely being replenished by infiltrating BM emigrants; were also found to display high degrees of similarity with foetal-derived steady-state MØs (3.7% and 2.9% DEGs respectively, Figure 3.23C). This suggested that refilling BM-derived monocytes were able to adopt the gene expression signatures of 'original' Frl TRMØs in the local tissue niches after integration, despite their divergent origins.

Hierarchical clustering also generated heatmaps of the top 50 DEGs with log 2-fold difference between SS and replenished MØs in individual organs (Figure 3.24A) and common across all three organs (Figure 3.24B).

Individually, and across all organs, the overall difference was that post-infection MØs still retained a more activated gene expression profile compared to steady-state MØs. Some of the commonly upregulated genes included antigen presentation MHC-II genes (red bracket - *H2-Ea-ps*, *H2-Eb1*, *H2-Ab1*, *H2-Aa*, *H2-DMb*, *H2-DMb2*) and their related genes *CD74* (Figure 3.24B, bottom). *CD163L1* which encodes a scavenger receptor [328] was also similarly upregulated in all tissues relative to their steady-state expression, with strongest fold-change in the spleen (Figure 3.24B, top).

Figure 3.23: Repopulating BM emigrants have similar gene signatures as originally seeded tissue-resident macrophages

RNA sequencing analyses of sorted macrophages from steady-state (SS) and recovered infected (rIN) WT mice. Relationships between SS and rIN macrophages in spleen, lung and liver of recovered mice are shown by (A) hierarchical clustering dendrograms, (B) principle component analysis (PCA1 axis: spleen, PCA2 axis: liver and PCA 3: lung) and (C) pie charts depicting percentage of DEGs (inset) in total number of analysed genes. Full DEG list for each organ is shown in Supplementary Figures. Hierarchical clustering and principal component analysis were performed with Log2-transformed FPKM values. Comparisons of pre- and post-infection MØ were performed using limma. DEGs were selected using Benjamini-Hochberg adjusted values of < 0.05 .

Figure 3.24 Tissue-resident macrophages retain a more activated genetic profile after recovery from *Py1.1GFP* infection

Heatmaps of top 50 DEGs in pre- and post-infection MØ. (A) Individual heatmaps showing DEGs of SS and recovered infected MØs (rIN) in spleen (left), liver (middle) and lung (right). (B) Heatmap comparison of top 50 common DEGs shared amongst SS and rIN MØs in spleen, liver and lung. Yellow arrows and red bracket represent common upregulated DEGs of interest in the three organs. Sample groups are denoted at the top with their respective triplicates. Dendrograms on the left depict relationships between genes, and DEG names are labelled on the right. Log2 fold change in gene expression is denoted by colour scale, negative -2 (blue) to positive +2 (red). Hierarchical clustering of pre- and post-infection MØ were performed using Ward's method with half-square Euclidean from adjusted p-values.

4. Discussion

4.1 Clec9A-DTR mouse model is a robust system to study roles of Clec9A⁺ DCs in *Plasmodium* infection

We showed that the Clec9A-DTR mouse is an efficient model that allows for specific depletion of Clec9A⁺ DCs in a multitude of organs, including those commonly affiliated with malaria pathology – spleen, liver and lung. Importantly, the specificity of this depletion was emphasized by intact cDC2 and pDC populations; as well as unperturbed macrophage and monocyte subsets in these organs. There was no apparent toxicity observed in test animals upon DT administration. Hence, this mouse model is advantageous over broad depletion systems like CD11c-DTR or clodronate liposomes, in allowing us to specifically study the role of Clec9A⁺ DCs in the context of, but not limited to, *PccAS* infection.

4.2 Clec9A⁺ DCs are key drivers influencing downstream immune responses against *Plasmodium chabaudi chabaudi* AS infection

4.2.1 Absence of Clec9A⁺ DCs dysregulates induction of T_{h1} proinflammatory responses and T effector polyfunctionality in *PccAS* infection

Although activation status was not significantly different in the CD4⁺ T_{EFF} compartment, CD8⁺ T_{EFF} conversely were drastically impaired, recapitulating findings reported by Guermonprez *et al* [329]. This may be attributed to reciprocal CD4⁺ T_{EFF} activation by the non-depleted cDC2 subset, while CD8⁺ T_{EFF} activation is defective due to absence of cross-presenting Clec9A⁺ cDC1s. In contrast, KLRG1⁺ CD4⁺ and CD8⁺ T_{EFF} were all uniformly reduced in the afflicted organs of Clec9A-DTR mice. KLRG1 expression associated with T_{h1} responses is dependent on upstream IL-12 signalling predominantly from cDC1s, and subsequent T_{bet} induction in T_{EFF} [288, 296, 330-332]. The significant prevalence of KLRG1⁺ CD4⁺ and CD8⁺ T_{EFF} observed in WT mice indicates a greater capacity to terminally differentiate into activated T_{h1} effector cells in response to *PccAS* infection, which is impaired in the absence of Clec9A⁺ DCs.

The impact of Clec9A⁺ DC absence also extended beyond impairment in T_{h1} T_{EFF} differentiation, to the functionality of these T_{EFF} cells. Splenic, as

well as peripheral lung and liver acute CD4⁺ and CD8⁺ T_{EFF} proinflammatory responses and polyfunctionality were also significantly compromised in Clec9A⁺ DC-deficient mice. Polyfunctionality impairment in cytokine production was most evident in lower numbers of IFN γ ⁺ and GzB⁺ T_{EFF}, corresponding with defective CD4⁺ T_{H1} proinflammatory responses. Our observations in Clec9A-DTR mice are reminiscent of studies done in CD4⁺ T-cell KO, IFN γ KO and IL-12-KO models; with pronounced parasitemia, defective effector T-cell responses and mortality [191, 203, 204, 219, 224, 226, 227].

The contributions of CD8⁺ CTLs in *PccAS* and *P.yoelii* infection however, are far less distinguished as CD4⁺ T_H and their redundancy alluded to, due to lack of MHC I expression on iRBCs, and ability of CD8⁺-deficient mice to clear acute infection without exacerbated phenotypes [226, 232]. A recent study by Imai *et al* reported a novel mechanism by which CD8⁺ CTLs assisted in protection against blood-stage *Py17XNL* infection by induction of phosphatidylserine (PS) expression on the surface of infected erythroblasts, thus facilitating detection and phagocytosis by macrophages [333]. Although not their preferred tropism, *PccAS* can also infect erythroblasts and reticulocytes. The drastic reduction in CD8⁺ CTL absolute numbers and IFN γ /GzB production observed in Clec9A⁺ DC-deficient mice can likewise impede their capability in parasite clearance via this mechanism in *PccAS* infection.

Recently, distinct subsets of CD4⁺ T_{H1} cells have been described to simultaneously produce anti-inflammatory cytokines, such as IL-10 together with IFN γ or TNF α . The role of IL-10 alone as a reciprocal regulator of IFN γ is well-established, and its deficiency results in severe immunopathology or mortality in malaria and other diseases [259, 261, 334]. These seemingly paradoxical IFN γ ⁺IL-10⁺ CD4⁺ T_{EFF}, newly termed as T-regulatory 1 cells (T_{RT}) cells [335], have been reported in a plethora of infection models spanning *Mycobacterium tuberculosis*, *Toxoplasma gondii* and influenza, and are crucial for host survival by self-limiting immunopathology imparted by strong proinflammatory responses [299, 300, 336]. These subsets are also present in CD8⁺ CTL and activated NK-cell populations during infectious conditions [301, 304].

In malarial infections, IFN γ ⁺IL-10⁺ CD4⁺ T-cells reported in mice [300, 337, 338] and humans [339], likewise have the indispensable function of controlling immunopathology. IL-27 signalling is recently described to be required for IL-10 production in these cells [300]. An elegant study by Lönnberg *et al* using single-cell RNA sequencing and bioinformatics approaches revealed that malaria T_{ri} cells are indeed, directly differentiated from responding CD4⁺ T_{H1} cells [340]. In our model, we also observed substantial CD4⁺ T_{EFF}, as well as CD8⁺ CTL and NK-cell populations co-producing IFN γ and IL-10, in afflicted organs of both groups of infected mice; but they were present in significantly higher numbers in WT controls. To our knowledge so far, IFN γ ⁺IL-10⁺ NK-cells have not been reported in *Plasmodium* models, and here we showed that they are also present in this model of chronic malaria infection.

4.2.2 An intact innate immune compartment compensates for polyfunctional proinflammatory responses in the absence of Clec9A⁺ DCs

However, absence of Clec9A⁺ DCs did not completely abrogate the T_{H1} proinflammatory response to *PccAS*, suggesting compensatory roles from non-depleted cDC2s or other APCs in T_{EFF} activation and polyfunctionality. Moreover, innate immune compartments in Clec9A-DTR mice, comprising of CD4⁺CD8⁻ non-classical T-lymphocytes predominantly found to be $\gamma\delta$ T-cells (data not shown), and NK-cells, were less drastically impaired in proportion and absolute numbers of polyfunctional IFN γ ⁺ effector cells, which could also contribute to parasite control and clearance.

$\gamma\delta$ T-cells have multiple roles in *Plasmodium* infection, being first responders that can be directly activated by invading parasites, potent producers of IFN γ , and perpetuators of the IL-12/IFN γ feedforward loop by acting on cDCs [216, 341]. In a non-lethal *P.berghei* XAT model, $\gamma\delta$ T-cells have been shown to activate DCs via IFN γ production and utilizing the CD40L-CD40 signalling pathway to stimulate IL-12 production from DCs, which subsequently induces CD4 T_{H1} responses; hence enhancing anti-*Plasmodium* immunity [342, 343].

The contributions of NK-cells, like $\gamma\delta$ T-cells, to anti-*Plasmodium* immunity also lie in their production of IFN γ and TNF α , and not direct cytotoxicity.

Studies in *P.berghei* ANKA and *PccAS* have elucidated reciprocal activation of DCs and NK-cells that enhances IL-12 and IFN γ production in response to the parasites [213, 344]. This IFN γ -dependent, NK-cell mediated DC maturation and activation has been shown to be necessary for DC induction of robust CD4 $^{+}$ T $_{H1}$ responses in *PccAS* infection [214]. Stimulation of cDC2 by IFN γ from $\gamma\delta$ T-cells and NK-cells could trigger to some extent, the proinflammatory responses necessary in controlling acute infection in Clec9A-DTR mice; albeit dampened in comparison to WT controls.

4.2.3 Germinal center reactions, B-cell responses and Ig production are not abrogated in absence of Clec9A $^{+}$ DCs

During *PccAS* biphasic infection, the predominantly T $_{H1}$ acute proinflammatory response transitions to an IL-10/IL-21-dominant environment; once believed to be T $_{H2}$ -mediated, but now inclined towards T $_{FH}$ -mediated humoral immunity [225, 241, 345].

For the onset of humoral and antibody-mediated immunity, antibody production by activated B-cells must occur in germinal centers (GCs); specialized anatomical structures found in secondary lymphoid tissues. A plethora of functions occur in GCs – B-cell affinity maturation, somatic hypermutation, class-switch recombination of pathogen-specific antibodies, and selection of high affinity B-cells which eventually will differentiate into long-lived memory B or plasma cells required for long-term humoral immunity [164, 346, 347]. Follicular T helper cells (T $_{FH}$) are a distinct subset of CD4 $^{+}$ T $_{H}$ cells exhibiting high heterogeneity and phenotypic plasticity; regulated by the master transcriptional factor B-cell lymphoma 6 (Bcl-6), and are essential in the induction, maintenance and survival of GCs. GC B-cells are also reciprocally required for sustained T $_{FH}$ survival and function [316, 320, 348, 349]. The vastly interconnected relationships between GC B-cells and T $_{FH}$ would perhaps best be discussed together, rather than separate entities, in the context of our results.

Despite the severely impaired CD4 $^{+}$ T $_{EFF}$ in our model of *PccAS* infection, GC reactions (CD38 lo GL-7 $^{+}$ IgM $^{+}$ or IgG $^{+}$ B-cells) by proportion were not defective in Clec9A-DTR mice. Additionally, IgD $^{-}$ IgM $^{+}$ and IgG $^{+}$ B-cell subsets, an indicator of Ig class-switching, were also proportionately

present in Clec9A-DTR mice. In our experiments, we were unable to robustly stain for IL-21, the canonical T_{FH} effector cytokine, in CD4⁺ T_{EFF} or T_{FH} *ex vivo* (data not shown) by flow cytometry. Indeed, commonly in literature, IL-21 is alternatively assessed by IL-21 reporter mice, qPCR mRNA fold expression changes or after long *ex vivo* stimulation with α CD3 and α CD28. However, GC B-cell antibody production and subsequent CSR is largely dependent on IL-21 produced by T_{FH} [318], hence, our observations of GL7⁺ or IgG⁺ splenic B-cells, and *Plasmodium*-specific Ig in the serum indirectly confirmed intact IL-21-associated effector functions in both WT and Clec9A-DTR mice. Proportionately, Bcl-6⁺ CXCR5⁺ PD-1⁺ T_{FH} were not significantly reduced in Clec9A-DTR mice, suggesting the absence of Clec9A⁺ DCs did not impair their ability to initiate T_{FH} differentiation, despite the requirement for DC priming in early T_{FH} induction [350, 351]. Clec9A-DTR mice had an intact CD11b⁺ cDC2 compartment at numbers higher than WT mice (Figure 3.1), and these DCs with their superior MHC-II presentation capabilities, could compensate for early CD4 T_{FH} induction and differentiation, as reported by Shin *et al* and Li *et al* [352, 353].

GC T_{FH} are commonly regarded as the highest differentiation state for T_{FH} as they express maximal levels of Bcl6 and surface markers (e.g. CXCR5⁺⁺⁺ PD-1⁺⁺⁺ ICOS⁺⁺⁺), and are anatomically located in GC follicles to directly provide B-cell help [243, 354]. Activated T_{FH} subsets found outside of, at borders of T-B zones or cycling between follicles; termed “pre-GC T_{FH}”, express varying levels of these markers and are also functionally capable, but mainly drive extrafollicular B-cell responses[355].

By virtue of this surface phenotype, we did observe substantial Bcl6⁺ or Bcl6⁺ CXCR5⁺ subsets in infected WT and Clec9A-DTR mice aside from GC T_{FH} (Bcl6⁺ CXCR5⁺ PD-1⁺), which could provide B-cell help to generate extrafollicular plasmablast responses. It appeared in both *PccAS*-infected WT and Clec9A-DTR mice, robust CD38^{lo} GL-7⁺ GC reactions, CD23⁺ CD38^{hi} B_{MEM} precursor and CD38^{lo} CD138⁺ plasmablast differentiation were happening concurrently. It is likely these CD138⁺ plasmablasts are a mixed population of extrafollicular (non-GC) PBs and GC-derived PBs that are in the process of fully differentiating to plasma cells and migrating to splenic or bone marrow niches for long-term survival. Although extrafollicular

plasmablast/plasma cells typically are shorter-lived and produce lower affinity antibodies [347, 354, 356], *PccAS* infection nonetheless has been reported to generate both long-lived memory B and plasma cells from a single episode [190, 228]; and we in agreement do observe formation of phenotypically similar IgD⁻ CD38^{lo} CD138⁺ plasmablasts and IgD⁻ CD38^{hi} B_{MEM} precursors; hence some progeny from the mixed populations could potentially progress to become long-lived memory B and plasma cells.

4.2.4 Absence of Clec9A⁺ DCs diminishes T_{h1}-like effector TFH and proinflammatory signals required for class switch recombination to more protective T_{h1} IgG2 and IgG3

Class switching recombination of *Plasmodium*-specific Ig was detected by proxy via serum isotype ELISA. The distinct Ig isotype polarization between IgG2c-dominant WT and IgG1-dominant Clec9A-DTR serum clearly indicated differences in CSR that were not visible through total IgG production. Antibody CSR in B-cells requires the enzyme activation-induced cytidine deaminase (AID) and Ig transcripts sequentially undergoes irreversible deletional transition from IgM → IgG → IgE → IgA in mice. IgG1 is the first and most abundant class-switched IgG isotype [357, 358]. It has been well-established that specific cytokines are involved in specific Ig class-switching, such as IL-4 for IgG1/IgE and IFN γ for IgG2a/IgG2b/IgG3 [311, 313, 359]. IgG2c is an IgG2a analogue (but not equivalent) found in genetic backgrounds such as C57BL/6, and CSR to this isotype is similarly regulated by IFN γ [360-362]. The proinflammatory IL-12 and IFN γ -dominant systemic and local tissue environments prevalent in *PccAS*-infected WT mice correlated well with high levels of *Plasmodium*-specific IgG2c, IgG2b and IgG3 generated, as these cytokines also repress IgG1 production. IFN γ mediating IgG2 isotype CSR in B-cells can come from multiple sources of IFN γ ⁺ cells such as NK-cells or CD4⁺/CD8⁺ T_{EFF}, in T-dependent or T-independent pathways [311, 363, 364]. Furthermore, infected WT CXCR5⁺ PD-1⁺ GC effector T_{FH} in close contact with GC B-cells specifically produced significantly greater amounts of IFN γ than Clec9A-DTR mice, which could directly influence CSR to T_{h1} isotypes more efficiently than extrafollicular CD4⁺ and CD8⁺ T_{EFF}. Interestingly, the appearance of polysaccharide/carbohydrate-binding T_{h1} IgG3 in both groups implies a

fraction of *Plasmodium*-specific Ig were produced under T-independent conditions [365, 366]. In contrast, IFN γ -deficient Clec9A-DTR mice expectedly had high T_{h2} IgG1 and minimal T_{h1} IgG2/IgG3 isotype titers; and although we were unable to directly stain for *ex vivo* CD4⁺ T_{EFF}/T_{FH} IL-4 (or IL-21) production, the manifestation of downstream outcomes indicates their successful induction.

Although all Ig isotypes are capable of opsonization and mediating phagocytosis via Fc receptor binding on phagocytes, T_{h1} Ig isotypes are associated with the greatest enhanced cytophilic properties [189, 367]. In particular, IgG2a/IgG2c are most potent in mediating antibody-dependent cellular toxicity (ADCC) and complement fixing for macrophages [368-370]. The markedly higher titers of cytophilic IgG2c / IgG2b and IgG3 in *PccAS*-infected WT mice could increase macrophage-dependent clearance or lysis of infected RBCs and free parasites, hence controlling recrudescence parasite loads more efficiently than IgG1-predominant Clec9A-DTR mice. Similarly, in human *Plasmodium falciparum* malaria, the most cytophilic isotype IgG1 is also shown to preferentially activate macrophage effector functions [371]. This correlates well with our serum passive transfer results, where recipients of WT infected serum developed delayed and lower magnitude of blood parasitemia than Clec9A-DTR infected serum recipients. Indeed, the protective effects of T_{h1} IgG2a/IgG2c or IgG3 have also been reported in other malaria models such as *Plasmodium yoelii* [372], non-lethal *Plasmodium berghei* XAT [373] infections, and as the dominant immune memory isotype during recall responses [190].

4.2.5 The T_{h1} - T_{FH} relationship conundrum

Cumulative evidence irrefutably supports the importance of T_{h1} proinflammatory responses in controlling acute *Plasmodium* infection. However, a large body of existing evidence also details the detrimental effects of overtly strong T_{h1} responses on establishment of humoral immunity and disease outcomes, particularly the repression of T_{FH} differentiation. Recent studies by Ryg-Cornejo *et al* [112] and Ioannidis *et al* [374] have shown that T_{h1} proinflammatory responses in severe *Plasmodium berghei* ANKA infections impair development of T_{FH} / GC reactions and hence protective humoral immunity, by disrupting T_{FH}

differentiation. In human *Plasmodium falciparum* malaria, a strong proinflammatory response likewise favours production and expansion of T_{H1} -like CXCR3⁺ T_{FH} , which differentiate into less functional effectors upon memory recall [375]. On the other spectrum, excessive IFN γ promotes spontaneous pathogenic T_{FH} and GC formation, a key feature of many autoimmune diseases including systemic lupus erythematosus [376]. T_{bet} , the master regulator of T_{H1} fate and IFN γ , is also an inhibitor of Bcl-6 [377, 378]. Suppression of T_{H1} T_{bet} and IFN γ / TNF α / IL-10 augments T_{FH} differentiation, GC formation and production of antigen-specific, class-switched antibodies, positively correlating with improved outcomes in various infection models [112, 379, 380]. A series of recent studies have also identified novel roles of innate Type I interferons (e.g. IFN α) in regulating T_{H1} and T_{FH} responses, in some context through promoting T_{H1} differentiation [310, 381, 382]. It is currently unclear how the new axis of IFN α signalling fits into our study but could warrant further investigation, since its effects are believed to be mediated by cDCs [310] and CD4⁺ T-cells [382], two cell types that were intimately affected in Clec9A⁺ DC-deficient mice. This T_{H1} - T_{FH} relationship is further complicated by intermediate developmental states that share transcriptional and phenotypical characteristics of both populations, such as early T_{FH} -like precursors which have the bifurcating potential to differentiate into either T_{H1} and T_{FH} cells [340, 377], and T_H effectors that produce both IFN γ and IL-21 [223, 375].

In lieu of these findings, Hansen *et al* has proposed a specific model encompassing murine and human malaria, in which heavily T_{H1} -polarized responses skew T_{FH} towards T_{H1} effector type that exhibits inefficient B-cell help, resulting in suboptimal GC and antibody responses that undermines humoral immunity against *Plasmodium* infection and hence exacerbates severity of disease [348]. This emerging concept appears to be supported by the higher (though not significant) proportion of GL-7⁺ GC B-cells and CD138⁺ plasmablasts seen in Clec9A-DTR mice (Figure 3.9), which suggests unimpaired T_{FH} responses compared to WT mice, in conditions of IFN γ ⁺ T_{H1} / IFN γ ⁺IL10⁺ T_{H1} deficiency. Additionally, despite the overall significantly reduced numbers of T_{FH} , Clec9A-DTR mice did not show severely impacted *Plasmodium*-specific Ig production, but rather

preferential IgG1 isotype skewing due to diminished local IFN γ production from T_{h1}-like T_{FH} or other cell types.

PccAS malaria does not appear to fully follow the paradigm proposed by Hansen *et al*, T_{h1} polarization and IFN γ still have indispensable roles in both acute and humoral immunity, triggering potent T_{EFF} responses and ultimately producing T_{h1} isotype antibodies and immune memory to confer protection [229, 241, 383], as demonstrated by our Clec9A-DTR model. This apparent disparity amongst T_{FH} induction and downstream effects in different models; even amongst *Plasmodium* infections, indicate that resultant observations are highly context-dependent, reiterating the complex intertwined relationship, plasticity and intricacy of the T_{h1} - T_{FH} axis.

4.2.6 Clec9A⁺ DC deficiency results in persistent T effector and memory precursor impairment during recrudescence *PccAS* infection

During recrudescence phase, Clec9A-DTR mice continued to show impaired CD3⁺ T-lymphocyte cellularity. A large proportion of the significantly higher numbers of CD3⁺ cells in WT mice were CD4⁺CD8⁻ T-cells, which included $\gamma\delta$ and NKT cells. Although we did not further profile this fraction and investigate their potential contributions, this observation is consistent with recent studies in *Plasmodium chabaudi* infections demonstrating late effector $\gamma\delta$ T-cell expansion in chronic malaria, distinct from the IFN γ -producing $\gamma\delta$ during acute infection [215]. These newly-described $\gamma\delta$ populations have preferential $\gamma\delta$ -TCR arrangements [384] and novel effector functions in M-CSF production, which may augment parasite clearance via cross-talks with myeloid cells during recrudescence [385]. Hence, in our infection model, drastically-expanded $\gamma\delta$ T-cells present in WT mice could be an additional factor contributing to enhanced parasite control, which is impaired in Clec9A⁺ DC-deficient mice and might warrant further investigation.

Clec9A-DTR mice also strikingly presented with significantly lesser numbers of T_{EFF} than WT during recrudescence. CD4⁺ T_{EFF} can be further delineated into early T_{EFF}, intermediate T_{EFF} and late T_{EFF}, based on the surface expression of CD44, CD27, CD62L and CD127; in a linear progress of differentiation as described by Stephens *et al* [115]. They also showed that

CD4⁺ CD127⁺ T_{MEM} subsets can likewise be delineated into T_{CM}, early T_{EM} and late T_{EM} differentiation states [115, 224]. All three subsets of T_{EFF} were clearly observed in CD4⁺ T-cells of WT and Clec9A-DTR mice. CD4⁺ T_{EFF} INT and T_{EFF} L, which was reported to have heightened cytokine production [115], were the most abundant and significantly increased subsets in WT mice compared to Clec9A-DTR mice, comprising about 80% of CD4⁺ T_{EFF}. Additionally, in a follow-up study, Opata *et al* reported all three T_{EFF} subsets were found to have protective capacities as evidenced by rapid proinflammatory cytokine production and protection against *PccAS* challenge after adoptive transfer of B5 TCR Tg-specific effector cells into RAG KO hosts [386].

Intriguingly, CD4⁺ T_{EFF}E, which showed only a modest increase in WT compared to Clec9A-DTR mice, is also found with the unique ability to generate memory lineage, in a parallel linear differentiation towards central memory (T_{CM}), to early (T_{EME}) and late (T_{EM}L) T effector memory cells [115, 387]. This decision to progress along T_{EFF} differentiation or revert towards T_{CM} → T_{EM} pathway is dependent on environmental signals and antigenic stimulation, and not an isolated phenomenon in malaria; similar observations have been reported in *Listeria*, influenza or model antigen models [388]. Although we did not explicitly assess functionality in CD4[±] T_{EFF} subsets, the significantly higher numbers in WT mice could contribute to overall better protection against recrudescence infection than deficient Clec9A-DTR mice.

It can be argued that at this timepoint of ongoing infection, identification of “T memory” (T_{MEM}) may not be prudent, and we recognize that true T_{MEM} studies, which typically involve assessment of subsets upon curation of disease or pathogen re-challenge, were not carried out in this entire study. However, Opata *et al* also reported that early T_{EFF}E parallel differentiation into T_{CM} → T_{EM} was already programmed to occur during acute *PccAS* infection, in concordance with early T_{EFF}E → late T_{EFF}L differentiation by peak parasitemia and beyond [115, 386-388]. It is generally accepted in literature that T_{CM} are long-lived with high differentiation potential and T_{EM} are superior at effector functions, but at the expense of longevity, hence one of the factors contributing to temporal decay of malaria protective immunity.

[224, 386, 387]. This proposed model of bi-linear differentiation was supported by numerous other studies in viral and bacterial infection models in both humans and mice, and highlights heterogeneity and plasticity of effector and memory T-cell differentiation and generation [388]. WT mice had more T_{CM} and late T_{EM}L than Clec9A-DTR mice; though overall T_{MEM} populations were eclipsed by T_{EFF} which were present in much greater abundance.

Coupled with work by Stephens *et al* [224] and Zander *et al* [389] describing that T_{h1} or T_{h1}-like CD4⁺ effector T_{EM} generated during acute infection survive and are maintained for humoral immunity; as well as the capability of early T_{EFF} in generating T_{CM} and T_{EM} proposed by Opata *et al* [115], a new angle in the inability of Clec9A⁺ DC-deficient mice in controlling recrudescence arising from their impaired acute IFN γ ⁺ IL-10⁺ CD4⁺ T_{EFF} responses, could be revealed.

Therefore, we reiterated established findings that some CD4⁺ T_{MEM} subsets could be differentiated within the primary *PccAS* infection itself, and at this recrudescence timepoint, T_{EFF} subsets still dominated the response in infected animals, with Clec9A⁺ DC-deficient animals showing impairment compared to WT mice. Altogether, the continued attenuated CD4⁺ T_{EFF} and possibly T_{EM} response, coupled with skewed predominance of less cytophilic *Plasmodium*-specific IgG1, could result in diminished parasite clearance and hence significantly higher recrudescence in experimental animals depleted of Clec9A⁺ DCs.

4.3 Caveats and limitations in the Clec9A-DTR *PccAS* infection model

To conclude this section, we discuss some of the caveats and limitations in our study, as well as possible future experiments to lend credence to our current data.

One limitation in the interpretation of our data would be that GCs and T_{FH} data were presented as flow cytometry, which do not provide the microanatomical, structural and spatial visualization that immunohistochemistry staining and microscopy offers. As GC B-cells and T_{FH} interactions require direct cell-to-cell contact [390, 391], these visualization techniques will provide invaluable insights into GC-T_{FH}

development in our *PccAS* infection model, especially in lieu of the emerging concepts of T_{H1} and T_{R1} -mediated suppression of T_{FH} differentiation.

The myeloid compartment and its effector functions were also not investigated in-depth. Despite induction of robust GC/ T_{FH} and *Plasmodium*-specific Ig responses in the wake of diminished T_{H1} responses and $IFN\gamma^+$, *Clec9A*-DTR mice remained more susceptible to *PccAS* infection. T_{H1} responses are also known to strongly activate macrophages and monocytes to exert effector functions like cytokine production, phagocytosis and intracellular killing, although the exact mechanisms and pathways are not fully understood. $IFN\gamma$ was shown to be critical in inducing the differentiation of $IL-17R\alpha^+$ $cKit^+$ atypical myelolymphoid progenitors in the bone marrow that preferentially generate myeloid lineages, along with rapid $CCR2$ -dependent egress of myeloid precursors that undergo extramedullary myelopoiesis in the spleen, both processes giving rise to myeloid effector cells with substantial parasite clearance capabilities and contributions during *PccAS* infection [219, 248]. A recent study from Fontana *et al* also elucidated a novel mechanism of T_{H1} $CD4^+$ cells in inducing expansion and activation of $CD169^+$ macrophages, splenic red pulp macrophages and emigrating monocytes via production of the myeloid-associated cytokine, Macrophage Colony Stimulating Factor (MCSF) [392]. A generic transcriptional signature of these MCSF⁺ $CD4^+$ T_{H1} cells include T_{bet} expression, $IFN\gamma$ and $IL-10$ production; suggesting they are a sub-population of the immunoregulatory $IFN\gamma^+IL-10^+$ T_{H1}/T_{R1} cells described by various groups [300, 337, 338, 340, 393]. Moreover, $IFN\gamma$ -dependent cytophilic IgG2c/IgG3 isotypes were also substantially present during recrudescence, coinciding temporally with the expansion of myeloid cells post-peak parasitemia; and could both work in concert for more efficient clearance of *PccAS* parasites compared to *Clec9A*⁺ DC-deficient animals.

The protective capacity of the generated *Plasmodium*-specific Ig and the correlation with isotype is also currently unknown, as no epitope characterization or antigen specificity studies were done. Future experiments profiling the myeloid compartment subset and assessing the activation, expansion and phagocytic activity of macrophages/monocytes in

the absence of Clec9A⁺ DCs and impaired T_{H1}/ IFN γ responses could bridge the current gaps in our data. Lastly, re-challenge and long-term memory recall studies will also be helpful in determining the true roles of Clec9A⁺ DCs in influencing downstream formation of memory cells, their effector functions and the protective effects of *Plasmodium*-specific Ig generated.

4.4 Organ-specific tissue-resident macrophages are replenished with different kinetics during acute *Plasmodium yoelii* infection

4.4.1 Emptied embryonically-seeded TRM $\emptyset$ niches in spleen and liver are partially replenished by BM-derived monocytic progenitors that differentiate into stable, bona fide TRM $\emptyset$ s

Macrophages have important effector roles in controlling and clearing parasites in various *Plasmodium* models [107, 247, 249, 253, 394, 395]. We showed that Frl tissue-resident M $\emptyset$ s do briefly disappear (or significantly decline in lung AM), but returned quickly with different kinetics between organs.

Splenic Frl TRM $\emptyset$ described in our study consists of RPM $\emptyset$ and CD169⁺ MMM [38, 41, 42]. Both subsets of TRM $\emptyset$ s are anatomically well-positioned and readily accessible to sample circulating blood and capture bloodborne pathogens, including *Plasmodium* parasites [161, 396]. During *Plasmodium* infection, splenic microarchitecture is altered and the clearly demarcated marginal zones and T/B-cell zones (under steady-state conditions) are prolongedly disrupted, affecting the dispersion and distribution of immune cells [169, 174-177].

In a chronic *PccAS* malaria model, Beattie *et al* reported the progressive disappearance of splenic marginal zone MMMs and MZMs from 4 d.p.i., and up till 60 d.p.i., these M $\emptyset$ subsets were still dramatically reduced compared to steady-state levels. Krucken *et al* also partially reiterated these findings in *PccAS*, where they reported complete MZM, but not MMM, loss and RPM $\emptyset$ s redistribution into white pulp following spleen closure after peak infection [175]. *Py1.1GFP* infection triggered Frl TRM $\emptyset$ disappearance, in concert with massive infiltration of inflammatory monocytes at 4 d.p.i,

likewise observed by Sponaas *et al* in a *P.chabaudi* model, and was described as a potent early anti-*Plasmodium* mechanism [247]. By 7 d.p.i, monocyte-derived and minorly, local Frl TRMØs had begun to refill the emptied niche. Although we did not explicitly stain for MZ MØs in this work, F480^{hi} Frl could potentially contain MMMs, as there was a subset of CD169⁺ MØs present, supported by an earlier published work using CD169-DTR mice which depletes CD169⁺ MØs, and was validated by immunofluorescence staining [253]. Our results did not agree with the long disappearance of MØs reported by Beattie and colleagues. This could be explained by less severe *Py1.1GFP* (~15% iRBCs in reporter mice) infection compared to *PccAS* (~ 30% iRBCs), as supported by similarly less exacerbated phenotypes in the milder *P.chabaudi adami* [182] and *P.yoelii* 17XNL [160].

There is marked inflammation during *Plasmodium* infection, and the severity and associated immune responses depends on strain and species. A correlation between severity of inflammation and period of TRMØ disappearance is also seen in non-pathogenic acute inflammation models. Davies and colleagues reported in zymosan-induced peritonitis, that despite the expected massive infiltration of Ly6C^{hi} inflammatory monocytes which differentiated into MØ, only TRMØ undergo intense proliferation to rapidly replenish the population, with little contribution from inflammatory MØ [397]. Our results in contrast, evidently showed the partial contributions of both BM progenitors and TRMØ, which could be due to context-dependent responses, since *Py1.1GFP* infection has much prolonged inflammation compared to zymosan-induced peritonitis. The same group later showed that BM-originated inflammatory MØs are generated in tandem with TRMØs, but persisted in proliferation beyond timepoints where TRMØs have halted self-renewal [66], which are more in line with our results.

Our fate-mapping studies revealed efficient refilling of splenic RPMØ and MMM by BM-originated progenitors during *Py1.1GFP* infection, which gradually differentiated into F4/80^{hi}CD11b^{lo} cells phenotypically resembling *bona fide* TRMØs. This partial differentiation occurred in competition with small proportion of surviving embryonic TRMØs, but at lower levels than the

liver KCs. Splenic Fc γ R TRM ϕ YFP-labelling steadily increased over the course of *Py1.1GFP* infection, following their replenishment after disappearance from BM progenitors. These differentiated TRM ϕ were stable long-term as YFP labelling and turnover kinetics in replenished fraction remained constant even after three months post-infection. Similarly, in a hemolysis model, RPM ϕ loss is refilled by BM-derived circulating monocytes that were able to adopt functional attributes of original RPM ϕ s and settle as long-term residents in the spleen [82].

Liver Kupffer cells, are the most abundant and long-lived TRM ϕ s with strategic anatomical placement within the hepatic sinusoids, overlaying the sinusoidal endothelial cells. Resident KCs partake in sentinel and homeostatic functions - sensing and orchestrating responses against blood-borne toxins, bacteria, and parasites; as well as RBC clearance and iron recycling, much like splenic TRM ϕ s [398-400]. Two populations of phenotypically and functionally-similar KCs have been identified, the sessile KCs with FL monocyte/HSC origins and a very minor population of BM-derived KCs proposed to be populated during the narrow neonatal window [51, 52, 59, 60, 398, 401, 402]. KCs also self-maintain in unperturbed conditions and only receive circulatory monocyte refilling following events like infection or transplantation [53, 69, 92, 93, 398, 403]. Our steady-state data consistently showed relative YFP labelling at about <15%, in line with two populations of distinct ontogeny. KCs showed markedly quicker replenishment upon disappearance than spleen RPM ϕ /MMMs and lung AM ϕ s during *Py1.1GFP* infection. In an acetaminophen-induced liver acute injury study, ablated KCs are also quickly replenished within two days in WT mice by local and infiltrating BM-derived precursors; and even CCR2⁻ mice refilled the populations by three days, demonstrating potent KC proliferation in the induced deficit of monocytes [404]. This observation may be an intrinsic consequence of the well-known physiological regeneration and reparation prowess of the liver in medicine and research, compared to other organs.

Liver KCs after early disappearance (3 d.p.i) in *Py1.1GFP* infection were mostly rapidly replenished by inflammatory monocytes (from 4 d.p.i.) as

evident by high YFP labelling, with refilling kinetics similar to the spleen. In two other infection models, *Listeria monocytogenes* and *Salmonella enterica*, phagocytic KCs undergo necroptosis and are rapidly refilled by inflammatory monocytes; which differentiate to both inflammatory MØs and anti-inflammatory KCs in response to IL-33/IL-4 production from neighbouring accessory cells, thus replenishing the necroptotic 'original' KCs in liver tissue [72]. Furthermore, in their study BM progenitors contribute fully to replenishment with no input from resident KCs; although we observed incomplete BM origins (<100% YFP labelling), indicating partial replenishment from embryonic KCs, perhaps due to the less severe pathology as *L.monocytogenes* and *S.enterica* solely infect KCs and cause massive necroptosis [72, 405]. The Clec4F-DTR KC depletion mouse model developed by Scott *et al* likewise also showed Ly6C^{hi} inflammatory monocytic replenishment of ablated KCs and restoration to steady-state levels by 7 days post-depletion [401]. These monocyte-derived KCs adopt full functional profiles of *bona fide* KCs, including their self-renewal properties in seeded tissues. In the event of partial depletion, both monocyte-derived and surviving resident KCs repopulated the emptied niche in tandem, with greater contribution from BM progenitors [401]. The transient 'activated' MØs with higher CD11b and CD11c expression we observed during KC refilling are reminiscent of a similar transient phenotype in an iron recycling model, where influx of monocyte-derived MØs rapidly differentiate into KCs that phagocytose RBCs and process heme for iron stores. This phenomenon is also reported in pathophysiological RBC conditions such as haemolytic anemia [406]. However, it does not appear that their transient KCs are stably replenished long-term unlike in *Py1.1GFP* infection, as fate-mapping studies showed their disappearance and no evidence of retention after RBC clearance. Taken together, our results support the findings of You *et al*, Bleriot *et al* and Scott *et al* in monocytic capability to re-establish tissue-resident KCs after necroptosis or depletion during liver inflammation and infection.

4.4.2 Depleted lung alveolar MØs almost exclusively self-renew with negligible input from BM-derived circulatory monocytes during acute *Py1.1GFP* infection

Alveolar MØs in our model was almost entirely devoid of YFP labelling (<5%) under steady-state conditions, in line with established work about self-maintenance of FL monocyte-derived lung TRMØs, with nearly no accessibility of circulatory monocytes [43, 53, 68, 69, 86, 92, 407]. During *Py1.1GFP* infection with its severe inflammation, AMØs did not completely disappear, unlike spleen RPMØs/MMMs and liver KCs. Though YFP labelling increases (<10%) as AMØs are refilled, the magnitude of labelling is far from that displayed by spleen or liver TRMØs; further highlighting some, but very minor contributions from BM-derived progenitors. Importantly, despite the massive influx of FcR γ inflammatory monocytes, the lung AMØ niche remained generally inaccessible to external responders, likely due to their inability to penetrate the alveolar epithelium. Thus, we extended these observations to a *Plasmodium* parasitic model and reiterate the hallmark of exclusive self-renewal in this tissue niche.

Even depletion of lung AMØs, which normally in other tissues would be replenished by BM-derived inflammatory monocytes, conversely resulted in local TRMØ proliferation [69]. Only in the event of serious genotoxic insult such as irradiation, will AMØs be majorly replenished by circulatory monocytes [69, 403].

4.5 Rapidly-responding Ly6C^{hi} inflammatory monocytes and monocyte-derived MØs massively infiltrate afflicted tissues and contribute to *Py1.1GFP* clearance

Monocytes are perhaps the most versatile and mobile cells of the MPS, with the capacity to rapidly generate heterogeneous subsets of monocyte-derived macrophages and dendritic cells, depending on the specific insult or inflammation. Two major subsets have been identified in the blood, the Ly6C^{hi} inflammatory and Ly6C^{lo} non-classical monocytes. Ly6C⁺ inflammatory monocytes from BM are CCR2⁺ and respond rapidly in large quantities to infection or injury. Ly6C^{lo} CXCR3⁺ 'resident' monocytes patrol

the endothelium and do not display the same propensity for homing to inflamed tissues [3, 50, 88, 89, 91].

4.5.1 *Ly6C^{hi} inflammatory monocytes rapidly and massively infiltrate organs upon *Py1.1GFP* infection*

Frl inflammatory monocytes in organs expectedly increased dramatically in proportion during *Py1.1GFP* infection, even at early timepoints (3 – 4d.p.i.) when the % iRBCs and blood-stage parasites are very low. This suggests a remarkable sensitivity of myeloid compartment to detect iRBCs and *Plasmodium* parasites. In early infection when haemozoin deposits or antibodies are not abundant yet, it is likely parasite recognition is mediated by *Plasmodium*-specific proteins on circulating or sequestered iRBCs, bioactive products like glycosylphosphatidylinositol (GPI) [408], or inflammatory toxins such as TatD-like DNase [409] and tyrosine-tRNA synthase [410]. These molecules initiate the secretion of inflammatory cytokines, upregulation of vascular endothelium adhesion molecules and trigger rapid expansion and differentiation of monocytes / MØs, in both murine and human malaria models [249, 411-413].

This rapid wave of BM and reservoir-mobilized monocytes has been well-documented in infection, inflammation and acute injury models outside of malaria [90, 91]. Egress of CCR2⁺ migrating monocytes has been documented in *PccAS* murine malaria, influx of which coincides with rapid decline in acute parasitemia. These CD11b^{hi} Ly6C^{hi} monocytes after entry into splenic niche are phagocytic, MHCII⁺ and some down-regulate CD11b expression, a phenotype that is more in line with differentiation into Frl F480^{hi} MØs than Tip-DCs [247]. Typically, this response wanes as inflammation or infection is resolved, though in chronic disease or injury like atherosclerosis, both Ly6C^{hi} inflammatory and Ly6C^{lo} monocytes progressively increase and remain high in circulation and afflicted tissues [414, 415]. We observed similarly, highest monocytic infiltration occurred in the organs before peak parasitemia and subsequently waned, except in lung which showed persistent inflammatory monocyte influx at 14 d.p.i and beyond. Soon after infiltration, the declining Frl TRMØs were gradually

refilled and YFP labelling in these cells increase significantly, confirming the partial, but substantial contribution of BM-derived monocytes in replenishing F480^{hi} TRMØs in the spleen and liver, but not lung; that also exhibited long-term stability rather than transient refilling. These results however are only in partial agreement with Hashimoto *et al*, wherein we reiterate TRMØs are indeed self-maintaining in steady state; but upon DT ablation, *Toxoplasma* and influenza infections, and even grievous genotoxic irradiation, they conversely report TRMØs were replenished mainly by local proliferation of TRMØ precursors overtime with little input from circulatory monocytes [69]. The latter finding was only true in the lung, but not spleen and liver in our acute malaria model, which was more in line with the findings of Epelman and colleagues, wherein cardiac MØs replenished from Ly6C^{hi} BM-derived monocytes after clodronate depletion, are subsequently permanently established as the 'new' functional TRMØs, which could self-maintain once sufficiently repopulated [59].

4.5.2 Concerted efforts of FrII inflammatory monocytes and monocyte-derived / other tissue-resident MØs in resolution of Py1.1GFP infection

Regardless of origin, FrI MØs (original and refilled) and FrII monocytes/ MØs unanimously contribute to *Plasmodium* clearance during the ascending, peak and descending phases of infection via opsonized / non-opsonized phagocytosis, cytokine production and antibody-dependent cellular inhibition (ADCI) [249]. This is a distinct opsonizing mechanism performed only by monocytes, requiring low amounts of cytophilic Ig (unlike pathogen neutralization) and results in the release of soluble mediators that arrest *Plasmodium* proliferation in the local proximity [197], and has also been documented in human *P.falciparum* malaria [252, 412].

Although MØs are important in the generation of immune induction and inflammation, they also have functional duality in the resolution and curbing of pathogenic inflammation resulting from these immune responses. Tissue-resident macrophages (FrI MØs) in various organs are known to adopt anti-inflammatory M2-like phenotypes in steady-state ('original'), as well as after resolution of infection or injury to restore homeostasis ('original' or BM

replenished), regardless of embryonic or BM ontogeny [71, 72, 79, 416-418].

We observed large deposits of hemozoin, the insoluble by-product from *Plasmodium* heme detoxification in the organs and myeloid cells of our mouse model [419, 420]. Hemozoin, although biochemically inert, is regarded as a cellular 'danger' signal and induces the NLRP3 inflammasome, hence is a strong advocate of inflammation during *Plasmodium* infections [419-423]. In this context, the observed late expansion of mixed population FrlIc MØs (and to some extent Frl TRMØs, particularly liver), might align to differentiation of M2-like anti-inflammatory MØs. Together with the replenished Frl TRMØs with their default M2 programming, this could be an ongoing response to dampen immunopathogenic inflammation induced by accumulated hemozoin deposits and attempts to restore homeostasis in the malaria-afflicted organs.

4.6 BM monocyte-derived MØs are phenotypically and transcriptionally similar to *bona fide* embryonic tissue-resident MØs

By now, our own results and multiple convincing, elegant studies have shown that inflammatory monocytes can refill TRMØ compartments normally undergoing self-maintenance, when steady-state homeostasis is perturbed. However, the capacity to do so also consequentially leads to one pressing question - besides phenotypical, how similar were their transcriptional, and subsequently functional, profiles to *bona fide* TRMØs? We showed in *Py1.1GFP* infection, the overall transcriptional profiles of replenished BM-derived TRMØs in recovered mice were very similar to steady-state 'original' TRMØs, as supported by low number of DEGs and close hierarchical clustering as well as PCA analysis. Several other studies have shown similar trends. Gibbings *et al* report extremely low level of DEGs (~0.1%) in their lung AMØ transplant model upon clodronate depletion or irradiation, between donor and host AMØs. These reconstituted AMØs are not only transcriptionally, but also functionally similar, as assessed by comparable phagocytic ability and cytokine production [424]. Our results were also in accordance with the microarray data of Scott and colleagues,

who showed low number of DEGs and close PCA clustering of monocyte-derived and steady-state KCs after DT depletion [401]. There is also growing evidence supporting strong involvement of the unique tissue niches or microenvironments, rather than ontogeny, in influencing the differentiation, transcriptional, epigenetic and functional programs of replenishing TRMØs [45, 81, 425]. Hence, multiparameter and high dimensional bioinformatics analyses such as UMAPs, PCA and heatmaps are increasingly useful in our understanding of the complexities of myeloid ontogeny and differentiation.

In-depth analysis into our small number of DEGs revealed that post-infection TRMØs presented with an overall more activated genetic profile than steady-state based on upregulated MHC-II genes and CD163-L1. CD163-L1 is a homolog of the well-studied multifunctional CD163 scavenger receptor [328], primarily involved in haemoglobin capture, mediating anti-inflammatory responses and exerting anti-bacterial functions [426, 427]. CD163-L1 is also an endocytic scavenger receptor, expressed exclusively on mammalian TRMØs (CD163-L1^{hi}) / monocytes (CD163-L1⁺), and is highly expressed in steady-state spleen WP and RPMØs but only weakly in liver KCs and lung AMØs [428, 429]. Interestingly, it does not bind to CD163 ligands like haptoglobin-haemoglobin complex and bacterial PAMPs [428, 429]. Moeller *et al* has described IL-6 and IL-10-mediated induction of CD163-L1; hence supporting anti-inflammatory and immunomodulating effects similar to CD163, on a proposed subset of CD163-L1⁺ 'regulatory' MØs [429]. Upregulation of CD163-L1 and related MHC-II genes were highest in spleen and liver, which correlates with high deposited haemozoin in these organs upon recovery, inducing inflammation and the resultant more activated phenotype. CD163-L1 expression, with its purported anti-inflammatory functions, could be upregulated in response to curtail ongoing low-grade inflammation from these deposited haemozoin deposits after *Py1.1GFP* infection. Our results are in line with notion of tissue microenvironment imprinting on TRMØs described by Lavin *et al* [45], Gosselin *et al* [81] and other researchers.

Although our number of DEGs were not as low as reported by Scott *et al* and Gibbings *et al*, it could be due to the context-dependent differences between MØ depletion and full-blown *Plasmodium* infection; which would induce greater inflammation, immune response and tissue restorative pathways, and hence more divergent genetic expression profiles than the former. Nevertheless, our results overall support phenotypical and transcriptional similarity of replenished TRMØs to 'original' foetal counterparts; as well as influence of niche-specific and microenvironment cues on the 'imprinting' of these repopulating TRMØs, rather than purely derived from ontogeny.

4.7 *Plasmodium* infection as a new precedent for the general mechanism of beneficial cell suicide

Tissue-resident macrophages have been documented to temporarily disappear during injury, inflammation and certain acute and chronic infections, such as leishmaniasis and malaria. This 'macrophage disappearance reaction' has been attributed to migration, apoptosis and necroptosis after phagocytosis, inflammation or other pathogen clearance-related functions [107, 405, 430-433]. It has long been assumed to be a detrimental consequence of innate immunity that curtails the responding pool of MØs to pathogens or injury [72, 433, 434]. However, emerging data has shed new perspectives and a proposed alternative model of 'beneficial cell suicide', where TRMØs die in a tightly-regulated manner after pathogen uptake, to allow for infiltration of BM-derived monocytes. Hence, this altruistic suicide makes way for potentially microbicidal inflammatory monocytes and MØs better equipped to combat infection, rather than homeostatic TRMØs which are more inclined towards immunoregulatory and anti-inflammatory phenotypes [72, 434, 435]. This process is fundamentally different from silent death by apoptosis; rather, necroptosis or pyroptosis are lytic and strongly immunogenic, lending credence to the theory that this deliberate suicide is meant to efficiently trigger and recruit downstream immune effectors. Additionally, it confers kamikaze effects in killing or trapping some invading pathogens, particularly those that infect TRMØs (i.e. *L.monocytogenes*), together with host cells before

reinforcements arrive [434-437]. In support of this, a recent study described a subset of migratory F480^{hi} CD169⁺ Tim-4⁺ TRMØ, potentially implicated in T_{REG} differentiation, were highly susceptible to Tim-4-mediated apoptosis, particularly in situations of oxidative stress (i.e inflammation). Although the mode of death here is by apoptosis, it was speculated that the fragility of these TRMØ was also a response mechanism in alleviating undesired immunoregulatory effects under inflammatory stress, usually an indicator of infection, so beneficial inflammatory responses can be amplified [417]. It is possible our *Py1.1GFP* infection model is another example of beneficial cell suicide, as both spleen and liver compartments which showed TRMØ disappearance, was then majorly and rapidly repopulated with BM-derived monocytes. Moreover, we have also earlier discussed that *Plasmodium* infection leads to high degree of inflammation during parasite replication in the acute phase and from metabolic by-products, which could altogether trigger necroptosis/pyroptosis of TRMØ for incoming inflammatory MØs. Undoubtedly, this strategy also risks immunopathology if uncontrolled, or depending on the nature or context of oxidative stress, i.e. during transplantation and tissue grafting.

5. Summary

Overall, our findings are remarkably similar to the phenotype of IL-12p40 KO mice described by Stevenson and Su nearly a decade ago [289]. A very recent study utilizing the newly created Pb-TII CD4 transgenic T-cell line and XCR-1-DTR transgenic mouse model recapitulated similar findings and roles of CD8⁺ DCs in CD4⁺ and CD8⁺ T-cell priming in the *PbA* ECM model [438].

In summary, Clec9A⁺ DCs is a key upstream driver in the orchestration of effective downstream immune responses against *Plasmodium chabaudi chabaudi* infection. We highlighted the important role IL-12 producing Clec9A⁺ DCs have in activating CD4⁺ and CD8⁺ T effector responses and the resultant T_{H1} effector cytokine polyfunctionality required for control of acute *PccAS* infection, largely mediated by IFN γ . Even as their direct priming function became refractory past peak infection, the compounding effects of their absence strongly influenced the downstream establishment of *Plasmodium*-specific Ig CSR, generation of protective T_{H1} isotype Ig and T effector functions important for parasite control and clearance during recrudescence infection.

Following up, we also reported that *Plasmodium* infection results in inflammation-induced disappearance of tissue-resident macrophages, similar to observations by Beattie *et al* [176] and Krucken *et al* [175]. Investigation of myeloid subset dynamics in response by traditional and high-dimensional, multi-parameter analyses revealed organ and subset-specific kinetics. Overall, the early disappearance of TRM $\emptyset$ s was inversely correlated with massive infiltration of BM-derived Ly6C^{hi} inflammatory monocytes. Fate-mapping studies further revealed these monocytes differentiate to inflammatory M $\emptyset$ s and partially replenished the emptied TRM $\emptyset$ niches in the spleen and liver, but were unable to repopulate the lung alveolar M $\emptyset$ s which had the capacity to self-renew by local proliferation. We summarized the refilling kinetics of spleen, liver and lung during *Py1.1GFP* infection in Figure 5.1.

Importantly, multiple transcriptional analyses showed high degree of similarity between the genetic profiles of BM monocyte-derived TRM $\emptyset$ s and

'original' embryonic TRMØs, but also reiterated effects of niche microenvironment imprinting, depending on the context of stimuli. Moreover, replenished TRMØs displayed stable longevity up to 90 d.p.i. after resolution of infection. Taken together, we show that BM emigrants are able to replenish emptied tissue niches in the context of *Plasmodium* infection, and their progeny are able to be imprinted with the molecular and transcriptional characteristics of the specific tissue microenvironment for functional specialization, despite different ontogeny origins.

Figure 5.1: Replenished tissue-resident MØs, except the lung, have dual origins during *Py1.1GFP* infection

Illustrations show the refilling kinetics of tissue-resident MØs in the spleen (top), liver (middle) and lung (bottom) during *Py1.1GFP* infection.

Top and middle – In the steady-state spleen and liver, embryonic-seeded tissue-resident MØs are maintained mostly by self-renewal and local proliferation, with minimal contribution from BM-derived monocytes. Inflammation and injury induced by *Plasmodium* infection results in

/pyroptosis of embryonic tissue-resident MØs and rapid recruitment of Ly6C^{hi} inflammatory monocytes which differentiate to MØs. Together with surviving embryonic MØs, both subsets replenish the depleted tissue-resident MØ compartment. Upon resolution of *Py1.1GFP* and inflammation, replenished dual origin (YFP⁺ and YFP⁻) tissue-resident MØs self-renew by local proliferation long-term.

Bottom – In the steady-state, fetal liver-derived lung alveolar MØs self-maintain their population. During *Py1.1GFP* infection, despite depletion of alveolar MØs and massive infiltration of Ly6C^{hi} monocytes, alveolar MØs gradually repopulate their numbers by self-renewal, with negligible input from inflammatory monocytes. After recovery, most lung alveolar MØs remain YFP⁻ and of embryonic origin.

6. Significance of studies on current malaria immunological research

6.1 Clec9A⁺ DCs and their roles in anti-*Plasmodium* immunity

At the point it was conceived, the study utilizing the Clec9A-DTR mouse model was the first to show the absence of a specific myeloid cell, the CD8⁺/CD103⁺ cDC1 subset, and its roles in a chronic model of blood stage malaria, *Plasmodium chabaudi chabaudi* AS. Although dendritic cells have been extensively studied in malarial infections, most studies have relied on broad depletion systems that remove both cDC1 and cDC2, as well as some other macrophages, resulting in an altered physiological state emptied of crucial myeloid populations, which may exacerbate phenotypes for interpretation. This study highlights the direct and indirect effects Clec9A⁺ DCs exert on shaping the anti-*Plasmodium* immune responses, a far-reaching cascade that emphasizes their key roles in controlling the outcome of chronic *Plasmodium chabaudi chabaudi* AS malaria infection. It compliments previous work done elucidating their immunopathogenic roles in the *Plasmodium berghei* ANKA model of cerebral malaria via activation of CD8⁺ CTLs and inflammation [257].

We have shown that Clec9A⁺ DCs, aside from influencing T_{H1} proinflammatory responses, also exerted effects on effector T_{FH} and subsequent antibody CSR in GC and extrafollicular B-cell responses. The human counterpart to mouse Clec9A⁺ CD8⁺/CD103⁺ DCs are CD141⁺ cDC1s, which also express Clec9A⁺ and XCR1⁺ and are specialized in cross-presentation [439, 440]. Various groups have also demonstrated the potent induction of humoral and antibody responses from targeting the Clec9A receptor in mice [441, 442], non-human primates [443] and *in vitro* human CD141⁺ DCs [444], making this molecule an attractive target for vaccine development. In the context of human malaria, Clec9A⁺ BDCA3⁺ DCs have been isolated in the circulation of Kenyan children afflicted with severe malaria syndromes [445]. Attempts at successful malaria vaccines have so far eluded researchers due to the complexity of acquisition of, and temporal decay of protective memory in humans and experimental animals [95, 386, 446]. Moreover, recent studies in both murine and humans have elucidated further complexities in T_{H1} - T_{FH} developmental axis, and with current agreement of the crucial roles of T_{FH} in initiating and sustaining anti-

Plasmodium humoral immunity, investigating the involvement of Clec9A⁺ DC in mediating T_{H1} - T_{FH} responses would be valuable for understanding the roles these cells play in humoral immunity. The knowledge generated could be useful for the strategic design of more efficient malaria vaccines or therapeutics via targeting the Clec9A receptor, or boosting T_{FH} responses for more effective humoral immunity without compromising T_{H1} responses for curbing acute inflammation.

6.2 Tissue-resident MØ repopulation kinetics during *Plasmodium* infection

This study to our knowledge, is the first to describe the kinetics and dynamics of tissue-resident MØ ontogeny in *Plasmodium* infection. Tissue-resident MØ ontogeny has in recent years garnered strong attention and rapid expansion of exciting research due to the heterogeneity and functional importance of these MØs. It is well accepted now that most steady-state TRMØs are seeded by embryonic precursors and require little replenishment from adult BM monocytes [38, 50, 58]; but in circumstances of extenuating injury, inflammation or infection, previously self-maintaining organs may acquire BM-derived inflammatory monocytic replenishment of TRMØ compartments [3, 38, 91, 249, 447]. An ongoing question in this event would be how similar replenished TRMØs are compared to *bona fide* embryonic-seeded TRMØs, and the degree of influence that progenitor ontogeny, local tissue microenvironment and epigenetic landscaping have on the transcriptional, genetic and functional profiles of these BM-derived TRMØs. Ultimately, the ability to acquire functional specialization of the specific tissue niche is the most crucial outcome, regardless of ontogeny or origins. Although some seminal studies [45, 68, 401, 448] have explored this aspect, (reviewed here [75, 77, 416]) this field of myeloid research is still relatively new.

Acute *Plasmodium yoelii* malaria causes multi-organ pathology. Hence, this parasite strain coupled with our cKit^{MerCreMer/R26} reporter mice, allowed us the advantage to study tissue-resident MØ replenishment simultaneously in the spleen, liver and lung, in the setting of a systemic infection, unlike many studies in the field where only one organ compartment was focused on. The findings of this study adds depth to our previously published work in CD169⁺

tissue-resident MØs and their roles in frontline anti-*Plasmodium* immunity [253], and also reconciles a general observation of MØ disappearance seen in *Plasmodium*, bacterial and viral infections [176, 434]. In fact, this lends credence to the recently-termed beneficial cell suicide theory [435, 437], by providing a perspective from the parasitic infection axis, as reports were mainly from bacterial infection models. Altogether, our findings provide the first insight into this growing body of literature, bridging the research gap from the parasitic infection angle, and could further contribute to elucidating the complex, tissue-specific myeloid immune responses to *Plasmodium* infection.

6.3 Concluding remarks

Dendritic cells and macrophages have both overlapping and unique functions in *Plasmodium* infection. We present studies investigating the roles of Clec9A⁺ CD8⁺/CD103⁺ cDCs in chronic *Plasmodium chabaudi* *chabaudi* AS infection and myeloid dynamics and turnover in acute *Plasmodium yoelii* 1.1 infection. The absence of Clec9A⁺ cDCs profoundly influenced downstream cascade of anti-*Plasmodium* immune responses, generally resulting in impaired T_{H1} proinflammatory responses and effector T-cell polyfunctionality in both acute and recrudescence infection.

Foetal-derived tissue-resident macrophages necrotize in a proposed beneficial cell suicide to allow for infiltration of inflammatory monocytes to efficiently combat *Plasmodium* infection. Inflammatory monocytes then refill the emptied MØ compartment together with surviving TRMØs, resulting in a mixed ontogeny repopulation of the tissue niches. Phenotypical and transcriptional profiles of replenished TRMØs are very similar to *bona fide* TRMØs, but also maintains a profile plasticity dependent or influenced by the local niche microenvironment and signalling cues. Altogether, we illustrate here additional insights into the modulation of innate and adaptive immune responses, by specific myeloid cell subsets during *Plasmodium* infection.

7. Appendix

7.1. Cell phenotype profile

Myeloid cells			
Cell Type	Cell Profile	Function(s)	Remarks
cDC1	CD45 ⁺ , CD11c ^{hi} , MHC-II ^{hi} , CD24 ⁺ , *CD103 ⁺ , **CD8 ⁺ , Clec9A ⁺	IL-12 production Cross-presentation CD8 ⁺ CTL activation	*non-lymphoid origin **lymphoid origin
cDC2	CD45 ⁺ , CD11c ^{hi} , MHC-II ^{hi} , CD24 ⁺ , CD11b ⁺	CD4 ⁺ T-cell activation IL-4, IL-5, IL-6 production	
pDC	CD45 ⁺ , CD11c ^{lo} , B220 ⁺ , Ly6C ⁺ , SiglecH ⁺	Type I IFN production	
F4/80 ⁺ MØ (FrI)	CD45 ⁺ , CD169 ⁺ , CD11b ^{lo} , F4/80 ⁺	Phagocytosis Cytokine production Tissue homeostasis	Varying levels of F4/80 ⁺ expression
Monocytes (FrII)	CD45 ⁺ , CD11b ⁺ , Ly6C ^{hi} , *MHC-II ⁺	Patrolling and infiltration Phagocytosis	*transitioning monocytes → MØ
Neutrophils (FrIII)	CD45 ⁺ , CD11b ⁺ , Ly6C ^{int} , Ly6G ⁺	Phagocytosis	

Appendix table 1: Myeloid cell phenotype profiles

T/NK-cells			
Cell Type	Cell Profile	Function(s)	Remarks
Naïve CD4 ⁺ / CD8 ⁺ T	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ or CD8 ⁺ , CD62L ^{hi}		
Activated CD4 ⁺ / CD8 ⁺ T	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ or CD8 ⁺ , CD62L ^{lo} , CD44 ^{hi} , *KLRG-1 ⁺	Effector responses	* More pronounced on CD8 ⁺ CTL
CD4 ⁺ T _{h1}	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ , IFNγ ⁺ , *IL-10 ⁺ , TNFα ⁺	Proinflammatory responses Immunoregulation	* IFNγ ⁺ , IL-10 ⁺ T _{h1} = T _{r1} cells
CD8 ⁺ CTL	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ , IFNγ ⁺ , *IL-10 ⁺ , Granzyme A ⁺ , Granzyme B ⁺ , TNFα ⁺	Proinflammatory responses Cytolytic killing	* Minimal production

NKT-cells	CD45 ⁺ , CD3 ⁺ , NK1.1 ⁺ , IFN γ ⁺ , Granzyme A ⁺ , Granzyme B ⁺	Proinflammatory responses	
NK-cells	CD45 ⁺ , NK1.1 ⁺ , IFN γ ⁺ , *IL-10 ⁺ , TNF α ⁺ , Granzyme A ⁺ , Granzyme B ⁺	Proinflammatory responses Cytolytic killing Immunoregulation	* Subset of NK
$\gamma\delta$ T-cells	CD45 ⁺ , CD3 ⁺ , $\gamma\delta$ ⁺ , IFN γ ⁺ , Granzyme A ⁺ , Granzyme B ⁺	Proinflammatory or anti-inflammatory responses	
CD4 ⁺ T _{FH}	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ , CXCR5 ⁺ , Bcl-6 ⁺ , CD44 ^{hi} , PD-1 ⁺	Extrafollicular antibody production	
GC CD4 ⁺ T _{FH}	CD45 ⁺ , CD3 ⁺ , CD4 ⁺ , CXCR5 ^{hi} , Bcl-6 ⁺ , CD44 ^{hi} , PD-1 ^{hi} , IL-21 ⁺ , *IFN γ ⁺	Supports GC B-cell reactions, antibody production and CSR	* T _{H1} -like effector T _{FH} subset

Appendix table 2: T and NK-cell phenotype profiles

B-cells			
Cell Type	Cell Profile	Function(s)	Remarks
B-cells	CD45 ⁺ , B220 ⁺ , CD19 ⁺	Antibody production	
GC B-cells	CD45 ⁺ , B220 ⁺ , CD19 ⁺ , CD38 ^{lo} , GL-7 ⁺ , *IgG ⁺ , *IgM ⁺	GC reactions for antibody production Sustains T _{FH} survival CSR and affinity maturation	*Surface-bound IgG and IgM as proxy of active class-switching
Plasmablast / plasma cells	CD45 ⁺ , B220 ⁺ , CD19 ⁺ , CD38 ^{lo} , CD138 ⁺ , IgG ⁺ , IgM ⁺	Antibody production	
B _{mem} precursors	CD45 ⁺ , B220 ⁺ , CD19 ⁺ , CD38 ^{hi} , CD21/CD23 ⁻ , IgG ⁺ , IgM ⁺	Memory B-cell formation	

Appendix table 3: B-cell phenotype profiles

Memory T precursors			
CD4 ⁺ T _{CM}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , CD127 ⁺ , CD62L ⁺	T central memory precursors	
CD4 ⁺ early T _{EM}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , CD127 ⁺ , CD27 ⁺	Early T effector memory precursors	
CD4 ⁺ late T _{EM}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , CD127 ⁺	Late T effector memory precursors	Differentiated from early precursors

CD4 ⁺ early T _{EFF}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , CD62L ⁺ , CD27 ⁺	Early effector T cells	
CD4 ⁺ intermediate T _{EFF}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , CD27 ⁺ , *KLRG- 1 ⁺	Transitioning effector T cells	Differentiated from early precursors *small subset
CD4 ⁺ late T _{EFF}	CD45 ⁺ , CD3 ⁺ , CD44 ⁺ , CD4 ⁺ , *KLRG-1 ⁺	Late effector T cells	Differentiated from intermediate precursors Terminally differentiated *small subset

Appendix table 4: Memory T precursor phenotype profiles

7.2. Antibody mastermix panels

1. Depletion DC and myeloid		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD11c	PE Cy7	1:600
MHC II	BV421	1:1000
CD11b	BUV395	1:600
CD8 / CD103	PerCP Cy5.5	1:600
CD24	APC	1:600
Siglec-H	FITC	1:500
F4/80	PE	1:600
Ly6C	BV605	1:600

1. Activation markers		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	BV421	1:600
CD3	BUV737	1:600
CD4	APC Cy7 / APC/Fire™ 750	1:500
CD8	PerCP Cy5.5	1:600
NK1.1	BV605	1:600
CD62L	APC	1:600
CD69	PE	1:600
CD44	FITC	1:600
PD-1	BUV395	1:600
CD43	PE Cy7	1:600

2. Cell subset profiling		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD3	BUV737	1:600
CD4	Alexa Fluor 488 / FITC	1:500
CD8	PerCP Cy5.5	1:600
NK1.1	BV605	1:600
γδ TCR	APC	1:600
CD11c	PE Cy7	1:600
MHC II	BV421	1:1000
CD19	BUV395	1:600

3. T effector polyfunctionality		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD3	BUV737	1:600
CD4	Alexa Fluor 488 / FITC	1:500
CD8	BUV395	1:600
NK1.1	BV605	1:600
TNFα	APC	1:600
IL-10	PE Cy7	1:600
Granzyme B	BV421	1:400
Granzyme A	PerCP Cy5.5	1:800
IFNγ	PE-CF594	1:800

4. T follicular helper cells 2		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD3	BUV737	1:600
CD4	APC Cy7 / APC/Fire™ 750	1:600
CD8	PerCP Cy5.5	1:600
CD44	FITC	1:500
PD-1	PE	1:600
CXCR5	Biotin	1:500
Streptavidin	BUV395	1:500
Bcl-6	BV421	1:300

5. T follicular helper cells effector functionality		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD3	BUV737	1:600
CD4	APC/Fire™ 750	1:600
CD8	PerCP Cy5.5	1:600
PD-1	PE	1:600
CXCR5	Biotin	1:500
Streptavidin	BUV395	1:500
Bcl-6	BV421	1:300
IFNγ	FITC	1:500
IL-21	APC	1:300

6. Germinal center and plasma B-cells		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD3	BUV737	1:600
B220	FITC	1:500
CD19	BUV395	1:600
CD23 / CD27	PerCP Cy5.5	1:600
CD38	BV421	1:600
CD138	PE	1:300
GL-7	APC	1:600
IgM	APC Cy7	1:400
IgD	PE Cy7	1:800
IgG	BV605	1:600

7. T _{MEM} and T _{EFF}		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	BV421	1:600
CD3	BUV737	1:600
CD4	APC Cy7 / APC/Fire™ 750	1:600
CD8	BUV395	1:600
CD27	PerCP Cy5.5	1:600
CD62L	PE	1:600
CD44	FITC	1:500
CD127	APC	1:300
KLRG-1	BV605	1:600
CCR7	Biotin	1:500
Streptavidin	PE Cy7	1:500

8. Myeloid		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD11c	PE Cy7	1:600
MHC II	BV421	1:1000
CD11b	BUV395	1:600
F4/80	PE	1:600
Ly6C	BV605 / PerCP Cy5.5	1:600
Ly6G/YFP	FITC	1:500/Inducible
CD169	APC	1:30

9. Liver capsular MØ		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD11c	BV605	1:600
MHC II	BV421	1:1000
CD11b	BUV395	1:600
F4/80	PE	1:600

Ly6C	PerCP Cy5.5	1:800
CD64	APC	1:30
Tim-4	PE Cy7	1:600
YFP	FITC	Inducible

10. MØ profiling		
Marker	Fluorochrome	Dilution
FVS	BV510	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD11c	PE Cy7	1:600
MHC II	BV421	1:1000
CD11b	BUV395	1:600
F4/80	PE	1:600
Ly6C	BV605 / PerCP Cy5.5	1:600 / 1:800
Ly6G	FITC	1:500
CD169	APC / eFluor 660	1:30 / 1:600
CD8	APC	1:600
CD3	Alexa Fluor 647	1:600
CD19	APC	1:600
CCR3	APC	1:600
CCR2	APC	1:100
MerTK	APC	1:100
cKit	APC	1:600
CD209a	APC	1:600
CD64	APC	1:600
Siglec-F	APC	1:600
CD34	eFluor 660 Control	1:600

1. Myeloid sorting panel 1		
Marker	Fluorochrome	Dilution
CD45	APC Cy7 / APC/Fire™ 750	1:600
MHCII	FITC	1:1500
CD11c	PE Cy7	1:600
CD11b	APC	1:600
F480	PE	1:600
Ly6C	PerCP Cy5.5	1:800

2. Myeloid sorting panel 2		
Marker	Fluorochrome	Dilution
FVS	DAPI	1:1000 / 10 ⁶ cells
CD45	APC Cy7 / APC/Fire™ 750	1:600
CD11c	PE Cy7	1:600
MHC II	APC	1:1000
CD11b	BUV395	1:600
F4/80	PE	1:600
Ly6C	PerCP Cy5.5	1:800
YFP / Ly6G	FITC	Inducible / 1:500

7.3. Supplementary Figures

Supplementary Figure 1: Clec9A⁺ DC depletion results in significant reduction of CD3⁺ T-cell subsets, particularly effector T-cells in spleen, lung and liver

Detailed bar charts showing numbers of all CD3⁺ and CD3⁻ cell subsets profiled in spleen (top row), lung (middle row) and liver (bottom row) of SS (white bars), *PccAS*-infected WT (black bars) and *Clec9A*-DTR mice (blue bars) at 9 d.p.i. CD3⁺ fraction comprises of CD4⁺, CD8⁺, $\gamma\delta$ - ($\gamma\delta$ TCR⁺), NK- (CD3⁺ NK1.1⁺) and double-negative (DN – CD4⁻CD8⁻ $\gamma\delta$ - NK1.1⁻) T-cells; while CD3⁻ fraction includes NK1.1⁺ NK-cells, CD19⁺ B-cells, CD11c^{hi} MHCII^{hi} DCs and CD11c⁺ myeloid cells. Data shown are representative of 3 independent experiments with n=3-4 mice/group, 6-10 weeks old. Statistical significance was determined using unpaired Student's t-test, ns = not significant, *p < 0.05, **p < 0.01 and ***p < 0.001.

A

B

Supplementary Figure 2: Clec9A⁺ DC depletion results in severely impaired splenic CD4 T_{Eff} polyfunctionality.

Clec9A-DTR CD4 T_{Eff} are significantly less polyfunctional than WT. (A) Gating strategy of live CD45⁺ polyfunctional immune effector cells in SS and *PccAS*-infected WT and Clec9A-DTR mice at 9 d.p.i. Representative plots from the spleen show PMA/ionomycin stimulated CD3⁺ CD4⁺, CD8⁺, CD4⁺CD8⁺ T-cells, CD3⁺ NK1.1⁺ NK cells and remaining CD3⁺ NK1.1⁺ mixed B/myeloid populations assayed for production of the cytokines IFN γ , TNF α , IL-10 and granzymes A (GzA) and B (GzB). (B) SPICE pie charts depict polyfunctional unstimulated splenic CD4 T_{Eff} in WT (left) and Clec9A-DTR (right) mice. Cytokines and cytolytic granules Granzyme A, Granzyme B, IFN γ , IL-10 and TNF α are represented as coloured arcs as per figure legend(s) over SPICE pies. Overlapping arcs and pie slices correspond to multiple cytokine-producing and the proportional heterogeneity of cell subpopulations respectively. SPICE pies are generated from cumulative data of unstimulated samples from 2 independent experiments, with n=7-8 mice/group, 6-10 weeks of age. Statistical significance is defined by the heterogeneity between two SPICE pies, analyzed using in-built Monte Carlo method, with *p < 0.05, **p < 0.01 and ***p < 0.001.

Supplementary Figure 3: Depletion of Clec9A⁺ DCs does not impair B-cell cellularity but results in differential polarization of *Plasmodium*-specific Ig isotypes in *PccAS*-infected mice

(A) Bar charts show absolute numbers of total live B220⁺ (left) and B220⁺ CD19⁺ (right) B-cells in SS (white), WT (black) and Clec9A-DTR (blue) mice at 14-17 d.p.i. Data shown in SEM are cumulative of three independent experiments, n = 3-4/group, 6-10 weeks old. (B) Pooled sera is pre-screened to ensure accurate Ig polarization respective to WT and Clec9A-DTR profiles. Dot graphs show *Plasmodium*-specific Ig isotypes in donor SS (white), WT (black) or Clec9A-DTR (blue) day 14 – 15 immune sera before. Data in SEM is representative of 2 experiments, n = 10 mice/group, 6 – 8 weeks old. Statistical significance was determined using 2-way ANOVA with Bonferroni's multiple comparisons post-test, *p < 0.05, **p < 0.01, and ***p < 0.001.

Supplementary Figure 4: CD3⁺ T-cell subsets remain severely depleted in the absence of Clec9A⁺ DCs during recrudescent *PccAS* infection.

Flow cytometry analysis of CD3⁺ and effector CD4⁺ T-cell subsets during *PccAS* recrudescent infection. (A) Bar charts show total numbers of CD3⁺ and CD3⁺ cell subsets profiled in spleen, lung and liver of SS (white bars), *PccAS*-infected WT (black bars) and Clec9A-DTR mice (blue bars). Top row, shown in order - total CD3⁺, CD4⁺, CD8⁺, CD4⁺CD8⁺ double-negative (DN) T-cells; and bottom row – activated CD44⁺ CD4⁺ and CD8⁺ T-cells were gated from live CD45⁺ cells. (A-B) SS (white bars), *PccAS*-infected WT (black bars) and Clec9A-DTR mice (blue bars) harvested during recrudescence, between days 16 – 18 p.i. Data shown in SEM are cumulative of 3 independent experiments with n=3-4 mice/group, 6-10 weeks old. Statistical significance was determined using Mann Whitney's test, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Supplementary Figure 5: KLRG1⁺ CD4 T_{EFF} are present during recrudescence *PccAS* infection and reduced in Clec9A⁺ DC-deficient mice

Flow cytometry analysis of CD3⁺ CD4⁺/CD8⁺ T_{MEM} and T_{EFF} subsets during *PccAS* recrudescence infection (16 – 18 d.p.i). Representative plots of KLRG1⁺ CD27⁺/CD27⁺ CD4 T_{EFF} (top). Percentage (middle) and absolute numbers (bottom) of KLRG1⁺ CD4 T_{EFF} subsets in spleen, lung and liver liver of SS (white bars), *PccAS*-infected WT (black bars) and Clec9A-DTR mice (blue bars).

Supplementary Figure 6: Tissue-resident macrophages are differentially repopulated in organs of WT Balb/c mice during *Py1.1GFP* infection

Myeloid population kinetics in Balb/c during *Py1.1GFP* infection. (A-C) Curves show WT Balb/c % kinetics of Frl (red, top row) TRM, Frlai (blue, 2nd row) monocytes, Frlb (green, 3rd row) and Frlc (purple, bottom row) in (A) spleen, (B) lung and (C) liver from 3 - 35 d.p.i. Data shown are SEM values calculated relative to total % CD11b⁺ myeloid cells; and cumulative of 2 independent experiments with n=3-4 mice/group, 6-10 weeks old upon TAM treatment. Statistical significance was determined using Mann Whitney's test against steady-state baseline (D0): *p < 0.05, **p < 0.01, ***p<0.001 and ****p<0.0001.

8. References

1. Yona, S. and S. Gordon, *From the Reticuloendothelial to Mononuclear Phagocyte System – The Unaccounted Years*. *Frontiers in Immunology*, 2015. **6**: p. 328.
2. van Furth, R., et al., *The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells*. *Bull World Health Organ*, 1972. **46**(6): p. 845-52.
3. Yona, S. and S. Jung, *Monocytes: subsets, origins, fates and functions*. *Current Opinion in Hematology*, 2010. **17**(1): p. 53-59.
4. Geissmann, F., S. Jung, and D.R. Littman, *Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties*. *Immunity*, 2003. **19**(1): p. 71-82.
5. Fogg, D.K., et al., *A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells*. *Science*, 2006. **311**(5757): p. 83-87.
6. Onai, N., et al., *Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow*. *Nature Immunology*, 2007. **8**: p. 1207.
7. Hettinger, J., et al., *Origin of monocytes and macrophages in a committed progenitor*. *Nature Immunology*, 2013. **14**: p. 821.
8. Gordon, S., A. Plüddemann, and F. Martinez Estrada, *Macrophage heterogeneity in tissues: phenotypic diversity and functions*. *Immunological Reviews*, 2014. **262**(1): p. 36-55.
9. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease*. *Nature*, 2013. **496**: p. 445.
10. Reis e Sousa, C., *Activation of dendritic cells: translating innate into adaptive immunity*. *Current Opinion in Immunology*, 2004. **16**(1): p. 21-25.
11. Reis e Sousa, C., *Dendritic cells in a mature age*. *Nat Rev Immunol*, 2006. **6**(6): p. 476-483.
12. Shortman, K. and Y.J. Liu, *Mouse and human dendritic cell subtypes*. *Nat Rev Immunol*, 2002. **2**.
13. Mildner, A. and S. Jung, *Development and Function of Dendritic Cell Subsets*. *Immunity*, 2014. **40**(5): p. 642-656.
14. Zhang, J., et al., *Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors*. *Blood*, 2006. **107**(9): p. 3600.
15. Spaulding, E., et al., *STING-Licensed Macrophages Prime Type I IFN Production by Plasmacytoid Dendritic Cells in the Bone Marrow during Severe Plasmodium yoelii Malaria*. *PLoS Pathogens*, 2016. **12**(10): p. e1005975.
16. Siegal, F.P., et al., *The Nature of the Principal Type 1 Interferon-Producing Cells in Human Blood*. *Science*, 1999. **284**(5421): p. 1835-1837.
17. Cisse, B., et al., *Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development*. *Cell*, 2008. **135**(1): p. 37-48.
18. Reynolds, G. and M. Haniffa, *Human and Mouse Mononuclear Phagocyte Networks: A Tale of Two Species?* *Frontiers in Immunology*, 2015. **6**: p. 330.
19. Carotta, S., et al., *The Transcription Factor PU.1 Controls Dendritic Cell Development and Flt3 Cytokine Receptor Expression in a Dose-Dependent Manner*. *Immunity*, 2010. **32**(5): p. 628-641.
20. McKenna, H.J., et al., *Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells*. *Blood*, 2000. **95**(11): p. 3489-3497.
21. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages*. *Nature*, 2000. **404**: p. 193.
22. den Haan, J.M.M., S.M. Lehar, and M.J. Bevan, *Cd8⁺ but Not Cd8⁺ Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo*. *The Journal of Experimental Medicine*, 2000. **192**(12): p. 1685-1696.
23. Poulin, L.F., et al., *DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues*. *Blood*, 2012. **119**(25): p. 6052-62.
24. Sancho, D., et al., *Identification of a dendritic cell receptor that couples sensing of necrosis to immunity*. *Nature*, 2009. **458**(7240): p. 899-903.

25. Arora, P., et al., *A Single Subset of Dendritic Cells Controls the Cytokine Bias of Natural Killer T Cell Responses to Diverse Glycolipid Antigens*. *Immunity*, 2014. **40**(1): p. 105-116.
26. Seillet, C., et al., *CD8⁺ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3*. *Blood*, 2013. **121**(9): p. 1574-1583.
27. Hacker, C., et al., *Transcriptional profiling identifies Id2 function in dendritic cell development*. *Nature Immunology*, 2003. **4**: p. 380.
28. Hildner, K., et al., *Batf3 Deficiency Reveals a Critical Role for CD8⁺ Dendritic Cells in Cytotoxic T Cell Immunity*. *Science*, 2008. **322**(5904): p. 1097-1100.
29. Ginhoux, F., et al., *The origin and development of nonlymphoid tissue CD103⁺ DCs*. *The Journal of Experimental Medicine*, 2009. **206**(13): p. 3115-3130.
30. Wu, L., et al., *RelB Is Essential for the Development of Myeloid-Related CD8⁺ Dendritic Cells but Not of Lymphoid-Related CD8⁺ Dendritic Cells*. *Immunity*, 1998. **9**(6): p. 839-847.
31. Lewis, Kanako L., et al., *Notch2 Receptor Signaling Controls Functional Differentiation of Dendritic Cells in the Spleen and Intestine*. *Immunity*, 2011. **35**(5): p. 780-791.
32. Suzuki, S., et al., *Critical roles of interferon regulatory factor 4 in CD11b^{high}CD8⁺ dendritic cell development*. *Proceedings of the National Academy of Sciences*, 2004. **101**(24): p. 8981-8986.
33. Vander Lugt, B., et al., *Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation*. *Nature Immunology*, 2013. **15**: p. 161.
34. Gao, Y., et al., *Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells*. *Immunity*, 2013. **39**(4): p. 722-732.
35. Persson, Emma K., et al., *IRF4 Transcription-Factor-Dependent CD103⁺CD11b⁺ Dendritic Cells Drive Mucosal T Helper 17 Cell Differentiation*. *Immunity*, 2013. **38**(5): p. 958-969.
36. Mildner, A., S. Yona, and S. Jung, *Chapter Three - A Close Encounter of the Third Kind: Monocyte-Derived Cells*, in *Advances in Immunology*, K.M. Murphy and M. Merad, Editors. 2013, Academic Press. p. 69-103.
37. Serbina, N.V., et al., *TNF/ iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection*. *Immunity*, 2003. **19**(1): p. 59-70.
38. Davies, L.C., et al., *Tissue-resident macrophages*. *Nature Immunology*, 2013. **14**: p. 986.
39. Shi, C. and E.G. Pamer, *Monocyte recruitment during infection and inflammation*. *Nat Rev Immunol*, 2011. **11**(11): p. 762-774.
40. Yona, S. and S. Jung, *Monocytes: subsets, origins, fates and functions*. *Curr Opin Hematol*, 2010. **17**(1): p. 53-9.
41. den Haan, J.M.M. and G. Kraal, *Innate Immune Functions of Macrophage Subpopulations in the Spleen*. *Journal of Innate Immunity*, 2012. **4**(5-6): p. 437-445.
42. Kohyama, M., et al., *Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis*. *Nature*, 2008. **457**: p. 318.
43. Joshi, N., J.M. Walter, and A.V. Misharin, *Alveolar Macrophages*. *Cellular Immunology*, 2018.
44. Hashimoto, D., J. Miller, and M. Merad, *Dendritic Cell and Macrophage Heterogeneity In Vivo*. *Immunity*, 2011. **35**(3): p. 323-335.
45. Lavin, Y., et al., *Tissue-Resident Macrophage Enhancer Landscapes Are Shaped by the Local Microenvironment*. *Cell*, 2014. **159**(6): p. 1312-1326.
46. Lavin, Y., et al., *Regulation of macrophage development and function in peripheral tissues*. *Nature Reviews Immunology*, 2015. **15**: p. 731.
47. van Furth, R. and Z.A. Cohn, *THE ORIGIN AND KINETICS OF MONONUCLEAR PHAGOCYTES*. *The Journal of Experimental Medicine*, 1968. **128**(3): p. 415-435.
48. Czernielewski, J., P. Vaigot, and M. Prunieras, *Epidermal Langerhans cells--a cycling cell population*. *J Invest Dermatol*, 1985. **84**(5): p. 424-6.
49. Yamada, M., M. Naito, and K. Takahashi, *Kupffer cell proliferation and glucan-induced granuloma formation in mice depleted of blood monocytes by strontium-89*. *J Leukoc Biol*, 1990. **47**(3): p. 195-205.

50. Ginhoux, F. and S. Jung, *Monocytes and macrophages: developmental pathways and tissue homeostasis*. Nat Rev Immunol, 2014. **14**(6): p. 392-404.
51. Hoeffel, G., et al., *C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages*. Immunity, 2015. **42**(4): p. 665-78.
52. Gomez Perdiguero, E., et al., *Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors*. Nature, 2015. **518**(7540): p. 547-551.
53. Schulz, C., et al., *A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells*. Science, 2012. **336**(6077): p. 86-90.
54. Perdiguero, E.G. and F. Geissmann, *The development and maintenance of resident macrophages*. Nature Immunology, 2015. **17**: p. 2.
55. Varol, C., A. Mildner, and S. Jung, *Macrophages: Development and Tissue Specialization*. Annual Review of Immunology, 2015. **33**(1): p. 643-675.
56. Bain, C.C., et al., *Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice*. Nat Immunol, 2014. **15**(10): p. 929-937.
57. Tamoutounour, S., et al., *Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin*. Immunity, 2013. **39**(5): p. 925-38.
58. Ginhoux, F. and M. Williams, *Tissue-Resident Macrophage Ontogeny and Homeostasis*. Immunity, 2016. **44**(3): p. 439-449.
59. Epelman, S., et al., *Embryonic and Adult-Derived Resident Cardiac Macrophages Are Maintained through Distinct Mechanisms at Steady State and during Inflammation*. Immunity, 2014. **40**(1): p. 91-104.
60. Sheng, J., C. Ruedl, and K. Karjalainen, *Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells*. Immunity, 2015. **43**(2): p. 382-93.
61. Hoeffel, G. and F. Ginhoux, *Ontogeny of Tissue-Resident Macrophages*. Frontiers in Immunology, 2015. **6**(486).
62. Bertrand, J.Y., et al., *Three pathways to mature macrophages in the early mouse yolk sac*. Blood, 2005. **106**(9): p. 3004-11.
63. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages*. Science, 2010. **330**(6005): p. 841-5.
64. Verrou, C., et al., *Comparison of the Tamoxifen Regulated Chimeric Cre Recombinases MerCreMer and CreMer*, in *Biological Chemistry*. 1999. p. 1435.
65. Metzger, D., et al., *Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase*. Proceedings of the National Academy of Sciences, 1995. **92**(15): p. 6991-6995.
66. Davies, L.C., et al., *Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation*. Nature Communications, 2013. **4**: p. 1886.
67. Sieweke, M.H. and J.E. Allen, *Beyond Stem Cells: Self-Renewal of Differentiated Macrophages*. Science, 2013. **342**(6161).
68. Williams, M., et al., *Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF*. The Journal of Experimental Medicine, 2013. **210**(10): p. 1977-1992.
69. Hashimoto, D., et al., *Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes*. Immunity, 2013. **38**(4): p. 792-804.
70. Wang, Y., et al., *IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia*. Nature Immunology, 2012. **13**: p. 753.
71. Jenkins, S.J., et al., *Local Macrophage Proliferation, Rather than Recruitment from the Blood, Is a Signature of T_H2 Inflammation*. Science, 2011. **332**(6035): p. 1284-1288.
72. Blieriot, C., et al., *Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection*. Immunity, 2015. **42**(1): p. 145-58.
73. DeKoter, R.P. and H. Singh, *Regulation of B Lymphocyte and Macrophage Development by Graded Expression of PU.1*. Science, 2000. **288**(5470): p. 1439-1441.
74. Imperato, M.R., et al., *The RUNX1–PU.1 axis in the control of hematopoiesis*. International Journal of Hematology, 2015. **101**(4): p. 319-329.

75. T'Jonck, W., M. Guillems, and J. Bonnardel, *Niche signals and transcription factors involved in tissue-resident macrophage development*. Cellular Immunology, 2018.
76. Zhang, D.E., et al., *The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor*. Molecular and Cellular Biology, 1994. **14**(1): p. 373-381.
77. Guillems, M. and C.L. Scott, *Does niche competition determine the origin of tissue-resident macrophages?* Nat Rev Immunol, 2017. **17**(7): p. 451-460.
78. Mass, E., et al., *Specification of tissue-resident macrophages during organogenesis*. Science, 2016. **353**(6304).
79. Geissmann, F., et al., *Development of Monocytes, Macrophages, and Dendritic Cells*. Science, 2010. **327**(5966): p. 656-661.
80. Gautier, E.L., et al., *Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages*. Nature Immunology, 2012. **13**: p. 1118.
81. Gosselin, D., et al., *Environment Drives Selection and Function of Enhancers Controlling Tissue-Specific Macrophage Identities*. Cell, 2014. **159**(6): p. 1327-1340.
82. Haldar, M., et al., *Heme-Mediated SPI-C Induction Promotes Monocyte Differentiation into Iron-Recycling Macrophages*. Cell, 2014. **156**(6): p. 1223-1234.
83. A-Gonzalez, N. and A. Castrillo, *Origin and specialization of splenic macrophages*. Cellular Immunology, 2018.
84. A-Gonzalez, N., et al., *The nuclear receptor LXR α controls the functional specialization of splenic macrophages*. Nature Immunology, 2013. **14**: p. 831.
85. Miyake, Y., et al., *Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens*. J Clin Invest, 2007. **117**(8): p. 2268-78.
86. van de Laar, L., et al., *Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages*. Immunity, **44**(4): p. 755-768.
87. Schneider, C., et al., *Induction of the nuclear receptor PPAR- γ by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages*. Nature Immunology, 2014. **15**: p. 1026.
88. Soehnlein, O. and L. Lindbom, *Phagocyte partnership during the onset and resolution of inflammation*. Nature Reviews Immunology, 2010. **10**: p. 427.
89. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells*. Annual Review of Immunology, 2009. **27**(1): p. 669-692.
90. Swirski, F.K., et al., *Identification of splenic reservoir monocytes and their deployment to inflammatory sites*. Science, 2009. **325**(5940): p. 612-6.
91. Robbins, C.S. and F.K. Swirski, *The multiple roles of monocyte subsets in steady state and inflammation*. Cellular and Molecular Life Sciences, 2010. **67**(16): p. 2685-2693.
92. Yona, S., et al., *Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis*. Immunity, 2013. **38**(1): p. 79-91.
93. Tacke, F. and H.W. Zimmermann, *Macrophage heterogeneity in liver injury and fibrosis*. Journal of Hepatology, 2014. **60**(5): p. 1090-1096.
94. Cox, F.E.G., *History of the discovery of the malaria parasites and their vectors*. Parasites & Vectors, 2010. **3**: p. 5-5.
95. Nlinwe, O.N., et al., *T-cell responses against Malaria: Effect of parasite antigen diversity and relevance for vaccine development*. Vaccine, 2018. **36**(17): p. 2237-2242.
96. Winzeler, E.A., *Malaria research in the post-genomic era*. Nature, 2008. **455**(7214): p. 751-756.
97. Prudencio, M., M.M. Mota, and A.M. Mendes, *A toolbox to study liver stage malaria*. Trends Parasitol, 2011. **27**(12): p. 565-74.
98. Lamb, T.J., et al., *Insights into the immunopathogenesis of malaria using mouse models*. Expert Rev Mol Med, 2006. **8**(6): p. 1-22.
99. Renia, L., et al., *Vaccination against malaria with live parasites*. Expert Rev Vaccines, 2006. **5**(4): p. 473-81.

100. Mueller, I., et al., *Key gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite*. The Lancet Infectious Diseases. **9**(9): p. 555-566.
101. Graewe, S., et al., *Chronicle of a death foretold: Plasmodium liver stage parasites decide on the fate of the host cell*. FEMS Microbiology Reviews, 2011. **36**(1): p. 111-130.
102. Prudencio, M., A. Rodriguez, and M.M. Mota, *The silent path to thousands of merozoites: the Plasmodium liver stage*. Nat Rev Microbiol, 2006. **4**(11): p. 849-56.
103. Bertolino, P. and D.G. Bowen, *Malaria and the liver: immunological hide-and-seek or subversion of immunity from within?* Front Microbiol, 2015. **6**: p. 41.
104. Sturm, A., et al., *Manipulation of Host Hepatocytes by the Malaria Parasite for Delivery into Liver Sinusoids*. Science, 2006. **313**(5791): p. 1287-1290.
105. Baer, K., et al., *Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature*. PLoS Pathog, 2007. **3**(11): p. e171.
106. Soulard, V., et al., *Plasmodium falciparum full life cycle and Plasmodium ovale liver stages in humanized mice*. Nature Communications, 2015. **6**: p. 7690.
107. Stevenson, M.M. and E.M. Riley, *Innate immunity to malaria*. Nat Rev Immunol, 2004. **4**(3): p. 169-80.
108. Cowman, A.F., D. Berry, and J. Baum, *The cellular and molecular basis for malaria parasite invasion of the human red blood cell*. The Journal of Cell Biology, 2012. **198**(6): p. 961-971.
109. Langhorne, J., et al., *The relevance of non-human primate and rodent malaria models for humans*. Malaria Journal, 2011. **10**: p. 23-23.
110. Craig, A.G., et al., *The role of animal models for research on severe malaria*. PLoS Pathog, 2012. **8**(2): p. e1002401.
111. Hansen, D.S., *Inflammatory Responses Associated with the Induction of Cerebral Malaria: Lessons from Experimental Murine Models*. PLoS Pathogens, 2012. **8**(12): p. e1003045.
112. Ryg-Cornejo, V., et al., *Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation*. Cell Reports, 2016. **14**(1): p. 68-81.
113. Stephens, R., R.L. Culleton, and T.J. Lamb, *The contribution of Plasmodium chabaudi to our understanding of malaria*. Trends in parasitology, 2012. **28**(2): p. 73-82.
114. Su, Z., et al., *Opsonin-Independent Phagocytosis: An Effector Mechanism against Acute Blood-Stage Plasmodium chabaudi AS Infection*. Journal of Infectious Diseases, 2002. **186**(9): p. 1321-1329.
115. Opata, M.M., et al., *Early effector cells survive the contraction phase in malaria infection and generate both central and effector memory T cells*. J Immunol, 2015. **194**(11): p. 5346-54.
116. Liehl, P., et al., *Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection*. Nat Med, 2014. **20**(1): p. 47-53.
117. Fernandez-Ruiz, D., et al., *Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection*. Immunity, 2016. **45**(4): p. 889-902.
118. Keitany, G.J., et al., *Immunization of mice with live-attenuated late liver stage-arresting Plasmodium yoelii parasites generates protective antibody responses to preerythrocytic stages of malaria*. Infect Immun, 2014. **82**(12): p. 5143-53.
119. Sack, B.K., et al., *Mechanisms of stage-transcending protection following immunization of mice with late liver stage-arresting genetically attenuated malaria parasites*. PLoS Pathog, 2015. **11**(5): p. e1004855.
120. Minkah, N.K., C. Schafer, and S.H.I. Kappe, *Humanized Mouse Models for the Study of Human Malaria Parasite Biology, Pathogenesis, and Immunity*. Front Immunol, 2018. **9**: p. 807.
121. Joyner, C., J.W. Barnwell, and M.R. Galinski, *No more monkeying around: primate malaria model systems are key to understanding Plasmodium vivax liver-stage biology, hypnozoites, and relapses*. Frontiers in Microbiology, 2015. **6**: p. 145.
122. Moreno, A., et al., *Plasmodium coatneyi in rhesus macaques replicates the multisystemic dysfunction of severe malaria in humans*. Infect Immun, 2013. **81**(6): p. 1889-904.

123. Beignon, A.-S., R. Le Grand, and C. Chapon, *In vivo imaging in NHP models of malaria: Challenges, progress and outlooks*. Parasitology International, 2014. **63**(1): p. 206-215.
124. Herrera, S., et al., *Aotus monkeys: their great value for anti-malaria vaccines and drug testing*. International Journal for Parasitology, 2002. **32**(13): p. 1625-1635.
125. Koehler, J.W., et al., *Altered Immune Responses in Rhesus Macaques Co-Infected with SIV and Plasmodium cynomolgi: An Animal Model for Coincident AIDS and Relapsing Malaria*. PLOS ONE, 2009. **4**(9): p. e7139.
126. Yan, G., et al., *Genome sequencing and comparison of two nonhuman primate animal models, the cynomolgus and Chinese rhesus macaques*. Nature Biotechnology, 2011. **29**: p. 1019.
127. Wiseman, R.W., et al., *Major histocompatibility complex genotyping with massively parallel pyrosequencing*. Nature Medicine, 2009. **15**: p. 1322.
128. Blokhuis, J.H., et al., *The mosaic of KIR haplotypes in rhesus macaques*. Immunogenetics, 2010. **62**(5): p. 295-306.
129. Sack, B.K., et al., *Model for in vivo assessment of humoral protection against malaria sporozoite challenge by passive transfer of monoclonal antibodies and immune serum*. Infect Immun, 2014. **82**(2): p. 808-17.
130. Huang, J., et al., *An AAV vector-mediated gene delivery approach facilitates reconstitution of functional human CD8⁺ T cells in mice*. PLoS One, 2014. **9**(2): p. e88205.
131. Huang, J., et al., *Human immune system mice immunized with Plasmodium falciparum circumsporozoite protein induce protective human humoral immunity against malaria*. J Immunol Methods, 2015. **427**: p. 42-50.
132. Mikolajczak, S.A., et al., *Plasmodium vivax liver stage development and hypnozoite persistence in human liver-chimeric mice*. Cell Host Microbe, 2015. **17**(4): p. 526-35.
133. Rongvaux, A., et al., *Development and function of human innate immune cells in a humanized mouse model*. Nat Biotechnol, 2014. **32**(4): p. 364-72.
134. Duffier, Y., et al., *A humanized mouse model for sequestration of Plasmodium falciparum sexual stages and in vivo evaluation of gametocytidal drugs*. Sci Rep, 2016. **6**: p. 35025.
135. Powell, R.D. and J.V. McNamara, *INFECTION WITH CHLOROQUINE-RESISTANT PLASMODIUM FALCIPARUM IN MAN: PREPATENT PERIODS, INCUBATION PERIODS, AND RELATIONSHIPS BETWEEN PARASITEMIA AND THE ONSET OF FEVER IN NONIMMUNE PERSONS**. Annals of the New York Academy of Sciences, 1970. **174**(2): p. 1027-1041.
136. Sauerwein, R.W., M. Roestenberg, and V.S. Moorthy, *Experimental human challenge infections can accelerate clinical malaria vaccine development*. Nature Reviews Immunology, 2010. **11**: p. 57.
137. Engwerda, C.R., et al., *Experimentally induced blood stage malaria infection as a tool for clinical research*. Trends in Parasitology, 2012. **28**(11): p. 515-521.
138. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination*. Nature Medicine, 2016. **22**: p. 614.
139. Bijker, E.M., et al., *Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity*. Proc Natl Acad Sci U S A, 2013. **110**(19): p. 7862-7.
140. Dups, J.N., M. Pepper, and I.A. Cockburn, *Antibody and B cell responses to Plasmodium sporozoites*. Frontiers in Microbiology, 2014. **5**(625).
141. Offeddu, V., et al., *Naturally acquired immune responses against Plasmodium falciparum sporozoites and liver infection*. International Journal for Parasitology, 2012. **42**(6): p. 535-548.
142. Bijker, E.M., et al., *Cytotoxic markers associate with protection against malaria in human volunteers immunized with Plasmodium falciparum sporozoites*. J Infect Dis, 2014. **210**(10): p. 1605-15.
143. Mordmüller, B., et al., *Sterile protection against human malaria by chemoattenuated PfSPZ vaccine*. Nature, 2017. **542**: p. 445.
144. Hoffman, S.L., et al., *Protection of Humans against Malaria by Immunization with Radiation-Attenuated Plasmodium falciparum Sporozoites*. The Journal of Infectious Diseases, 2002. **185**(8): p. 1155-1164.
145. Kisalu, N.K., et al., *A human monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on the parasite*. Nature Medicine, 2018. **24**: p. 408.

146. Matuschewski, K., *Vaccines against malaria—still a long way to go*. The FEBS Journal, 2017. **284**(16): p. 2560-2568.
147. Polhemus, M.E., et al., *Evaluation of RTS,S/AS02A and RTS,S/AS01B in Adults in a High Malaria Transmission Area*. PLOS ONE, 2009. **4**(7): p. e6465.
148. Olotu, A., et al., *Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children*. New England Journal of Medicine, 2016. **374**(26): p. 2519-2529.
149. *Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial*. Lancet, 2015. **386**(9988): p. 31-45.
150. Sissoko, M.S., et al., *Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial*. Lancet Infect Dis, 2017. **17**(5): p. 498-509.
151. Miller, L.H., et al., *The pathogenic basis of malaria*. Nature, 2002. **415**(6872): p. 673-9.
152. Hora, R., et al., *Cerebral malaria - clinical manifestations and pathogenesis*. Metab Brain Dis, 2016.
153. Deroost, K., et al., *Hemozoin induces hepatic inflammation in mice and is differentially associated with liver pathology depending on the Plasmodium strain*. PLoS One, 2014. **9**(11): p. e113519.
154. Epiphonio, S., et al., *VEGF promotes malaria-associated acute lung injury in mice*. PLoS Pathog, 2010. **6**(5): p. e1000916.
155. Morse, H.C., 3rd, et al., *Cells of the marginal zone--origins, function and neoplasia*. Leuk Res, 2001. **25**(2): p. 169-78.
156. Mebius, R.E. and G. Kraal, *Structure and function of the spleen*. Nat Rev Immunol, 2005. **5**(8): p. 606-16.
157. Saadia, R., *The Complete Spleen: Structure, Function, and Clinical Disorders*. 2nd ed. Canadian Journal of Surgery, 2002. **45**(3): p. 226-226.
158. Kraal, G., *Cells in the marginal zone of the spleen*. Int Rev Cytol, 1992. **132**: p. 31-74.
159. Groom, A.C., E.E. Schmidt, and I.C. MacDonald, *Microcirculatory pathways and blood flow in spleen: new insights from washout kinetics, corrosion casts, and quantitative intravital videomicroscopy*. Scanning Microsc, 1991. **5**(1): p. 159-73; discussion 173-4.
160. Weiss, L., U. Geduldig, and W. Weidanz, *Mechanisms of splenic control of murine malaria: Reticular cell activation and the development of a blood-spleen barrier*. American Journal of Anatomy, 1986. **176**(3): p. 251-285.
161. Borges da Silva, H., et al., *Splenic Macrophage Subsets and Their Function during Blood-Borne Infections*. Front Immunol, 2015. **6**: p. 480.
162. Chow, A., et al., *CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress*. Nat Med, 2013. **19**(4): p. 429-36.
163. Hey, Y.Y. and H.C. O'Neill, *Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function*. J Cell Mol Med, 2012. **16**(11): p. 2611-9.
164. Bannard, O. and J.G. Cyster, *Germinal centers: programmed for affinity maturation and antibody diversification*. Current Opinion in Immunology, 2017. **45**: p. 21-30.
165. Qiu, C.H., et al., *Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens*. J Immunol, 2009. **182**(7): p. 4127-36.
166. Kang, Y.S., et al., *SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran*. Int Immunol, 2003. **15**(2): p. 177-86.
167. Oliver, A.M., et al., *Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses*. European Journal of Immunology, 1997. **27**(9): p. 2366-2374.
168. Martin, F. and J.F. Kearney, *Marginal-zone B cells*. Nature Reviews Immunology, 2002. **2**: p. 323.
169. Engwerda, C.R., L. Beattie, and F.H. Amante, *The importance of the spleen in malaria*. Trends in Parasitology, 2005. **21**(2): p. 75-80.

170. Balogh, P. and Á. Lábadi, *Structural Evolution of the Spleen in Man and Mouse*, in *Developmental Biology of Peripheral Lymphoid Organs*, P. Balogh, Editor. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 121-141.
171. Huang, X., et al., *Differential Spleen Remodeling Associated with Different Levels of Parasite Virulence Controls Disease Outcome in Malaria Parasite Infections*. mSphere, 2016. **1**(1).
172. Del Portillo, H.A., et al., *The role of the spleen in malaria*. Cell Microbiol, 2012. **14**(3): p. 343-55.
173. Safeukui, I., et al., *Retention of Plasmodium falciparum ring-infected erythrocytes in the slow, open microcirculation of the human spleen*. Blood, 2008. **112**(6): p. 2520-8.
174. Achtman, A.H., et al., *Plasmodium chabaudi chabaudi infection in mice induces strong B cell responses and striking but temporary changes in splenic cell distribution*. J Immunol, 2003. **171**(1): p. 317-24.
175. Krücken, J., et al., *Massive Destruction of Malaria-Parasitized Red Blood Cells despite Spleen Closure*. Infection and Immunity, 2005. **73**(10): p. 6390-6398.
176. Beattie, L., et al., *CD8+ T Lymphocyte-Mediated Loss of Marginal Metallophilic Macrophages following Infection with Plasmodium chabaudi chabaudi AS*. The Journal of Immunology, 2006. **177**(4): p. 2518-2526.
177. Cadman, E.T., et al., *Alterations of Splenic Architecture in Malaria Are Induced Independently of Toll-Like Receptors 2, 4, and 9 or MyD88 and May Affect Antibody Affinity*. Infection and Immunity, 2008. **76**(9): p. 3924-3931.
178. Wyler, D.J., T.C. Quinn, and L.T. Chen, *Relationship of alterations in splenic clearance function and microcirculation to host defense in acute rodent malaria*. J Clin Invest, 1981. **67**(5): p. 1400-4.
179. Martin-Jaular, L., et al., *Strain-specific spleen remodelling in Plasmodium yoelii infections in Balb/c mice facilitates adherence and spleen macrophage-clearance escape*. Cellular Microbiology, 2010. **13**(1): p. 109-122.
180. Buffet, P.A., et al., *The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology*. Blood, 2011. **117**(2): p. 381-92.
181. Schnitzer, B., et al., *Pitting function of the spleen in malaria: ultrastructural observations*. Science, 1972. **177**(4044): p. 175-7.
182. Yadava, A., et al., *Trafficking of Plasmodium chabaudi adami-infected erythrocytes within the mouse spleen*. Proceedings of the National Academy of Sciences, 1996. **93**(10): p. 4595-4599.
183. Aichele, P., et al., *Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses*. J Immunol, 2003. **171**(3): p. 1148-55.
184. Seiler, P., et al., *Crucial role of marginal zone macrophages and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection*. Eur J Immunol, 1997. **27**(10): p. 2626-33.
185. Bernhardt, C.A., et al., *CD169+ macrophages are sufficient for priming of CTLs with specificities left out by cross-priming dendritic cells*. Proc Natl Acad Sci U S A, 2015. **112**(17): p. 5461-6.
186. Joffre, O.P., et al., *Cross-presentation by dendritic cells*. Nat Rev Immunol, 2012. **12**(8): p. 557-569.
187. Wykes, M., et al., *Dendritic cell biology during malaria*. Cellular Microbiology, 2007. **9**(2): p. 300-305.
188. Cockburn, I.A. and F. Zavala, *Dendritic cell function and antigen presentation in malaria*. Current Opinion in Immunology, 2016. **40**: p. 1-6.
189. Mota, M.M., et al., *Acute Plasmodium chabaudi chabaudi Malaria Infection Induces Antibodies Which Bind to the Surfaces of Parasitized Erythrocytes and Promote Their Phagocytosis by Macrophages In Vitro*. Infection and Immunity, 1998. **66**(9): p. 4080-4086.
190. Ndungu, F.M., et al., *Functional memory B cells and long-lived plasma cells are generated after a single Plasmodium chabaudi infection in mice*. PLoS Pathog, 2009. **5**(12): p. e1000690.
191. Su, Z. and M.M. Stevenson, *IL-12 Is Required for Antibody-Mediated Protective Immunity Against Blood-Stage Plasmodium chabaudi AS Malaria Infection in Mice*. The Journal of Immunology, 2002. **168**(3): p. 1348-1355.
192. Oster, C.N., L.C. Koontz, and D.J. Wyler, *Malaria in asplenic mice: effects of splenectomy, congenital asplenia, and splenic reconstitution on the course of infection*. Am J Trop Med Hyg, 1980. **29**(6): p. 1138-42.

193. Bach, O., et al., *Falciparum malaria after splenectomy: a prospective controlled study of 33 previously splenectomized Malawian adults*. Trans R Soc Trop Med Hyg, 2005. **99**(11): p. 861-7.
194. Yap, G.S. and M.M. Stevenson, *Differential requirements for an intact spleen in induction and expression of B-cell-dependent immunity to Plasmodium chabaudi* AS. Infect Immun, 1994. **62**(10): p. 4219-25.
195. Belnoue, E., et al., *Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment*. J Immunol, 2004. **172**(4): p. 2487-95.
196. Miller, Jessica L., et al., *Interferon-Mediated Innate Immune Responses against Malaria Parasite Liver Stages*. Cell Reports, 2014. **7**(2): p. 436-447.
197. Mac-Daniel, L. and R. Menard, *Plasmodium and mononuclear phagocytes*. Microb Pathog, 2015. **78**: p. 43-51.
198. Terzakis, J.A., et al., *Exoerythrocytic Merozoites of Plasmodium berghei in Rat Hepatic Kupffer Cells*. The Journal of Protozoology, 1979. **26**(3): p. 385-389.
199. Spencer, A.J., et al., *The Threshold of Protection from Liver-Stage Malaria Relies on a Fine Balance between the Number of Infected Hepatocytes and Effector CD8⁺ T Cells Present in the Liver*. The Journal of Immunology, 2017. **198**(5): p. 2006-2016.
200. Chakravarty, S., et al., *CD8⁺ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes*. Nat Med, 2007. **13**(9): p. 1035-41.
201. Radtke, A.J., et al., *Lymph-node resident CD8α⁺ dendritic cells capture antigens from migratory malaria sporozoites and induce CD8⁺ T cell responses*. PLoS pathogens, 2015. **11**(2): p. e1004637-e1004637.
202. Obeid, M., et al., *Skin-draining lymph node priming is sufficient to induce sterile immunity against pre-erythrocytic malaria*. EMBO molecular medicine, 2013. **5**(2): p. 250-263.
203. Su, Z. and M.M. Stevenson, *Central Role of Endogenous Gamma Interferon in Protective Immunity against Blood-Stage Plasmodium chabaudi* AS Infection. Infection and Immunity, 2000. **68**(8): p. 4399-4406.
204. Meding, S.J., et al., *Role of gamma interferon during infection with Plasmodium chabaudi chabaudi*. Infection and Immunity, 1990. **58**(11): p. 3671-3678.
205. King, T. and T. Lamb, *Interferon-γ: The Jekyll and Hyde of Malaria*. PLoS Pathogens, 2015. **11**(10): p. e1005118.
206. Haque, A., et al., *Type I IFN signaling in CD8[−] DCs impairs Th1-dependent malaria immunity*. The Journal of Clinical Investigation, 2014. **124**(6): p. 2483-2496.
207. Haque, A., et al., *Type I interferons suppress CD4⁺ T-cell-dependent parasite control during blood-stage Plasmodium infection*. European Journal of Immunology, 2011. **41**(9): p. 2688-2698.
208. Montes de Oca, M., et al., *Type I Interferons Regulate Immune Responses in Humans with Blood-Stage Plasmodium falciparum Infection*. Cell Reports, 2016. **17**(2): p. 399-412.
209. Seixas, E., et al., *Direct activation of dendritic cells by the malaria parasite, Plasmodium chabaudi chabaudi*. Eur J Immunol, 2001. **31**.
210. Langhorne, J., et al., *Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection*. Immunological Reviews, 2004. **201**(1): p. 35-47.
211. Borges da Silva, H., et al., *In Vivo Approaches Reveal a Key Role for DCs in CD4⁺ T Cell Activation and Parasite Clearance during the Acute Phase of Experimental Blood-Stage Malaria*. PLOS Pathogens, 2015. **11**(2): p. e1004598.
212. Amorim, K.N.S., et al., *Dendritic Cells and Their Multiple Roles during Malaria Infection*. Journal of Immunology Research, 2016. **2016**: p. 11.
213. Ryg-Cornejo, V., et al., *NK cells and conventional dendritic cells engage in reciprocal activation for the induction of inflammatory responses during Plasmodium berghei ANKA infection*. Immunobiology, 2013. **218**(2): p. 263-271.
214. Ing, R. and M.M. Stevenson, *Dendritic Cell and NK Cell Reciprocal Cross Talk Promotes Gamma Interferon-Dependent Immunity to Blood-Stage Plasmodium chabaudi AS Infection in Mice*. Infection and Immunity, 2009. **77**(2): p. 770-782.
215. Inoue, S.-I., et al., *Roles of IFN-γ and γδ T Cells in Protective Immunity Against Blood-Stage Malaria*. Frontiers in Immunology, 2013. **4**: p. 258.

216. Weidanz, W.P., et al., *$\gamma\delta$ T Cells but Not NK Cells Are Essential for Cell-Mediated Immunity against Plasmodium chabaudi Malaria*. Infection and Immunity, 2010. **78**(10): p. 4331-4340.
217. Sam, H. and M.M. Stevenson, *Early IL-12 p70, but not p40, production by splenic macrophages correlates with host resistance to blood-stage Plasmodium chabaudi AS malaria*. Clinical and Experimental Immunology, 1999. **117**(2): p. 343-349.
218. McCall, M.B.B. and R.W. Sauerwein, *Interferon- γ —central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria*. Journal of Leukocyte Biology, 2010. **88**(6): p. 1131-1143.
219. Belyaev, N.N., et al., *Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria*. Nat Immunol, 2010. **11**(6): p. 477-85.
220. Lundie, R.J., et al., *Blood-stage Plasmodium infection induces CD8(+) T lymphocytes to parasite-expressed antigens, largely regulated by CD8 α (+) dendritic cells*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(38): p. 14509-14514.
221. Wong, K.A. and A. Rodriguez, *Plasmodium infection and endotoxin shock induce the expansion of regulatory dendritic cells*. Journal of immunology (Baltimore, Md. : 1950), 2008. **180**(2): p. 716-726.
222. Voisine, C., et al., *Classical CD11c+ dendritic cells, not plasmacytoid dendritic cells, induce T cell responses to Plasmodium chabaudi malaria*. International Journal for Parasitology, 2010. **40**(6): p. 711-719.
223. Carpio, V.H., et al., *IFN- γ and IL-21 Double Producing T Cells Are Bcl6-Independent and Survive into the Memory Phase in Plasmodium chabaudi Infection*. PLoS ONE, 2015. **10**(12): p. e0144654.
224. Stephens, R. and J. Langhorne, *Effector memory Th1 CD4 T cells are maintained in a mouse model of chronic malaria*. PLoS Pathog, 2010. **6**(11): p. e1001208.
225. Perez-Mazliah, D. and J. Langhorne, *CD4 T-cell subsets in malaria: TH1/TH2 revisited*. Front Immunol, 2014. **5**: p. 671.
226. Süss, G., et al., *Roles of CD4- and CD8-bearing T lymphocytes in the immune response to the erythrocytic stages of Plasmodium chabaudi*. Infection and Immunity, 1988. **56**(12): p. 3081-3088.
227. Langhorne, J., B. Simon-Haarhaus, and S.J. Meding, *The role of CD4+ T cells in the protective immune response to Plasmodium chabaudi in vivo*. Immunol Lett, 1990. **25**(1-3): p. 101-7.
228. Nduati, E.W., et al., *Distinct Kinetics of Memory B-Cell and Plasma-Cell Responses in Peripheral Blood Following a Blood-Stage Plasmodium chabaudi Infection in Mice*. PLoS ONE, 2010. **5**(11): p. e15007.
229. Perez-Mazliah, D., et al., *Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria*. PLoS Pathog, 2015. **11**(3): p. e1004715.
230. Spence, P.J. and J. Langhorne, *T cell control of malaria pathogenesis*. Current Opinion in Immunology, 2012. **24**(4): p. 444-448.
231. Lundie, R.J., et al., *Blood-stage Plasmodium infection induces CD8+ T lymphocytes to parasite-expressed antigens, largely regulated by CD8 α (+) dendritic cells*. Proc Natl Acad Sci U S A, 2008. **105**(38): p. 14509-14.
232. Podoba, J.E. and M.M. Stevenson, *CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage Plasmodium chabaudi AS*. Infection and Immunity, 1991. **59**(1): p. 51-58.
233. Howland, S.W., et al., *Brain microvessel cross-presentation is a hallmark of experimental cerebral malaria*. EMBO Mol Med, 2013. **5**(7): p. 916-31.
234. Sponaas, A.M., et al., *Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells*. J Exp Med, 2006. **203**(6): p. 1427-33.
235. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties*. Annual Review of Immunology, 1989. **7**(1): p. 145-173.
236. Langhorne, J., et al., *Frequencies of CD4+ T cells reactive with Plasmodium chabaudi chabaudi: distinct response kinetics for cells with Th1 and Th2 characteristics during infection*. International Immunology, 1989. **1**(4): p. 416-424.

237. Meding, S.J. and J. Langhorne, *CD4⁺ T cells and B cells are necessary for the transfer of protective immunity to Plasmodium chabaudi chabaudi*. Eur J Immunol, 1991. **21**(6): p. 1433-8.
238. Stevenson, M.M. and M.F. Tam, *Differential induction of helper T cell subsets during blood-stage Plasmodium chabaudi AS infection in resistant and susceptible mice*. Clinical & Experimental Immunology, 1993. **92**(1): p. 77-83.
239. von Der Weld, T. and J. Langhorne, *The Roles of Cytokines Produced in the Immune Response to the Erythrocytic Stages of Mouse Malaria*. Immunobiology, 1993. **189**(3): p. 397-418.
240. Perez-Mazliah, D. and J. Langhorne, *CD4 T-Cell Subsets in Malaria: TH1/TH2 Revisited*. Frontiers in Immunology, 2014. **5**: p. 671.
241. Pérez-Mazliah, D., et al., *Follicular Helper T Cells are Essential for the Elimination of Plasmodium Infection*. EBioMedicine, 2017. **24**: p. 216-230.
242. Reinhardt, R.L., H.-E. Liang, and R.M. Locksley, *Cytokine-secreting follicular T cells shape the antibody repertoire*. Nature Immunology, 2009. **10**: p. 385.
243. Ramiscal, R.R. and C.G. Vinuesa, *T-cell subsets in the germinal center*. Immunol Rev, 2013. **252**(1): p. 146-55.
244. Dufaud, C.R., L.J. McHeyzer-Williams, and M.G. McHeyzer-Williams, *Deconstructing the germinal center, one cell at a time*. Curr Opin Immunol, 2017. **45**: p. 112-118.
245. Cannons, J.L., K.T. Lu, and P.L. Schwartzberg, *T follicular helper cell diversity and plasticity*. Trends in Immunology, 2013. **34**(5): p. 200-207.
246. Soon, M.S.F. and A. Haque, *Recent Insights into CD4⁺ Th Cell Differentiation in Malaria*. The Journal of Immunology, 2018. **200**(6): p. 1965-1975.
247. Sponaas, A.-M., et al., *Migrating monocytes recruited to the spleen play an important role in control of blood stage malaria*. Blood, 2009. **114**(27): p. 5522-5531.
248. Belyaev, N.N., et al., *Extramedullary Myelopoiesis in Malaria Depends on Mobilization of Myeloid-Restricted Progenitors by IFN- γ Induced Chemokines*. PLOS Pathogens, 2013. **9**(6): p. e1003406.
249. Chua, C.L.L., et al., *Monocytes and macrophages in malaria: protection or pathology?* Trends in Parasitology, 2013. **29**(1): p. 26-34.
250. Langhorne, J., et al., *Immunity to malaria: more questions than answers*. Nat Immunol, 2008. **9**(7): p. 725-732.
251. Torre, D., *Early Production of γ -Interferon in Clinical Malaria: Role of Interleukin-18 and Interleukin-12*. Clinical Infectious Diseases, 2009. **48**(10): p. 1481-1482.
252. Jafarshad, A., et al., *A Novel Antibody-Dependent Cellular Cytotoxicity Mechanism Involved in Defense against Malaria Requires Costimulation of Monocytes Fc γ RII and Fc γ RIII*. The Journal of Immunology, 2007. **178**(5): p. 3099-3106.
253. Gupta, P., et al., *Tissue-Resident CD169⁺ Macrophages Form a Crucial Front Line against Plasmodium Infection*. Cell Reports. **16**(6): p. 1749-1761.
254. Riley, E.M. and V.A. Stewart, *Immune mechanisms in malaria: new insights in vaccine development*. Nat Med, 2013. **19**(2): p. 168-78.
255. Stephens, R., F.M. Ndungu, and J. Langhorne, *Germinal centre and marginal zone B cells expand quickly in a second Plasmodium chabaudi malaria infection producing mature plasma cells*. Parasite Immunol, 2009. **31**(1): p. 20-31.
256. Rénia, L., et al., *Cerebral malaria: Mysteries at the blood-brain barrier*. Virulence, 2012. **3**(2): p. 193-201.
257. Piva, L., et al., *Cutting edge: Clec9A⁺ dendritic cells mediate the development of experimental cerebral malaria*. J Immunol, 2012. **189**(3): p. 1128-32.
258. Amante, F.H., et al., *Immune-Mediated Mechanisms of Parasite Tissue Sequestration during Experimental Cerebral Malaria*. The Journal of Immunology, 2010. **185**(6): p. 3632.
259. Sanni, L.A., et al., *Cerebral Edema and Cerebral Hemorrhages in Interleukin-10-Deficient Mice Infected with Plasmodium chabaudi*. Infection and Immunity, 2004. **72**(5): p. 3054-3058.
260. Li, C., et al., *Pathology of Plasmodium chabaudi chabaudi Infection and Mortality in Interleukin-10-Deficient Mice Are Ameliorated by Anti-Tumor Necrosis Factor Alpha and Exacerbated by Anti-Transforming Growth Factor β Antibodies*. Infection and Immunity, 2003. **71**(9): p. 4850-4856.

261. Li, C., I. Corraliza, and J. Langhorne, *A Defect in Interleukin-10 Leads to Enhanced Malarial Disease in Plasmodium chabaudi chabaudi Infection in Mice*. Infection and Immunity, 1999. **67**(9): p. 4435-4442.
262. Linke, A., et al., *Plasmodium chabaudi chabaudi: Differential Susceptibility of Gene-Targeted Mice Deficient in IL-10 to an Erythrocytic-Stage Infection*. Experimental Parasitology, 1996. **84**(2): p. 253-263.
263. Bennett, C.L. and B.E. Clausen, *DC ablation in mice: promises, pitfalls, and challenges*. Trends Immunol, 2007. **28**(12): p. 525-31.
264. Van Rooijen, N., *The liposome-mediated macrophage 'suicide' technique*. J Immunol Methods, 1989. **124**(1): p. 1-6.
265. Burnett, S.H., et al., *Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene*. J Leukoc Biol, 2004. **75**(4): p. 612-23.
266. Sauer, B. and N. Henderson, *Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase*. New Biol, 1990. **2**(5): p. 441-9.
267. Saito, M., et al., *Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice*. Nat Biotechnol, 2001. **19**(8): p. 746-50.
268. Collier, R.J., *Diphtheria toxin: mode of action and structure*. Bacteriol Rev, 1975. **39**(1): p. 54-85.
269. Gill, D.M. and L.L. Dinius, *Observations on the Structure of Diphtheria Toxin*. Journal of Biological Chemistry, 1971. **246**(5): p. 1485-1491.
270. Tittel, A.P., et al., *Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice*. Nature Methods, 2012. **9**: p. 385.
271. Buch, T., et al., *A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration*. Nature Methods, 2005. **2**: p. 419.
272. van Blijswijk, J., B.U. Schraml, and C.R.e. Sousa, *Advantages and limitations of mouse models to deplete dendritic cells*. European Journal of Immunology, 2013. **43**(1): p. 22-26.
273. Heath, W.R. and F.R. Carbone, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces*. Nat Immunol, 2009. **10**(12): p. 1237-1244.
274. Schlitzer, A. and F. Ginhoux, *Organization of the mouse and human DC network*. Current Opinion in Immunology, 2014. **26**: p. 90-99.
275. Haniffa, M., M. Collin, and F. Ginhoux, *Ontogeny and Functional Specialization of Dendritic Cells in Human and Mouse*. Advances in Immunology, 2013. **120**: p. 1-49.
276. Merad, M., et al., *The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting*. Annual Review of Immunology, 2013. **31**(1): p. 563-604.
277. Banchereau, J., et al., *Immunobiology of dendritic cells*. Annu Rev Immunol, 2000. **18**.
278. Moser, M. and K.M. Murphy, *Dendritic cell regulation of TH1-TH2 development*. Nat Immunol, 2000. **1**(3): p. 199-205.
279. Schlitzer, A. and F. Ginhoux, *DNGR-ing the dendritic cell lineage*. EMBO Reports, 2013. **14**(10): p. 850-851.
280. Purnama, C., et al., *Transient ablation of alveolar macrophages leads to massive pathology of influenza infection without affecting cellular adaptive immunity*. Eur J Immunol, 2014. **44**(7): p. 2003-12.
281. Malleret, B., et al., *A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development*. Sci. Rep., 2011. **1**.
282. Picelli, S., et al., *Full-length RNA-seq from single cells using Smart-seq2*. Nat Protoc, 2014. **9**(1): p. 171-81.
283. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner*. Bioinformatics, 2013. **29**(1): p. 15-21.
284. Liao, Y., G.K. Smyth, and W. Shi, *featureCounts: an efficient general purpose program for assigning sequence reads to genomic features*. Bioinformatics, 2014. **30**(7): p. 923-30.
285. Harrow, J., et al., *GENCODE: producing a reference annotation for ENCODE*. Genome Biol, 2006. **7 Suppl 1**: p. S4.1-9.
286. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing*. Journal of the Royal Statistical Society. Series B (Methodological), 1995. **57**(1): p. 289-300.
287. Bianchi, R., et al., *Autocrine IL-12 Is Involved in Dendritic Cell Modulation Via CD40 Ligation*. The Journal of Immunology, 1999. **163**(5): p. 2517-2521.

288. Wilson, D.C., S. Matthews, and G.S. Yap, *IL-12 Signaling Drives CD8⁺ T Cell IFN- γ Production and Differentiation of KLRG1⁺ Effector Subpopulations during *Toxoplasma gondii* Infection*. The Journal of Immunology, 2008. **180**(9): p. 5935-5945.
289. Su, Z. and M.M. Stevenson, *IL-12 is required for antibody-mediated protective immunity against blood-stage Plasmodium chabaudi AS malaria infection in mice*. J Immunol, 2002. **168**(3): p. 1348-55.
290. Guillemins, M., et al., *Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny*. Nat Rev Immunol, 2014. **14**(8): p. 571-578.
291. Guernonprez, P., et al., *Antigen Presentation and T Cell Stimulation by Dendritic Cells*. Annual Review of Immunology, 2002. **20**(1): p. 621-667.
292. Lindell, D.M., et al., *Distinct Compartmentalization of CD4(+) T-Cell Effector Function Versus Proliferative Capacity during Pulmonary Cryptococcosis*. The American Journal of Pathology, 2006. **168**(3): p. 847-855.
293. Ernst, D.N., et al., *The age-associated increase in IFN-gamma synthesis by mouse CD8⁺ T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression*. The Journal of Immunology, 1993. **151**(2): p. 575-587.
294. Ziegler, S.F., F. Ramsdell, and M.R. Alderson, *The activation antigen CD69*. Stem Cells, 1994. **12**(5): p. 456-65.
295. Reiley, W.W., et al., *Distinct functions of antigen-specific CD4 T cells during murine Mycobacterium tuberculosis infection*. Proceedings of the National Academy of Sciences, 2010. **107**(45): p. 19408-19413.
296. Joshi, N.S., et al., *Inflammation Directs Memory Precursor and Short-Lived Effector CD8⁺ T Cell Fates via the Graded Expression of T-bet Transcription Factor*. Immunity. **27**(2): p. 281-295.
297. Li, C. and J. Langhorne, *Tumor necrosis factor alpha p55 receptor is important for development of memory responses to blood-stage malaria infection*. Infect Immun, 2000. **68**(10): p. 5724-30.
298. von der Weid, T., et al., *The immune response to Plasmodium chabaudi malaria in interleukin-4-deficient mice*. Eur J Immunol, 1994. **24**(10): p. 2285-93.
299. Jankovic, D., D.G. Kugler, and A. Sher, *IL-10 production by CD4(+) effector T cells: a mechanism for self-regulation*. Mucosal immunology, 2010. **3**(3): p. 239-246.
300. Freitas do Rosário, A.P., et al., *IL-27 promotes IL-10 production by effector Th1 CD4(+) T cells: a critical mechanism for protection from severe immunopathology during malaria infection()*. Journal of immunology (Baltimore, Md. : 1950), 2012. **188**(3): p. 1178-1190.
301. Sun, J., et al., *Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10*. Nature medicine, 2009. **15**(3): p. 277-284.
302. Seder, R.A., P.A. Darrah, and M. Roederer, *T-cell quality in memory and protection: implications for vaccine design*. Nat Rev Immunol, 2008. **8**(4): p. 247-258.
303. Duvall, M.G., et al., *Polyfunctional T cell responses are a hallmark of HIV-2 infection*. European Journal of Immunology, 2008. **38**(2): p. 350-363.
304. Perona-Wright, G., et al., *Systemic but Not Local Infections Elicit Immunosuppressive IL-10 Production by Natural Killer Cells*. Cell Host & Microbe, 2009. **6**(6): p. 503-512.
305. Lee, S.-H., et al., *Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection*. The Journal of Experimental Medicine, 2009. **206**(10): p. 2235.
306. He, J., et al., *Circulating precursor CCR7(lo)PD-1(hi) CXCR5⁺ CD4⁺ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure*. Immunity., 2013. **39**.
307. Weidanz, W.P., et al., *Plasmodium chabaudi adami: use of the B-cell-deficient mouse to define possible mechanisms modulating parasitemia of chronic malaria*. Exp Parasitol, 2005. **111**(2): p. 97-104.
308. Vinuesa, C.G., et al., *T cells and follicular dendritic cells in germinal center B-cell formation and selection*. Immunol Rev., 2010. **237**.
309. Good-Jacobson, K.L., et al., *PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells*. Nat Immunol., 2010. **11**.

310. Sebina, I., et al., *IFNAR1-Signalling Obstructs ICOS-mediated Humoral Immunity during Non-lethal Blood-Stage Plasmodium Infection*. PLoS Pathogens, 2016. **12**(11): p. e1005999.
311. Peng, S.L., S.J. Szabo, and L.H. Glimcher, *T-bet regulates IgG class switching and pathogenic autoantibody production*. Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5545-50.
312. Xu, W. and J.J. Zhang, *Stat1-Dependent Synergistic Activation of T-bet for IgG2a Production during Early Stage of B Cell Activation*. The Journal of Immunology, 2005. **175**(11): p. 7419-7424.
313. Oakley, M.S., et al., *T-bet modulates the antibody response and immune protection during murine malaria*. European Journal of Immunology, 2014. **44**(9): p. 2680-2691.
314. Crotty, S., *T follicular helper cell differentiation, function, and roles in disease*. Immunity, 2014. **41**.
315. Vogelzang, A., et al., *A Fundamental Role for Interleukin-21 in the Generation of T Follicular Helper Cells*. Immunity, 2008. **29**(1): p. 127-137.
316. Yu, D., et al., *The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment*. Immunity, 2009. **31**(3): p. 457-468.
317. Nurieva, R.I., et al., *Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages*. Immunity, 2008. **29**(1): p. 138-149.
318. Spolski, R. and W.J. Leonard, *IL-21 and T follicular helper cells*. International Immunology, 2010. **22**(1): p. 7-12.
319. Fazilleau, N., et al., *Follicular Helper T Cells: Lineage and Location*. Immunity, 2009. **30**(3): p. 324-335.
320. Crotty, S., *Follicular Helper CD4 T Cells (TFH)*. Annual Review of Immunology, 2011. **29**(1): p. 621-663.
321. Baaten, B.J.G., et al., *CD44 Regulates Survival and Memory Development in Th1 Cells*. Immunity, 2010. **32**(1): p. 104-115.
322. Hans, D. and A.A. K., *Control of CD4+ T-cell memory by cytokines and costimulators*. Immunological Reviews, 2006. **211**(1): p. 23-38.
323. Henson, S.M. and A.N. Akbar, *KLRG1—more than a marker for T cell senescence*. AGE, 2009. **31**(4): p. 285-291.
324. Gwyer Findlay, E., et al., *IL-27 Receptor Signaling Regulates Memory CD4+ T Cell Populations and Suppresses Rapid Inflammatory Responses during Secondary Malaria Infection*. Infection and Immunity, 2014. **82**(1): p. 10-20.
325. Robbins, S.H., et al., *Differential Regulation of Killer Cell Lectin-Like Receptor G1 Expression on T Cells*. The Journal of Immunology, 2003. **170**(12): p. 5876.
326. Levine, J.H., et al., *Data-Driven Phenotypic Dissection of AML Reveals Progenitor-like Cells that Correlate with Prognosis*. Cell, 2015. **162**(1): p. 184-97.
327. Becht, E., et al., *Dimensionality reduction for visualizing single-cell data using UMAP*. Nature Biotechnology, 2018. **37**: p. 38.
328. PrabhuDas, M.R., et al., *A Consensus Definitive Classification of Scavenger Receptors and Their Roles in Health and Disease*. The Journal of Immunology, 2017. **198**(10): p. 3775-3789.
329. Guermonprez, P., et al., *Inflammatory Flt3L is essential to mobilize dendritic cells and for T cell responses during Plasmodium infection*. Nature medicine, 2013. **19**(6): p. 730-738.
330. Sallin, M.A., et al., *Th1 Differentiation Drives the Accumulation of Intravascular, Non-protective CD4 T Cells during Tuberculosis*. Cell Reports, 2017. **18**(13): p. 3091-3104.
331. Marple, A., et al., *Cutting Edge: Helminth Coinfection Blocks Effector Differentiation of CD8 T Cells through Alternate Host Th2- and IL-10-Mediated Responses*. The Journal of Immunology, 2017. **198**(2): p. 634.
332. Kallies, A. and K.L. Good-Jacobson, *Transcription Factor T-bet Orchestrates Lineage Development and Function in the Immune System*. Trends in Immunology, 2017. **38**(4): p. 287-297.
333. Imai, T., et al., *Cytotoxic activities of CD8+ T cells collaborate with macrophages to protect against blood-stage murine malaria*. eLife, 2015. **4**: p. e04232.
334. Couper, K.N., D.G. Blount, and E.M. Riley, *IL-10: The Master Regulator of Immunity to Infection*. The Journal of Immunology, 2008. **180**(9): p. 5771-5777.
335. Zeng, H., et al., *Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance*. Cellular And Molecular Immunology, 2015. **12**: p. 566.

336. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells*. Nat Rev Immunol, 2010. **10**(3): p. 170-181.
337. Freitas do Rosario, A.P. and J. Langhorne, *T cell-derived IL-10 and its impact on the regulation of host responses during malaria*. Int J Parasitol, 2012. **42**(6): p. 549-55.
338. Couper, K.N., et al., *IL-10 from CD4(+)CD25(-)Foxp3(-)CD127(-) Adaptive Regulatory T Cells Modulates Parasite Clearance and Pathology during Malaria Infection*. PLoS Pathogens, 2008. **4**(2): p. e1000004.
339. Jagannathan, P., et al., *IFN γ /IL-10 Co-producing Cells Dominate the CD4 Response to Malaria in Highly Exposed Children*. PLOS Pathogens, 2014. **10**(1): p. e1003864.
340. Lönnberg, T., et al., *Single-cell RNA-seq and computational analysis using temporal mixture modeling resolves T_H1/T_{FH} fate bifurcation in malaria*. Science Immunology, 2017. **2**(9).
341. Langhorne, J., P. Mombaerts, and S. Tonegawa, *alpha beta and gamma delta T cells in the immune response to the erythrocytic stages of malaria in mice*. International immunology, 1995. **7**(6): p. 1005-1011.
342. Seixas, E.M.G. and J. Langhorne, *$\gamma\delta$ T Cells Contribute to Control of Chronic Parasitemia in Plasmodium chabaudi Infections in Mice*. The Journal of Immunology, 1999. **162**(5): p. 2837-2841.
343. Inoue, S.-I., et al., *Enhancement of dendritic cell activation via CD40 ligand-expressing $\gamma\delta$ T cells is responsible for protective immunity to Plasmodium parasites*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(30): p. 12129-12134.
344. Mohan, K., P. Moulin, and M.M. Stevenson, *Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection*. The Journal of Immunology, 1997. **159**(10): p. 4990-4998.
345. H., A.A., et al., *Malaria-specific antibody responses and parasite persistence after infection of mice with Plasmodium chabaudi chabaudi*. Parasite Immunology, 2007. **29**(9): p. 435-444.
346. Dufaud, C.R., L.J. McHeyzer-Williams, and M.G. McHeyzer-Williams, *Deconstructing the germinal center, one cell at a time*. Current Opinion in Immunology, 2017. **45**: p. 112-118.
347. Suan, D., C. Sundling, and R. Brink, *Plasma cell and memory B cell differentiation from the germinal center*. Current Opinion in Immunology, 2017. **45**(Supplement C): p. 97-102.
348. Hansen, D.S., et al., *Emerging concepts in T follicular helper cell responses to malaria*. International Journal for Parasitology, 2017. **47**(2-3): p. 105-110.
349. Vinuesa, C.G., et al., *Follicular Helper T Cells*. Annual Review of Immunology, 2016. **34**(1): p. 335-368.
350. Goenka, R., et al., *Cutting Edge: Dendritic Cell-Restricted Antigen Presentation Initiates the Follicular Th Cell Program but Cannot Complete Ultimate Effector Differentiation*. The Journal of Immunology, 2011.
351. Choi, Youn S., et al., *ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6*. Immunity, 2011. **34**(6): p. 932-946.
352. Shin, C., et al., *CD8 α ^{hi} Dendritic Cells Induce Antigen-Specific T Follicular Helper Cells Generating Efficient Humoral Immune Responses*. Cell Reports, 2015. **11**(12): p. 1929-1940.
353. Li, J., et al., *EBI2 augments Tfh cell fate by promoting interaction with IL-2-quenching dendritic cells*. Nature, 2016. **533**: p. 110.
354. Mesin, L., J. Ersching, and Gabriel D. Vitoria, *Germinal Center B Cell Dynamics*. Immunity, 2016. **45**(3): p. 471-482.
355. Lee, S.K., et al., *B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells*. The Journal of Experimental Medicine, 2011. **208**(7): p. 1377-1388.
356. MacLennan Ian, C.M., et al., *Extrafollicular antibody responses*. Immunological Reviews, 2003. **194**(1): p. 8-18.
357. Chaudhuri, J. and F.W. Alt, *Class-switch recombination: interplay of transcription, DNA deamination and DNA repair*. Nature Reviews Immunology, 2004. **4**: p. 541.
358. Matthews, A.J., et al., *Chapter One - Regulation of Immunoglobulin Class-Switch Recombination: Choreography of Noncoding Transcription, Targeted DNA*

- Deamination, and Long-Range DNA Repair*, in *Advances in Immunology*, F.W. Alt, Editor. 2014, Academic Press. p. 1-57.
359. Finkelman, F.D., et al., *Lymphokine Control of In Vivo Immunoglobulin Isotype Selection*. Annual Review of Immunology, 1990. **8**(1): p. 303-333.
 360. Zhang, Z., T. Goldschmidt, and H. Salter, *Possible allelic structure of IgG2a and IgG2c in mice*. Molecular Immunology, 2012. **50**(3): p. 169-171.
 361. Martin, R.M., J.L. Brady, and A.M. Lew, *The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice*. Journal of Immunological Methods, 1998. **212**(2): p. 187-192.
 362. Barr, T.A., et al., *B Cell Intrinsic MyD88 Signals Drive IFN- γ Production from T Cells and Control Switching to IgG2c*. The Journal of Immunology, 2009.
 363. Gerth, A.J., L. Lin, and S.L. Peng, *T-bet regulates T-independent IgG2a class switching*. International Immunology, 2003. **15**(8): p. 937-944.
 364. Mohr, E., et al., *IFN- γ produced by CD8 T cells induces T-bet-dependent and-independent class switching in B cells in responses to alum-precipitated protein vaccine*. Proceedings of the National Academy of Sciences, 2010. **107**(40): p. 17292-17297.
 365. Snapper, C.M., et al., *Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens*. The Journal of Experimental Medicine, 1992. **175**(5): p. 1367-1371.
 366. Gavin, A.L., et al., *Cutting Edge: Identification of the Mouse IgG3 Receptor: Implications for Antibody Effector Function at the Interface Between Innate and Adaptive Immunity*. The Journal of Immunology, 1998. **160**(1): p. 20-23.
 367. Ralph, P., et al., *All classes of murine IgG antibody mediate macrophage phagocytosis and lysis of erythrocytes*. The Journal of Immunology, 1980. **125**(5): p. 1885.
 368. Snapper, C.M. and W.E. Paul, *Interferon- γ and B Cell Stimulatory Factor-1 Reciprocally Regulate Ig Isotype Production*. Science, 1987. **236**(4804): p. 944-947.
 369. Germann, T., et al., *Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo*. Eur J Immunol, 1995. **25**(3): p. 823-9.
 370. Ezekowitz, R.A., M. Bampton, and S. Gordon, *Macrophage activation selectively enhances expression of Fc receptors for IgG2a*. The Journal of Experimental Medicine, 1983. **157**(2): p. 807.
 371. McIntosh, R.S., et al., *The importance of human Fc γ RI in mediating protection to malaria*. PLoS Pathog, 2007. **3**(5): p. e72.
 372. White, W.I., C.B. Evans, and D.W. Taylor, *Antimalarial antibodies of the immunoglobulin G2a isotype modulate parasitemias in mice infected with Plasmodium yoelii*. Infection and Immunity, 1991. **59**(10): p. 3547-3554.
 373. Waki, S., et al., *Interferon-gamma and the induction of protective IgG2a antibodies in non-lethal Plasmodium berghei infections of mice*. Parasite Immunology, 1995. **17**(10): p. 503-508.
 374. Ioannidis, L.J., et al., *Monocyte- and Neutrophil-Derived CXCL10 Impairs Efficient Control of Blood-Stage Malaria Infection and Promotes Severe Disease*. The Journal of Immunology, 2016. **196**(3): p. 1227-1238.
 375. Obeng-Adjei, N., et al., *Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children*. Cell Reports, 2015. **13**(2): p. 425-439.
 376. Lee, Sau K., et al., *Interferon- γ Excess Leads to Pathogenic Accumulation of Follicular Helper T Cells and Germinal Centers*. Immunity, 2012. **37**(5): p. 880-892.
 377. Nakayamada, S., et al., *Early Th1 Cell Differentiation Is Marked by a Tfh Cell-like Transition*. Immunity, 2011. **35**(6): p. 919-931.
 378. Jogdand, G.M., S. Mohanty, and S. Devadas, *Regulators of Tfh Cell Differentiation*. Frontiers in Immunology, 2016. **7**(520).
 379. Guthmiller, J.J., et al., *Cutting Edge: IL-10 Is Essential for the Generation of Germinal Center B Cell Responses and Anti-Plasmodium Humoral Immunity*. The Journal of Immunology, 2016.
 380. Zander, R.A., et al., *PD-1 Co-inhibitory and OX40 Co-stimulatory Crosstalk Regulates Helper T Cell Differentiation and Anti-Plasmodium Humoral Immunity*. Cell Host Microbe, 2015. **17**(5): p. 628-41.

381. Haque, A., et al., *Type I IFN signaling in CD8- DCs impairs Th1-dependent malaria immunity*. The Journal of clinical investigation, 2014. **124**(6): p. 2483-2496.
382. Zander, R.A., et al., *Type I Interferons Induce T Regulatory 1 Responses and Restrict Humoral Immunity during Experimental Malaria*. PLOS Pathogens, 2016. **12**(10): p. e1005945.
383. Wikenheiser, D.J., et al., *The Costimulatory Molecule ICOS Regulates Host Th1 and Follicular Th Cell Differentiation in Response to Plasmodium chabaudi chabaudi AS Infection*. The Journal of Immunology, 2016. **196**(2): p. 778-791.
384. Inoue, S.-I., et al., *Preferentially expanding V γ 1+ $\gamma\delta$ T cells are associated with protective immunity against Plasmodium infection in mice*. European Journal of Immunology, 2017. **47**(4): p. 685-691.
385. Mamedov, M.R., et al., *A Macrophage Colony-Stimulating-Factor-Producing $\gamma\delta$ T Cell Subset Prevents Malarial Parasitemic Recurrence*. Immunity, 2018. **48**(2): p. 350-363.e7.
386. Opat, M.M., et al., *Protection by and maintenance of CD4 effector memory and effector T cell subsets in persistent malaria infection*. PLoS Pathog, 2018. **14**(4): p. e1006960.
387. Opat, M.M. and R. Stephens, *Chronic Plasmodium chabaudi Infection Generates CD4 Memory T Cells with Increased T Cell Receptor Sensitivity but Poor Secondary Expansion and Increased Apoptosis*. Infect Immun, 2017. **85**(3).
388. Michael, M.O. and S. Robin, *Early Decision: Effector and Effector Memory T Cell Differentiation in Chronic Infection*. Current Immunology Reviews, 2013. **9**(3): p. 190-206.
389. Zander, R.A., et al., *Th1-like Plasmodium-Specific Memory CD4+ T Cells Support Humoral Immunity*. Cell Reports, 2017. **21**(7): p. 1839-1852.
390. Liu, D., et al., *T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction*. Nature, 2014. **517**: p. 214.
391. Pérez-Mazliah, D., et al., *Disruption of IL-21 Signaling Affects T Cell-B Cell Interactions and Abrogates Protective Humoral Immunity to Malaria*. PLOS Pathogens, 2015. **11**(3): p. e1004715.
392. Fontana, M.F., et al., *Macrophage Colony Stimulating Factor Derived from CD4+ T Cells Contributes to Control of a Blood-Borne Infection*. PLOS Pathogens, 2016. **12**(12): p. e1006046.
393. Engwerda, C.R., S.S. Ng, and P.T. Bunn, *The Regulation of CD4+ T Cell Responses during Protozoan Infections*. Frontiers in Immunology, 2014. **5**(498).
394. Terkawi, M.A., et al., *Depletion of Phagocytic Cells during Nonlethal Plasmodium yoelii Infection Causes Severe Malaria Characterized by Acute Renal Failure in Mice*. Infection and Immunity, 2016. **84**(3): p. 845-855.
395. Bastos, K.R., et al., *Impaired macrophage responses may contribute to exacerbation of blood-stage Plasmodium chabaudi chabaudi malaria in interleukin-12-deficient mice*. J Interferon Cytokine Res, 2002. **22**(12): p. 1191-9.
396. Mebius, R.E. and G. Kraal, *Structure and function of the spleen*. Nat Rev Immunol, 2005. **5**(8): p. 606-616.
397. Davies, L.C., et al., *A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation*. European Journal of Immunology, 2011. **41**(8): p. 2155-2164.
398. Klein, I., et al., *Kupffer cell heterogeneity: functional properties of bone marrow-derived and sessile hepatic macrophages*. Blood, 2007. **110**(12): p. 4077-4085.
399. Heymann, F., et al., *Liver inflammation abrogates immunological tolerance induced by Kupffer cells*. Hepatology, 2015. **62**(1): p. 279-291.
400. Krenkel, O. and F. Tacke, *Liver macrophages in tissue homeostasis and disease*. Nat Rev Immunol, 2017. **17**(5): p. 306-321.
401. Scott, C.L., et al., *Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells*. 2016. **7**: p. 10321.
402. Sawai, Catherine M., et al., *Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals*. Immunity, 2016. **45**(3): p. 597-609.
403. Epelman, S., Kory J. Lavine, and Gwendalyn J. Randolph, *Origin and Functions of Tissue Macrophages*. Immunity, 2014. **41**(1): p. 21-35.
404. You, Q., et al., *Role of hepatic resident and infiltrating macrophages in liver repair after acute injury*. Biochemical Pharmacology, 2013. **86**(6): p. 836-843.

405. Robinson, N., et al., *Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium*. Nat Immunol, 2012. **13**(10): p. 954-62.
406. Theurl, I., et al., *On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver*. Nature Medicine, 2016. **22**: p. 945.
407. Tan, S.Y.S. and M.A. Krasnow, *Developmental origin of lung macrophage diversity*. Development, 2016. **143**(8): p. 1318-1327.
408. Schofield, L., et al., *Glycosylphosphatidylinositol toxin of Plasmodium up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction*. J Immunol, 1996. **156**(5): p. 1886-96.
409. Chang, Z., et al., *The TatD-like DNase of Plasmodium is a virulence factor and a potential malaria vaccine candidate*. Nature Communications, 2016. **7**: p. 11537.
410. Bhatt, T.K., et al., *Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses*. Nat Commun, 2011. **2**: p. 530.
411. Couper, K.N., et al., *Parasite-Derived Plasma Microparticles Contribute Significantly to Malaria Infection-Induced Inflammation through Potent Macrophage Stimulation*. PLOS Pathogens, 2010. **6**(1): p. e1000744.
412. Chimma, P., et al., *A Distinct Peripheral Blood Monocyte Phenotype Is Associated with Parasite Inhibitory Activity in Acute Uncomplicated Plasmodium falciparum Malaria*. PLOS Pathogens, 2009. **5**(10): p. e1000631.
413. Tachado, S.D., et al., *Signal transduction in macrophages by glycosylphosphatidylinositols of Plasmodium, Trypanosoma, and Leishmania: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties*. Proc Natl Acad Sci U S A, 1997. **94**(8): p. 4022-7.
414. Swirski, F.K., et al., *Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease*. Proc Natl Acad Sci U S A, 2006. **103**(27): p. 10340-10345.
415. Combadiere, C., et al., *Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice*. Circulation, 2008. **117**(13): p. 1649-57.
416. Ginhoux, F., et al., *New insights into the multidimensional concept of macrophage ontogeny, activation and function*. Nature Immunology, 2015. **17**: p. 34.
417. Thornley, T.B., et al., *Fragile TIM-4-expressing tissue resident macrophages are migratory and immunoregulatory*. The Journal of Clinical Investigation, 2014. **124**(8): p. 3443-3454.
418. Pyonteck, S.M., et al., *CSF-1R inhibition alters macrophage polarization and blocks glioma progression*. Nature Medicine, 2013. **19**: p. 1264.
419. Olivier, M., et al., *Malarial Pigment Hemozoin and the Innate Inflammatory Response*. Frontiers in Immunology, 2014. **5**: p. 25.
420. Boura, M., et al., *The hemozoin conundrum: is malaria pigment immune-activating, inhibiting, or simply a bystander?* Trends Parasitol, 2013. **29**(10): p. 469-76.
421. Urban, B.C. and S. Todryk, *Malaria pigment paralyzes dendritic cells*. Journal of Biology, 2006. **5**(2): p. 4.
422. Coban, C., et al., *Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin*. J Exp Med, 2005. **201**.
423. Corbett, Y., et al., *Involvement of Nod2 in the innate immune response elicited by malarial pigment hemozoin*. Microbes Infect, 2015. **17**(3): p. 184-94.
424. Gibbings, S.L., et al., *Transcriptome analysis highlights the conserved difference between embryonic and postnatal-derived alveolar macrophages*. Blood, 2015. **126**(11): p. 1357-1366.
425. Beattie, L., et al., *Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions*. Journal of Hepatology, 2016. **65**(4): p. 758-768.
426. Fabrick, B.O., et al., *The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria*. Blood, 2009. **113**(4): p. 887-892.
427. Kneidl, J., et al., *Soluble CD163 promotes recognition, phagocytosis and killing of Staphylococcus aureus via binding of specific fibronectin peptides*. Cellular Microbiology, 2012. **14**(6): p. 914-936.

428. González-Domínguez, é., et al., *CD163L1 and CLEC5A discriminate subsets of human resident and inflammatory macrophages in vivo*. Journal of Leukocyte Biology, 2015. **98**(4): p. 453-466.
429. Moeller, J.B., et al., *CD163-L1 Is an Endocytic Macrophage Protein Strongly Regulated by Mediators in the Inflammatory Response*. The Journal of Immunology, 2012. **188**(5): p. 2399-2409.
430. Lauder, S.N., et al., *Paracetamol reduces influenza-induced immunopathology in a mouse model of infection without compromising virus clearance or the generation of protective immunity*. Thorax, 2011. **66**(5): p. 368.
431. Engwerda, C.R., et al., *A Role for Tumor Necrosis Factor- α in Remodeling the Splenic Marginal Zone during *Leishmania donovani* Infection*. The American Journal of Pathology, 2002. **161**(2): p. 429-437.
432. Liddiard, K., et al., *Macrophage heterogeneity and acute inflammation*. European Journal of Immunology, 2011. **41**(9): p. 2503-2508.
433. Zychlinsky, A., M.C. Prevost, and P.J. Sansonetti, *Shigella flexneri induces apoptosis in infected macrophages*. Nature, 1992. **358**(6382): p. 167-9.
434. Di Paolo, Nelson C., et al., *The Transcription Factor IRF3 Triggers "Defensive Suicide" Necrosis in Response to Viral and Bacterial Pathogens*. Cell Reports, 2013. **3**(6): p. 1840-1846.
435. Ginhoux, F., C. Blieriot, and M. Lecuit, *Dying for a Cause: Regulated Necrosis of Tissue-Resident Macrophages upon Infection*. Trends in Immunology, 2017. **38**(10): p. 693-695.
436. Stephenson, H.N., A. Herzig, and A. Zychlinsky, *Beyond the grave: When is cell death critical for immunity to infection?* Current Opinion in Immunology, 2016. **38**: p. 59-66.
437. Wallach, D., et al., *Programmed necrosis in inflammation: Toward identification of the effector molecules*. Science, 2016. **352**(6281).
438. Fernandez-Ruiz, D., et al., *Development of a Novel CD4+ TCR Transgenic Line that Reveals a Dominant Role for CD8+ DC and CD40-Signaling in the Generation of Helper and CTL Responses to Blood Stage Malaria*. bioRxiv, 2017.
439. Poulin, L.F., et al., *Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8 α + dendritic cells*. J Exp Med, 2010. **207**(6): p. 1261-71.
440. Bachem, A., et al., *Superior antigen cross-presentation and XCR1 expression define human CD11c(+)CD141(+) cells as homologues of mouse CD8(+) dendritic cells*. The Journal of Experimental Medicine, 2010. **207**(6): p. 1273-1281.
441. Park, H.Y., et al., *Evolution of B cell responses to Clec9A-targeted antigen*. J Immunol, 2013. **191**(10): p. 4919-25.
442. Kato, Y., et al., *Targeting Antigen to Clec9A Primes Follicular Th Cell Memory Responses Capable of Robust Recall*. J Immunol, 2015. **195**(3): p. 1006-14.
443. Li, J., et al., *Antibodies targeting Clec9A promote strong humoral immunity without adjuvant in mice and non-human primates*. Eur J Immunol, 2015. **45**(3): p. 854-64.
444. Tullett, K.M., et al., *Targeting CLEC9A delivers antigen to human CD141+ DC for CD4+ and CD8+T cell recognition*. JCI Insight, 2016. **1**(7).
445. Urban, B.C., et al., *The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria*. Infect Immun, 2006. **74**(12): p. 6700-6.
446. Freitas do Rosário, A.P., et al., *Gradual Decline in Malaria-Specific Memory T Cell Responses Leads to Failure to Maintain Long-Term Protective Immunity to *Plasmodium chabaudi* AS Despite Persistence of B Cell Memory and Circulating Antibody*. The Journal of Immunology, 2008. **181**(12): p. 8344-8355.
447. Scott, C.L., et al., *Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells*. Nat Commun, 2016. **7**: p. 10321.
448. Zigmond, E., et al., *Infiltrating Monocyte-Derived Macrophages and Resident Kupffer Cells Display Different Ontogeny and Functions in Acute Liver Injury*. The Journal of Immunology, 2014. **193**(1): p. 344-353.

9. Author's publication(s)

1. Gupta, P., **Lai, S. M.**, Sheng, J., Tetlak, P., Balachander, A., Claser, C., Ruedl, C. (2016). Tissue-Resident CD169(+) Macrophages Form a Crucial Front Line against Plasmodium Infection. *Cell Reports*, 16(6), 1749-1761. doi:10.1016/j.celrep.2016.07.010
2. **Lai, S. M.**, Sheng, J., Gupta, P., Renia, L., Duan, K., Zolezzi, F., Ruedl, C. (2018). Organ-Specific Fate, Recruitment, and Refilling Dynamics of Tissue-Resident Macrophages during Blood-Stage Malaria. *Cell Reports*, 25(11), 3099-3109.e3093. doi:10.1016/j.celrep.2018.11.059

11. Oral and poster presentations

1. **Oral Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at FIMSA (Federation of Immunological Societies of Asia-Oceania) Meeting, Singapore, July 2015.
2. **Poster Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at 4th Singapore Malaria Network Meeting (SingMalNet), Nanyang Technological University, Singapore, February 2016.
3. **Poster Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at 8th International Singapore Symposium of Immunology, Singapore, May 2016.
4. **Poster Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at 5th SGSI PhD Student Retreat, 2016, Singapore.
5. **Poster Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at ICI 2016 (International Congress of Immunology), Melbourne, Australia, August 2016.
6. **Poster Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at Science Improving Lives Symposium, Nanyang Technological University, Singapore, February 2017
7. **Oral Presentation**, “Clec9A⁺ CD8⁺ Dendritic Cells are Key Players in Controlling *Plasmodium chabaudi chabaudi* Infection” at EMBL Conference: BioMalPar XIII: Biology and Pathology of the Malaria Parasite, Heidelberg, Germany, May 2017.
8. **Poster Presentation**, “Ontogeny of tissue-resident macrophages during blood-stage malaria” at ECI 2018 (European Congress of Immunology), Amsterdam, Netherlands, September 2018.