Inducing immunity to liver stage malaria through endogenous tissue resident memory cells

Ana Maria Valencia Hernandez

ORCID ID:
0000-0002-3907-5806

from Cali, Colombia

Submitted in total fulfilment of the requirements of the joint degree of Doctor of Philosophy (PhD)

of The Medical Faculty

The Rheinische Friedrich-Wilhelms-Universität Bonn

and

The Department of Microbiology and Immunology

The University of Melbourne

Bonn/Melbourne, 2020

Performed and approved by The Medical Faculty of The Rheinische Friedrich-Wilhelms-Universität Bonn and The University of Melbourne

1. Supervisor: Prof. Christoph Wilhelm, The Medical Faculty, The Rheinische Friedrich- Wilhelms-Universität Bonn

2. Supervisor: Prof. William Heath and Dr. Daniel Fernandez-Ruiz, The Department of Microbiology and Immunology, The University of Melbourne.

Month and year of the original thesis submission: June 2020
Month and year of the oral examination: November 2020

Institute of Clinical Chemistry and Clinical Pharmacology, Bonn,
Director: Prof. Gunther Hartmann

TABLE OF CONTENTS

Title page	I
Table of content	III
Abbreviations	VIII
List of tables	XII
List of figures	XIII
Abstract	XV
Declaration	XVII
Preface	XVIII
Acknowledgements	XIX
List of publications	XXI
CHAPTER ONE:	1
Literature review	
1.1 Malaria introduction	2
1.2 Biology of the malaria disease	3
1.2.1 Malaria-liver stage	3
1.2.2 Malaria-blood stage	8
1.3 Pathogenesis	10
1.3.1 Severe malaria	10
1.4 Naturally acquired immunity	12
1.5 Malaria vaccines	14
1.5.1 Blood stage vaccines	14
1.5.2 Transmission blocking vaccines	16
1.5.3 Pre-erythrocytic vaccines	16
1.5.4 Radiation-attenuated based vaccines	18
1.5.5 Genetically-attenuated parasites based vaccines	19
1.5.6 Chemoprophylaxis vaccination	19
1.6 Malaria liver-stage immunity	20
1.6.1 Innate response to liver stage infection	20
1.6.2 T cell priming	22

1.6.3 Memory CD8 T cells 24
1.7 Novel vaccine strategies 29
1.7.1 Prime-and-trap vaccine 30
1.7.2 Prime-target vaccine 33
1.7.3 CD8 epitopes derived from *P. berghei* ANKA 34
1.8 Adjuvants 35
1.8.1 Delivery systems 36
1.8.2 Immune potentiators 37
1.8.3 Endosomal sensors 38
1.8.4 Cytosolic sensors 42
1.8.5 Adjuvants for malaria vaccines 44
1.8.6 Adjuvants for T_{RM} cell formation 45
1.9 Thesis aims 47

CHAPTER TWO: 50
Materials and methods
2.1 Materials 51
2.1.1 Experimental models 51
2.1.2 Immunisation reagents 51
2.1.3 Cell biology reagents and materials 54
2.1.3.1 Antibodies 54
2.1.3.2 Cell biology chemicals and reagents 55
2.1.4 Molecular biology reagents 57
2.1.5 Specialised equipment 58
2.1.6 Software 58
2.2 Methods 59
2.2.1 Mice mosquitoes and parasites 59
2.2.2 Naïve CD8 T cell enrichment and adoptive transfer 60
2.2.3 Mice immunisations 60
2.2.3.1 Prime immunisation with Clec9A mAb 60
2.2.3.2 Adjuvant stimulation and preparation 61

2.2.3.3 Prime-and-trap vaccination 62
2.2.3.4 RAS vaccination 62
2.2.4 Challenge experiments with live PbA sporozoites 62
2.2.5 Organ processing for T cell analysis 63
2.2.6 Flow cytometry 63
2.2.7 Enumeration of antigen-specific CD8 T cells in naïve repertoire 64
2.2.8 Enzyme-linked immunosorbent assay (ELISA) 65
2.2.8.1 Plasma preparation 65
2.2.8.2 Organ processing 65
2.2.8.3 ELISA to detect IFNα 65
2.2.9 Quantitative real-time PCR 66
2.2.9.1 RNA isolation 66
2.2.9.2 cDNA synthesis 66
2.2.9.3 Real-time PCR 67
2.2.10 Statistical analysis 67

CHAPTER THREE:
Characterization of the memory response induced by immunisation with an epitope from the ribosomal protein L6 (RPL6) of *P. berghei* ANKA. 69
3.1 Introduction 70
3.2 Results 74
3.2.1 Potency of αClec9A-Pb-1 priming on PbT-I cell expansion and memory formation in spleen and liver 74
3.2.2 Endogenous response and protection induced by immunisation with the Pb-1 antigen 80
3.2.3 Induction of memory CD8 T cells using the Pb-1 epitope in prime-and-trap vaccination 86
3.2.4 Analysis of sterile protection against sporozoite infection by prime-and-trap vaccination using the Pb-1 antigen 94
3.3 Discussion 100

3.3.1 Novel MHC-I restricted epitope derived from *P. berghei* ANKA 100
3.3.2 PbT-I cell expansion in response to immunisation targeting the Pb-1 epitope 102
3.3.3 Endogenous response and protection induced by immunisation with the Pb-1 antigen 103
3.3.4 Sterile protection against malaria induced by prime-and-trap vaccination using the Pb-1 epitope 105

CHAPTER FOUR:
Analysis of nucleic acid-based adjuvants for liver T_{RM} cell formation induced by Clec9A targeted immunisation. 108
4.1 Introduction 109
4.1.2 The adjuvants 110
4.2 Results 115
4.2.1 TLR9 ligands are potent at inducing liver T_{RM} cell formation 115
4.2.2 High dose of agonists enhanced liver T_{RM} cells formation 118
4.2.3 CpG-ligands preferentially induce liver T_{RM} cell formation 122
4.2.4 CpG-combo has no significant effect on the liver TRM cell formation induced by RAS 124
4.2.5 DOTAP enhances the effect of the CpG-ligands on the formation of liver T_{RM} cells 128
4.2.6 Assessment of adjuvants for type I IFN production: Control experiments 134
4.3 Discussion 139
4.3.1 CpG as an adjuvant for CD8 T cell expansion 139
4.3.2 Other agonists 143

CHAPTER FIVE:
General discussion 148
5.1 Context 149

5.2 Summary of main findings 150
5.3 A protective malaria epitope 151
5.4 Adjuvants for liver T_{RM} cell formation 156
5.5 Challenges for the induction of liver inflammation 158
5.6 CpG favour liver T_{RM} cell formation 163
5.7 Too much of a good thing? 165
5.8 The translational potential of ‘prime-and-trap’ 167
5.9 T_{RM}-based vaccines for humans 170
5.10 Concluding remarks 172

REFERENCES 174

CURRICULUM VITAE 245

ABBREVIATIONS

Special characters

%	Percent
#	Number
°C	Degree Celsius
α	alpha
β	beta
γ	gamma
µg	Microgram
µg	Microlitre

A

A.	*Anopheles*
Ab	Antibody
AMA1	Apical membrane antigen-1
αClec9A-Pb-1	Rat anti-Clec9A mAb genetically coupled to the Pb-1 epitope
APC	Antigen presenting cell

B

B6	C57Bl/6 mice
BSA	Bovine serum albumin fraction V

C

CSP	Circumsporozoite protein
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDN	Cyclic dinucleotides
cGAS	Cyclic GMP–AMP (cGAMP) synthase
ChAd	Chimpanzee adenovirus
ChAd63	Chimpanzee adenovirus serotype 63
CpG	Cytosine-phosphate-Guanine
CTL	Cytotoxic T Lymphocytes
CTV	Cell-Trace Violet™
cVac	Chemoprophylaxis vaccination
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor

D

D	Day
DC	Dendritic cells
dLN	Draining Lymph node
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA

E

ECM	Experimental cerebral malaria
e.g.	*exempli gratia*

F

FCS	Fetal Calf Serum
FNRG	FAH-/- NOD Rag1-/- IL2Rγ^{NULL}

G

GAP	Genetically-attenuated parasites
GFP	Green fluorescent protein

H

HBSS	Hanks' buffered salt solution
HBV	*Hepatitis B virus*
HPLC	*High-performance liquid chromatography*
HPV	*Human papilloma virus*
Hr	Hour(s)
HSV	Herpes Simplex Virus

I

i.e.	*Id est*
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscular
iRBC	Infected red blood cells
IRF	Interferon-Regulatory Factor
iRT	Indexed retention time
ISG	Interferon-Stimulated-Gene
i.v.	Intravenously

K

KC	Kupfer cells
KLRG1	Killer cell lectin-like receptor subfamily G member 1
KO	Knock out

L

LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LN	Lymph node
LPS	Lipopolysaccharide
LSA1	Liver-stage antigen 1
LSAP2	Liver stage-associated protein 2

M

MAVS	Mitochondrial antiviral-signalling
MDA5	Melanoma Differentiation-Associated gene 5
MAb	Monoclonal antibody
ME	Multi-epitope string
MHC	Major Histocompatibility Complex
MSP1	Merozoite surface protein-1
MVA	Modified vaccinia virus Ankara
MyD88	Myeloid Differentiation factor 88

N

NCY	NCYDFNNI
NF-κB	Nuclear Factor kappa B
NK	Natural killer
Np	Nanoparticles
NVY	NVYDFNLL

O

ODN	Oligodeoxynucleotides
OVA	Ovalbumin

P

P.	*Plasmodium*
PAMP	Pathogen-Associated Molecular Pattern
PbA	*Plasmodium berghei* ANKA
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic Cell
PD-1	Programmed death 1
PE	Pre-erythrocytic
PfCSP	*P. falciparum* CSP
PfSPZ	*P. falciparum* sporozoites
PI	Propidium iodine
(p.i.)	Post infection
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern-Recognition Receptor

R

RAS	Radiation-attenuated sporozoites
rAAV	Recombinant adeno-associated virus
RBC	Red blood cells
RIG-I	Retinoic acid Inducible Gene I
RPL6	Ribosomal protein L6
RT	Room temperature

T

T_{CM}	Central memory T (cells)

TCR	T cell receptor
T_{EM}	Effector memory T (cells)
Th1	Type 1 T helper
Th2	Type 2 T helper
Tim-3	T-cell immunoglobulin mucin 3
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAP	Thrombospondin-related adhesion protein
TRIF	TIR-domain-containing adapter protein inducing interferon IFN-β
T_{RM}	Tissue-resident memory T (cells)

S

SLO	Secondary lymphoid organs
ssRNA	Single stranded RNA

W

WHO	World Health Organization

LIST OF TABLES

CHAPTER TWO:

Materials and methods

Table 2.1 List of the experimental organisms and strains used in this study 51

Table 2.2 List of the immunisation reagents used in this study 51

Table 2.3 List of antibodies 54

Table 2.4 List of cell biology reagents and materials 55

Table 2.5 List of molecular biology reagents and materials 57

Table 2.6 List of specialised equipment 58

Table 2.7 List of software packages 58

Table 2.8 List of primers used for real-time PCR 67

CHAPTER THREE:

Characterization of the memory response induced by immunisation with an epitope from the ribosomal protein L6 (RPL6) of *P. berghei* ANKA.

Table 3.1 Amino acid sequence of the RPL6 protein 71

CHAPTER FOUR:

Effect of various nucleic acids as adjuvants for the liver TRM cell formation induced by Clec9A targeted immunisation

Table 4.1 Characteristics of the adjuvants tested during the first round of experiments 113

Table 4.2 Characteristics of the adjuvants that were complexed in transfection reagents 116

LIST OF FIGURES

CHAPTER ONE:

Literature review

Figure 1.1	Plasmodium life cycle within the mammalian host	9
Figure 1.2	Subtypes of memory T cells	28
Figure 1.3	Prime-and-trap vaccine	32
Figure 1.4	Immune potentiators	41

CHAPTER THREE:

Characterization of the memory response induced by immunisation with an epitope from the ribosomal protein L6 (RPL6) of *P. berghei* ANKA.

Figure 3.1	Effect of αClec9A-Pb-1 priming on the expansion of PbT-I cells in the spleen	76
Figure 3.2	Kinetics of the PbT-I cell expansion and memory formation after αClec9A-Pb-1 priming	78
Figure 3.3	Kinetics of CXCR6+ PbT-I cells in αClec9-Pb-1 primed mice	79
Figure 3.4	The naive Pb-1-specific CD8 T cell repertoire is very large	81
Figure 3.5	Expansion of Pb-1-specific CD8 T cells in response to blood stage and RAS infection	83
Figure 3.6	Immunisation with αClec9-Pb-1 mAb protects B6 mice against sporozoite challenge	85
Figure 3.7	Prime-and-trap-Pb-1 induced high numbers of PbT-I T_{RM} cells in the liver	88
Figure 3.8	Prime-and-trap-Pb-1 induced high numbers of T_{RM} cells in the liver of B6 mice with endogenous repertoire	90
Figure 3.9	Adverse effect of very high doses of rAAV-Pb-1 on the liver T_{RM} cell formation	93
Figure 3.10	Effect of high doses of rAAV-Pb-1 on the induction of protection in mice vaccinated with Prime-and-trap-Pb-1	95

Figure 3.11 Prime-and-trap-Pb-1 induces more than 90% protection in B6 mice against sporozoite challenge. 97
Figure 3.12 Delayed trapping with prime-and-trap-Pb-1. 99

CHAPTER FOUR:

Effect of various nucleic acids as adjuvants for the liver T_{RM} cell formation induced by Clec9A targeted immunisation

Figure 4.1 CpG-combo is a potent adjuvant for the induction of liver memory cells 115
Figure 4.2 RIG-I ligand and CpG 2006 also induce great numbers of memory cells in liver 119
Figure 4.3 Mice body weight after priming with high dose of adjuvants 121
Figure 4.4 High dose of CpG-2006 is very potent at inducing liver T_{RM} cell formation 123
Figure 4.5 CpG-ligands are the best at inducing T_{RM} cell formation 125
Figure 4.6 CpG-combo has no significant effect on the liver T_{RM} cell formation induced by RAS vaccination 127
Figure 4.7 DOTAP enhances the effect of the CpG-ligands on the formation of liver T_{RM} cells 129
Figure 4.8 DOTAP has an adverse effect on the formation of T_{RM} cells induced by RAS (preliminary results) 131
Figure 4.9 DOTAP has no significant effect on the sterile protection induced by prime-and-trap targeting the Pb-1 epitope 133
Figure 4.10 Adjuvants induce type I IFN production, preliminary experiment 136
Figure 4.11 Adjuvants induce type I IFN production; late control experiment 138

ABSTRACT

Tissue-resident memory CD8 T (T_{RM}) cells provide effective tissue surveillance and can respond rapidly to infection due to their strategic location. Within the liver, T_{RM} cells can induce effective protection against liver-stage *Plasmodium* infection. Recently, members from our group identified a highly immunogenic peptide (named Pb-1) within the putative 60S ribosomal protein L6 of *P. berghei* ANKA. Experiments conducted and presented in this thesis aimed to assess the suitability of Pb-1 for the induction of endogenous liver T_{RM} cells that confer sterilizing protection in B6 mice. To this end, a series of different immunisation strategies targeting the Pb-1 epitope were implemented and specific CD8 T cell responses were assessed. Results revealed that the number of naïve specific CD8 T cell precursors for the Pb-1 epitope was very large. Substantial expansion and formation of specific liver T_{RM} cells was achieved by two different immunisation strategies: i) Single injection with Clec9A mAb plus adjuvant and ii) Prime-and-trap, both targeting the Pb-1 epitope. While mice vaccinated with Clec9A mAb developed partial protection, almost all mice vaccinated with prime-and-trap targeting Pb-1 were sterily protected against liver-stage challenge.

Inflammation favours the formation T_{RM} cells and adjuvants can affect their numbers. Accordingly, a second focus of this thesis sought to investigate how to enhance liver T_{RM} cell formation by using TLR and RIG-I-like receptors agonists as adjuvants. For this, 8 different agonists were assessed for the generation of liver T_{RM} cells induced by Clec9A targeted immunisation with the Pb-1 epitope. Data from this screen showed that CpG-based adjuvants were most effective at inducing the formation of T_{RM} cells in the livers of vaccinated mice and that the transfection reagent DOTAP enhanced this effect. Based on this understanding, we then investigated the potential of CpG and its encapsulation in DOTAP to improve T_{RM} cell generation by other vaccination strategies. Surprisingly, these studies revealed that CpG-based adjuvants did not improve liver T_{RM} cell

generation by vaccination with radiation-attenuated sporozoites. The basis for this outcome is discussed.

Altogether, these findings provide insights into elements that favour the generation of protective liver T_{RM} cells; information that can be used for the design of T_{RM} cell-based subunit vaccines against *Plasmodium* infection.

DECLARATION

The work presented in this thesis was conducted at The Rheinische Friedrich-Wilhelms-Universität Bonn, in the laboratory of Dr. Winfried Barchet and at The University of Melbourne, in the laboratory of Prof. William Heath. This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Council) within the Bonn & Melbourne Research and Graduate School (Immunosciences, IRTG2168). Ana Maria Valencia Hernandez was supported by the Deutsche Forschungsgemeinschaft, The Medical Faculty of the The Rheinische Friedrich-Wilhelms-Universität Bonn, the Melbourne International Research Scholarship and grants from the National Health and Medical Research Council (NHMRC) of the Australian Government.

This is to certify that,

(i) the thesis comprises only my original work towards the PhD except where indicated in the preface;

(ii) due acknowledgement has been made in the text to all other material used;

(iii) the thesis is less than 100,000 word limit in length, exclusive of tables, maps, bibliographies and appendices as approved by the Research Higher Degrees Committee.

Melbourne, October 2020 Ana Maria Valencia Hernandez

PREFACE

My contribution to the experiments within each chapter was as follows:

Chapter 3: 90%
Chapter 4: 100%

I acknowledge the important contributions of these people:

Chapter 3:
Results of Figure 3.5 (**A.**) Dr. Nazanin Ghazanfari (5%) and (**B.**) Dr. Maria Menezes (5%).

Research declaration

Some of the content of Chapter 2 and Chapter 3 is also represented elsewhere in the reference below.

Valencia-Hernandez AM, Ng WY, Ghazanfari N, Ghilas S, de Menezes MN, Holz LE, et al. A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver TRM Cell-Mediated Immunity against Malaria in Mice. Cell Host Microbe 2020:1–13. doi:10.1016/j.chom.2020.04.010.

ACKNOWLEDGEMENTS

The work presented in this thesis was only possible with the support of several people, whom I would like to thank sincerely.

To begin, I would like to express my sincere gratitude to my supervisors Winfried Barchet, Bill Heath and Daniel Fernandez-Ruiz. To Winfried, thank you very much for giving me the opportunity to participate in this joint PhD program. Thanks for giving me the freedom to pursue new (sometimes crazy) ideas and for your assistance establishing new collaborations across campus and Germany and thank you for your support making a malaria lab within your nucleic-acid sensing lab, thank you. To Bill, thank you for your continuous support, for teaching me how to be clear about scientific arguments and for modelling my writing so I can deliver a clear message. To Daniel, thank you for all your help during the transition times, for showing me with patience how everything works in the lab and for your optimism. Beside my supervisors, I would like to thank the members of my PhD thesis committee, Profs. Thomas Gebhardt and Achim Hörauf.

My special gratitude goes to all our collaborators. Special thanks to Ann-Kristin Müller and Roland Frank for providing malaria-infected mosquitoes in Germany. My gratitude to Dr. Stephan Garbe in the department for clinical radiation at the University Hospital Bonn and to the research group of Profs. Irene Caminschi, Mireille Lahoud, Nicole La Gruta, Patrick Bertolino, Geoff McFadden for providing essential training and reagents.

I would also like to thank present and former members of the AG Barchet group, Heath lab, Mackay lab, Mueller lab. It was a great pleasure working in such a supportive environment. I am particular thankful to Patrick Müller for all your help with my project. I would also like to acknowledge all the members of the animal and flow cytometry facility from Melbourne and Bonn.

I would like to thank the Bonn and Melbourne IRTG2168 coordination offices. Thanks for your continuous support throughout the past years.

I would also like to thank my fellow IRTG2168 students for all your support, the great trips, the fun at parties and friendship. During this journey I encountered numberless challenges and even when I was feeling that I had to face them all by myself, incredibly warm people were always there giving me emotional support and true friendship. To Fabian, Fernando, Gordon, Kasia, Leo, Maria, Max, Natalia, Raissa, Santi (the worst), Sonia, Svencito, I tressure them very deeply. Special thanks to my dearest friend Gayathri for being there always willing to listen in a non-judgemental way and for always knowing what to say when I needed it.

Este trabajo no seria posible sin el apoyo de mi familia. Gracias a mis papas y a mi hermano por su apoyo incondicional. Infinitas gracias por sus palabras de aliento en momentos difíciles. Por la dedicación y la guía continua. Por las risas y los viajes interminables en avión. Dedico este trabajo a lo mucho que los extraño y los amo.

Por último, ¡pero por encima de todo GRACIAS a DIOS!, gracias por las oportunidades y por las fuerzas para terminar este proyecto.

LIST OF PUBLICATIONS

1. Ghilas S., **Valencia-Hernandez, A. M.**, Enders, M. H., Heath, W. R & Fernandez-Ruiz, D. (2020). Resident memory T cells and their role within the liver. *International Journal of Molecular Sciences*, 1-15. https://doi.org/10.3390/ijms21228565

2. **Valencia-Hernandez, A. M.**, Ng, W. Y., Ghazanfari, N., Ghilas, S., de Menezes, M. N., Holz, L. E., Huang, C., English, K., Naung, M., Tan, P. S., Tullett, K. M., Steiner, T. M., Enders, M. H., Beattie, L., Chua, Y. C., Jones, C. M., Cozijnsen, A., Mollard, V., Cai, Y., … Fernandez-Ruiz, D. (2020). A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver TRM Cell-Mediated Immunity against Malaria in Mice. *Cell Host & Microbe*, 1–13. https://doi.org/10.1016/j.chom.2020.04.010

3. Holz, L. E., Chua, Y. C., de Menezes, M. N., Anderson, R. J., Draper, S. L., Compton, B. J., Chan, S. T. S., Mathew, J., Li, J., Kedzierski, L., Wang, Z., Beattie, L., Enders, M. H., Ghilas, S., May, R., Steiner, T. M., Lange, J., Fernandez-Ruiz, D., **Valencia-Hernandez, A. M**., … Heath, W. R. (2020). Glycolipid-peptide vaccination induces liver-resident memory CD8+ T cells that protect against rodent malaria. *Science Immunology*, *5*(48), 1–14. https://doi.org/10.1126/sciimmunol.aaz8035.

4. Masic, A., **Valencia-Hernandez, A. M**., Hazra, S., Glaser, J., Holzgrabe, U., Hazra, B., & Schurigt, U. (2015). Cinnamic acid bornyl ester derivatives from valeriana wallichii exhibit antileishmanial in vivo activity in leishmania major-infected BALB/c Mice. *PLoS ONE*, *10*(11), 1–20. https://doi.org/10.1371/journal.pone.0142386

CHAPTER 1

Literature review

1.1 Introduction:

Malaria is a disease caused by protozoan parasites of the Plasmodium spp. Six plasmodial species infect humans, and *Plasmodium falciparum* and *P. vivax* are responsible for the largest number of malaria cases (Cowman et al., 2016; Phillips et al., 2017; White et al., 2014). Over the past years, the international community have undertaken great efforts to prevent, control and eliminate malaria. In 2016, the World Health Organization (WHO) reported that 57 countries reduced their malaria cases by 75% compared to 2000. For the African region, the location with the highest burden worldwide, mortality fell 66% among all age groups and by 71% among children under five; the group with the highest mortality (WHO, 2016). Insecticide-treated bed nets, antimalarial medication and indoor residual insecticide spraying, were the three scaled-up interventions that effectively reduced malaria burden (WHO, 2016).

Additionally, in 2016, the WHO and other international partners established a target for 2030 to achieve at least 90% reduction in global malaria mortality and cases compared to 2015. Despite these encouraging results, the WHO revealed in 2019 that there was no significant reduction on the global malaria cases from 2015-2017. On the contrary, high endemic areas reported a growing number of cases for the same period of time, which translated to an estimated 217 and 219 million cases in 2016 and 2017, respectively (WHO, 2018). Thus, there is an urgent need for strategies that contribute to the reduction of malaria cases. Besides the current strategies to accomplish the objectives towards a malaria free-world, the international community have focused their funding into five research areas: drugs, basic research, vaccines, vector control and diagnostics, in that order of priority (WHO, 2018).

The literature review of this thesis is focused on vaccine development, especially those strategies that target the liver-stage of the infection. In the first part of this chapter, general information about the life cycle of the parasite,

pathogenesis and how humans acquired immunity in endemic areas will be revised.

1.2 Biology of the malaria disease:

Plasmodium parasites have a complex life cycle characterized by alternation within two different hosts; female *Anopheles* mosquitoes and vertebrate hosts. Thus, parasites undergo a series of substantial cellular differentiations that allows them to survive in different organs, temperatures and environments within those hosts.

Malaria transmission and epidemiology relies greatly on the vector fitness. Longevity, density and resilience to environmental challenges determine the efficiency of the transmission by *Anopheles spp.* (Smith and McKenzie, 2004). *A. gambiae* is a robust and efficient vector distributed in sub-Saharan Africa, where *P. falciparum* predominates (Gething et al., 2011; Snow et al., 2005). In this high endemic region, infectious bites can reach 1000 per year. Morbidity and mortality are prevalent in children > 5 years, and clinical immunity to malaria can be developed during adulthood (Dondorp et al., 2008; Von Seidlein et al., 2012).

During the blood meal of an infected mosquito, sporozoites – the motile form of the parasite – are injected into the skin of the mammalian host. After they reach the bloodstream and infect hepatocytes, parasites are released to the blood stream once again where they replicate asexually. Sexual forms, which are able to infect mosquitoes later develop from blood stage parasites and the cycle is complete. The life cycle of the parasite is depicted in Figure 1.1.

1.2.1 Malaria liver stage

Experimental murine malaria infection starts with a blood meal of a malaria infected female *Anopheles* mosquito during which approximately 50-100 highly

motile sporozoites are inoculated into the skin of the murine host (Kebaier et al., 2009). Within a couple of hours, approximately half of the parasites glide out of the bite site and invade dermal blood or lymphatic vessels (Fig. 1.1) (Amino et al., 2006; Kebaier et al., 2009; Sidjanski and Vanderberg, 1997). The sporozoites that remain at the bite site beyond 3 hours have a decreased motility and are eventually destroyed by host factors (Amino et al., 2006; Kebaier et al., 2009). Trap-like protein (TLP), highly expressed by sporozoites (Howick et al., 2019; Moreira et al., 2008), has been found to be important during migration from the skin to the blood vessels, as mutant sporozoites fail to traverse epithelial cells *in vitro* and have lower infectivity *in vivo* (Moreira et al., 2008). Once in the blood stream, sporozoites can rapidly access the liver to stablish infection (Yamauchi Lucy M. et al., 2007).

Before infecting hepatocytes, sporozoites cross the liver sinusoidal barrier by traversing through fenestrated endothelial cells and Kupffer cells (KC) (Tavares et al., 2013). Cell traversal activity involves breaching the host cell plasma membrane, gliding through the cytosol forming transient vacuoles and exiting the host cell again (Mota et al., 2001; Risco-Castillo et al., 2015). It has been recently demonstrated that GAPDH, a protein highly expressed across different stages of the *Plasmodium* life cycle, including sporozoites (Howick et al., 2019; Lindner et al., 2013), interacts with the KC receptor CD68 and promotes cell traversal (Cha et al., 2016). Cell-passage alters the plasma membrane integrity and can lead to cell death, preventing clearance by the KC, which might be exploited as a mechanism of immune evasion (Tavares et al., 2013). In fact, antibodies blocking *Plasmodium* GAPDH reduces hepatocyte invasion by sporozoites (Cha et al., 2016). Some other proteins involved in cell traversal include the sporozoite microneme protein essential for traversal (SPECT), SPECT2 and the cell traversal protein for ookinetes and sporozoites (CelTOS). SPECT is expressed by liver-infective sporozoites and it is required for the cell-passage through the liver sinusoidal cell layer (Ishino et al., 2004). SPECT2 is important for the egress of sporozoites from the transient vacuoles, which is thought to be associated with the presence of a pore forming complex domain

within the protein (Risco-Castillo et al., 2015). CelTOS is produced by liver-infective sporozoites, important for cell-passage activity *in vitro* and hepatocyte invasion *in vivo* (Kariu et al., 2006). Sporozoites can also traverse multiple hepatocytes before invading the final hepatocyte where they further develop.

During the last few decades, several molecular mechanisms involved in the host-parasite interactions during hepatocyte invasion have been described. Here, some of them will be discussed. Sporozoites are densely coated with several copies of the circumsporozoite protein (CSP) which is highly expressed by sporozoites and liver-stage parasites and is required for binding to highly sulphated forms of the heparan sulphate proteoglycans (HSPG) on the surface of hepatocytes (Herrera et al., 2015; Pinzon-Ortiz et al., 2001). After sporozoite binding to HSPG, CSP is cleaved by the parasite kinase CDPK-6, a step thought to be involved in a switch from migration to invasion mode of the sporozoite (Coppi et al., 2011, 2007). The thrombospondin-related anonymous protein (TRAP) is a transmembrane protein expressed by liver-infective sporozoites. It also binds HSPG and it is important for gliding motility *in vitro* and hepatocyte invasion *in vivo* (Kappe et al., 1999; Sultan et al., 1997). TRAP requires specific cleavage by rhomboid proteases to ensure the active invasion of hepatocytes by sporozoites (Baker et al., 2006; Ejigiri et al., 2012). The apical membrane antigen-1 (AMA-1) is known as a merozoite protein important for erythrocyte invasion (Anders et al., 1998; Thomas et al., 1984; Triglia et al., 2000). Additionally, AMA-1 is also expressed by sporozoites and while it seems to be dispensable for *P. berghei* liver infection (Giovannini et al., 2011), blocking antibodies can reduce hepatocyte invasion by *P. falciparum* sporozoites (Silvie et al., 2004; Yang et al., 2017).

A major component of the hepatocyte infection is the establishment of the parasitophorous vacuole membrane (PVM) of hepatocyte origin that sustain the further development of the parasite inside the host cell (Meis et al., 1983). The PVM not only provides a barrier between the developing parasite and the host cell cytoplasm but also mediates acquisition of nutrients. Some of the parasitic

proteins involved in the establishment of the PVM include P52 and P36 (Ishino et al., 2005; Labaied et al., 2007; van Schaijk et al., 2008). From the host side, three proteins expressed by hepatocytes have been found to play a role in cell invasion and the formation of the PVM. Transgenic mice and human cell lines lacking the scavenger receptor BI and the tetraspanin CD81, as well as the use of blocking antibodies can reduce sporozoite cell infection and affect the establishment of the PVM (Rodrigues et al., 2008; Silvie et al., 2007, 2003). Furthermore, antibodies binding the hepatocytic transmembrane receptor Ephrin 2 (EphA2) reduce the formation of the PVM and the subsequent development within the hepatocyte (Kaushansky et al., 2015). Evidence suggests that the *Plasmodium* protein P36 interacts with EphA2 (Kaushansky et al., 2015).

Once sporozoites have established hepatocyte invasion and reside within the PV, the PVM is extensively modified and parasites began to dedifferentiate into liver trophozoites (Jayabalasingham et al., 2010). Some of the proteins involved in the PVM modification include UIS3 and UIS4. They are highly transcribed by salivary sporozoites and translated during *Plasmodium* liver-stage development (Howick et al., 2019; Kaiser et al., 2004). Indeed, deletion of either UIS3 or UIS4 leads to early liver stage arrest but has no effect in the initial sporozoite invasion (Jobe et al., 2007; Mueller et al., 2007, 2005). Another protein present on the PVM is Hep17 in rodent malaria, also known as exported protein 1 (EXP1) in *P. falciparum* (Charoenvit et al., 1995; Doolan et al., 1996). This protein is not only expressed by schizonts and trophozoites during blood stage development but also by merozoites and liver stage parasites (Charoenvit et al., 1995; Howick et al., 2019). Defective parasites fail to develop intracellularly and it has been shown that EXP1 is important for nutrient intake (Mesén-Ramírez et al., 2019).

As liver trophozoites mature, they differentiate into schizonts containing merozoites (Fig. 1.1). PVM-associated proteins expressed during late liver stage development include liver specific protein 1 (LISP1) and LISP2 (Ishino et al., 2009; Orito et al., 2013). LISP1 is dispensable for blood stage and mosquito

development and it is required for PVM rupture of mature schizonts and the release of merozoites (Ishino et al., 2009). LISP2 can be exported to the host cytoplasm through the PVM and is important for merozoite development, as mutants have a reduce capacity to invade erythrocytes (Orito et al., 2013). It has been estimated in mice that up to 30,000 merozoites are released from every schizont into the bloodstream (Baer et al., 2007). The liver stage is usually asymptomatic, lasting approximately 2 days in mice and about 7 to 10 days in humans (Sturm et al., 2006). During *P. vivax* and *P. ovale* infections, parasites can remain in the liver for months or years in a dormant state (called the hypnozoite) that can cause relapses (Krotoski et al., 1982; Markus, 2011; Mikolajczak et al., 2015; Phillips et al., 2017; Soulard et al., 2015).

It is important to consider that during *Plasmodium* development, parasites display different sets of proteins at different stages of infection (Howick et al., 2019). Some of the proteins are stage-specific while others are expressed at multiple stages of the parasite development (Franke-Fayard et al., 2004; Koning-Ward et al., 1999). As a consequence of differences in temporal protein expression, antigens derived from individual proteins will also be subject to temporally regulated presentation. In this sense, the kinetics of presentation of each antigen is a dynamic process. Some antigens are only expressed by sporozoites, and as a consequence these may be presented shortly after hepatocyte invasion, but their presentation may wane as the antigen source is depleted. Other antigens, for example those derived from proteins only expressed by liver-stage parasites, might not be presented immediately upon sporozoite entry into hepatocytes as some time will be needed for protein synthesis. Presentation of this type of protein may thus be limited to the mid to late stages of liver-stage development. Blood-stage proteins will be expressed by merozoites as they develop at the end of the liver stage and might therefore represent antigens presented late in the liver stage. Finally, other proteins constitutively expressed in multiple stages might be presented both early and later during the liver stage infection. Thus, T cell control of liver-stage infection may be shaped not only by antigen expression levels, but by the kinetics of

antigen presentation for proteins expressed at different stages of the pre-erythrocytic development.

1.2.2 Malaria blood stage

During the blood stage, parasite replication inside red blood cells (RBC) is extreme, cell death is abundant, and symptoms begin to occur. Merozoite invasion of RBC is a fast three-step process – involving pre-invasion, internalization and echinocytosis (Weiss et al., 2015) – that happens in approximately 2 minutes. The pre-invasion step is induced by low affinity protein interactions and the second step is mediated by several specific ligand-receptor interactions of molecules expressed on the surface of merozoites and host erythrocytes (Paul et al., 2015; Weiss et al., 2015). The echinocytosis occurs after internalization, when newly infected RBC (iRBC) shrink and adopt a shape with spikes.

Within erythrocytes, parasites remain in a parasitophorous vacuole and export a large number of effector proteins into the host cell cytoplasm (Charpian and Przyborski, 2008; Maier et al., 2009; Tilley et al., 2008). These molecules modulate parasite nutrient intake and cytoadherence of the host cell. Cell adhesion allows *P. falciparum*-infected erythrocytes to sequester in major organs, preventing circulation to the spleen, increasing the adhesion to other erythrocytes, and potentially leading to blood flow obstruction linked to severe clinical outcomes (Boddey and Cowman, 2013; Spillman et al., 2015). Malaria blood stage is a cyclic process of four differentiation steps (Fig. 1.1). During the first 5 h, the invading merozoite form a ring and start exporting proteins. From 10 to 40 h, parasites develop in trophozoites characterized by the enlargement of the food vacuole and maximized hemoglobin consumption. Next, parasites undergo nuclear division forming the schizont (40–48 h). Lastly, the host cell membrane collapses, realizing between 16 and 32 *P. falciparum* merozoites per infected erythrocyte and the cycle starts again (Boddey and Cowman, 2013; Spillman et al., 2015). In humans, after a week of asexual replication, when the

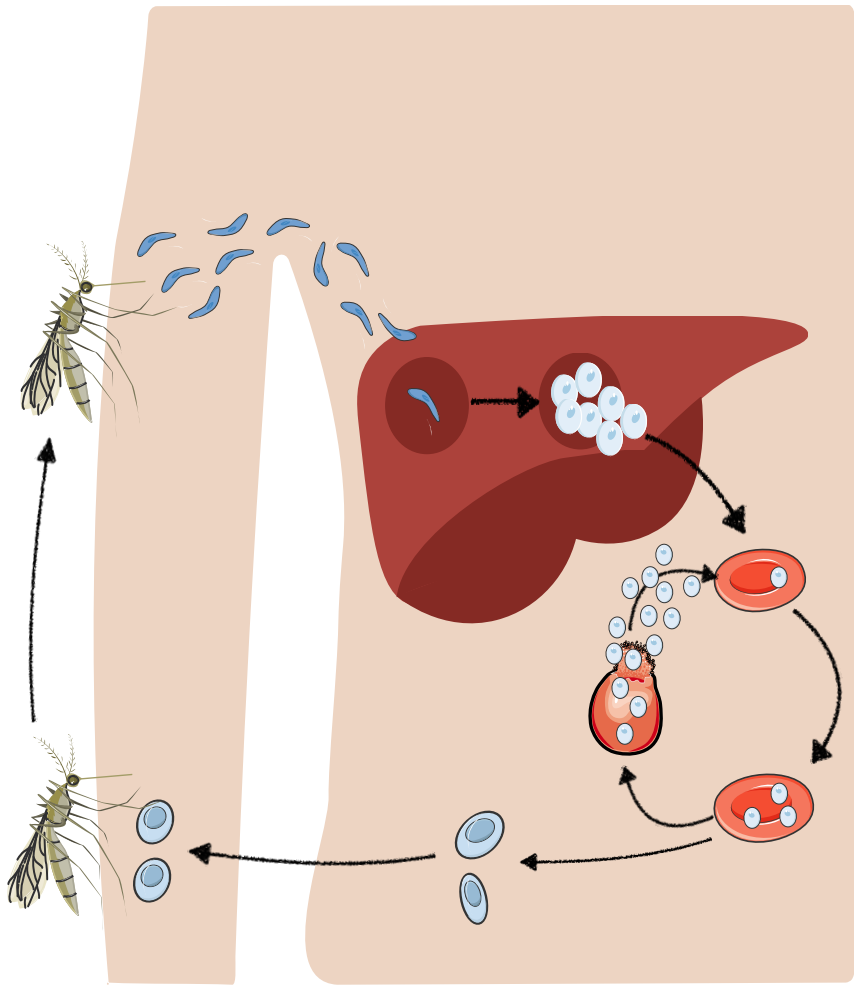

Figure 1.1. Plasmodium life cycle within the mammalian host. During the blood meal of a female infected mosquito, sporozoites are injected into the skin of the mammalian host. Within a few hours sporozoites reach the bloodstream and migrate to the liver. Before infecting hepatocytes, sporozoites cross the liver sinusoidal barrier by traversing through endothelial cells and KC (Tavares et al., 2013). Only a few sporozoites establish infection within hepatocytes, where they mature into schizonts producing merozoites that are then released into the bloodstream. The liver stage is usually asymptomatic, lasting approximately 2 days in mice and about 7 to 10 days in humans (Cowman et al., 2016). During blood stage infection, merozoites replicate asexually inside erythrocytes in a cyclic manner realising more merozoites each time with the capacity to infect other RBC. In humans, after a week of asexual replication, when the number of parasites in the blood is high cell death is abundant, and symptoms begin to occur. Eventually, some merozoites undergo a special differentiation step to become gametocytes with the capacity to infect mosquitoes. Within the mosquito gut, haploid gametes fuse to form zygotes (Sinden, 1983), which develop into oocysts, that produce sporozoites. Then they migrate to the salivary glands of the mosquito and get injected into the mammalian host during a blood meal to complete the cycle.

number of parasites in the blood stream reaches around 100 million, it is possible to detect these parasites by microscopy of blood smears and the symptoms begin to occur (Simpson et al., 2002).

Some merozoites undergo a special differentiation step to become gametocytes, a longer-lived sexual form with the capacity to infect mosquitoes (Fig. 1.1). Within the mosquito gut, haploid male and female gametes fuse to generate zygotes (Sinden, 1983), that develop into oocysts, which in turn divide asexually (Vlachou et al., 2006). Oocysts then release sporozoites that migrate to the salivary glands to start the transmission cycle again (Josling and Llinás, 2015).

1.3 Pathogenesis:

Uncomplicated malaria causes fever, mild anemia and jaundice, which is more common in adults, while an enlarged spleen and liver is more common in infants (Bartoloni and Zammarchi, 2012). Episodes of high fever have been associated with the systemic release of pro-inflammatory cytokines including tumour necrosis factor alpha (TNFα) (Karunaweera et al., 1992; Wijesekera et al., 1996). Hemozoin, a detoxification product derived from the lysis of the haemoglobin, is accumulated in the parasite food vacuole inside infected erythrocytes (Coronado et al., 2014). One study revealed that after erythrocytes rupture releasing several merozoites, the hemozoin is up taken by myeloid cells, inducing the production of TNF-α in a toll-like receptor 9 (TLR9)-dependent manner (Coban et al., 2005).

1.3.1 Severe malaria

Complicated malaria cases and mortality depend on age and transmission intensity. Most deaths in sub-Saharan Africa happen in children under 5 years of age where transmission is high. Severe malaria may develop in non-treated or

under-treated malaria and is mainly caused by extensive sequestration of infected erythrocytes in capillaries of vital organs. Four different syndromes are the main manifestation of severe malaria, cerebral malaria, severe anemia, respiratory distress and acidosis (MacKintosh et al., 2004; Wassmer et al., 2015).

Cerebral malaria can cause coma and although the precise pathogenic mechanisms remain to be understood, several studies suggest that the sequestration of iRBC is associated with limited blood flow through the brain capillaries and coma (MacPherson et al., 1985; Pongponratn et al., 2003; Silamut et al., 1999). Additionally, autopsy reports have demonstrated substantial accumulation of platelets in microvasculature of brains from children that died from cerebral malaria (Dorovini-Zis et al., 2011; Grau et al., 2003; Hochman et al., 2013). Blood-brain barrier disruption and brain swelling have also been implicated in the pathogenesis of human cerebral malaria (Dorovini-Zis et al., 2011; Greiner et al., 2015; Seydel et al., 2015). Based on murine studies, a simplified model has been suggested to explain the mechanisms underlying cerebral malaria (Ghazanfari et al., 2018). In this model, sequestration of iRBC and accumulation of platelets in the brain vasculature occurs first. This process is amplified by the intravascular accumulation of monocytes, neutrophils, T cells and natural killer (NK) cells in brains of mice with experimental cerebral malaria (ECM) (Belnoue et al., 2008, 2002; Hansen et al., 2007). This accumulation increases blood pressure and the risk of blood-brain barrier breakdown, leading to edema and swelling of the brain (Ghazanfari et al., 2018).

Severe anemia is mainly developed by infants and is caused by overlapping processes including excessive splenic removal of uninfected RBC (Buffet et al., 2011), lysis of iRBC by schizogony disruption (Perkins et al., 2011; Price et al., 2001), and ineffective erythropoiesis (Dörmer et al., 1983; Phillips et al., 1986). Respiratory distress is particularly present in pregnant woman infected with *P. falciparum* or *P. vivax* (Nosten et al., 1999; Taylor et al., 2012). It has been suggested that pulmonary edema may be mediated by inflammation driven by endothelial and cytoadherence damage (Charoenpan et al., 1990; Corbett et al.,

1989; Pongponratn et al., 2003). Metabolic acidosis develops from the excessive production of organic acids, such as lactic acid, that result from anaerobic glycolysis in tissues and organs with low supply of blood and oxygen (Maitland and Newton, 2005). Patients with acidic breathing have poor prognosis, as at this stage acidosis is suggestive of major organ failure (Hanson et al., 2010; Marsh et al., 1995).

1.4 Naturally acquired immunity:

Despite decades of efforts to control malaria, it still is a major public health concern, which can damage economic development in many disadvantaged communities. Aspects that limit the progress in the field are: (i) the development of parasite resistance to almost all antimalarial drugs, (ii) the mosquito resistance to vector insecticides and the impracticality of those interventions for application in certain regions lacking economic support, (iii) the social instability in war and conflict areas where the health system is fragile, (iv) and failure to develop an efficient vaccine.

The complex life cycle of the parasite and the variety of clinical manifestations of the disease in the host, represent a major challenge to understand how people in endemic regions become protected against severe malaria, and hence, how to develop effective vaccine strategies. It has been suggested that two major factors contribute to the development of naturally acquired immunity, the age of the host and the parasite transmission intensity (Doolan et al., 2009).

Virtually all naïve individuals of all ages develop clinical malaria at relatively low levels of parasitemia (numbers of circulating parasites) during the first infection (Doolan et al., 2009; Langhorne et al., 2008). In the case of *P. falciparum*, naturally acquired immunity to clinical malaria develops with age in countries with high parasite transmission. Remarkably, children under 6

months of age are protected from severe malaria through inhibitory factors found in the breast milk (Kassim et al., 2000), and the passive transfer of maternal immunoglobulin (Ig) G before birth (Hviid and Staalsoe, 2004). Morbidity and mortality increases after 6 months of age and cerebral malaria peaks in infants ranging from 2 to 4 years of age and is usually associated with high levels of parasitemia (Okiro et al., 2009; Reyburn et al., 2005). Beyond 5 years of age, the risk of severe malaria begins to diminish although parasitemia levels may still be high. During adolescence, clinical disease frequency decreases, deaths caused by malaria are rare and the average number of circulating parasites decreases as well (Carneiro et al., 2010; Griffin et al., 2015; Roca-Feltrer et al., 2010). Adults become asymptomatic carriers with low parasitemia levels (Doolan et al., 2009; Marsh and Kinyanjui, 2006). In adults, naturally acquired immunity protects against clinical malaria by maintaining low parasitemia levels, helping to prevent the onset of severe disease (Baird et al., 2003; Owusu-Agyei et al., 2001). Sterilizing immunity, i.e. no blood stage parasites, never develops and clinical immunity is not long lasting (Doolan et al., 2009; Keegan and Dushoff, 2013). Importantly, studies on communities migrating from low to hyper-endemic areas revealed that adults developed naturally acquired immunity more rapidly than children (Baird, 1995; Baird et al., 1998), suggesting that immunity depends on intrinsic factors related to age, like immune system maturity (Kurtis et al., 2001).

The intensity of parasite transmission also influences the development of acquired immunity. In areas of low to intermediate transmission, the acquisition of clinical immunity is delayed (Carneiro et al., 2010; Marsh and Kinyanjui, 2006). Children living in these areas remain at a constant risk of severe malaria even after 5 years of age (Carneiro et al., 2010; Mbogo et al., 1995, 1993; Rodriguez-Barraquer et al., 2018; Snow et al., 1997). Some adults may develop severe malaria in rare cases when transmission intensity transitions from high to extremely high (Doolan et al., 2009; Marsh and Kinyanjui, 2006).

The exact immunological mechanisms involved in the development of naturally acquired immunity remain to be elucidated. Many studies have reported

a correlation between IgG antibodies (Ab) against blood-stage parasites and the reduced risk of severe malaria (Dodoo et al., 2001; Magistrado et al., 2007; Ofori et al., 2002; Yone et al., 2005). Ab against sporozoites, the liver-stage parasitic form, seem to have a limited or negligible role in naturally acquired immunity (Hoffman et al., 1987; Schofield et al., 1987). In fact, immune adults from highly endemic areas were able to control parasitemia and avoid symptoms after direct injection of blood-stage parasites (Bruce-chwatt, 1963; Langhorne et al., 2008).

1.5 Malaria vaccines:

As mentioned previously, exceptional progress has been made to control malaria and a substantial reduction in mortality has been achieved in endemic countries. The scale up of interventions such as the distribution of antimalarial drugs, insecticide-treated bed nets, and indoor insecticide spraying, are the major contributors to this progress (WHO, 2016). However, emerging resistance to both insecticides and antimalarial medications have stalled the mortality reduction and threaten the sustainability of these interventions (WHO, 2018). Thus, the development of a highly efficacious vaccine is urgent to complement the current malaria elimination efforts.

Despite decades of intense research, a highly effective malaria vaccine for application in endemic areas remains elusive (Coelho et al., 2017; Draper et al., 2018). Currently, a wide range of vaccine approaches are in pre-clinical development and clinical trials. These strategies target different stages of the *Plasmodium*-life cycle, including pre-erythrocytic (PE) forms, blood-stage parasites and gametocytes.

1.5.1 Blood-stage vaccines:

The rationale behind this type of vaccines is that they aim at triggering a similar immune response induced by naturally acquired immunity. These strategies face several challenges related to immune evasion mechanisms, such

as antigenic variation of both free merozoites as well as in the surface proteins of infected erythrocytes (Ferreira et al., 2004).

While subunit approaches targeting merozoite derived antigens have failed to confer protection (Miura, 2016), whole parasite strategies have conferred immunity in murine and primate models (Good et al., 2013; Raja et al., 2016). In these studies, blood-stage parasites were chemically attenuated by incubation with compounds that irreversibly alkylates the DNA of the parasite (Good et al., 2013). Immunised mice developed long-lasting protection against other strains and *Plasmodium* and it was mediated by CD4 T cells (Good et al., 2013; Raja et al., 2017, 2016). Promising results in *Aotus* monkeys using chemically attenuated blood-stage *P. falciparum* (De et al., 2016) prompted the implementation of a pilot experiment in malaria-naïve volunteers. Preliminary results revealed that the vaccine is safe, well tolerated, and that it induces the production of homologous and heterologous antibodies as detected in the sera of participants (Stanisic et al., 2018).

In addition to the whole parasite approach, recent studies on the novel antigen *P. falciparum* RH5 have demonstrated its capacity to induce neutralizing Ab that block merozoite invasion *in vitro* (Douglas et al., 2015, 2011; Illingworth et al., 2019). The production of neutralizing Ab was also observed *in vivo* in studies with *Aotus* monkeys (Hjerrild et al., 2016). Now this protein is being targeted in phase I and II clinical trials. The expression of the RH5 protein has proven to be particularly difficult and therefore a different approach has been taken to successfully target it. For this, a replication-deficient chimpanzee adenovirus (ChAd) and an attenuated modified vaccinia virus Ankara (MVA) have been engineered to encode the RH5 *P. falciparum* protein sequence (Payne et al., 2017). Preliminary results in malaria naïve volunteers confirmed the induction of RH5 Ab, B cells and T cell responses, and some degree of growth inhibition *in vitro* mediated by Ab found in the sera of vaccinated volunteers (Payne et al., 2017).

1.5.2 Transmission-blocking vaccines:

Vaccines that aim at inducing immunity against parasite stages that infect mosquitoes, e.g. gametocytes, are known as transmission-blocking vaccines. Less progress has been made in this field but there are some antigens that have been subjected to investigation in the recent years. In animal models, epitopes of the Pfs25 surface protein expressed in mosquito stage of *P. falciparum* have demonstrated capacity to induce Ab with transmission blocking activity (Miura et al., 2007). In preliminary human trials, soluble Pfs25 has proved to be poorly immunogenic and in formulation with the adjuvant Montanide1 ISA 51, induced unexpected adverse events (Wu et al., 2008). During a second effort, Pfs25 was conjugated to the carrier protein ExoProtein A from *Pseudomonas aeruginosa* and formulated together with the adjuvant Alhydrogel® (Radtke et al., 2017; Talaat et al., 2016). While this vaccine was well tolerated, functional activity of Ab in serum was only achieved after four vaccine doses and Ab titres waned rapidly after each dose (Talaat et al., 2016).

1.5.3 Pre-erythrocytic (PE) vaccines:

PE vaccine strategies are directed to induce immune responses against sporozoites prior to hepatocyte invasion and liver-stage parasites before being released to the blood stream. These vaccine strategies have priority as they i) avoid the occurrence of disease symptoms, ii) stop the development of clinical malaria and iii) block transmission. Prevention of egress to the blood-stage is called sterilizing immunity.

Immunization against PE stages has been investigated since the 1960s in murine models (Nussenzweig et al., 1967) and 1975 when Clyde and collaborators achieved protection in human volunteers that were previously immunized by bites from irradiated mosquitoes (Clyde 1990). At that time, vaccination was only possible via mosquito bites, which represented a major obstacle for the design and implementation of safer approaches. During the

1980s, efforts switched to a more suitable and safer approach based on the production of recombinant proteins derived from sporozoites, for use as subunit vaccines.

To date, the most advanced sub-unit vaccine candidate for *P. falciparum* sporozoites is the RTS,S/AS01. In fact, a pilot implementation program for this vaccine is currently being undertaken in Ghana, Kenya and Malawi (Adepoju, 2019). This vaccine targets the CSP of *P. falciparum* (PfCSP) and is a virus-like particle with several copies of the C-terminal domain of PfCSP fused to hepatitis B virus surface antigen and a potent liposomal adjuvant called AS01 (RTSS Clinical Trials Partnership, 2015). The RTS,S/AS01 vaccine demonstrated partial clinical immunity during a phase III clinical study performed in seven countries of sub-Saharan Africa (RTSS Clinical Trials Partnership, 2015). For a period of two years, children (5-17 months age) and young infants (6-12 weeks age) were vaccinated with 3 doses of RTS,S/AS01 or control vaccine. In children, vaccine efficacy against clinical and severe malaria was 36% and 32%, respectively; while in young infants it only reached 25% and 17%. Importantly, results also indicated that the protection wanes rapidly over time (Olotu et al., 2013). In fact, vaccine efficacy after 7 years, in a small cohort of children enrolled in the phase II, was only 4.4% (Olotu et al., 2016). Antibodies against the CSP protein have been implicated as the potential mechanism mediating the immunity induced by the RTS,S/AS01 vaccine (Oyen et al., 2017; White et al., 2015).

Other PE vaccines are based on administration of whole attenuated sporozoites and have demonstrated high efficacy in mice and humans. These strategies include immunisation with radiation-attenuated sporozoites, genetically attenuated parasites and infection with living sporozoites in conjunction with chemoprophylaxis.

1.5.4 Radiation-attenuated sporozoites (RAS) based vaccines:

Early studies in mice and humans demonstrated the efficacy of RAS to confer protection through mosquito bites (Clyde, 1990; Nussenzweig et al., 1967). Since then, substantial efforts have been made to develop a safe product that can be used in clinical trials. The PfSPZ vaccine (*P. falciparum* sporozoite) are sporozoites manually isolated from the salivary glands of infected and irradiated mosquitoes, aseptic and cryopreserved (Hoffman et al., 2010).

In 2013, Seder and collaborators demonstrated that whereas four doses protected 33% of the volunteers, five doses of 1.35 x 10^5 PfSPZ protected 100% of the subjects. They also showed that high doses of RAS enhanced protection since 55% of individuals vaccinated with four doses of 2.7 x 10^7 PfSPZ remained protected after 21 weeks of vaccination (Seder et al. 2013). These results suggest that there is a critical threshold of RAS numbers required to achieve effective immunity. The researchers also showed that immunity was correlated with Ab titres and specific T cell responses (Seder et al. 2013). Moreover, the PfSPZ vaccine must be administered intravenously as other routes have limited immunogenicity (Ishizuka et al., 2016). While these studies demonstrated substantial protection in malaria-naïve volunteers, RAS vaccination demonstrated limited efficacy in malaria-exposed subjects from Mali and Equatorial Guinea (Olotu et al., 2018; Sissoko et al., 2017). Two major factors may influence the immunity mediated by RAS vaccination in malaria-exposed subjects. Firstly, malaria-naïve volunteers were protected against homologous challenge, while subjects from Mali and Equatorial Guinea were exposed to naturally transmitted heterologous parasites (Epstein et al., 2017). Second, some studies support the idea that pre-existing blood-stage infections are able to induce immune suppression, thus affecting the establishment of appropriate immune responses against sporozoites (Rénia and Goh, 2016).

1.5.5 Genetically-attenuated parasites (GAP) based vaccines:

Contrary to RAS, genetically-attenuated parasites arrest at specific stages of the pre-erythrocytic development. It has been demonstrated in murine models that late arrest of liver-stage parasites potentiates protection by enhancing the breadth of the immune response (Butler et al., 2011). Pilot experiments in humans have demonstrated that administration of a triple knock out (KO) parasite (known as PfGAP3KO) through mosquito bites was safe and able to induce the production of inhibitory Ab to sporozoites found in the sera of vaccinated volunteers (Kublin et al., 2017). The PfGAP3KO parasite lacks the *P. falciparum p52*, *p36* and *sap1* genes which induces early pre-erythrocytic arrest (Kublin et al., 2017; Mikolajczak et al., 2014). Engineering human parasites that arrest late during liver-stage development has been difficult because these parasites often become unviable in the mosquito. Moreover, there are safety concerns related to breakthrough infections with GAP that could lead to blood stage infections. However, substantial efforts are currently being made to provide such vaccine (Vaughan et al., 2018)

1.5.6 Chemoprophylaxis vaccination (cVac):

These strategies involve infections with susceptible live wild type sporozoites under host drug treatment, which eliminates blood stage parasites. This approach is considered one of the most potent strategies since it allows full liver development – increasing the spectrum of antigens that the host is exposed to. In fact, murine studies have demonstrated the superior capacity of cVac to induce protection, since fewer and lower doses were required to induce sterilizing immunity compared to RAS immunisation (Belnoue et al., 2004). Clinical trials confirmed the superior capacity of cVac as only three doses of 5.12×10^4 sporozoites were sufficient to confer 100% protection (Mordmüller et al., 2017). This is 10 to 100 times less than with the PfSPZ immunisations (Seder et al. 2013). Assessment the cVac approach in endemic areas is underway.

1.6 Malaria liver-stage immunity:

While whole parasite-based vaccines are efficient at inducing protection in humans, these strategies have major limitations that question their suitability for routine use in endemic areas. Some of those challenges are associated with technical and logistical issues of large scale sporozoite production and distribution in endemic areas. There are also safety issues related to breakthrough infections with GAP or the use of a live fully virulent pathogen that demands strict attachment to drug administration regimes, as is the case with cVac.

Animal studies using pre-erythrocytic vaccines have provided essential knowledge about the immune mechanisms that govern the induction of sterilizing immunity. Deep understanding of these mechanisms provide important insights for the design and development of vaccine strategies that are more suitable for implementation in endemic areas.

The next section of the chapter will be focused on how animal studies have revealed memory CD8 T cell responses as the major mediator of sterilizing immunity. Additionally, innate mechanisms involved in the recognition of liver-stage parasites will be briefly discussed.

1.6.1 Innate response to liver-stage infection:

For many years, the liver-stage of malaria was considered immunologically silent because it is asymptomatic, and the liver is considered as an immunoprivileged organ (Prudêncio et al., 2006). Recent murine studies have proved this is not the case, showing that liver-stage parasites are recognised after infection (Epiphanio et al., 2008; Leiriao et al., 2005; Liehl et al., 2014; Van De Sand et al., 2005). While parasites inside hepatocytes are poorly recognized by macrophages and dendritic cells (DC), hepatocytes recognize them through cytosolic pattern recognition receptors (PRR) and are able to initiate an antipathogen type I interferon (IFN) response (Liehl et al., 2014; Miller et al.,

2014). Liehl and collaborators showed that the innate sensing of *Plasmodium* nucleic acids activates the innate immune response and restricts parasite replication in the liver. They first found that the replication of parasites inside mouse livers was accompanied by a significant induction of interferon stimulated genes (ISGs) compared to non-infected mice. Additionally, they showed that *Plasmodium* RNA triggered a type I IFN response that is dependent of melanoma differentiation-associated protein 5 (MDA5) and independent of retinoic acid-inducible gene I (RIG-I), TLR3 and TLR4. In the same study, they also found that live sporozoites induced a higher type I IFN response compared to heat inactivated or live irradiated parasites (Liehl et al., 2014). The activation of type I IFN response leads to a wide range of events, including not only expression of ISG, but also production of chemokines that mediate recruitment of macrophages, neutrophils and lymphocytes to the site of infection. It also induces generation of IFN-γ that promotes infiltration of NK cells to the liver (Liehl et al., 2014; Miller et al., 2014).

NK cells are important effector cells of the innate immune system and defend the host during the early phase of pathogenic infections (Korbel et al., 2004). Studies with a murine malaria model have shown that after *P. yoelli* sporozoite infection, the numbers of NK cells decreases in the spleen and increases in the liver (Roland et al., 2006). Functional *in vitro* assays have revealed their cytotoxic capacity to inhibit parasite development within hepatocytes but not against blood-stage parasites (Roland et al., 2006). NKT cells express effector mechanisms similar to NK cells and a semi-invariant T cell receptor (TCR). In steady state, 20 to 30% of murine liver lymphocytes are NKT cells (Bendelac et al., 1997), and when activated, they exert potent antitumor responses (Nakagawa et al., 1998). Infection with high numbers of genetically-attenuated *P. yoelli* sporozoites lead to the recruitment of NKT cells to the liver (Miller et al., 2014). When they were stimulated *in vivo* by treating mice with α-galactosylceramide (α-GalCer), an NKT cell-specific ligand, they mediated reduction of liver-stage parasite development for a short period of time (Gonzalez-Aseguinolaza et al., 2000). Moreover, it has been also suggested that

activation of NKT cells promote the accumulation of parasite-specific CD8 T cells (Gonzalez-Aseguinolaza et al., 2000; Korten et al., 2005). Even though NKT cells were initially suggested to reduce liver-stage development, studies in CD1d KO mice, which lack NKT cells, have shown that CD8 T cell immune responses and levels of protection induced by RAS vaccination is independent of NKT cells (Carvalho et al., 2002; Overstreet et al., 2011; Romero et al., 2001).

1.6.2 T cell priming:

During natural infection by mosquito bites, T cell activation is first detected in the skin draining lymph node (LN) at 48 h post infection and not in spleen, liver or liver-draining LN (Chakravarty et al., 2007). The removal of the skin draining LN prior to mosquito bite-infection reduced the number of activated T cells in the liver by over 60%; and in the absence of both LN and spleen, this reduction reached 80% (Chakravarty et al., 2007). Remarkably, sporozoites can be detected by PCR in draining LN as soon as 3 h post intradermal inoculation (Yamauchi Lucy M. et al., 2007). After intradermal inoculation of murine sporozoites, antigen-specific CD8 T cells cluster around lymph-node resident XCR1 cDC in draining LN at early time points (Radtke et al., 2015). Using immunofluorescence microscopy, Radtke et al. revealed that the two cells are in direct physical contact, indicating that CD8 T cell priming may be mediated by LN-resident CD8+ DC. Interestingly, when sporozoite antigen presentation is restricted to the skin draining LN (by using a mouse strain in which sporozoites cannot establish liver stage infection), T cell priming at this site was sufficient to induce protective immunity against malaria (Obeid et al., 2013). Priming of CD8 T cells has been observed in the spleen and liver after intravenous (i.v.) administration of sporozoites. In these studies, activated $CD8^+$ T cells accumulate in spleen between days 4 and 5 after i.v. injection with either live sporozoites or RAS (Lau et al., 2011; Sano et al., 2001).

Although, priming of CD8 T cells can occur in the liver (Bertolino et al., 2001), the role of liver-resident cells as antigen presenting cells (APC) of

Plasmodium antigens is controversial. Liver DC are located in close proximity to portal veins, relatively far from non-fenestrated endothelial cell layer (Lau and Thomson, 2003). With this distribution it is unlikely liver DC can mediate intrahepatic naïve CD8 T cell activation (Holz et al., 2010). Additionally, hepatic DC exhibit an immature phenotype, suggesting these cells collect antigen from blood circulating in the liver and then migrate to draining LN for further maturation and effector functions, rather than inducing T cell activation *in situ* (Thomson et al., 2002). In hepatocytes, the sporozoite protein CSP can be degraded and presented on major histocompatibility complex (MHC) I to CD8 T cells (Bongfen et al., 2007; Cockburn et al., 2011), however they cannot prime the naïve CD8 T cells. Recently, a population of liver monocyte-derived $CD11c^+$ APC was found to acquire *Plasmodium* parasites from dying infected hepatocytes (Kurup et al., 2019). Importantly, hepatic $CD11c^+$ cells harbouring *Plasmodium* parasites were also found in the liver draining LN where they primed CD8 T cells responses to liver-stage malaria antigens (Kurup et al., 2019).

Following vaccination, naïve CD8 T cells can be primed and then form memory T cells in the liver for detection of infected hepatocytes when natural infection occurs. Although exclusive liver homing molecules remained to be identified, it has been suggested that CXCL16, which is highly expressed by liver sinusoidal endothelial cells, may influence the retention of immune cells in this organ, including NK T cells expressing CXCR6 (Geissmann et al., 2005). In a murine vaccination study, wild-type (WT) mice were transferred with WT or *Cxcr6*$^{-/-}$ OT-I cells, transgenic CD8 T cells specific for an ovalbumin (OVA) antigen, and then vaccinated with RAS expressing SIINFEKL, the antigen recognised by OT-I cells. Results revealed 75% reduction of OT-I cell numbers in the liver of mice transferred with CXCR6-deficient cells compared to mice receiving WT cells (Tse et al., 2014). Moreover, two studies have demonstrated the importance of antigen presentation by hepatocytes for the immunity mediated by CD8 T effector cells. During the first study, bone marrow chimera experiments revealed that APC are essential for priming of parasite-specific CD8 T cells and are dispensable for CD8 T cell effector function in the liver, which is mediated by hepatocytes (Chakravarty

et al., 2007). For the second study, transporters associated with antigen processing (TAP) knockout mice, which lack antigen presentation by APC were co-transferred with infected hepatocytes and effector CSP-specific T cells. These mice were efficiently protected from infection, suggesting that effector cells are able to exert their function by hepatic antigen presentation independently of APC antigen presentation (Balam et al., 2012).

1.6.3 Memory CD8 T cells:

Early studies in 1980s first demonstrated the critical role of CD8 T cell in immunity mediated by RAS vaccination (Schofield et al., 1987; Weiss et al., 1988). Since then, several animal studies have confirmed these findings (Cockburn et al., 2014; Doll et al., 2016; Epstein et al., 2011; Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Nganou-Makamdop et al., 2012). Other reports also confirmed that CD8 T cells are crucial for the immunity induced by a GAP vaccine based on a double-knockout *P. berghei* parasite for *UIS3* and *UIS4*, which are highly expressed by sporozoites (Jobe et al., 2007).

The number of infected hepatocytes under a normal course of infection has been estimated to be only 1 in one million cells in mice and one in 100 million cells in humans (Van Braeckel-Budimir and Harty, 2014). Accordingly, the generation of highly effective parasite-specific CD8 T cells required for the surveillance of the whole liver and the elimination of every single infected hepatocyte (prerequisite of sterilizing immunity) is essential. In 2008, Schmidt and collaborators induced sterilizing immunity in BABL/c mice by immunisations with mature DC coated with a *P. berghei* antigen and boosting with a recombinant attenuated *L. monocytogenes* strain expressing the same antigen. They found that sterilizing immunity was correlated with the number of specific CD8 T cells and estimated that the threshold of specific cells is one million in the spleen and 20,000 in the liver to achieve protection against malaria. These numbers correspond to a 100-1000-fold higher dose of specific T cells required for sterilizing immunity against bacterial and viral infections (Schmidt et al., 2008).

In the context of a primary infection, productive activation of naïve T cells induces clonal expansion, providing a large army of specific effector cells (Joshi et al., 2007). After the elimination of infected cells, the effectors contract and memory T cells survive – providing long-term protection against reinfection (Sarkar et al., 2008). In fact, parasite-specific short-lived effector cells peak in spleen shortly after *P. yoelii* infection and decrease with clearance, while memory precursor effector cells remain and develop into true memory T cells over time (Chandele et al., 2011). Memory cells are functionally and phenotypically different and can be grouped into central, effector and tissue resident memory T cells (Fig. 1.2).

Central memory T (T_{CM}) cells home to secondary lymphoid organs (SLO), express high levels of CD62L and CCR7, have the ability to circulate to the blood, retaining highly proliferative features, providing a stem-like pool of antigen-specific memory T cells (Sallusto et al., 1999). Little evidence supports the idea that T_{CM} cells have an important role in immunity against sporozoites (Holz et al., 2016).

Effector memory T (T_{EM}) cells express low levels of CD62L and CCR7, important molecules for homing to SLO, but possess receptors that allow them to enter non-lymphoid tissues (Sallusto et al., 1999). These cells patrol the body and are loaded with proteins to exert several effector functions. In C57BL/6 (B6) mice vaccinated with *P. berghei* RAS, the majority of circulating memory cells (96%) are T_{EM} cells (Berenzon et al., 2003). BALB/c mice, which are relatively easy to protect, generate high frequencies of CSP-specific T_{EM} cells, whereas B6 mice, which are more difficult to protect, produce a lower proportion of specific T_{EM} cells and these are not specific for CSP (Schmidt et al., 2010).

Tissue-resident memory T cells (T_{RM} cells) are found in several organs and tissues including intestines (Masopust et al., 2010), skin (Gebhardt et al., 2009), brain (Wakim et al., 2010), lungs (Anderson et al., 2012; Teijaro et al., 2011), female reproductive tract (Shin and Iwasaki, 2012), salivary glands

(Hofmann and Pircher, 2011), thymus (Hofmann et al., 2013), liver (Fernandez-Ruiz et al., 2016; Tse et al., 2013), kidneys, pancreas and heart (Casey et al., 2012). T_{RM} cells remain in the tissues, maintaining an effective local surveillance at sites of previous infection, especially at barrier sites in the body (Steinbach et al., 2018). These cells have been effectively studied in parabiosis experiments where the circulatory systems of two mice are joined, circulatory cells balanced between the mice while a proportion of T cells do not recirculate and thus are tissue resident (Jiang et al., 2012; Klonowski et al., 2004). In the case of secondary infections, the strategic location of the T_{RM} cells allows them to mediate effector responses more rapidly than their counterpart, the circulating memory cells (Mueller and Mackay, 2016). T_{RM} cells have a unique molecular profile that distinguishes them from the circulatory memory cells. They express low levels of Killer cell lectin-like receptor subfamily G member 1 (KLRG1) (Gebhardt et al., 2009; Mackay et al., 2012a; Steinert et al., 2015). Additionally, high levels of CD103 has been observed on many cells with some exceptions (Carbone, 2015), and CD69, which is also a marker of recent activation (Gebhardt et al., 2009). The differential expression of these markers promotes the residency of these cells in different tissues.

T_{RM} cells exert effective killing of infected cells through many effector mechanisms, including the expression of granzyme B (Casey et al., 2012; Mackay et al., 2012b; Schenkel et al., 2014) and the production of proinflammatory cytokines such as IFN-γ (Glennie et al., 2015; Schenkel et al., 2013), promoting the recruitment of circulating T cells and B cells (Schenkel et al., 2014). The production of IFN-γ can also alert neighbouring cells and promote the upregulation of innate functions that prevent viral infections (Gil et al., 2001; Karst et al., 2003; Kimura et al., 1994; Shrestha et al., 2006). Upon re-activation with cognate stimulus, CD8 T_{RM} also express TNFα and promote DC maturation, leading to granzyme B upregulation in NK cells (Schenkel et al., 2014). The presence of T_{RM} cells has been associated with tumour and infection control (Masopust and Soerens, 2019). The generation and maintenance of T_{RM} cells is

the target of intensive research efforts for the development of more potent vaccines.

Liver T_{RM} cells have been recently described and differently from T_{RM} cells from other tissues, these cells are found in the blood patrolling the liver sinusoids and are much faster than skin T_{RM} cells (Fernandez-Ruiz et al., 2016; Mackay et al., 2016; Steinert et al., 2015). Intravital microscopy and sequencing data of after RAS vaccination, have revealed that the elimination of *Plasmodium*-infected hepatocytes is associated with the effector function of CD8 T cells that display matching features with T_{RM} cells (Cockburn et al., 2013; Tse et al., 2013). In fact, Fernandez-Ruiz and collaborators confirmed by parabiosis experiments in B6 mice that *P. berghei* ANKA (PbA) RAS induced the formation of liver T_{RM} cells (Fernandez-Ruiz et al., 2016). They first investigated the transcriptional profile of different memory T cell subpopulations present in the liver and the spleen after vaccination with PbA RAS. A specific subpopulation only present in the liver was found to express CXCR6, CD69, CXCR3 and low levels of KLRG1 and CD62L, common markers for T_{RM} cells (Fernandez-Ruiz et al., 2016). Subsequently, parabiosis experiments confirmed the tissue resident status of this subpopulation and intravital images showed that they patrol the liver sinusoids. Importantly, formation of large numbers of liver T_{RM} cells can be promoted through different vaccination strategies (Fernandez-Ruiz et al., 2016). Depletion using anti-CXCR3 antibodies abrogated the sterile protection mediated by vaccination, providing evidence that liver T_{RM} cells are essential for protection against liver-stage parasites (Fernandez-Ruiz et al., 2016).

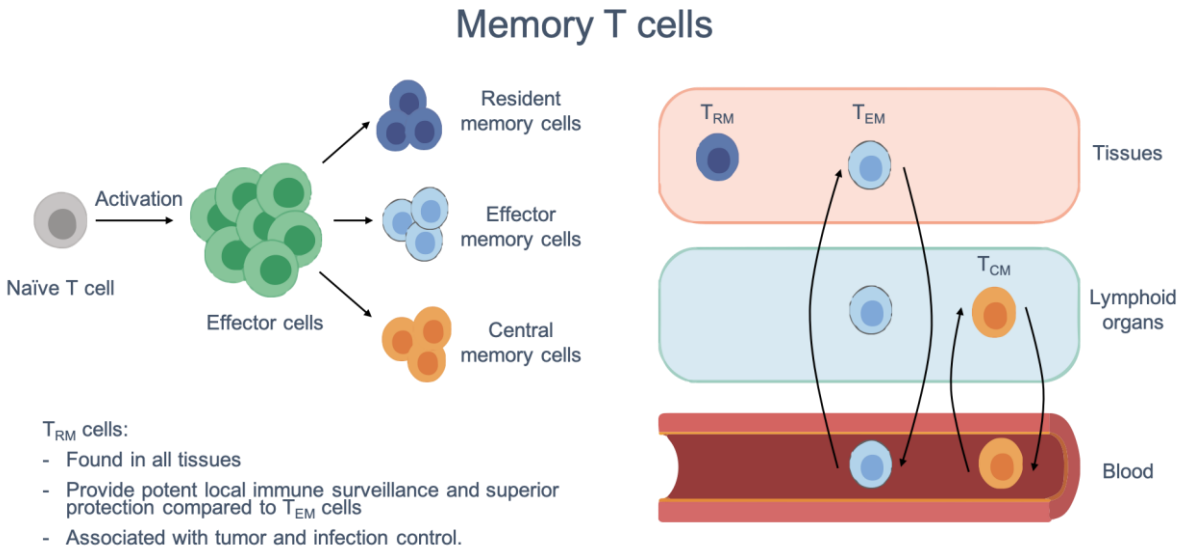

Figure 1.2. Subtypes of memory T cells. In the context of a primary infection, productive activation of naïve T cells induces clonal expansion, providing a large army of specific effector cells. After the elimination of infected cells, the effectors contract and memory T cells survive providing long-term protection against reinfection. Memory cells are functionally and phenotypically different and can be grouped into central, effector and tissue resident memory T cells. T_{CM} cells home to SLO, express high levels of CD62L and CCR7, have the ability to circulate to the blood, retaining highly proliferative features, providing a stem-like pool of antigen-specific memory T cells (Sallusto et al., 1999). T_{EM} cells express low levels of CD62L and CCR7, important molecules for homing to SLO, but possess receptors that allow them to enter non-lymphoid tissues (Sallusto et al., 1999). These cells patrol the body loaded with proteins to exert several effector functions. T_{RM} cells remain in the tissues, maintaining an effective local surveillance at sites of previous infection, especially at barrier sites in the body (Steinbach et al., 2018). They can be virtually found in all tissues and induce a superior protection compared to effector memory cells. The presence of T_{RM} cells has been associated with tumour and infection control.

In healthy adults, γδ T cells constitute 1 to 5 % of circulating lymphocytes, whereas higher frequencies can be found in epithelial-rich tissues, such as the reproductive track, skin and intestine (Hayday, 2000; Holtmeier and Kabelitz, 2005). These cells display features that are common for innate and adaptive immune cells and the exact biological functions remain to be elucidated (Carding and Egan, 2002). Emerging studies have suggested the importance of γδ T cells in the induction of protective responses mediated by RAS vaccination in humans and murine models (Ishizuka et al., 2016; Zaidi et al., 2017).

All these data demonstrate that sterile protection against malaria mediated by vaccination largely depends on the formation of parasite-specific memory CD8 T cells that kill infected hepatocytes displaying cognate antigen. Of the several memory CD8 T cell subpopulations, liver T_{RM} cells appear to be essential for sterile protection induced by RAS vaccination against sporozoite challenge (Fernandez-Ruiz et al., 2016). These cells are strategically located in liver sinusoids to provide potent surveillance and are armed with effector mechanisms to eliminate infected cells. Because liver T_{RM} cell formation can be induced through vaccination, the design of strategies aiming at the generation of effective T_{RM} cells is a priority.

1.7 Liver-stage subunit vaccines:

Liver-stage subunit vaccines are designed to generate CD8 T cell immune responses against specific antigens derived from liver-stage parasites. These vaccines overcome the manufacturing challenges of whole-parasite approaches but face their own difficulties, such as limitations in the number of known immunogenic and protective CD8 T cell epitopes derived from *Plasmodium* parasites (Draper et al., 2018; Schmidt et al., 2010). Noting the shortage of known epitopes, this section of the chapter will cover the existing development of liver-stage subunit vaccines.

Viral vectored vaccines are to-date the most clinically tested liver-stage subunit vaccines, since they are known to be particularly effective at inducing T cell responses. They use replication-deficient viral particles, in particular recombinant chimpanzee adenovirus serotype 63 (ChAd63) for priming and modified vaccinia Ankara (MVA) for boosting (Ewer et al., 2015). These viral particles are modified to encode for the TRAP linked to a multi-epitope string (ME-TRAP). The ME is a string of T and B cell epitopes from several expressed during pre-erythrocytic stage (Ewer et al., 2015; Gilbert et al., 1997), and TRAP is a protein that plays an important role in hepatocyte cell invasion (Akhouri et al., 2008). Vaccine efficacy ranges from 20% to 25% in malaria naïve volunteers (Ewer et al., 2013), and was found to reduce the risk of infection in adults in Kenya by 67% during 8 weeks of monitoring (Ogwang et al., 2015). However, no efficacy was observed in Senegalese adults (Mensah et al., 2016). Vaccination of children in two cohorts from Gambia and Burkina Faso revealed high immunogenicity (Bliss et al., 2017), but no efficacy was observed in the highly endemic malaria transmission cohort from Burkina Faso (Tiono et al., 2018). Interestingly, other studies in malaria naïve subjects revealed that co-administration of ChAd63-MVA encoding for other well-known epitopes, such as apical membrane antigen-1 (AMA1) and merozoite surface protein-1 (MSP1) together with the ME-TRAP vaccine, failed to improve protection (Hodgson et al., 2015; Sheehy et al., 2012).

Another clinically tested strategy uses DNA plasmids for priming and adenovirus for boosting (Sedegah et al., 2011; Tamminga et al., 2011; Wang et al., 2001; Webster et al., 2005). Immunisations with DNA plasmids and adenovirus vector encoding for CPS and AMA1 resulted in 27% of sterile protection in malaria naïve volunteers; this immune response was associated to effector CD8 T cells (Chuang et al., 2013).

1.7.1 Prime-and-trap vaccine:

“Prime-and-trap” is a three-component vaccine strategy used to induce parasite-specific liver T_{RM} cell formation through the priming of naïve immune cells and

the trapping of those cells in the liver (Fig. 1.3) (Fernandez-Ruiz et al., 2016). The first component is based on the use of an αClec9A monoclonal Ab (mAb) coupled to a relevant epitope to achieve epitope delivery to conventional DC-1 (cDC1) (Caminschi et al., 2008; Lahoud et al., 2011); which is the main antigen presenting cell stimulating T cells in malaria (Fernandez-Ruiz et al., 2017; Lau et al., 2014). The Clec9A molecule, also known as DNGR-1, improves cross-presentation of dead-cell-associated antigens (Ahrens et al., 2012; Iborra et al., 2012; Sancho et al., 2008). Its expression is restricted to DCs and is highly expressed by CD8+ DCs and CD103+ DCs in mice and in humans by the BDCA-3+ DCs (Caminschi et al., 2008; Desch et al., 2011; Huysamen et al., 2008; Sancho et al., 2008; Schreibelt et al., 2012). Moreover, the in vivo delivery of exogenous antigens by targeting Clec9A with mAb has been exploited successfully in the context of tumour therapy and vaccine development (Caminschi et al., 2012a; Palucka et al., 2010; Tacken et al., 2007). Importantly, cytotoxic T lymphocyte (CTL) responses in mice injected with αClec9A coupled to specific antigens is mediated by co-injection with adjuvants such as cytosine-phosphate-Guanine (CpG), polyinosinic-polycytidylic acid (p I:C) and anti-CD40 (Caminschi et al., 2012b; Lahoud et al., 2011; Sancho et al., 2008). In the absence of adjuvant, this form of immunisation leads to MHC-II presentation, follicular helper T cell activation and efficient antibody production, but no CD8 T cell response (Caminschi et al., 2012b; Kato et al., 2015; Lahoud et al., 2011; Li et al., 2015).

The second component of the prime and trap vaccine is a recombinant adeno-associated virus (rAAV) engineered to selectively infect hepatocytes, inducing the presentation of antigen on hepatocytes and trapping of the T cells to the liver. This vector is also modified to express the relevant epitope under the control of a hepatocyte-specific promoter (Tay et al., 2014). This enables the presentation of antigen on hepatocytes to facilitate the trapping of the T cells to the liver for formation of T_{RM} cells. The third and last component, is a CpG adjuvant used for the licensing of DC involved in $CD8^+$ T cell priming (Lahoud et al., 2011) and to promote a proinflammatory liver-microenvironment

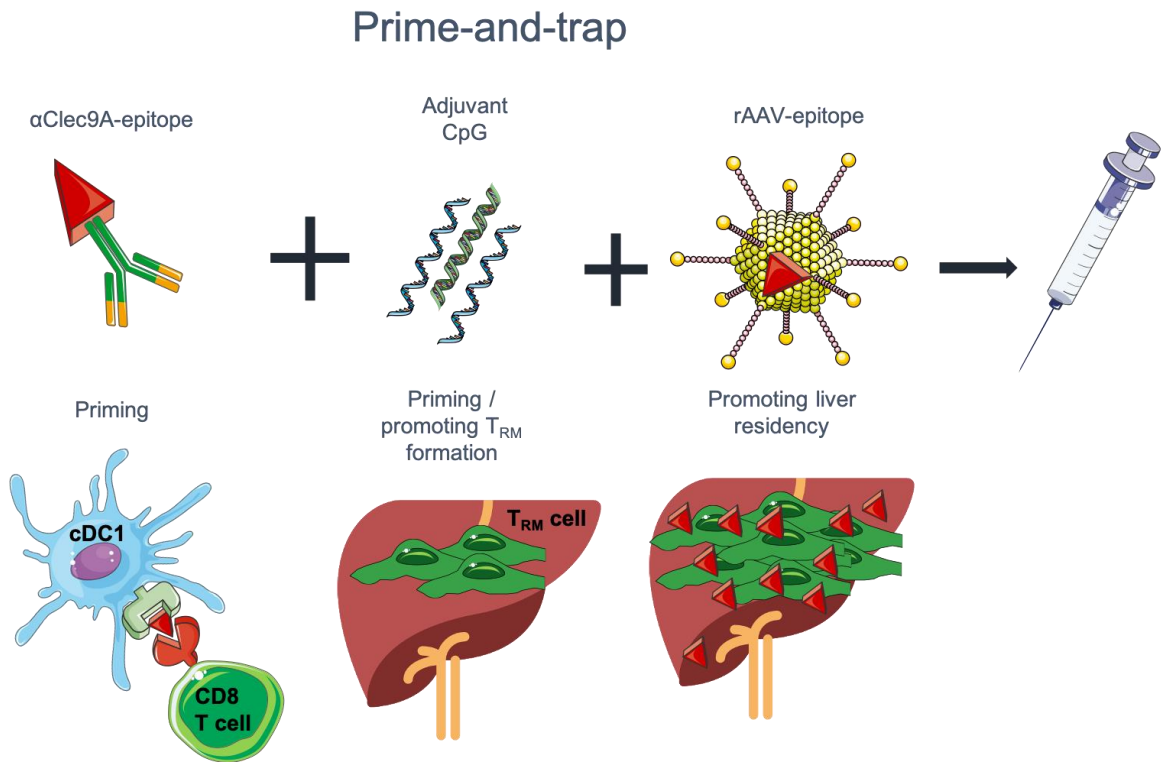

Figure 1.3. Prime-and-trap vaccine. Prime-and-trap is a three-component vaccine strategy for the induction of liver T_{RM} cell formation. The first component is an αClec9A mAb coupled to a relevant epitope for delivery to cDC1. The adjuvant CpG is used for the licensing of DC and for the induction of a proinflammatory liver-microenvironment that promotes the accumulation and formation of T_{RM} cells. The trap component is based on the use of a recombinant adeno-associated virus (rAAV) that expresses the antigen in the liver enhancing the differentiation of T_{RM} cells in the liver. Three components are delivered i.v. to improve targeting the antigen to the liver.

(Huang et al., 2013), which aids T_{RM} cell formation (Holz et al., 2018; Mackay et al., 2012a). Importantly, all three components are delivered i.v., which has been associated with improved protection (Seder et al., 2013). Fernandez-Ruiz and collaborators demonstrated that relatively high numbers of liver T_{RM} cells can be generated by prime-and-trap vaccination and that these cells can effectively confer sterilizing immunity (Fernandez-Ruiz et al., 2016).

1.7.2 Prime-target vaccine:

The prime-target approach (Gola et al., 2018) is similar to “prime-and-trap” and uses a modified adenovirus the for priming step and nanoparticles (Np) or a modified viral vector (MVA) for cell targeting to the liver. In detail, mice were primed intramuscularly (i.m.) with a modified adenovirus expressing OVA and two weeks later Np containing OVA were administrated i.v. (Gola et al., 2018). They observed increased numbers of specific-CD8 T cells expressing IFN-γ in the livers of vaccinated mice, as well as the formation of cell clusters displaying T_{RM} cell markers, and colocalization of IFN-γ^+ granzyme B^+ CD8 T cells in proximity to sporozoites. Vaccinated mice were sterilely protected from challenge with sporozoites expressing OVA and protection was abrogated by the use of anti-CD8-depleting Ab (Gola et al., 2018). In an effort to translate this approach using clinically relevant epitopes, they also investigated protection induced by the *P. falciparum* antigens TRAP, liver-stage antigen 1 (LSA1) and liver stage-associated protein 2 (LSAP2). For this, the ChAd63 vector was engineered to express the respective antigens, mice were primed i.m. and two weeks mice received either same ChAd63 i.v. or MVA i.m. for targeting the CD8 T cells to the liver. Three weeks later mice were challenged with *P. berghei* sporozoites expressing the relevant *P. falciparum* antigens (Gola et al., 2018). Again, mice were sterilely protected, these encouraging results prompted a phase I clinical trial to assess the safety and immunogenicity of this approach by vaccinating volunteers i.v. with ChAd63.ME-TRAP (Gola et al., 2018). Preliminary results of this phase I clinical trial showed that intravenous administration of ChAd63.ME-TRAP was safe and well tolerated. Moreover, they also showed that IFN-γ

production measured by enzyme-linked immunospot (ELISPOT) picked at day 14 after vaccination and was still detectable on day 42 in volunteers that received the highest dose of the viral vector (5 x 10^{10} vp) (Gola et al., 2018).

1.7.3 CD8 epitopes derived from PbA:

As previously mentioned, one of the major limitations for the development of liver-stage subunit vaccines is the scarcity of known protective T cell epitopes derived from *Plasmodium* parasites. The paucity of known epitopes could be partially explained by the difficulty in isolating infected hepatocytes and then identify epitopes via elution and mass spectrometry. As mentioned before, under a normal course of liver-stage infection, the percentage of infected hepatocytes is low (Van Braeckel-Budimir and Harty, 2014), and therefore the number of MHC I molecules that can be obtained from these cells is also low. Recently, efforts have been made to generate a source of sufficient epitopes, e.g. via use of humanized liver-chimeric mouse models and liver-stage cell culture methods (Longley et al., 2015).

To date, only two MHC-I restricted epitopes from PbA have been described to confer some protection against liver stage infection in B6 mice, a mouse strain that has been considered difficult to protect (Doolan and Hoffman, 2000; Schmidt et al., 2010). The first characterized epitope was the $PbTRAP_{130\text{-}138}$. Hafalla and collaborators found that B6 mice vaccinated with a modified adenovirus expressing $PbTRAP_{130\text{-}138}$ showed 95% reduction in liver parasite load, but this vaccine did not induce sterile protection (Hafalla et al., 2013).

The second epitope, from $PBANKA\_1031000_{631\text{-}639}$, is termed Kb-17 and is contained within a protein of unknown function; the MIF4G-like protein (Pichugin et al., 2018; Speake et al., 2016). Pichugin and colleagues showed that B6 mice primed intramuscularly with Kb-17-DNA plasmid and boosted intravenously with a recombinant adenovirus expressing Kb-17 had 7-fold less

liver parasite burden compared to control mice, but no sterile protection was observed (Pichugin et al., 2018).

PbT-I cells are transgenic MHC I-restricted cells specific for PbA, which can confer sterile protection against sporozoite challenge in B6 mice (Lau et al., 2014). These cells have the ability to respond to blood-stage PbA, *P. yoelii* XNL and *P. chabaudi*, and to PbA RAS parasites. Initial attempts to identify the authentic epitope recognized by the PbT-I cells were unsuccessful (Lau et al., 2014). From initial screenings, an agonist mimic epitope for activation of PbT-I cells was identified but was not present in the *Plasmodium berghei* genome (Fernandez-Ruiz et al., 2016). This epitope was then used as the target antigen for the prime-and-trap vaccine developed by Fernandez-Ruiz and collaborators (Fernandez-Ruiz et al., 2016). They adoptedly transferred B6 mice with PbT-I cells and vaccinated them with αClec9A mAb conjugated to the mimic epitope, together with CpG and the modified adenovirus expressing this epitope. Mice immunised with this prime-and-trap vaccine formed substantial numbers of liver PbT-I T_{RM} cells and developed high levels of sterile protection against PbA (Fernandez-Ruiz et al., 2016). The authentic cognate epitope for the PbT-I cells has now been identified – and Chapter 3 of this thesis will focus on the characterization of the memory response induced by prime-and-trap immunisation using this epitope.

1.8 Adjuvants:

As a general principle, the majority of vaccines administered to humans already contain or are formulated together with an adjuvant. Vaccine adjuvants are substances that enhance the immunogenicity of a vaccine when added or mixed to it (Apostólico et al., 2016; Di Pasquale et al., 2015). They are used for several purposes including (i) to reduce the number of doses and amount of antigen required to induce protective immunity, (ii) increase the rate of

seroconversion and induce protective responses more rapidly (Coffman et al., 2010).

In many cases, adjuvants were widely used based on empirical data due to the lack of understanding of the mechanisms of action. However, over the last years, several mechanisms of action have been described. Adjuvants enhance immunity by (i) modulating the kinetics of the vaccine release, increasing the antigen lifetime, (ii) stimulating the release of cytokines by immune or non-immune cells, (iii) increasing antigen uptake and maturation of APC, and (iv) inducing local inflammation for cellular recruitment (Awate et al., 2013; Coffman et al., 2010; Di Pasquale et al., 2015). Based on the mechanism of action, adjuvants can be classified into delivery systems and immune potentiators.

1.8.1 Delivery systems

These types of adjuvants are employed as antigen carriers and they can induce local inflammation for the recruitment of immune cells to the site of injection. These adjuvants include aluminium salts, microparticles and lipid particles. Alum is used with vaccines against hepatitis A, B, diphtheria/tetanus/pertussis (DTP), human papillomavirus (HPV) and *Haemophilus influenza* type B (FDA, 2018). For several years it was thought that alum works by inducing the formation of a depot of antigen reducing the rate of antigen release (Gupta et al., 1995). On the contrary, recent studies revealed that independently of the depot formation, alum can activate an innate immune response by stimulating the NLRP3/NALP3 inflammasome complex (Eisenbarth et al., 2008; Kool et al., 2008). Moreover, adjuvants that contain aluminium preferentially induce humoral over cellular immune responses (Lindblad, 2004).

Microparticles including virus-like particles are generated by structural viral proteins and are non-infective and non-replicative (Huret et al., 2013). They can deliver antigen directly to immune cells such as DC (Bosio et al., 2004; Da Silva

et al., 2007), activate B cells (Zhang et al., 2009), induce cross-presentation to CD8 T cells (Dalba et al., 2007), and thus promote a broad range of humoral and cellular immune responses (Buonaguro et al., 2002; Smith et al., 2013). Some vaccines against hepatitis B (GlaxoSmithKline, 2012) and HPV (GlaxoSmithKline, 2015) are based in virus-like particles. Poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA) are biodegradable polymers that can form micro and nanoparticles to encapsulate antigen and adjuvants in same particle, increasing the delivery efficiency (Danhier et al., 2012). PLGA particles are taken up by endocytosis and can reach the cytosol soon after administration (Vasir and Labhasetwar, 2007). They have also been associated with increased uptake by APC (Beaudette et al., 2009), and antigen cross-presentation (Shen et al., 2006). PLGA particles have been used in clinical trials to deliver antigens from *Bacillus anthracis* (Manish et al., 2013) and hepatitis B virus (HBV) (Thomas et al., 2011).

MF59 is a water-in-oil emulsion and the mechanism of action remains unknown. It induces powerful cellular and humoral responses (Stephenson et al., 2005) by stimulating macrophages, monocytes and DC to secrete chemokines which in turn induce leukocyte recruitment (Seubert et al., 2008). MF59 is currently used as adjuvant for the flu vaccine (Wilkins et al., 2017) and it was approved for the H1N1 pandemic vaccine (O'Hagan et al., 2013). AS03 is an oil-in-water emulsion that contains α-tocopherol and induces the production of NF-κB, recruitment of immune cells and the induction of high Ab titres (Garçon et al., 2012; Morel et al., 2011). AS03 has shown promising results in clinical trials of vaccines against influenza in immunised children (Díez-Domingo et al., 2015).

1.8.2 Immune potentiators

Immune potentiators activate innate immune responses by stimulating pattern-recognition receptors (PRR) that are widely expressed on immune and non-immune cells. Some of those PRR include TLR, nucleotide-binding oligomerization domain-like receptors (NLR) and RIG-I like receptors (RLRs). Binding of PRR with their respective agonists triggers the activation and

maturation of immune cells which in turn modulate the production of cytokines and chemokines that ultimately can lead to induction of adaptive immune responses (Akira et al., 2006; Kumar et al., 2011). For the following paragraphs, the function of several PRR ligands will be discussed in the context of vaccine adjuvants. Based on their location, PRR can be classified as plasma membrane, endosomal or cytosolic sensors (Fig. 1.4).

TLR4 is expressed on the plasma membrane of human macrophages and dendritic cells (Vaure and Liu, 2014). A broad range of pathogen-associated molecular patterns (PAMP) are able to trigger TLR4 mediated responses. However, the most common ligand is lipopolysaccharide (LPS) which enhances type 1 T helper (Th1) immune responses (Steinhagen et al., 2011). Stimulation of TLR4 signals via the adaptor protein myeloid differentiation primary response protein 88 (MYD88) and the TIR-domain-containing adapter protein inducing interferon IFN-β (TRIF), which in turn lead to the production of cytokines (Vaure and Liu, 2014). LPS is quite toxic, even at low doses, therefore safe TRL4 ligands has been recently designed. Monophosphoryl lipid A (MPL) is a non-toxic LPS derivate that induces the production of high amounts of TNFα, IL-12 and IFN-γ (Mata-Haro et al., 2007). AS04 is an approved adjuvant consisting of MPL and alum used in vaccines against stage IV melanoma (Melacine®) (Cluff, 2013), HPV (Giannini et al., 2006) and HBV (Tong et al., 2005), and is part of clinical trials for leishmaniasis, malaria and Herpes antigens (NIH, 2020).

1.8.3 Endosomal sensors:

Endosomal PRR include TLR3, TLR7/8 and TLR9 (Fig. 1.4), which are selectively expressed by a subset of innate immune cells (Junt and Barchet, 2015). Poly I:C is a well-known TLR3 ligand that leads to NF-κB activation and IL-12 production but can also bind MDA5 inducing IFNα release (Kato et al., 2006; Kumar et al., 2006). Stimulation with p I:C activates DC for the upregulation of MHC II, production of IL-12 and type I IFN (Choi et al., 2012; Davey et al., 2010; Longhi et al., 2009). While IL-12 activates NK cells for the production of

IFN-γ, which in turn activates T and B cells (Lieberman et al., 1991), type I IFN responses have been associated with Th1 responses and antigen cross-presentation (Schulz et al., 2005). Altogether, p I:C modulates APC maturation, antigen presentation and ultimately T and B cell responses. Moreover, p I:C has been tested as an adjuvant for vaccines against several pathogens including HIV (Trumpfheller et al., 2008), dengue (Henriques et al., 2013) and cancer (Ammi et al., 2015). These studies demonstrated that p I:C enhances the generation of a robust immune response mediated by CD4 (Trumpfheller et al., 2008) and CD8 T cells (Henriques et al., 2013; Trumpfheller et al., 2008) in mice vaccinated with antigen DC-targeted antibodies, increasing protection (Henriques et al., 2013; Trumpfheller et al., 2008).

TLR7 detects single stranded (ss) GU-rich RNA (Lund et al., 2004) and is differentially expressed by human pDC and B cells, and by murine pDC, B cells, monocytes, cDC2 and neutrophils (Junt and Barchet, 2015). Ligand binding of TLR7 induces signalling through MyD88, activating the NF-κB pathway (Kawai and Akira, 2007). Imiquimod is a synthetically produced molecule that mimics ssRNA and can trigger TLR7 (Harrison et al., 1994; Shukla et al., 2010) and it has been associated with Th1, CD8 T cell and CTL responses (Stanley, 2002). Imiquimod has been approved for topical use in the treatment of skin conditions (Berman and Caperton, 2011) and as an adjuvant against melanoma cancer (Adams et al., 2008; Shackleton et al., 2004; Swetter et al., 2015).

CpG oligodeoxynucleotides (ODN) are synthetic DNA molecules containing unmethylated CG motifs able to trigger responses via TLR9. The pattern of TLR9 expression differs between humans and mice. For instance, in mice, TLR9 is expressed by B cells and various DC subtypes, including cDC and pDC (Edwards et al., 2003; Krieg et al., 1995), whereas in humans, TLR9 is mainly expressed by pDC and B cells (Hornung et al., 2002; Jarrossay et al., 2001; Kadowaki et al., 2001; Rothenfusser et al., 2001). Given these variations in the expression profile of TLR9 and the distinctive role played by diverse DC subtypes, different immunological outcomes could be expected in humans and

mice. Moreover, early studies have shown that CpG ODN sequences are species-specific, meaning that mice and humans have different optimal ligands (Bauer et al., 1999, 2001; Hartmann et al., 1999; Krieg et al., 1995). Therefore, it is important to carefully distinguish between types of CpG ODN, their respective roles and different immunological outcomes when used as vaccine adjuvants.

There are four types of CpG ODN, A-, B-, C- and P-class. The following data is based on studies investigating the role of different CpG ODN in B cells and pDC, which are known to express TLR9 in both, humans and mice (Edwards et al., 2003; Hornung et al., 2002; Kadowaki et al., 2001; Krieg et al., 1995). A-class CpG ODN induce strong production of IFNα by pDC, leading to the activation of cellular antiviral state as shown by studies performed in human PBMC and murine splenocytes (Guiducci et al., 2006; Hemmi et al., 2003). B-class ODN triggers pDC differentiation and the production of TNFα, which in turn leads to T cell activation. Additionally, B-class ODN induces B cells to proliferate and secrete IgM (Hartmann and Krieg, 2000; Hemmi et al., 2003; Rothenfusser et al., 2001; Verthelyi et al., 2001). C-class combines features from both A and B-class, as it stimulates B cells to secrete IL-6 and pDC to produce IFNα (Hartmann et al., 2003; Marshall et al., 2003). P-class ODN is the last described CpG ODN and has been shown to produce more IFNα, IL-6 and the CXCR3 ligand CXCL10 compared to B- or C-class ODN (Samulowitz et al., 2010). Stimulation with CpG ODN ultimately leads to the production of proinflammatory cytokines such as IL-6, IL-12, IL-18 and TNFα, inducing the maturation of DC and the upregulation of costimulatory molecules such as CD40, CD80 and CD86 (Bayik et al., 2016; Klinman, 2004).

From all the endosomal sensor ligands, CpG ODN have been extensively tested in several clinical trials. In fact, CpG ODN have been demonstrated to induce strong humoral responses in vaccine studies against HBV (Davis et al., 1998) and cellular T cell responses against *Leishmania* (Raman et al., 2012; Verthelyi et al., 2002), influenza (Beignon et al., 2002), anthrax (Minang et al., 2014) and tumours (Muraoka et al., 2010; Speiser et al., 2005; Stern et al., 2002). Moreover,

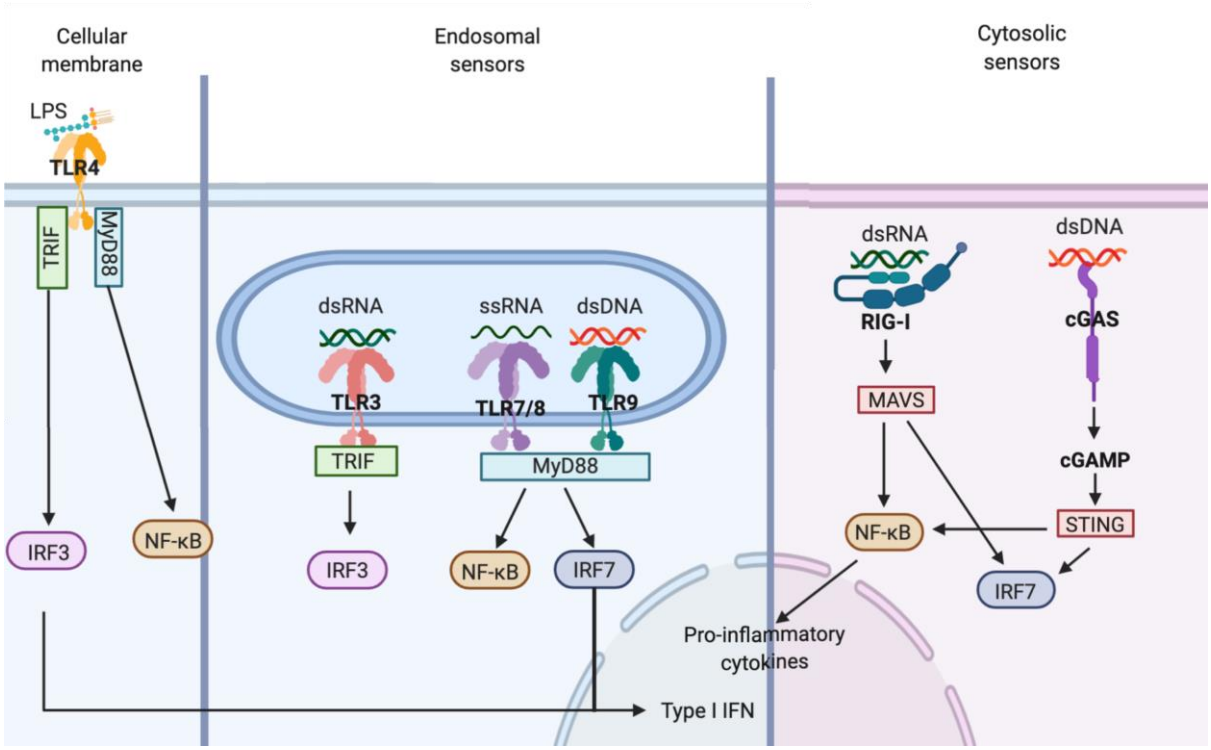

Figure 1.4 Immune potentiators: TLR4 is expressed on the plasma membrane and can get activated by LPS. Endosomal sensors include: TLR3 that recognizes dsRNA structures like p I:C, TLR7/8 binds to ssRNA and TLR9 which recognizes dsDNA with CpG motifs. Some of the best described cytosolic receptors include i) RIG-I-like receptors that get activated with dsRNA and signal through the adaptor protein MAVS; and ii) cGAS that upon recognition of dsDNA produces cGAMP, which is an activator of STING. TLR4 and TLR3 stimulates TRIF which in turn activates the transcription factor IRF3. TLR7/8, TLR9 and TLR4 signal through MyD88 protein leading to the activation of the transcription factors IRF7 and NF-κB, which can also get stimulated by MAVS and STING. Ultimately, activation of IRF3 and IRF7 induces the expression of type I IFN stimulated genes and NF-κB of pro-inflammatory cytokines. (Figure made in BioRender®).

CpG ODN also has the ability to promote the formation of intrahepatic aggregates of myeloid cells that facilitate the expansion of $CD8^+$ T cells in the liver (Huang et al., 2013).

1.8.4 Cytosolic sensors:

In contrast to the TLRs, cytosolic receptors are ubiquitously expressed in immune and non-immune cells. Cytosolic sensors include RIG-I-like receptors (RIR), cyclic GMP–AMP (cGAMP) synthase (cGAS), stimulator of interferon genes (STING), and absent in melanoma 2 (AIM2).

RIR are a family of DExD/H box RNA helicases that upon activation signal through a cascade pathway leading to an antiviral response mediated by type I IFN (Loo and Gale, 2011). RIG-I and MDA5 are members of this family. RIG-I recognizes short double stranded (ds) RNA bearing 5′ triphosphorylated or 5′ diphosphorylated ends (Hornung et al. 2006; Goubau et al. 2014); whereas self mRNA is excluded by the presence of a methyl group at the first base pair (Schuberth-Wagner et al. 2015). MDA5 binds p I:C, which mimics the structure of a high molecular weight dsRNA (Kato et al., 2008). After RIG-I and MDA5 engagement, the adaptor protein mitochondrial antiviral-signalling (MAVS) is triggered. This event leads to activation of the transcription factors interferon-regulatory factor 3 (IRF3), IRF7 and NF-κB, which in turn induce the production of IFNβ, IFNα and interferon stimulated genes (Loo and Gale, 2011). Studies have demonstrated that use of a RIG-I ligand as adjuvant for an influenza vaccine enhanced Ab affinity, conferred protection against challenge, and reduces the amount of antigen required for protection in mice (Kulkarni et al., 2014). Additionally, a recent report revealed that activation RIG-I agonists promotes cross-presentation enhancing CTL responses in mice vaccinated against an adenovirus (Hochheiser et al. 2016).

cGAS binds dsDNA and upon activation it produces cGAMP (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013), which functions as a second

messenger that binds the adaptor STING (Burdette et al., 2011; Burdette and Vance, 2013; Danilchanka and Mekalanos, 2013). Cyclic dinucleotides are PAMP derived from bacteria (Tamayo et al., 2007) and like cGAMP, can stimulate STING, which in turn activates the transcription factors NF-κB and IRF3 to induce type I IFN and cytokines (Tanaka and Chen, 2012; Wu et al., 2013). *In vivo* studies showed that mice treated with cyclic dinucleotides controlled bacterial infections with *Klebsiella pneumoniae* and *Staphylococcus aureus* by activating innate immune cells (Karaolis et al., 2007b, 2007a). Immunisation with the model antigen OVA in conjunction with cyclic dinucleotides as adjuvant enhanced T cell activation and Ab production (Li et al., 2013). Of clinical relevance, it has been demonstrated that cyclic dinucleotides used as adjuvant in murine vaccine studies promotes humoral immunuty against extracellular bacteria such as *S. aureus* and *Streptococcus pneumoniae* (Ebensen et al., 2007; Hu et al., 2009; Ogunniyi et al., 2008; Yan et al., 2009). Additionally, cyclic dinucleotides has been used as adjuvant for the generation of protective T cell responses against *Mycobacterium tuberculosis* (Van Dis et al., 2018), influenza virus (Madhun et al., 2011) and breast cancer metastasis (Chandra et al., 2014). Importantly, *in vitro* studies with human PBMC demonstrated that cGAMP derivatives enhance antigen specific expansion and functional maturation of effector CD8 T cells (Gutjahr et al., 2019).

Finally, AMI2 binds dsDNA, triggering the activation of the inflammasome machinery resulting in activation of caspase-1, the release of IL-1 and IL-18 and programmed cell death (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). Data from studies in *Aim2*-deficient mice suggest the inflammasome activation mediated by AIM2 is essential for host defence against the cytosolic bacteria *Francisella tularensis* and *Listeria monocytogenes* (Fernandes-Alnemri et al., 2010; Sauer et al., 2010) and DNA viruses such as vaccinia virus and mouse cytomegalovirus (Rathinam et al., 2010). Although this research implicates AIM2 as an important mediator of defence against infection, the role of AIM2 ligands as vaccine adjuvants have not been addressed.

1.8.5 Adjuvants for malaria vaccines:

While adjuvants from the delivery system category have been tested in several clinical trials of vaccines against malaria, only a few studies have investigated the effect of immune potentiators. Early studies in malaria naïve volunteers revealed that RTS,S vaccine in an oil-in-water emulsion enhanced the production of IgG compared to RTS,S containing alum. Importantly, when the vaccine was in emulsion plus the immune stimulant MPL (TLR4 ligand), vaccine efficacy was further enhanced (Stoute et al., 1997). In other studies including emulsion adjuvants, strong humoral and T cell responses were triggered, but they failed to enhance protection against sporozoite challenge or the trials had to be discontinued due to reactogenicity (Malkin et al., 2008; McCarthy et al., 2011; Pierce et al., 2010; Wu et al., 2008).

In a phase I clinical trial, epitopes of the CSP were co-expressed with HBV core structure particles that self-assemble into virus-like particles. Immunised volunteers developed high Ab titres and elicited CS-specific T cells that produce IFN-γ (Nardin et al., 2004). Pre-clinical studies in mice also revealed that immunisation with PLGA nanoparticles containing VMP001 *P. vivax* antigen induced Ab with enhanced avidity and affinity, and stronger T cell responses compared to the mice vaccinated with soluble antigen (Moon et al., 2012).

In the context of immune potentiators, studies in non-human primates revealed that p I:C used as vaccine adjuvant for CSP immunisation, enhanced the production of long-lasting high affinity IgG, and reduced the liver parasite load in mice (Tewari et al., 2010). Recently, in a macaque study, CpG or R848 (a TLR7/8 ligand) encapsulated in PLGA based particles were co-administered together with the poorly immunogenic Pfs25 antigen. Results demonstrated that both TLR-ligands enhanced the production of long-lasting Ab that also exhibit higher affinities, and higher numbers of B and CD4 T cells (Thompson et al., 2018). Additionally, in a phase I clinical trial against blood-stage *P. falciparum* malaria, CpG ODN was used as an adjuvant together with AMA1 antigen,

inducing significantly higher titres of IgG compared to participants that received vaccine alone (Mullen et al., 2008). The naturally occurring glycolipid, αGal-Cer, has been also used as an adjuvant for a whole parasite-based vaccine (Gonzalez-Aseguinolaza et al., 2002). αGal-Cer has the capacity to activate NKT cells which are present at high frequencies in the liver. It was shown that co-administration of this compound together with RAS enhances the number of IFN-γ producing splenocytes and vaccine efficacy in mice (Gonzalez-Aseguinolaza et al., 2002).

1.8.6 Adjuvants for T_{RM} cell formation:

There is minimal research that suggests immune potentiators can act as adjuvants for driving T_{RM} cell formation. Early studies demonstrated that in the absence of in situ antigen recognition, inflammation driven by external agents such as 1-fluoro-2,4-dinitrobenzene (DNFB) is enough for the generation of long-lasting T_{RM} cells in the skin (Mackay et al., 2012a). In fact, T_{RM} cell formation can be driven by inflammatory stimuli in the absence of cognate antigen in other tissues such as the female reproductive tract (Shin and Iwasaki, 2012), gut (Bergsbaken and Bevan, 2015), lung (Caminschi et al., 2019) and liver (Holz et al., 2018).

It has been demonstrated that CpG ODN enhance the formation of liver T_{RM} cells in mice vaccinated with antigen targeted to DC (Fernandez-Ruiz et al., 2016; Holz et al., 2018). This enhancement might occur because CpG ODN induces a pro-inflammatory microenvironment in the liver that supports the accumulation of CD8 T cells (Huang et al., 2013). To the author's knowledge there are not further studies on adjuvants that induce the formation of liver T_{RM} cells. CpG has been also used as adjuvant in a skin model, where mice were vaccinated subcutaneously with OVA and CpG ODN was administered epicutaneously as adjuvant. Results revealed that these vaccinated mice had an increased number of skin T_{RM} cells and enhanced protection against melanoma-

OVA expressing cells compared to mice immunised without CpG adjuvant (Lai et al., 2019).

Cytokines can also be used as immune potentiator adjuvants. In fact, it has been recently reported that mice immunised intranasally with adenoviral replication-deficient vectors expressing the influenza antigen hemagglutinin and nucleoprotein together with IL-1β developed more lung specific T_{RM} cells and increased immunity against viral challenge (Lapuente et al., 2018).

In a pre-clinical study in young macaques, a TLR7/8 agonist was assessed as an adjuvant for the generation of HIV-specific T_{RM} cells in the vagina. The prime and boost vaccine regime consisted of three components, first, viral vectors encoding for HIV-Gag protein for the generation of specific CD8 T cells, HIV-Env protein for the generation of Ab and PLGA nanoparticles containing a TLR7/8 agonist (Petitdemange et al., 2019). They demonstrated that macaques vaccinated with the three components (including the adjuvant) developed substantial protection against intravaginal challenge with heterologous HIV virus strain compared to macaques that received the Gag protein or the Env protein alone. Animals vaccinated with the three components developed higher numbers of vaginal T_{RM} cells and the protection was correlated with the production of Env-specific neutralizing Ab and Ab-dependent cell-mediated virus inhibition (Petitdemange et al., 2019).

1.9 Thesis aims:

Despite the substantial progress that has been made in controlling malaria, reduction in morbidity and mortality has stalled in the last 5 years. Therefore, malaria is still a major health public concern causing 400,000 deaths annually (WHO, 2018). One way to improve the control malaria is to develop an effective vaccine. Vaccines designed to stop parasite infection within the liver are ideal because they avoid the occurrence of symptoms associated with blood-stage infection and would also block transmission. Studies have demonstrated that pre-erythrocytic vaccines based on administration of whole attenuated sporozoites have high efficacy in animal models and humans (Butler et al., 2011; Clyde, 1990; Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Mordmüller et al., 2017; Nussenzweig et al., 1967; Seder et al., 2013; Tse et al., 2013). However, these vaccines face major limitations that question their suitability for routine use in endemic areas. Some of those challenges are associated with poor efficacy in malaria pre-exposed individuals, logistical limitations for large scale sporozoite production and safety concerns when using live parasites. Liver-stage subunit vaccines overcome many of these problems, in particular, the manufacture limitations and safety issues related to delivering infective sporozoites.

Plasmodium liver-stage infection has constrained biology and it is critical that a robust immune response in the liver is triggered to identify the few infected hepatocytes in the entire liver before parasites progress to the blood-stage and induce symptoms (one week in humans and 2 days in mice) (Sturm et al., 2006). Several studies have demonstrated that sterile protection mediated by vaccination largely depends on the formation of effective memory CD8 T cell responses in the liver (Cockburn et al., 2014; Doll et al., 2016; Epstein et al., 2011; Fernandez-Ruiz et al., 2016; Ishizuka et al., 2016; Nganou-Makamdop et al., 2012). Moreover, liver T_{RM} cells, a recently described subpopulation of memory T cells, have proved to be essential for protection against liver-stage challenge due to their strategic location and the potent surveillance they provide (Fernandez-Ruiz et al., 2016).

Recently, substantial efforts have been made to develop effective liver-stage subunit vaccines that promote the formation of liver associated CD8 T cells (Ewer et al., 2013; Fernandez-Ruiz et al., 2016; Gola et al., 2018). However, progress in this area is hampered by the limited number of identified protective CD8 T cell epitopes derived from *Plasmodium* parasites (Schmidt et al., 2010). To date, only two MHC-I restricted epitopes from PbA have been described to reduce liver parasite load in B6 mice and both failed to confer sterilising immunity (Hafalla et al., 2013; Pichugin et al., 2018). Accordingly, it is important to (i) identify new immunogenic MHC-I restricted epitopes and (ii) use those peptides in effective vaccine strategies that can generate robust liver CD8 T cell responses that in turn induce sterilizing immunity.

Our team was previously able to generate a transgenic CD8 T cell specific for PbA, which can be expanded and differentiated into liver T_{RM} cells to confer effective protection against sporozoite challenge in B6 mice (Fernandez-Ruiz et al., 2016). Recently, the cognate epitope for these transgenic cells has been identified and shown to be derived from the ribosomal protein L6 (RPL6) of *P. berghei* ANKA (Valencia-Hernandez et al., 2020). This epitope is termed Pb-1 in this thesis. Experiments conducted and presented in this thesis aim to investigate the suitability of Pb-1 for induction of protection through different immunisation strategies.

As previously mentioned, liver T_{RM} cells are effective and essential for protection against malaria liver-stage infection (Fernandez-Ruiz et al., 2016). Therefore, it is important to explore how to enhance the generation of liver T_{RM} cells. Vaccine immunogenicity is usually enhanced by adjuvants, but limited information is currently available about adjuvants or molecules that could enhance the formation of liver T_{RM} cells. Studies have shown that inflammation favours the formation of T_{RM} cells in general (Bergsbaken and Bevan, 2015; Caminschi et al., 2019; Mackay et al., 2012a; Shin and Iwasaki, 2012), thus molecules that generate local inflammation in the liver may be particularly effective at promoting liver T_{RM} formation. In fact, studies have shown that CpG

ODN promote local inflammation in the liver that supports the accumulation of CD8 T cells (Huang et al., 2013) and also enhances the generation of T_{RM} cells (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Based on these findings, we hypothesized that nucleic acid sensor agonists that generate liver inflammation can act as adjuvants for the formation of liver T_{RM} cell. To test this hypothesis, a series of synthetic nucleic acid agonists were assessed for the formation of T_{RM} cells in the liver of mice vaccinated with the novel epitope Pb-1.

More precisely, the studies contained within this dissertation address the following aims:

(1) To investigate the CD8 T cell memory responses mediated by different immunisation strategies using the Pb-1 epitope and to assess the suitability of these vaccines to evoke sterilizing immunity in mice with a normal endogenous T cell repertoire (Chapter 3).
(2) To analyse various nucleic acid sensor agonists as novel adjuvants for the induction of liver T_{RM} cell formation induced by antigen targeted immunisation (Chapter 4).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Experimental models

Table 2.1: List of the experimental organisms and strains used in this study. The table shows the experimental organisms and strains and the origin/source.

Organisms / Strains	Origin / Source
Mouse: C57BL/6 (H2-Db) (B6)	Janvier LABS (Australia and Germany), Charles River (Australia and Germany), Animal Resources Centre, Australia, bred in house at The Peter Doherty Institute for Infection and Immunity.
Mouse: PbT-I/uGFP	(Lau et al., 2014), bred in house at The Peter Doherty Institute for Infection and Immunity.
Anopheles stephensi (STE2/MRA-128)	The Malaria Research and Reference Reagent Resource Center; BEI resources, bred in house at the School of Biosciences, University of Melbourne
Plasmodium berghei ANKA (MRA-871)	The Malaria Research and Reference Reagent Resource Center; BEI resources, bred in house at the School of Biosciences, University of Melbourne.

2.1.2. Immunisation reagents

Table 2.2: List of the immunisation reagents used in this study. The table includes names, features, sequences and source of the immunisation reagents used in this study including adjuvants and transfection reagents.

Reagent	Sequence (5'-3')	Source
rAAV-NVF (recombinant Adeno-	rAAV encoding for the following sequence: NVFDFNNL	Patrick Bertolino, David G Bowen; University of Sydney

Associated Virus encoding NVF)		
Rat, anti-mouse Clec9A-NVY mAb (clone 24/04-10B4)	mAb linked to the following sequence: HSLSNVYDFNLLLERD	Irina Caminschi, Mireille H. Lahoud; Monash University
Rat, anti-mouse Clec9A-NVF mAb (clone 24/04-10B4)	mAb linked to the following sequence: STNVFDFNNLS	Irina Caminschi, Mireille H. Lahoud; Monash University
RAS γ-irradiated PbA sporozoites		Fresh sporozoites provided by Geoff McFadden and Anton Cozijnsen, University of Melbourne
Adjuvants		
cGAS-ligand (D3250 P*) dsDNA	AGCATAGGCCTATGCTG TGATGTATACATCAC 3' phosphorylated	Integrated DNA technologies, Coralville, IA, USA.
CpG-combo (CpG-2006 linked to CpG-21798) TLR9 ligand	T*C*G*T*C*G*T*T*T*T*G* T*C*G*T*T*T*T*G*T*C*G* T*T*T*CG*T*CG*A*CG*A* T*CG*G*C*G* (*Phosphorothioate backbone)	Integrated DNA Technologies, Coralville, IA, USA.
CpG-1668 (ODN 1668) TLR9 ligand	TCCATGACGTTCCTGAT GCT	Geneworks, Thebarton, SA, Australia
CpG-2006 (PTO 2006) TLR9 ligand	T*C*G*T*C*G*T*T*T*T*G* T*C*G*T*T*T*T*G*T*C*G* T*T* (*Phosphorothioate backbone)	Integrated DNA technologies, Coralville, IA, USA.
LPS		Sigma-Aldrich, USA

TLR4 ligand		
Poly (I:C) (p I:C) TLR3 ligand		GE Health Care, Chicago, IL, USA
RIG-I-ligand (IVT4) 5’ 3p ssRNA	5’- ppp GGGACGCUGACCCAGA AGAUCUACUAGAAAUAG UAGAUCUUCUGGGUCA GCGUCCC (p phosphate)	Winfried Barchet, Patrick Müller, University Hospital Bonn, Germany.
TLR7-ligand (R3023), GU rich ssRNA	5’- C*C*rUrCrCrUrCrCrUrCrCr GrGrGrGrGrGrGrGrGrG rGrGrG*G*G*G*G*G -3’ (*Phosphorothioate backbone)	Integrated DNA technologies, Coralville, IA, USA.
Transfection reagent		
DOTAP Liposomal transfection reagent N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate		Sigma-Aldrich, USA
Glucose (10% Ultra-pure)		Polyplus transfection SA, Illkirch-Graffenstaden, Francia
In-vivo Jet PEI ®		Polyplus transfection SA, Illkirch-Graffenstaden, Francia

2.1.3 Cell biology reagents and materials

2.1.3.1 Antibodies

Table 2.3: List of antibodies. The table shows the antibody name, clone, application and company used in this study.

Antibodies	Company	Application
Rat, anti-mouse CD4 (RM4-5)	BD Biosciences, San Jose, CA, USA	Flow cytometry
Rat, anti-mouse CD8α (53-6.7)	BD Biosciences, San Jose, CA, USA	Flow cytometry
Rat, anti-mouse CD11a (clone 2D7)	BD Biosciences, San Jose, CA, USA	Flow cytometry
Armenian hamster, anti-mouse TCRβ (H57-597)	BD Biosciences, San Jose, CA, USA	Flow cytometry
Rat, anti-mouse Vα8.3 TCR (B21.14)	BD Biosciences, San Jose, CA, USA	Flow cytometry
Rat, anti-human/mouse B220 (RA3-6B2)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Rat, anti-mouse CD11b (M1/70)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Rat, anti-human/mouse CD44 (IM7)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Rat, anti-mouse CD62L (MEL-14)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Armenian hamster, anti-mouse CD69 (H1.2F3)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Rat, anti-mouse F4/80 (BM8)	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry

Rat, anti-mouse CXCR6 (SA05D1)	BioLegend, San Diego, CA, USA	Flow cytometry
Mouse, anti-mouse NK1.1 (PK136)	BioLegend, San Diego, CA, USA	Flow cytometry
Live/Dead Aqua Blue	ThermoFisher Scientific, Waltham, MA, USA	Flow cytometry
Rat, anti-mouse Tim-3 (B8.2C12)	BioLegend, San Diego, CA, USA	Flow cytometry
Rat, anti-mouse PD1 (29F.1A12)	BioLegend, San Diego, CA, USA	Flow cytometry
Anti-mouse CD4 (GK 1.5)	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail
Anti-mouse F4/80	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail
Anti-mouse Gr1 (RB6-8C5)	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail
Anti-mouse I-A/E (M5/114)	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail
Anti-mouse Mac-1 (M1/80)	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail
Anti-mouse erythrocytes (Ter119)	Recombinant antibody facility, WEHI, Australia	CD8 T cell enrichment cocktail

2.1.3.2 Cell biology chemicals and reagents

Table 2.4 List of cell biology reagents and materials.

Reagents and materials	Company
Anti-PE microbeads	MACS, Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-APC microbeads	MACS, Miltenyi Biotec, Bergisch Gladbach, Germany
BioMag® Goat anti-rat IgG beads	Qiagen, Germany

Bovine serum albumin fraction V (BSA)	Sigma-Aldrich, USA
Cell strainer, 70 µm	MACS, Miltenyi Biotec, Bergisch Gladbach, Germany
Desatinib	Cell Signalling Technology, Danvers, MA, USA
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich, USA
EDTA	Sigma-Aldrich, USA
FACS buffer	PBS 5% w/v BSA, 5% EDTA 5mM.
Fetal Bovine Serum (FBS)	Gibco – Life Technologies
Glycerol	Sigma-Aldrich, USA
H2-K^b (for tetramers)	Jie Lin, The University of Melbourne
Hanks' buffered salt solution (HBSS)	Media preparation Unit – The Peter Doherty Institute for Infection and Immunity
Hoechst 33258	ThermoFisher Scientific, USA
LS column MACS	MACS Miltenyi Biotec, Bergisch Gladbach, Germany
Nylon mesh, 70 µm	Madison Filter Pty Ltd., Australia
Percoll®	GE Health Care, Chicago, IL, USA
Phosphate buffered saline (PBS)	Media preparation Unit – The Peter Doherty Institute for Infection and Immunity
Polypropylene tubes (5 ml) (in this thesis called FACS tubes)	BD Bioscience, USA
Propidium iodine (PI)	Sigma-Aldrich, USA
RPMI1640	Gibco – Life Technologies, Media preparation Unit – The Peter Doherty Institute for Infection and Immunity

RPMI 1640 – 2.5 % FBS (RPMI 2.5%)	RPMI 1640 supplemented with 2.5 % of heat inactivated FBS
Red blood cell lysis buffer (RBCL)	826 g/L NH_4Cl, 1g/L $KHCO_3$, 0.037g/L EDTA in dH_2O; pH7.3
Sphero blank calibrations beads (6.0 – 6.4 µm)	BD Bioscience, USA
Streptavidin-PE	ThermoFisher Scientific, Waltham, MA, USA
Trypan Blue	Sigma-Aldrich, USA

2.1.4 Molecular biology reagents and materials

Table 2.5 List of molecular biology reagents and materials.

Reagents and materials	Company
BD Microtainer® blood collection tubes	BD Bioscience, USA
Chloroform	Carl Roth GmbH, Karlsruhe, Germany
DBL™ Heparin Sodium (porcine mucous)	Hospira, Australia
Donkey anti-Rb-HRP	Jackson ImmunoResearch Laboratories, INC.
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
EvaGreen polymerase mix	Bio-Budget, Krefeld, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
Mouse IFNa	PBL assay sciences, USA
Oligo $(dT)_{18}$ primer (0.5 µg/µL)	ThermoFisher Scientific, Waltham, MA, USA
Rabbit polyclonal anti-IFNa	PBL assay sciences, USA
RevertAid RT Reverse Transcription kit	ThermoFisher Scientific, Waltham, MA, USA
RMMA-1 antibody	PBL assay sciences, USA

TMB substrate reagent set	BD Bioscience, USA
TRIzol®	ThermoFisher Scientific, Waltham, MA, USA
UltraPure™ DEPC-treated water	ThermoFisher Scientific, Waltham, MA, USA
Zirconia beads (2.0 mm)	Carl Roth GmbH, Karlsruhe, Germany

2.1.5 Specialised equipment

Table 2.6 List of specialised equipment.

Equipment	Company
FACS Canto	BD Immunocytometry Systems, San Jose, CA, USA
Fortessa or Fortessa X20	BD Immunocytometry Systems, San Jose, CA, USA
Precellys 24 homogenizer	Bertin Technologies
Cytation 3 Imaging reader	BioTek Instruments, Inc, USA
Quant Studio 5	Applied Biosystems, by Thermo Fischer Scientific

2.1.6 Software

Table 2.7 List of software packages.

Software	Company
FACSDiva	BD Immunocytometry Systems
Flowjo software	Tree Star, Ashland, OR, USA
GraphPad Prism 8	GraphPad Software, San Diego, CA, USA

2.2 Methods

2.2.1 Mice, mosquitoes and parasites:

All animal experiments were approved by the local government authorities LANUV, NRW, Germany (Animal ethics number Az. 84-02.04.2016.A403) and The University of Melbourne Animal Ethics Committee (Animal ethics number 1714302, 1814522). All protocols were performed in strict accordance with the recommendations of the national and institutional guidelines for the care and use of animals for scientific purposes.

Mice used in this study were bred and maintained in specific pathogen-free conditions at the House for Experimental Therapy and the Institute for Experimental Immunology at the Hospital University Bonn, Germany and at the animal facility of the Department of Microbiology and Immunology, at The University of Melbourne, Australia.

Female C57BL/6 (B6) and PbT-I/uGFP (Lau et al., 2014) were used between 6-12 weeks of age. Animals used for the generation of the sporozoites were 4-5-week-old male Swiss Webster mice purchased from the Monash Animal Services (Melbourne, Victoria, Australia) and maintained at the School of Botany, The University of Melbourne, Australia.

Anopheles stephensi mosquitoes (strain STE2/MRA-128 from The Malaria Research and Reference Reagent Resource Center) were reared and infected with *P. berghei* ANKA (*P. berghei*) as described by (Benedict, 1997). Sporozoites were dissected from mosquito salivary glands, resuspended in cold PBS, and either left untreated for challenge experiments or irradiated with 20,000 rads using gamma 60^{Co} source (Ramakrishnan et al., 2012). Sporozoites were kindly provided by our collaborators from the research group of Prof. Geoff McFadden, School of Biosciences, University of Melbourne; where Anton Cozijnsen and Vanessa Mollard bred, maintained and dissected the mosquitoes.

2.2.2 Naïve CD8 T cell enrichment and adoptive transfer

CD8 T cells were negatively enriched from naïve PbT-I/uGFP transgenic female mice (Lau et al., 2014). Cell suspensions were prepared from spleen and LN harvested in RPMI 2.5% through mechanical disruption and passaging through a 70 µm mesh. Cells were incubated for 2 min in filtered sterile red blood cell lysis (RBLC) buffer at room temperature (RT) to lyse red blood cells and then washed (centrifuged at 596 G, 5min, 4°C) in RPMI 2.5%. To enrich for CD8 T cells, suspensions were incubated in a cocktail (1 µL per 10^4 cells) of rat mAbs specific for erythrocytes (Ter119), I-A/E (M5/114), CD4 (GK 1.5), Gr1 (RB6-8C5), Mac-1 (M1/70) and F4/80 (F4/80) and incubated for 30 minutes on ice. Cells were then washed, resuspended in 400 µL of RPMI 2.5% and incubated with an equal volume of 3 x washed BioMag® sheep anti-rat IgG-coupled magnetic beads (used at 10:1 bead : cell ratio) for 20 minutes on constant rotation at 4 °C. Tubes were then loaded onto a cold magnetic rack for bead separation. Negatively enriched CD8 T cells were collected in the supernatant and resuspended in RPMI 2.5% and kept on ice until use. Cells were counted with trypan blue and purity was determined by flow cytometry. For this, a small aliquot of 100 µL of cell suspension was stained with anti-CD8 and anti-Vα8.3. Purity was determined as the percentage of PI^-, GFP^+, $CD8^+$, $Vα8.3^+$ cells of the total population. Cells were washed and resuspended in cold sterile PBS. Recipient naïve B6 mice were adoptedly transferred with 50,000 PbT-I/uGFP cells by intravenous injection of 200 µL of cell suspension (Fernandez-Ruiz et al., 2016).

2.2.3 Mice immunisations:

2.2.3.1 Prime immunisation with Clec9A mAb

Rat anti-muse Clec9A monoclonal antibodies (mAb) (clone 24/04-10B4) were genetically coupled to the HSLSNVYDFNLLLERD or STNVFDFNNLS peptides – containing the NVY mimic epitope or the $PbRPL6_{120-127}$ cognate epitope, respectively – via a 4 Alanine linker to generate the αClec9A-NVY (Fernandez-Ruiz et al., 2016) and αClec9A-NVF (in this thesis named: αClec9A-

Pb-1) mAb, respectively. The antibodies were kindly provided by our collaborators Prof. Irina Caminschi and Prof. Mirelle H. Lahoud, Monash University. B6 mice were injected i.v. with the indicated doses αClec9A-NVY and αClec9A-Pb-1 mAb plus indicated doses of adjuvants in a total volume of 200 µL. All mice were injected via the tail vein using a 29G insulin syringes.

2.2.3.2 Adjuvant stimulation and preparation

For the experiments described in chapter 3, B6 mice were i.v. injected with αClec9A-Pb-1 mAb plus an adjuvant: either 75 µg of CpG-combo, generated by linking (5' to 3') CpG-2006 to CpG-21798 (Samulowitz et al., 2010), or 50 µg of p I:C. For the experiments described in chapter 4, B6 mice were i.v. injected with 2 µg of αClec9A-Pb-1 mAb in the presence or absence of one of the following adjuvants in indicated doses: CpG-combo, p I:C, CpG-1668 (Fernandez-Ruiz et al., 2016; Holz et al., 2018) or LPS.

For chapter 4, mice were also i.v. injected with adjuvants complexed in transfection reagents, as described next. Different doses of cGAS-L and RIG-I-L, generated by *in vitro* retro-transcription (Winfried Barchet, University Hospital Bonn), were complexed in the transfection reagent *In-vivo* Jet-PEI® according to manufacturer instructions. In detail, in a ratio of 0.12 µL of *In-vivo* Jet-PEI® per 1 µg of adjuvant, cGAS-L or RIG-I-L were mixed with Jet-PEI® in a total volume of 100 µL of glucose (5% ultrapure) per mouse. This solution was mixed thoroughly and incubated at RT for 20 min. Additionally, CpG-1668 and two different doses of each, CpG-combo, CpG-2006 and TLR7-L were mixed thoroughly with DOTAP, in a ratio of 2.5 µg DOTAP per 1 µg of adjuvant and incubated for 20 min at RT (following manufacturer instructions). The complexed adjuvants were then mixed with 10 ng / µL of αClec9A-Pb-1 mAb, each mouse was injected with 200 µL of priming solution containing a total of 2 µg of mAb. Solution was kept on ice until injection.

2.2.3.3 Prime-and-trap vaccination

B6 mice were injected i.v. with the indicated doses αClec9A-Pb-1 mAb plus 75 µg of CpG-combo as adjuvant and different doses of recombinant adeno-associated virus expressing the Pb-1 epitope (rAAV-Pb-1). The prime-and-trap vaccination strategy was developed by (Fernandez-Ruiz et al., 2016) and in this thesis is used to target the Pb-1 epitope. It differs from the previous publication in that all three components are administered simultaneously, whereas previously the adenovirus was given one day post immunisation with αClec9A mAb.

Viral particles were produced and kindly provided by our collaborators Prof. Patrick Bertolino and David G Bowen, University of Sydney. rAAV were prepared and purified in house at the Centenary Institute or by the Vector and Genome Engineering Facility (at the Children Medial Research Institute, Sydney, Australia) over caesium chloride (CsCl)-density gradient centrifugation followed by dialysis as previously described by (Tay et al., 2014). rAAV-NVF – in this thesis named rAAV-Pb-1 – vector was generated by replacing the sequence encoding for the natural SIINFEKL epitope of OVA ($OVA_{257-264}$) contained in rAAV-OVA (Tay et al., 2014) with the sequence encoding for NVFDFNNL.

2.2.3.4 RAS vaccination

RAS were prepared by irradiating freshly isolated sporozoites with 20,000 rads using gamma 60^{Co} source (Ramakrishnan et al., 2012). Immediately after this, B6 mice were injected i.v. with 50,000 RAS in the absence or presence of 75 µg CpG-combo complexed or not in DOTAP, as previously described.

2.2.4 Challenge experiments with live PbA sporozoites

Vaccinated B6 mice were challenged i.v. with 200 freshly isolated PbA sporozoites per mouse resuspended in cold PBS. Mice were monitored daily for body condition until day 16 post infection (p.i.). Parasitemia was assessed according to (Fernandez-Ruiz et al., 2016). On days 6-8 and 10 p.i., 2 µL of blood were taken from the tail vain and resuspended in 100 µL of FACS buffer.

Parasitemia was assessed by incubating blood suspensions in 5 pg/mL Hoechst 33258 solution for 1 hour at 37°C. Parasites were detected from infected RBC by flow cytometry using a 405 violet laser and a 450/50 filter. Protection was considered sterile when no blood stage parasites had been detected within 10 days after challenge. Numbers over bars represent protected mice/total mice.

2.2.5 Organ processing for T cell analysis

Tissues were harvested in RPMI 2.5% at different time points after immunisation. Cell suspensions were generated from spleens and livers through mechanical disruption and passage through a 70 µm mesh and washed once with RPMI. Then, liver cell suspensions were resuspended in RT 35% isotonic Percoll and centrifuged at 500 G for 20 min at RT. (Fernandez-Ruiz et al., 2016; Holz et al., 2018).Spleen and liver cell suspensions were incubated for 2 min in 3 mL of RBLC at RT to lyse red blood cells, then washed and resuspended in cold FACS buffer (PBS 5% w/v BSA, 5% EDTA 5mM).

2.2.6 Flow cytometry

Cell surface immunostainings were performed according to standard protocols. After single cell suspensions were prepared from liver and spleen as described above, cells were stained with fluorophore-conjugated antibodies. H2-K^b-PbRPL6$_{120-127}$ and H2-D^b-PbTRAP$_{130-138}$ tetramers (Pb-1 and TRAP tetramer, respectively) were made in house. Tetramers were prepared by our collaborators Jie Lin and Prof. Andrew Brooks at the Peter Doherty Institute, University of Melbourne. Cell populations were identified based on forward-scatter area (FSC-A) and side-scatter area (SSC-A). Singlets were excluded based on FSC-A and forward-scatter height (FSC-H). Dead cells were excluded by propidium iodide (PI) staining. For the tetramer staining, $B220^+$, $NK1.1^+$ and $F4/80^+$ cells were excluded before gating on $tetramer^+$ CD8 T cells. For the analysis of memory CD8 T cell populations in the spleen and the liver, $tetramer^+$ or PbT-I/GFP^+ $CD8^+$ $CD44^{hi}$ cells were subdivided into T_{CM}, T_{EM} or T_{RM} based on CD69 and CD62L

expression (T_{CM} CD62L$^+$ CD69$^-$, T_{EM} CD62L$^-$ CD69$^-$ and T_{RM} CD62L$^-$ CD69$^+$). CXCR6 was included as an additional T_{RM} cell marker. In some instances, where indicated, cells were also stained with anti-PD1 and Tim-3 Ab, to determine an exhaustion-like phenotype. To enumerate cell numbers, Sphero blank calibrations beads (6-6.4μm) were diluted into FACS buffer containing PI and added to surface-stained cells immediately before acquisition of samples. The particles were distinguished from cells based on their differential size and granularity reflected by FSC-A, FSC-H and SSC-A representation. The number of cells detected by FACS was divided by the ratio of the number of particles added and the number of particles detected.

2.2.7 Enumeration of antigen-specific CD8 T cells in the naïve repertoire

Spleen, axillary, branchial, mesenteric, cervical and inguinal lymph nodes were harvested from naïve or immunised B6 mice (used as control). Single cell suspensions were produced by gently mashing the tissue through a 70 μm mesh and resuspending cells in FACS buffer. Incubation with 50 nM dasatinib (Cell Signalling Technology, Danvers, MA, USA) for 30 min at 37ºC was used to prevent TCR/tetramer internalization. Samples were stained with PE and APC-conjugated tetramer of the indicated specificity for 1 hour at room temperature. Solutions were then incubated with anti-PE and anti-APC conjugated magnetic microbeads, and tetramer-bound cells were enriched over a magnetic LS column MACS®. Enriched cells were then stained with fluorophore-conjugated antibodies specific for TCRβ, CD8, CD4, CD11b, CD11c, B220, F4/80, NK1.1 and CD62L. Dead cells were excluded by Live/Dead Aqua Blue or propidium iodide staining (La Gruta et al., 2010; Obar et al., 2008).

2.2.8 Enzyme-linked immunosorbent assay (ELISA)

2.2.8.1 Plasma preparation

Blood samples were collected from live mice by cheek bleeding on BD Microtainer® blood collection tubes or in 1,5 mL tubes containing 100 µL of heparin solution (200 IU/mL in RPMI). After 6 h or 10h as indicated, mice were killed, and blood was collected immediately by cardiac puncture. Samples were mixed by inversion and plasma was separated by centrifugation (10,000 G) for 10 min at RT. Plasma samples were stored at -20 ^{o}C until further use.

2.2.8.2 Organ processing

Spleens were cut in two and livers were perfused with cold PBS and two similar sized pieces of the main lobule were collected. The weight of each organ piece was determined for the normalization of protein content and levels of gene expression relative to organ weight. Tissues were collected in 1 mL tubes with 2.0 mm Zirconia beads, resuspended in RPMI supplemented with protease inhibitors or in TRIzol®, for ELISA assays or RNA extraction, respectively. Tissues were disrupted and homogenized in a Precellys 24 homogenizer. Tubes were shortly centrifuged at maximum speed and the supernatant was collected and stored at -20°C for ELISA assays or at -80°C until RNA extraction.

2.2.8.3 ELISA to detect IFNα

Murine IFNα (m IFNα) ELISA was performed in serum and homogenized spleen and liver tissue. Nunc-Immuno™ MicroWell™ 96-well high protein binding plates were coated with 50 µL of PBS containing 250 ng/ mL of RMMA-1 antibody overnight (ON) at 4°C. Unbound protein was washed by soaking the plates 3 to 5 times with wash buffer (0.05% Tween20 in PBS) and blocked for 3 h with 50 µL of PBS containing 1% BSA at RT. After washing the plates 3 to 5 times, 50 µL of sera (diluted 1:5 in PRMI) and undiluted tissue samples were loaded on the plate. Two technical replicates were loaded per sample; additionally, a twofold serial dilution of recombinant IFNα was also loaded and used to calculate a standard curve. Plates with samples and standard were incubated at 4°C ON. For the

detection, washed plates were incubated at RT for 4 h with 50 µL of antibody buffer (1% BSA, 0.05% Tween20 in PBS) containing rabbit polyclonal anti-mouse IFNα (1:1000) and after a subsequent washing, plates were incubated at RT for 3 h with donkey anti-rabbit HRP (diluted 1:2000 in antibody buffer). The wells were washed 6 to 12 times and HRP was detected by adding 100 µL of TMB detection reagent (substance A + B in equal volumes) and incubated in darkness for 30 min before measurement. The chemical reaction was stopped with 100 µL of 2N H_2SO_4 or 100 µL of 0.2 M HCl. Plates were analysed at 450/570 nm using Cytation 3 Imaging reader. The concentration of protein was calculated by linear regression of a standard curve.

2.2.9 Quantitative real-time PCR:

2.2.9.1 RNA isolation

Total RNA was isolated from homogenized liver and spleen tissue by phenol-chloroform extraction. To separate the aqueous phase containing RNA, 200 µL of chloroform were added to samples in TRIzol®, mixed thoroughly, incubated for 10 min on ice and centrifuged at 12,000 G for 15 min at 4°C. The upper aqueous phase was transferred to a new tube and nucleic acids precipitated by adding 400 µL isopropanol, mixed by inversion, incubated on ice for 10 min and centrifuged at 12,000 G for 10 min at 4°C. Supernatant was removed and pellet was washed (14,000 G for 5 min at 4°) twice with 70% ethanol. Supernatant was completely removed, and pellet was resuspended in 16 µL of UltraPure™ DEPC-treated water. RNA concentration was determined using Nanodrop 2000. DNA was eliminated by incubating RNA containing tubes with DNase 1 for 15 min at 37°C, followed by an inactivation step at 65°C for 5 min. Samples were stored at -80°C until use.

2.2.9.2 cDNA synthesis

The cDNA was synthetized from the extracted RNA with a RevertAid RT Reverse Transcription kit. In detail, 200 ng of RNA were resuspended in 13 µL of DEPC-treated water plus 1 µL of Oligo $(dT)_{18}$ primer (0.5 µg/µL) and incubated

for 10 min at 70°C. Samples were placed on ice and the reverse transcriptase mixture was added. This mixture contained 4 µL 5 x First Strand Buffer, 1 µL dNTP Mix (5nM each dNTP) and 1 µL RevertAid reverse transcriptase (200U/µL), per sample. cDNA synthesis was performed by incubating samples for 60 min at 42°C, then volume was adjusted to 60 µL in total and cDNA was stored at -20°C until use.

2.2.9.2 Real-time PCR

For real-time PCR a total of 2 µL of cDNA was mixed with 0.6 µL of 10 mM primer mix, 2 µL of Fast EvaGreen qPCR master mix in a total volume reaction of 10 µL. Reaction was performed in a Quant Studio 5 with the following cycle conditions: 95 °C for 2 minutes, 35 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The mRNA expression of each gene (*Ifi44* and *Irf7*) was normalized to the house keeping gene *Hprt* and the fold change relative expression was calculated using unstimulated controls as $2^{-\Delta\Delta CT}$.

Table 2.8 List of primers used for real-time PCR.

Name	Forward 5' → 3'	Reverse 5' → 3'
mHPRT	GCCCCAAAATGGTTAAGTT	CAAGGGCATATCCAACAACA
mIFI44	TCGATTCCATGAAACCAATCAC	CAAATCGCAGAATGCCATGTTTT
mIrf7	CTTCAGCACTTTCTTCCGAGA	TGTAGTGTGGTGACCCTTGC

2.2.10 Statistical analyses

Figures were generated using GraphPad Prism 8. Data are shown as mean values ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 8. The statistical test used is indicated in each figure legend. $P<0.05$ was considered to indicate statistical significance. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. Unless otherwise indicated, figures show

pooled data from all independent experiments performed. Statistical analyses of cell frequencies, percentage of iRBC and ratios were performed by comparing groups using a Kruskal- Wallis test with Dunn's multiple comparison post-test, as these data were considered non-parametric. Statistical comparisons of cell numbers in different groups were performed by log-transforming the data and using a Student's t-test (2 groups) or one-way ANOVA followed by Tukey's multiple comparisons test (>2 groups). Asterisks directly over groups denote statistical differences with the unvaccinated control group. Rates of sterile protection were compared using one-sided or two-sided Fisher's exact tests, for comparisons of immunized groups vs the unimmunized control group, or between immunized groups respectively.

CHAPTER 3:

Characterization of the memory response induced by immunisation with an epitope from the ribosomal protein L6 (RPL6) of *P. berghei* ANKA.

3.1 Introduction:

In 2014, members of our group developed a C57Bl/6 (B6) T cell receptor (TCR) transgenic line able to produce MHC class I-restricted cells specific for PbA. This TCR transgenic line, termed PbT-I, produced cells that respond to blood-stage PbA, *P. yoelii* XNL and *P. chabaudi*, and to liver-stage parasites after infection with PbA radiation attenuated sporozoites (RAS). PbT-I cells were also shown to confer sterile protection against PbA sporozoite challenge (Lau et al., 2014).

To identify the cognate antigen for the PbT-I cells, an octamer combinatorial peptide library was used to determine ideal residues required for activation of PbT-I cells (Lau et al., 2014). These residues were then used to screen rodent malaria parasite genes for closely matching sequences that were then synthesised and tested for activation of PbT-I cells. After screening 151 potential peptides, the NCYDFNNI (NCY) epitope was described as the leading peptide candidate recognized by the PbT-I cells. This was shown by assessing *in vivo* cell proliferation and IFN-γ production by PbT-I cells stimulated with this peptide (Lau et al., 2014). This epitope is part of a protein with unknown function, encoded on the genomes of PbA (PBANKA_0714500) and P. *chabaudi* (PCHAS_0723600). *In silico* analysis of the ortholog protein in *P. yoelii* (PY000568), however, revealed that this protein is truncated and does not contain the NCY epitope. This was later confirmed by sequencing the PY000568 gene within the *P. yoelii* genome (Valencia-Hernandez et al., 2020). Since PbT-I cells are able to respond to blood-antigen from *P. yoelii* infection, yet NCY was not present in this species genome, this indicated that NCY was not the authentic cognate peptide recognised PbT-I cells and that the authentic peptide antigen for the TCR remained to be identified.

From the initial screening of the octamer peptide library, another agonist epitope was identified, NVYDFNLL (NVY) (Fernandez-Ruiz et al., 2016). This is an antigen mimic epitope that is not present on the genome of *Plasmodium*

species but is able to induce the activation of the PbT-I cells. The NVY peptide was subsequently used as an antigen in a novel vaccine, termed “prime-and-trap” (described on Chapter 1) for the generation of PbT-I T_{RM} cells in the liver, which were shown to be crucial for protection against the PbA sporozoite challenge (Fernandez-Ruiz et al., 2016).

Subsequently, analysis of the sequences from *Plasmodium* peptide candidates similar to NVY together with mass spectrometry of eluted peptides from dendritic cells presenting blood-stage PbA parasites identified the authentic cognate peptide of the PbT-I cells. In detail, the protein basic local alignment search tool (Blastp) within the PlasmoDB platform was used to identify peptides similar to NVY in the proteomes of PbA, *P. yoelli* and *P. chabaudi*. As a result, the NVFDFNNL epitope, in this document denominated as Pb-1, was identified. The Pb-1 epitope is present within the 60S ribosomal protein L6-2 of PbA (RPL6) (Valencia-Hernandez et al., 2020). Very similar epitope sequences were found in the ortholog proteins of *P. yoelii* XNL and *P. chabaudi chabaudi* (Table 1.).

Table 3.1. Amino acid sequence of the RPL6 protein. Alignment to the Pb-1 epitope. Same amino acids appeared in yellow, different in blue.

Strain	Gene code	Protein sequence
P. berghei ANKA	PBANKA_1351900	MAKNTKSGASADDKKKTLKHYVIKGQK KTLTPVRAKKTIAKKYYGKKLASKKKYIV QRKMRKSIQVGKVAIILTGKHMGKRCIIT KVLKSGLLAVIGPYEVNGVPLKRVDPRY LIVTSTNVFDFNNLSQIKDKFIQAAERIN DEIFIKSIDIKKRQKKLLKNKNESLFMND VIQQIKEIRDSDPKMKRIKLLQKQLGDLL KPEISKDKMFRSYIKSKFTLRNNMSFHN IKF
P. yoelii XNL	PY02722	MAKNNKNTKSVVSSDDKKSLLKHYVIK GQHKILTPIQAKKTISKKHYGRKIASKKK YIVQRKMRKSIQIGKVAIILTGKHMGKRC

		IITKILNSGLLAVVGPYEVNGVPLKRVDP RYLIVTSTNVFDLNNLSQIKDKFIQTAEDI HDDIFIKSNDIKKRQKKLLKNKNESLFMN DVIQQIKEIRDSDPKMKKIKSLQKQLGDL LKPEMSKDKMLRSYLKSKFTLRNNMSF HTIKF
P. chabaudi chabaudi	PCHAS_1356500	MAKITKSTKSSKETKSVVPATGDKKNAL KHYVIKGQKKILTPVRAKKTISKKHYGRK LASKKKFVVQRKMRSSIQVGKVAIILTG KHMGKRCIIAKVLKSGLLAVVGPYEVNG VPLKRVDPRYLIVTSTNIFDFNNLSQVKE KFIQSAERINDEIFIKSMDVKKRQKKLLK NKNESLFMNDVIQKIKEIRDSDPKMKKIK LLQKELGDLLKPEISKDKMFRSYIKSKFT LRNNMSFHNMNF

The Pb-1 epitope as well as its orthologs from *P. yoelli* and *P. chabaudi* were tested for their capacity to stimulate PbT-I cells *in vitro*. Splenic dendritic cells (DC) coated with the different peptides were cultured with Cell-Trace Violet™ (CTV)-labelled PbT-I cells. All three peptides were able to induce proliferation of PbT-I cells and very importantly, the sensitivity to different peptide doses of the PbT-I cells to Pb-1 was equivalent to that of OT-I cells to $OVA_{257-264}$ (SIINFEKL) peptide (Valencia-Hernandez et al., 2020). This result supports the idea that Pb-1 is the authentic cognate antigen for the PbT-I cells.

To determine whether the Pb-1 epitope from PbA is naturally processed and presented by DC, mass spectrometry was utilised to identify epitopes eluted from H-$2K^b$ molecules of DC cells cultured with blood stage malaria parasites; an abundant source of antigens. First, eluted peptides were fractionated by *high-performance liquid chromatography* (HPLC); a small aliquot of each fraction was co-cultured with PbT-I cells and naive DC; then the activation and proliferation of PbT-I cells was determined by CD69 upregulation and CTV-labelling

respectively. Only one fraction triggered a PbT-I response (Valencia-Hernandez et al., 2020). Posteriorly, analysis by high-resolution liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) of the fraction of interest and the commercially synthesised Pb-1 peptide in presence of indexed retention time (iRT) peptides (Escher et al., 2012) demonstrated an overlap between the two retention time profiles (Valencia-Hernandez et al., 2020). These results indicate that Pb-1 is present in the fraction that activated PbT-I cells, implying that this peptide is a natural occurring parasite antigen presented on H-2K^{b} molecules by DC during PbA infection (Valencia-Hernandez et al., 2020).

As mentioned earlier, PbT-I T_{RM} cells in the liver are very effective at inducing immunity against PbA liver stage malaria. To this point, the generation of high numbers of liver T_{RM} cells had only been achieved by prime-and-trap vaccination using the NVY mimic peptide in combination with transferred PbT-I cells, i.e. endogenous T cells had not been tested (Fernandez-Ruiz et al., 2016). Based on the success of prime and trap vaccination using PbT-I cells, together with recent identification of the cognate and natural occurring antigen recognised by these cells; the objective of the following study was to determine if the prime-and-trap vaccine strategy was able to use the Pb-1 epitope to evoke memory T cell responses in the liver and induce protection in mice with a normal endogenous T cell repertoire e.g. in the absence of transgenic PbT-I cells.

In this chapter, a series of different immunisation experiments utilising the Pb-1 epitope for priming were implemented and specific CD8 T cell responses were assessed. This study revealed the suitability of the novel Pb-1 epitope for induction of protection through vaccination. Importantly, when Pb-1 was targeted for the prime-and-trap protocol, high levels of sterile protection were achieved in B6 mice in absence of transgenic cells, supporting the potential for translation of this approach.

3.2 Results:

3.2.1 Potency of αClec9A-Pb-1 priming on PbT-I cell expansion and memory formation in spleen and liver:

Rat anti-Clec9A monoclonal antibodies (mAb) genetically coupled to the Pb-1 epitope (αClec9A-Pb-1) were generated by our collaborators (Caminschi and Lahoud, Monash University). The expression of the Clec9A molecule is restricted to DC and is highly expressed by type 1 convention dendritic cells (cDC1) (Caminschi et al., 2008; Desch et al., 2011; Huysamen et al., 2008; Sancho et al., 2008; Schreibelt et al., 2012); which is the main antigen-presenting cell type responsible for stimulating T cells in malaria (Fernandez-Ruiz et al., 2017; Lau et al., 2014). αClec9A-Pb-1 was utilised to prime naive mice and study the memory CD8 T cell response to the Pb-1 peptide. Mechanistically, this reagent target cDC1 delivering the Pb-1 peptide. In the spleen, these cDC1 process and cross-present the peptide to naive PbT-I or specific Pb-1-CD8 T cells; inducing their activation and proliferation. Details of the immune responses triggered by antigen DC-targeted immunization via Clec9A were reviewed in Chapter 1. It is important to mention that, for most of the experiments performed in this chapter, the response of PbT-I cells was examined first. This was done to get clear evidence that the priming reagents were working before examining their effectiveness at stimulating the responses from endogenous cells.

To determine the optimal dose of αClec9A-Pb-1 for induction of maximal expansion of specific CD8 T cells, a titration experiment was carried out. First, 50,000 PbT-I cells were adoptively transferred intravenously into naive B6 mice. The next day, mice were primed with increasing doses of αClec9A-Pb-1 or αClec9A-NVY, the last was used as control for comparative purposes between the authentic cognate epitope, i.e. Pb-1 and the mimic epitope, i.e. NVY. Additionally, CpG was co-injected with the αClec9A reagent as adjuvant, which is required for induction of CD8 T cell responses (Lahoud et al., 2011) and also favours liver T_{RM} cell formation (Fernandez-Ruiz et al., 2016).

PbT-I cells from spleens were analysed on day 7 post priming, during the effector phase of the response, as these data provide a rapid indication of the effectiveness of the vaccine as well as an approximation of the proportion of memory cells that could be formed at a later time point. Results showed increasing expansion of PbT-I cells as the dose of the antibody increased (Fig. 3.1 B). In control unprimed mice (0 μg αClec9A-Pb-1), the mean number of cells per spleen was approximately one thousand, while at the highest dose of αClec9A-Pb-1 (16 μg per mouse) the number of cells reached approximately 6 million (a six thousand-fold increase). When compared to αClec9A-NVY, αClec9A-Pb-1 was significantly better at inducing the expansion of PbT-I cells at all doses tested (Fig. 3.1 C). Additionally, no plateau of PbT-I cell expansion was evident; further experiments were performed using 2 μg of αClec9A-Pb-1 as it had one of the smallest deviations and was deemed sufficient to generate substantial expansion of PbT-I cells. This dose had been previously used for the OVA system; for these experiments mice have been adoptively transferred with OT-I cells, one day later primed with 2 μg of αClec9A-SIINFEKL plus CpG; analyses at early and memory time points have revealed substantial expansion and memory cell formation of the OT-I cells (Holz et al., 2018).

The previous experiment revealed that PbT-I cells expand substantially in response to αClec9A-Pb-1 priming at an early stage of the response. To explore the capacity of this reagent to induce long-term responses to Pb-1, the kinetics of the PbT-I response were examined at memory time points. Mice were transferred with naive PbT-I cells and, the next day, primed with 2 μg of αClec9A-Pb-1 plus CpG or poly I:C as adjuvants. The effect of these adjuvants and others will be evaluated in more detail on Chapter 4.

Blood was collected on day 7 to measure the percentage of the PbT-I cell expansion and study the potential correlation of this initial expansion with the numbers of memory cells at later points. Spleens and livers were harvest from day 3 to day 35. For the analysis of memory formation, cells were labelled for

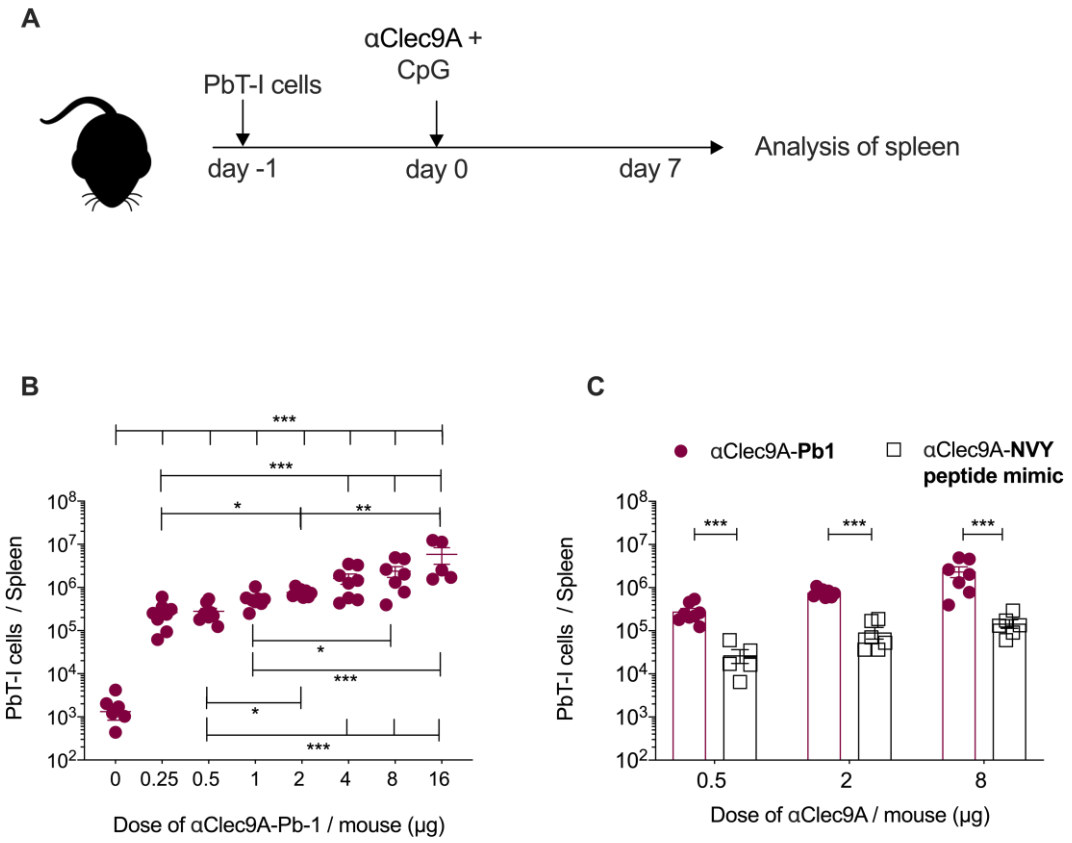

Figure 3.1. Effect of αClec9A-Pb-1 priming on the expansion of PbT-I cells in the spleen. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells. The next day, mice were immunised with increasing doses of αClec9A-Pb-1 or αClec9A-NVY plus 75µg of CpG. 7 days later, spleens were collected. Single cell suspensions were generated, and the number of PbT-I cells were determined by flow cytometry. A. Schematic of mice immunisation. B. Quantification of PbT-I cells (CD8+, GFP+ cells) in the spleen 7 days after priming with increasing doses of αClec9A-Pb-1. C. PbT-I cell expansion in the spleen 7 days after priming with 3 increasing doses of αClec9A-Pb-1 or αClec9A-NVY. The data on panel C. for the corresponding doses of αClec9A-Pb-1 are the same as shown in panel B. Results were pooled of two independent experiments. Data were log-transformed and compared by one-way ANOVA and Tuckey's multiple comparisons test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001. Two sets of statistical analysis were conducted. First, multiple comparisons were performed comparing all increasing doses. Second, the data of the three equivalent doses of αClec9A-Pb-1 vs. αClec9A-NVY were compared.

surface markers, including CD44, CD62L, CD69 and then analysed by flow cytometry; liver T_{RM} like cells were defined as CD44highCD62L-CD69+. Since CD69 is also a marker for activation after stimulation (González-Amaro et al., 2013), CXCR6 was included as an additional T_{RM} cell marker.

CpG and p I:C induced extensive expansion of PbT-I cells in blood on day 7 (Fig. 3.2 B). PbT-I cell expansion peaked on day 5 in the spleen, reaching in average of one million total PbT-I cells per spleen (Fig. 3.2 C). In the liver, p I:C induced a peak on day 5 with an average of 1.7 +/- 0.2 x 10^6 cells while CpG on day 7 generated 3.7 +/- 0.8 x 10^6 cells, which was significantly higher than p I:C on that day (Fig. 3.2 D). PbT-I cells slowly contracted over time in the spleen and liver. By day 35, the number of PbT-I cells in the liver was still significantly higher in mice primed with αClec9-Pb-1 mAb compared to naive mice, regardless of the adjuvant.

Moreover, CpG induced more T_{RM}-like cells in the liver compared to p I:C. In fact, CpG showed a significant greater capacity at generating PbT-I T_{RM} cells (CD44Hi, CD62L-, CD69+) on day 28 and 35 post immunisation compared with p I:C (Fig. 3.2 E). On day 35, mice immunised with CpG had approximately 8.1 +/- 0.9 x 10^4 PbT-I T_{RM} cells per liver. Interestingly, the numbers of T_{EM} cells in the spleen was similar for the two adjuvants over time (Fig. 3.2 F).

As analyses in Figure 3.2 only used CD62L and CD69 expression to identify liver T_{RM} cells, there was some possibility that CD69+ cells represented effector T cells that were recognising antigen and upregulating CD69. To further substantiate the preliminary identification of CD69+ cells in the liver as authentic T_{RM} cells, the expression of the marker CXCR6 was also investigated in some of the experiments. The number of T_{RM} like cells (CD62L-, CD69+) expressing CXCR6 was calculated for the different time points (Fig. 3.3 A).

These new analyses confirmed similar kinetics to those observed for CD69+, T_{RM}-like cells. The expression profile of CXCR6 peaked on day 7 and

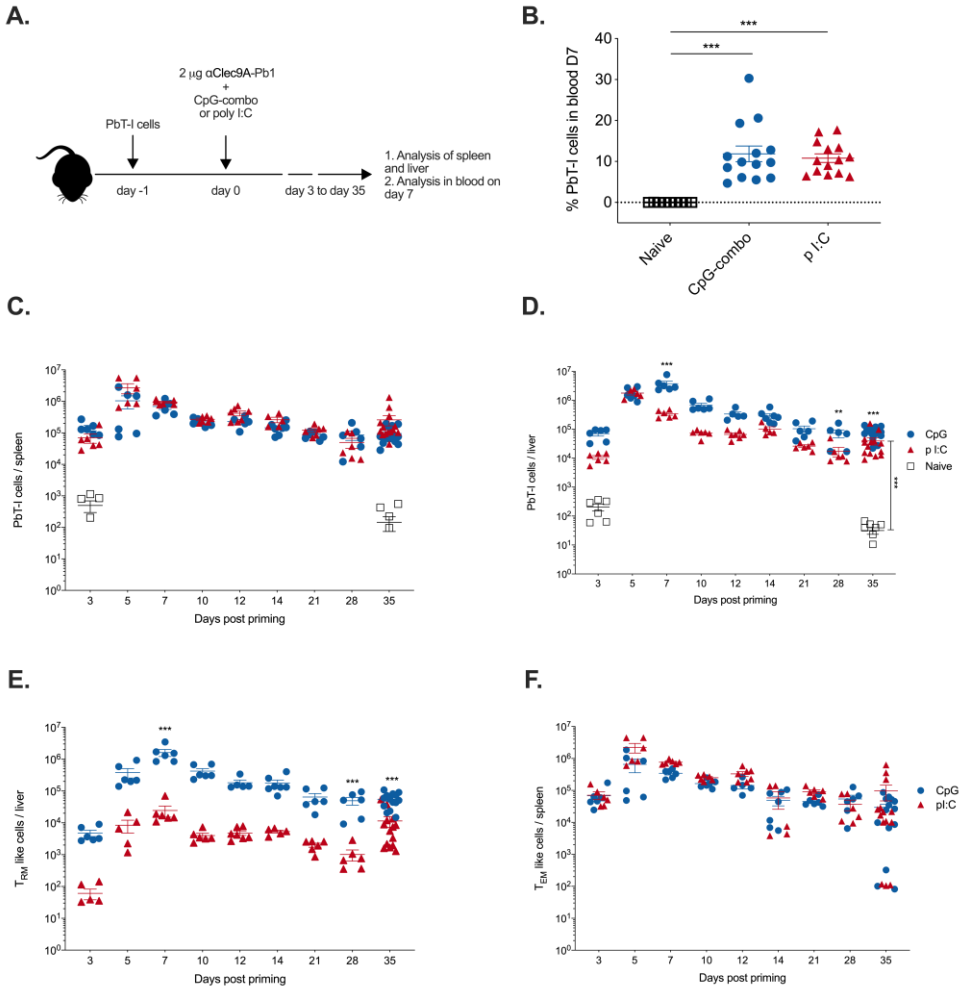

Figure 3.2. Kinetics of the PbT-I cell expansion and memory formation after αClec9A-Pb-1 priming. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and immunised with 2 μg of αClec9A-Pb-1 plus 75 μg of CpG or 50 μg of poly I:C. On day 7 after immunisation, mice were bled, and blood was analysed by flow cytometry. Spleens and livers were collected on various days up to day 35. Single cell suspensions were generated, and the number and memory status of PbT-I cells was determined by flow cytometry. A. Schematic of mice immunisation. B. Percentage of PbT-I cells (CD8+, GFP + cells) in the blood on day 7 post priming. C. and D. Total number of PbT-I cells in spleen and liver over time. E. and F. Kinetics of PbT-I T_{RM}-like cell numbers (CD8+, GFP+, $CD44^{Hi}$, CD62L-, CD69+) per liver and PbT-I T_{EM} cells (CD8+, GFP+, CD44Hi, CD62L-, CD69-) per spleen, respectively. Results were pooled of two independent experiments, except for day 35 which corresponds to a pool of 4 independent experiments. Data were compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (B.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (D. and E.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001. Data was analysed separately for each corresponding day and the values for CpG vs. poly I:C were compared.

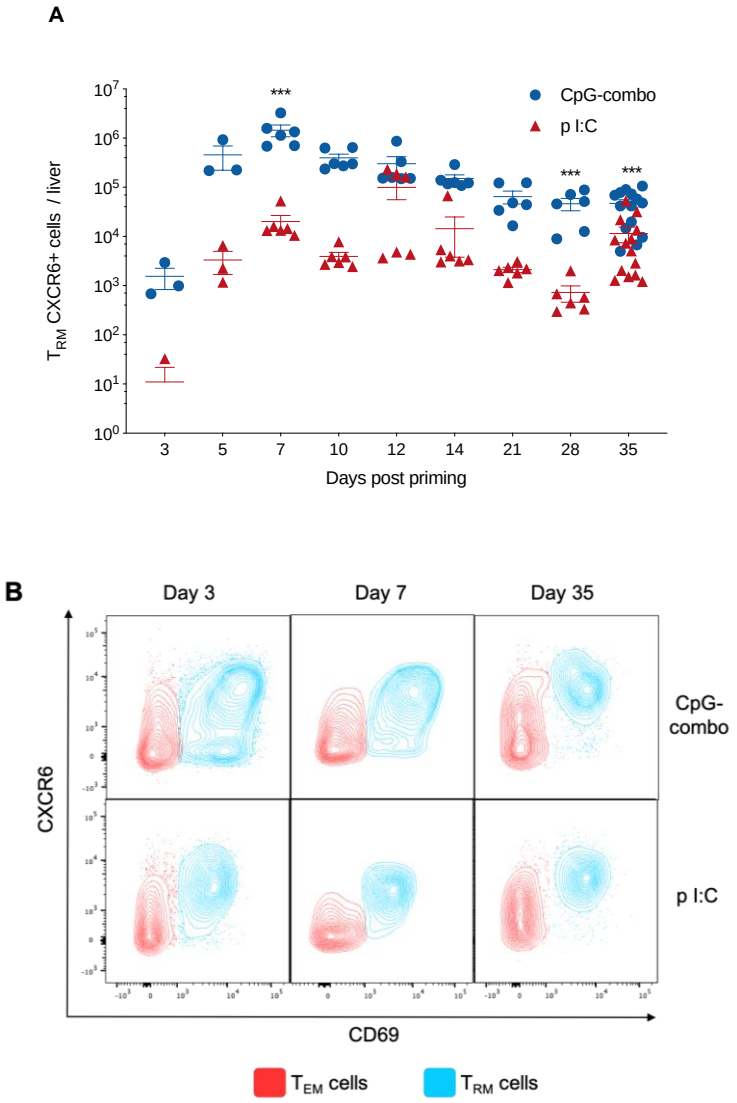

Figure 3.3. Kinetics of CXCR6+ PbT-I cells in αClec9-Pb-1 primed mice. Analysis of CXCR6 expression by flow cytometry from the previous experiment. A. Kinetics of the number of PbT-I T_{RM} cells expressing CXCR6 (CD8+, GFP+, $CD44^{Hi}$, CD62L-, CD69+, CXCR6+) per liver. B. Representative flow cytometry plots for the expression of CD69 and CXCR6 in T_{RM} and T_{EM} like cells at three different time points. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001. Data was analysed separately for each corresponding day and the values for CpG vs. poly I:C were compared.

slowly decreased until day 35. As expected, early after priming (day 3 – day 7) there was a population of T_{RM} like cells negative for the expression of CXCR6, whereas on day 35 almost all T_{RM} like cells express CXCR6 (Fig. 3.3 B). Again, CpG induced the highest number of T_{RM} CXCR6+ cells in the liver compared to p I:C.

Together these results suggest that immunisation with αClec9-Pb-1 mAb is able to induce the formation of memory PbT-I cells and that CpG has a greater capacity to induce the generation of liver T_{RM} cells than p I:C, as previously reported (Fernandez-Ruiz et al., 2016).

3.2.2 Endogenous response and protection induced by immunisation with the Pb-1 antigen:

After demonstrating the efficacy of αClec9-Pb-1 mAb at inducing PbT-I cell expansion and memory formation, it was sought to assess the capacity of this reagent to evoke an endogenous response in B6 mice.

It has been shown that the frequency of peptide-specific naive T cell precursor determines to a certain extend the magnitude and expansion of the subsequent T cell response after exposure to the specific peptide (Jenkins and Moon, 2012; Moon et al., 2007; Obar et al., 2008). Thus, the number of naive CD8 T cell precursors harbouring a TCR specific for the Pb-1 epitope was first determined.

For this purpose, single cell suspensions of the spleen and major lymph nodes of naive B6 mice were generated and tetramer-associated magnetic enrichment was applied to those single cell suspensions. Tetramers for the Pb-1 and the $TRAP_{130-138}$ epitopes were used. $PbTRAP_{130-138}$ is an H-$2D^b$-restricted epitope within the TRAP protein, identified as partially protective for B6 mice (Hafalla et al., 2013). Additional aspects of the TRAP protein

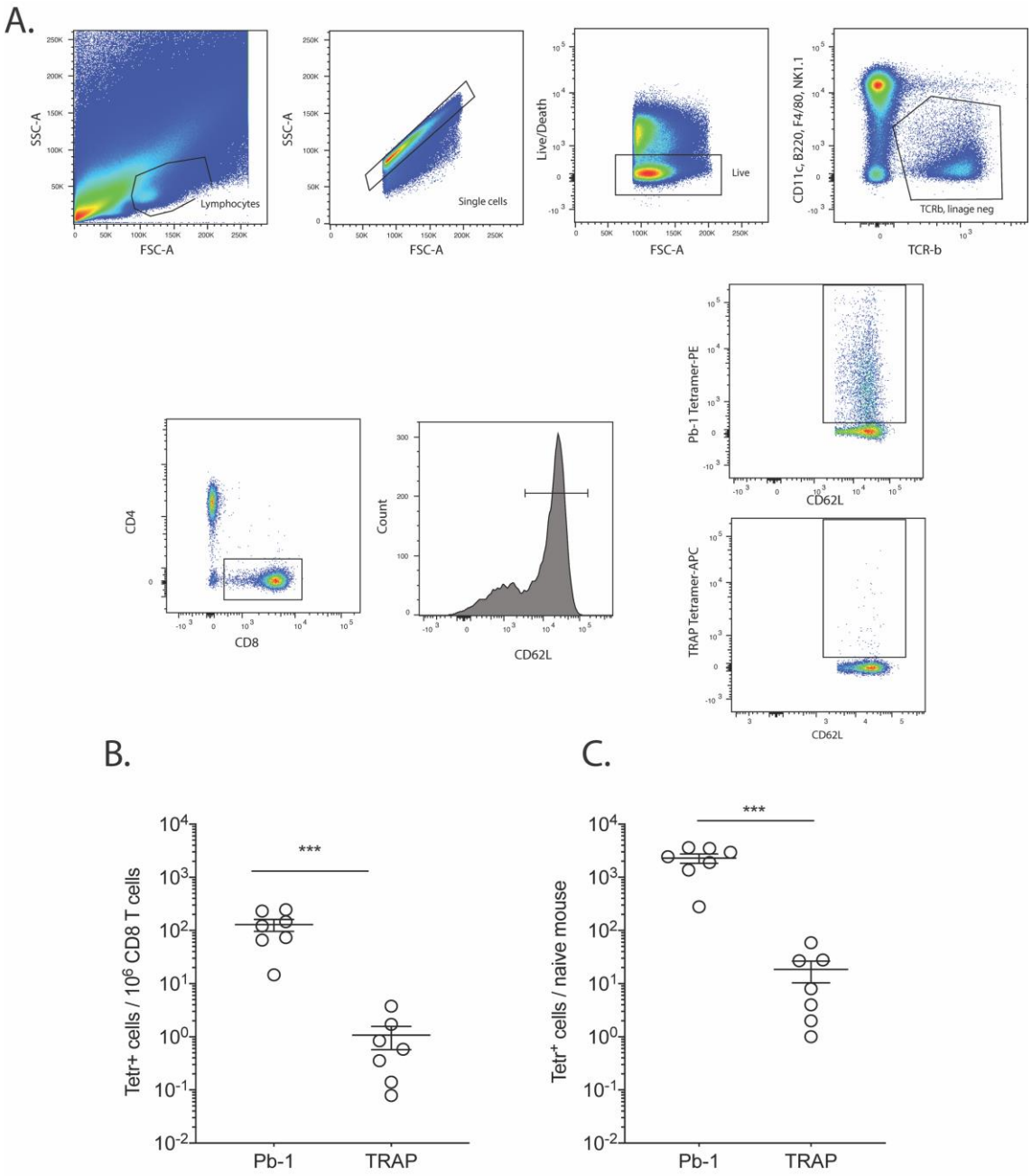

Figure 3.4. The naive Pb-1-specific CD8 T cell repertoire is very large. CD8 T cells specific for Pb-1 and the $TRAP_{130-138}$ epitopes were enriched from single cell suspensions of the spleen and major lymph nodes of naive B6 mice using specific tetramers. Cells were then stained for TCRβ, CD8, CD4, CD11c, B220, F4/80, NK1.1 and CD62L. A. Gating strategy on a representative sample: Naive tetramer-positive cells were defined as TCRβ+, linage$^-$, CD4$^-$, CD8$^+$, CD62L^{Hi} and Pb-1 tetramer-positive. Number of tetramer-positive cells per million CD8 T cells (B) and per mouse (C) are displayed. Results were pooled from two independent experiments. Data were log-transformed and compared by unpaired T-test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

and this particular epitope were reviewed on Chapter 1. The number of $TRAP_{130-138}$ precursors has been reported (Van Braeckel-Budimir et al., 2017); and was used here as an internal control for the technique. Cells were stained for TCRβ, CD8, CD4, CD11c, B220, F4/80, NK1.1 and CD62L plus either the tetramer for Pb-1 or for $TRAP_{130-138}$. Naive tetramer-positive cells were defined as TCRβ+, $linage^-$, $CD4^-$, $CD8^+$, $CD62L^{Hi}$ and tetramer-positive (Figure 3.4 A).

The frequency of TRAP-specific naive precursors ranged between 1 and 59 cells per mouse; with these numbers consistent with a previous report (Van Braeckel-Budimir et al., 2017). On the other hand, the average number of Pb-1-specific cells in naive B6 mice averaged 2.3 ± 0.4 x 10^3 cells per mouse, with variation ranging from just 278 to 3619 (Fig. 3.4 C). When compared to other well-known specificities such as $OVA_{257-264}$ or LCMV GP_{33-41} with 183 ± 33 and 358 ± 40 cells per mouse, respectively (Jenkins and Moon, 2012), the number of Pb-1-specific naive precursors can be considered as extremely high. This compares well to $GAP50_{40-48}$, another *P. berghei* epitope, recently reported to exhibit a large number of naïve precursors in the range of 2.9 ± 0.4 x 10^3 cells per mouse (Van Braeckel-Budimir et al., 2017).

Subsequently, the endogenous response of Pb-1-specific CD8 T cell response was studied in the context of natural infection. Data confirmed expansion of Pb1-specific precursors after blood-stage infection and memory formation in the liver after RAS immunisation. These experiments were performed by two postdoctoral researchers from our group, Maria Menezes and Nazanin Ghazanfari. For the blood infection experiment, naive B6 mice were infected with iRBC and 7 days later the number of tetramer-specific CD8 T cells was determined in spleen. For the RAS immunisation experiment, mice were injected with RAS and 25 days later, the number of specific memory cells was determined in the spleen and the liver. A large expansion of activated cells was observed in the spleen after blood stage compared to naive mice (Fig. 3.5 A), as was expansion of memory specific CD8 T cells in response to RAS immunisation (Fig. 3.5 B). The above findings showed that Pb-1-specific CD8 T cells could

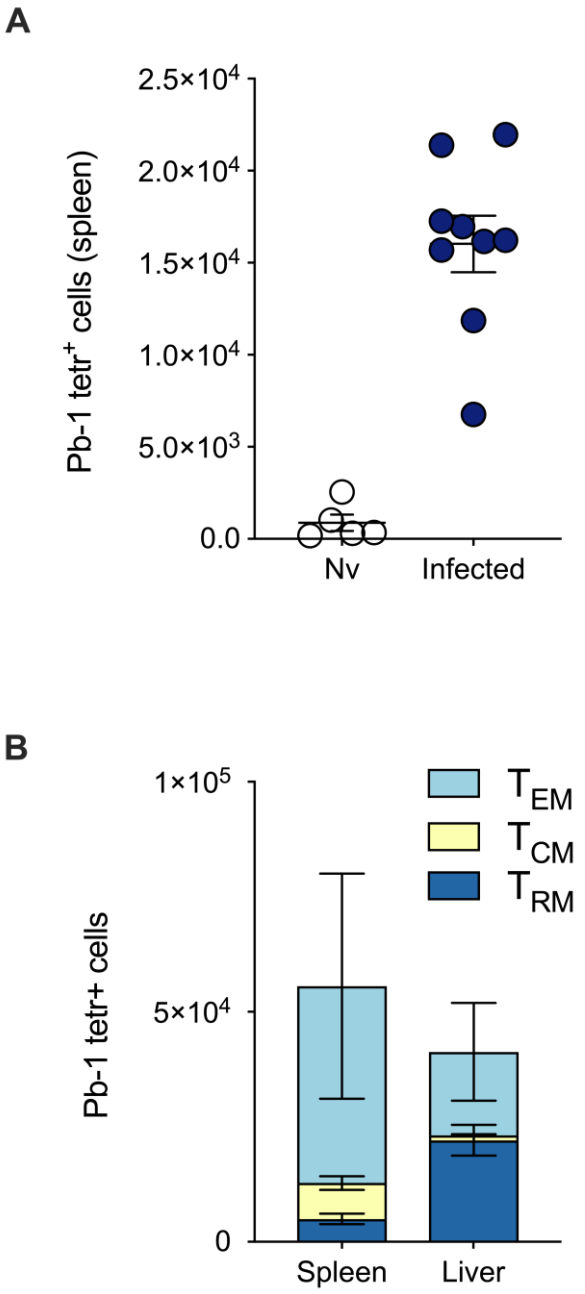

Figure 3.5. Expansion of Pb-1-specific CD8 T cells in response to blood stage and RAS infection. A. Mice were infected with 10^4 PbA iRBC, 7 days later spleens were dissected, and single cell suspensions were stained with Pb-1 tetramer and CD44. Activated Pb-1-specific CD8 T cells are displayed. B. Spleen and livers from mice primed with $5x10^4$ PbA RAS were collected 25 days post infection. Single cell suspensions were stained with Pb-1 tetramer, CD8, CD44, CD62L and CD69. Numbers of specific memory CD8 T cells subpopulations are displayed. Results were pooled from two independent experiments. Data were log-transformed and compared by unpaired T-test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001. Data obtained by Maria Menezes and Nazanin Ghazanfari modified from (Valencia-Hernandez et al., 2020).

respond to both blood-stage and RAS PbA in B6 mice but did not address the protective capacity of this response against liver-stage infection. The next series of experiments, therefore, addressed whether immunisation with αClec9-Pb-1 mAb could elicit a protective memory response against the liver stage of malaria through stimulation of the endogenous T cell repertoire.

Mice were immunised with 2 μg or 16 μg of αClec9-Pb-1 or 2 μg of αClec9-NVY plus CpG; some mice were transferred with PbT-I cells and used as control for the αClec9 mAb priming, while other mice were left without cell transfer to monitor the response of the endogenous repertoire. 35-46 days later, when the memory cells had been formed, spleens and livers of some mice were analysed by flow cytometry for memory cell formation; and the remaining mice were challenged intravenously with live sporozoites on day 35 (Fig. 3.6 A).

After the challenge, blood samples were assessed for the presence of parasites up to day 10 post-infection. Mice were considered sterile protected when no blood stage parasites were detected 10 days after challenge.

Mice transferred with PbT-I cells and immunised with 2 μg of αClec9-Pb1 + CpG generated similar numbers of liver T_{RM} cells to those that did not receive PbT-I cells and were immunized with 16 μg of αClec9-Pb1 + CpG (Fig. 3.6 B). In absence of PbT-I cells, mice vaccinated with high dose of Ab generated on average 1 +/- 0.2 x 10^5 tetramer-positive T_{RM} cells per liver. On the other hand, mice that did not receive PbT-I cells and were immunised with 2 μg of αClec9-Pb1 generated significantly less liver T_{RM} cells. A similar pattern was also observed for the other memory populations of T_{EM} and T_{CM} cells in the spleen and the liver (Fig. 3.6 C, D).

Seven days after challenge all naive control mice were parasitemic; mice that received PbT-I cells and were primed with 2 μg of αClec9-NVY as well as

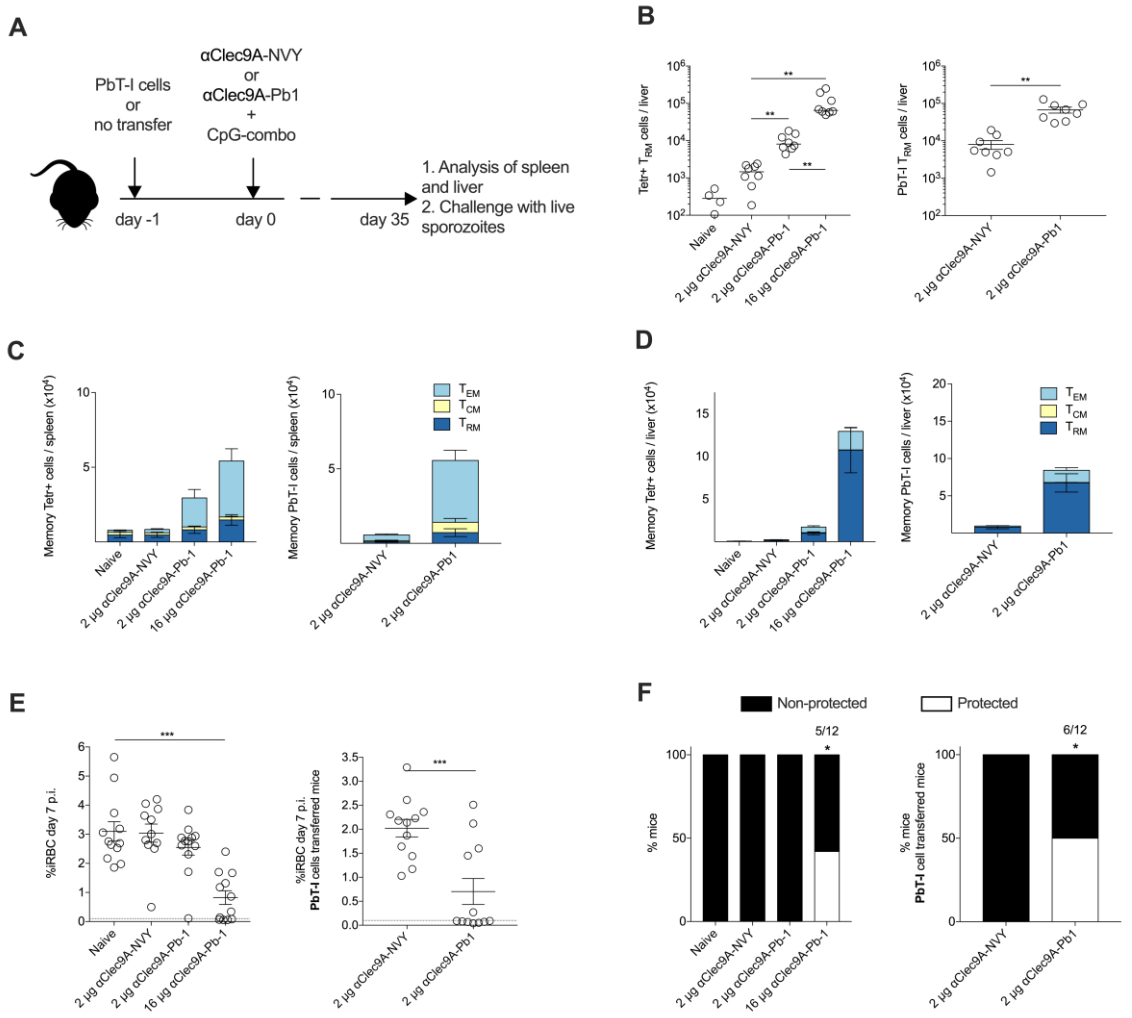

Figure 3.6 Immunisation with αClec9-Pb-1 mAb protects B6 mice against sporozoite challenge. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells or no cells and were immunised with αClec9-Pb-1 or αClec9-NVY plus 75 μg of CpG on the next day. Spleens and livers from 4 mice were collected on day 35 or 46. Single cell suspensions were generated and the number of PbT-I or tetramer-Pb1 positive cells and memory status were determined by flow cytometry. The remaining mice (n=6) were challenged intravenously with 200 PbA sporozoites on day 35. Mice were monitored daily for parasitemia from day 6 until day 10 after challenge, when the percentage of sterile protection was determined. A. Schematic for the protection experiment. B. Number of T_{RM} cells per liver determined as CD8+ GFP+ or Tetr+, CD44Hi, CD62L-, CD69+. C. and D. Number of the CD8+ memory cells in spleen and liver. E. Percentage of infected red blood cells (iRBC) in blood on day 7 post challenge. F. Percentage of sterile protection determined by absence of blood stage infection by day 15 after challenge. Results were pooled of two independent experiments. Data were log-transformed and compared by one-way ANOVA with Tukey's multiple comparisons test in (B.), using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (E.) or using Fisher's exact test (F.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

mice with no PbT-I cells immunised with either 2 μg of αClec9-NVY or 2 μg αClec9-Pb-1 were also positive for parasitemia on day 7 post infection. In contrast, 7 mice that received PbT-I cells and were primed with 2 μg αClec9-Pb1 and 5 mice with no PbT-I cells injected with 16 μg αClec9-Pb1 were negative for parasitemia (Fig. 3.6 E). Data from day 10, revealed that 41% of the mice with a normal endogenous T cell repertoire that were immunised with 16 μg of αClec9-Pb1 mAb plus CpG successfully controlled the liver infection and therefore were considered sterilely protected (Fig. 3.6 F).

This is the first time with this system that vaccinated B6 mice challenged with PbA liver-stage parasites become sterile protected in the absence of transgenic PbT-I cells. This suggests the Pb-1 epitope is an excellent antigen for the induction of protective immunity.

3.2.3 Induction of memory CD8 T cells using the Pb-1 epitope in prime-and-trap vaccination:

“Prime-and-trap” is a three-component vaccine strategy used to induce liver T_{RM} cell formation through the priming of naive immune cells and the trapping of those cells in the liver (Fernandez-Ruiz et al., 2016). The first component is based on the use of an αClec9 mAb coupled to a relevant epitope to achieve epitope delivery to cDC1 (Caminschi et al., 2008; Lahoud et al., 2011); which is the main antigen presenting cell stimulating T cells in malaria (Fernandez-Ruiz et al., 2017; Lau et al., 2014). The second component is a recombinant adeno-associated virus (rAAV) engineered to selectively infect hepatocytes (Tay et al., 2014), inducing the trapping of the T cells to the liver. This vector is also modified for the expression of the relevant epitope under the control of a hepatocyte-specific promoter. The third and last component, is a CpG adjuvant used for the licensing of DC involve in CD8 T cell priming (Lahoud et al., 2011) and to promote proinflammatory liver-microenvironment (Huang et al., 2013), which aids T_{RM} cell formation (Holz et al., 2018; Mackay et al., 2012) . In the past, it has been shown

that mice transferred with PbT-I cells and vaccinated with prime-and-trap using the mimic NVY epitope are sterilely protected against sporozoite challenge (Fernandez-Ruiz et al., 2016).

After confirming the efficacy of αClec9-Pb-1 mAb at promoting the expansion and memory formation of specific-endogenous CD8 T cells, the ability of the prime-and-trap vaccine to generate specific-endogenous liver T_{RM} cells and induce protection against malaria was assessed using the Pb-1 epitope. To achieve this goal, reagents containing the Pb-1 epitope, including αClec9 mAb and rAAV particles expressing the Pb-1 epitope, were generated.

The priming capacity of the rAAV-Pb-1 alone (i.e. “trap” only) on the endogenous repertoire was first investigated. Mice with a normal endogenous T cell repertoire or that had been transferred with PbT-I cells were injected with increasing doses of rAAV-Pb-1. Livers and spleens were collected on day 35 post-injection when the viral particles has been cleared (Tay et al., 2014).

Analysis of the organs failed to identify specific T cells (data not shown). Spleens and livers were collected and analysed by flow cytometry for the presence of PbT-I cells or Pb-1 tetramer-positive cells. Neither GFP+ nor tetramer-positive cells were detected on day 35 in spleen or liver, indicating that the rAAV-Pb-1 on its own had no effect on the priming and or recruiting of PbT-I cells or Pb-1-specific cells. Endogenous non-specific cells were also analysed, to determine if this vaccine component in any way disturbed these populations. No differences in the number of memory cells in the liver or spleen were observed (including T_{RM} cells) among groups injected with different doses of rAAV-Pb-1 or control naive mice.

While priming with rAAV-Pb-1 alone was ineffective, it was still possible that when combined with αClec9-Pb-1 mAb and CpG, liver T_{RM} formation might be boosted. To test this possibility, mice were transferred with PbT-I cells and were immunised with αClec9-Pb-1 mAb, CpG and rAAV-Pb-1 on the next day.

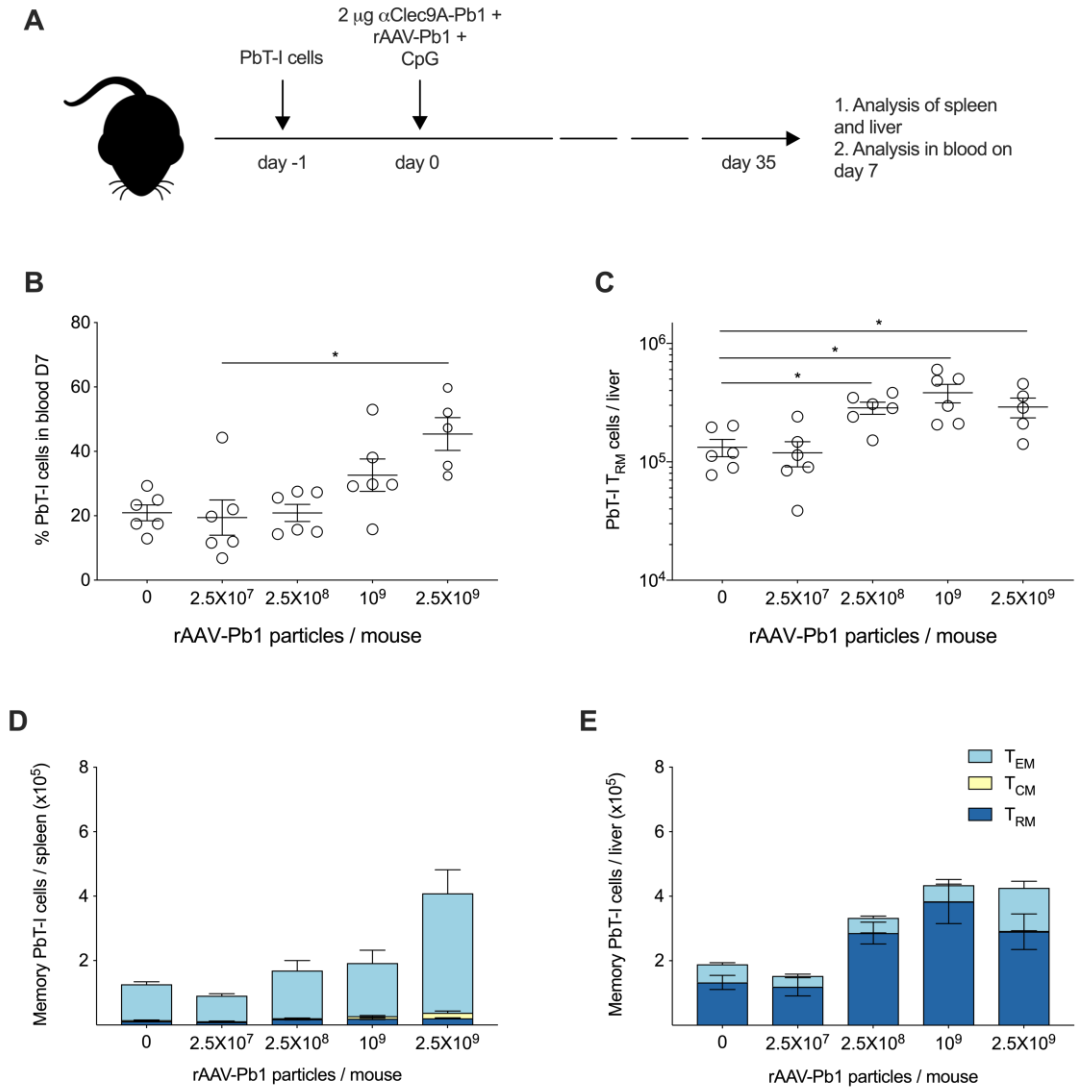

Figure 3.7. Prime-and-trap-Pb-1 induced high numbers of PbT-I T_{RM} cells in the liver. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and next day immunised with 2 μg of αClec9-Pb-1 plus 75 μg of CpG plus different doses of rAAV-Pb-1. Mice were bled on day 7 and number of PbT-I cells was determined by flow cytometry. Spleens and livers were collected on day 35, for the analysis of memory formation. A. Schematic of the mice immunisation B. Percentage of PbT-I cells in blood on day 7, calculated as the frequency of total CD8+ cells. Data were compared by one-way ANOVA and Kruskal-Wallis test with Dunn's multiple comparison post-test. C. Number of T_{RM} cells per liver. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test. C. and E. Number of the PbT-I CD8+ memory cells in spleen and liver, respectively. Results were pooled of two independent experiments. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

Four different doses of rAAV-Pb-1 were tested, ranging from $2.5x10^7$ to $2.5x10^9$ viral particles per mouse; and one group were primed with αClec9-Pb-1 mAb and CpG without rAAV-Pb-1. Livers and spleens were analysed on day 35.

On day 7 after priming, 20% of total CD8 T cells corresponded to PbT-I cells in the blood of mice immunised with αClec9-Pb-1 mAb and CpG alone; this percentage increased up to 60% in mice that received prime-and-trap with a dose of $2.5x10^9$ rAAV-Pb-1 viral particles (Fig. 3.7 B). The percentage of PbT-I cells in blood correlated roughly with the number of PbT-I T_{RM} cells in the liver on day 35, though a slight decrease in liver T_{RM} numbers occurred at the highest viral dose (Fig. 3.7 C). The maximum number of PbT-I T_{RM} cells per liver was achieved in mice vaccinated with 2 μg of αClec9-Pb-1 mAb, CpG and 10^9 rAAV-Pb-1, with a mean of 3.8 +/- 0.7 x 10^5 T_{RM} cells per liver. Mice vaccinated with a dose equal or higher than $2.5x10^8$ rAAV-Pb-1 formed significantly higher numbers of liver T_{RM} cells compared to mice that only got αClec9-Pb-1 mAb plus CpG (Fig. 3.7 C). Other memory subpopulations showed a similar pattern; in fact, increasing numbers of spleen T_{EM} cells formed in mice vaccinated with the highest doses of rAAV-Pb-1 (Fig 3.7 D, E).

These results demonstrated that indeed rAAV-Pb-1 is able to boost liver T_{RM} cell formation in the PbT-I system. Subsequently, the same effect was studied in mice with normal endogenous T cell repertoire i.e. no transferred PbT-I cells. This experiment assessed the expansion potential of Pb-1 specific precursor cells. In this case, mice were immunised with αClec9-Pb-1 mAb, CpG and increasing doses of rAAV-Pb-1. Stained cells from livers and spleens were analysed on day 35 (Fig. 3.8 A). Unlike the previous experiment, no differences were evident in the percentage of Pb-1 tetramer-positive cells in the blood on day 7 (Fig 3.8 B). However, on day 35 after immunisation, the number of liver T_{RM} cells increased with the amount of rAAV-Pb-1 virus administered (Fig. 3.8 C, E).

The maximum number of Pb-1 tetramer-positive T_{RM} cells per liver was achieved when mice were vaccinated with αClec9-Pb-1 mAb, CpG and $2.5x10^9$

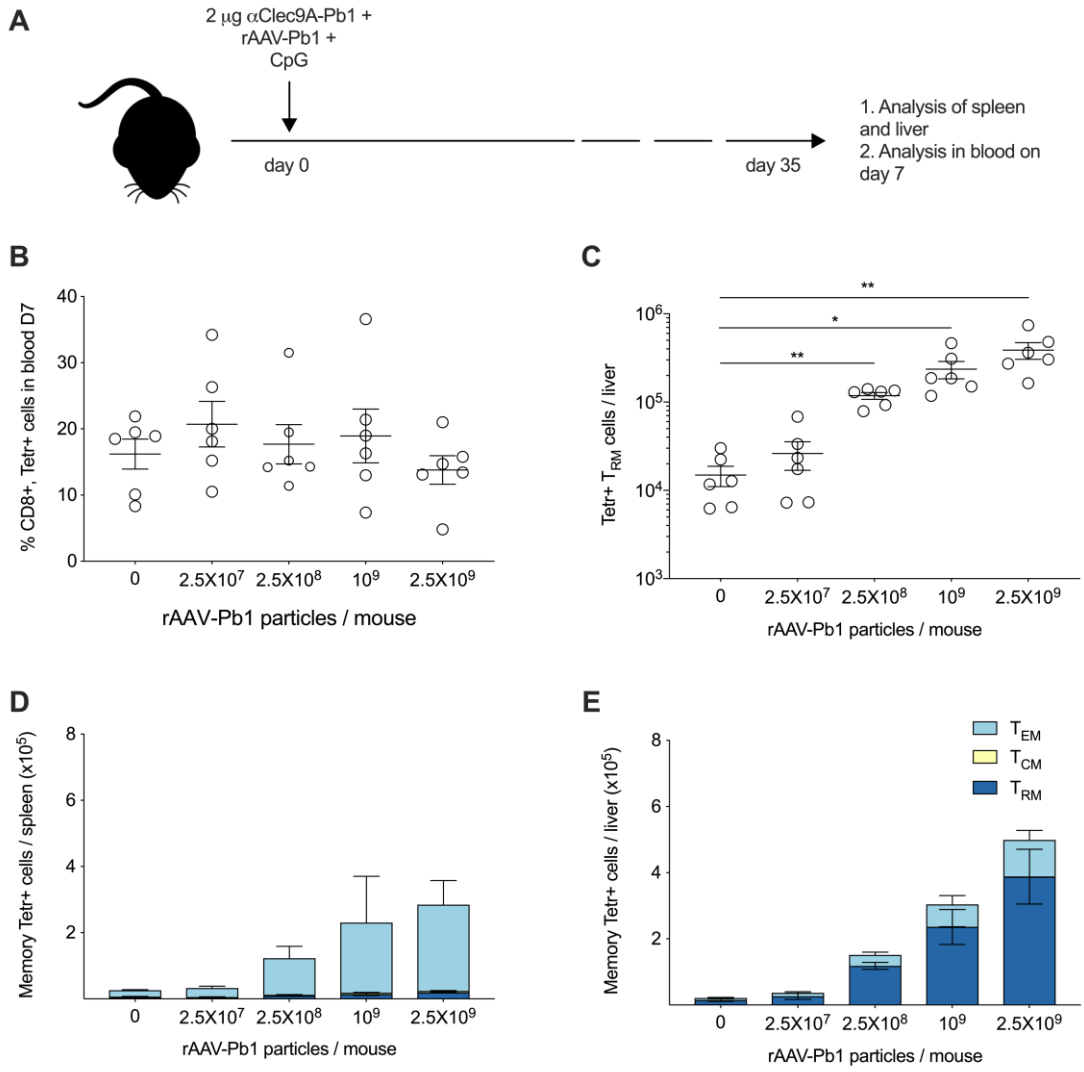

Figure 3.8. Prime-and-trap-Pb-1 induced high numbers of T_{RM} cells in the liver of B6 mice with endogenous repertoire. B6 mice were immunised with 2 μg of αClec9-Pb-1 plus 75 μg of CpG plus different doses of rAAV-Pb-1. Mice were bled on day 7 and number of Pb-1 tetramer-positive cells was determined by flow cytometry. Spleens and livers were collected on day 35, single cell suspensions were generated and the number of Pb-1 tetramer-positive cells and memory cell subset were determined by flow cytometry. A. Schematic of the mice immunisation B. Percentage of tetramer-positive cells in blood on day 7. C. Number of T_{RM} cells per liver. D. and E. Number of the Pb-1 tetramer-positive, CD8+ memory cells in spleen and liver, respectively. Results were pooled of two independent experiments. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

rAAV-Pb-1 (mean of $3.8x10^5$ T_{RM} cells per liver). Mice vaccinated with a dose equal or higher than $2.5x10^8$ rAAV-Pb-1 developed significantly higher liver T_{RM} cells compared to mice that only received αClec9-Pb-1 mAb plus CpG (Fig. 3.8 C).

Similar patterns were evident for the other memory sub-populations (Fig. 3.8 D, E). Together, these data confirmed the boosting capacity of rAAV-Pb-1 for the generation of liver T_{RM} cells in the context of prime-and-trap vaccination in B6 mice with normal endogenous T cell repertoire.

Previous studies have shown that numbers of specific liver T_{RM} cells correlate with levels of protection against sporozoite infection (Fernandez-Ruiz et al., 2016). Consequently, the next experiment sought to determine whether the number of cells could be increased by using even higher doses of the rAAV-Pb-1 virus. These experiments were performed only in mice with normal endogenous repertoire, in the absence of PbT-I cells. For this purpose, mice were immunised with αClec9-Pb-1 mAb, CpG and four different doses of rAAV-Pb-1, ranging from 10^9 to $3.3x10^{10}$ viral particles per mouse; and one group was primed only with αClec9-Pb-1 mAb and CpG. Livers and spleens were collected and processed for single cells staining and flow cytometry analysis on day 35 post immunisation.

Blood analysis on day 7 revealed a peak of Pb-1 specific CD8 T cells when injecting $3.3x10^9$ viral particles per mouse and lower numbers when using 10^{10} or more viral particles per mouse (Fig. 3.9 B). The numbers of T_{RM} cells in the livers mirrored this pattern, with a maximum mean of $3.2x10^5$ T_{RM} cells per liver at $3.3x10^9$ viral particles and significantly reduced mean of $5.7x10^3$ T_{RM} cells when injecting $3.3x10^{10}$ particles (Fig. 3.9 C). T_{EM} and T_{CM} cells in spleen and liver expanded and contracted in the same way as the liver T_{RM} cells (Fig. 3.9 D, E).

In 2014, Tay and collaborators described how persisting high levels of antigen expression by rAAV promote the silencing of the CTL function (Tay et al.,

2014). In these studies, mice injected with high doses of rAAV-mOVA showed significantly less specific killing, high levels of programmed death-1 (PD-1) and T-cell immunoglobulin mucin 3 (Tim-3) expression in liver CD8 T cells and low expression of IFN-γ upon *ex-vivo* restimulation (Tay et al., 2014). In fact, the expression of PD-1 has been associated with immunotolerance and CD8 T cell apoptosis mediated by PD-1 ligands in the context of chronic viral infections and persistence of antigen presentation (Golden-Mason et al., 2007; Okazaki and Honjo, 2006). Tim-3 is an inhibitory molecule induced during activation and involved in T-cell exhaustion and tolerance in cancers and chronic infections (Jones et al., 2008; Monney et al., 2002; Sabatos et al., 2003; Sakuishi et al., 2010; Sánchez-Fueyo et al., 2003). To investigate whether the reduction on the memory cell numbers that was observed at high doses of rAAV-Pb-1 is governed by a similar mechanism, the expression profile of Tim-3 and PD-1 was determined on day 35 after immunisation.

Indeed, activated specific CD8 T cells from the liver of mice vaccinated with high doses of rAAV-Pb-1 expressed significantly higher levels of PD-1 and Tim-3 (Fig 3.9 F, G). Very importantly, the expression of PD-1 was highly susceptible to small dose changes of the adenovirus. In fact, activated CD8 T cell from mice injected with $3.3x10^9$ rAAV-Pb-1 expressed significantly higher levels of PD-1 compared to mice that were injected with 10^9. Higher levels of Tim-3 were observed on mice that received 10^{10} or more viral particles. This result indicates that the levels of antigen expression greatly influence the numbers of liver T_{RM} cells generated, and therefore the doses of rAAV-Pb-1 should be considering carefully during the formulation of an efficient vaccine.

In summary, these findings revealed that prime-and-trap using the Pb-1 epitope as reagent induces very high numbers of liver specific T_{RM} cells in B6 mice with a normal endogenous repertoire or with transferred PbT-I cells. This number was maximized by immunisations with αClec9-Pb-1 mAb, CpG plus doses of rAAV-Pb-1 ranging from 10^9 to $3.3x10^9$ particles per mouse. However, raised levels of PD-1, a hallmark for T cell exhaustion (Okazaki and Honjo, 2006),

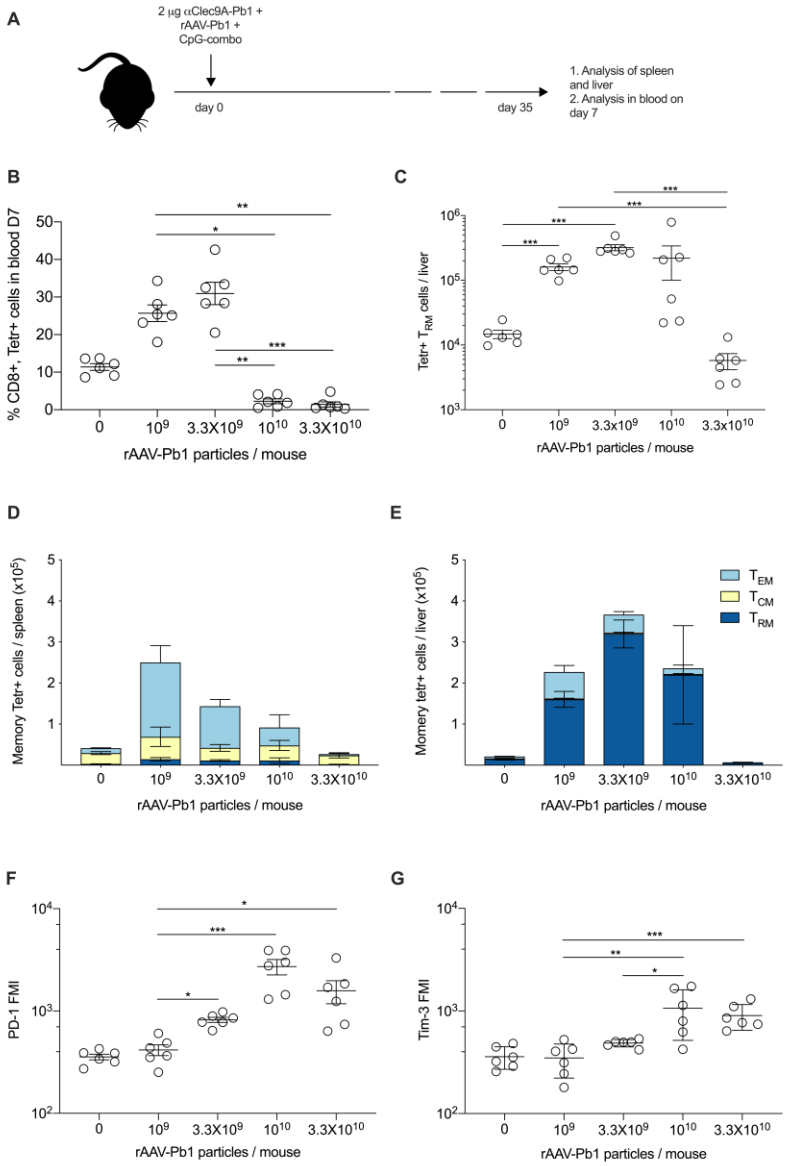

Figure 3.9. Adverse effect of very high doses of rAAV-Pb-1 on the liver T_{RM} cell formation. B6 mice were immunised with 2 μg of αClec9-Pb-1, 75 μg of CpG and different high doses of rAAV-Pb-1. Mice were bled on day 7 and number of Pb-1 tetramer-positive cells was determined by flow cytometry. Spleens and livers were collected on day 35, single cell suspensions were generated and the number of Pb-1 tetramer-positive cells and memory status were determined by flow cytometry. A. Schematic of the mice immunisation B. Percentage of tetramer-positive cells in blood on day 7; data were compared by Kruskal-Wallis test with Dunn's multiple comparison post-test. C. Number of T_{RM} cells per liver. D. and E. Number of the Pb-1 tetramer-positive, CD8+ memory cells in spleen and liver, respectively. Analysis of PD-1 and Tim-3 expression on activated (CD44Hi) liver CD8 T cells from mice vaccinated with prime-and-trap and high doses of rAAV-Pb-1. Fluorescence mean intensity for PD-1 (F.) and Tim-3 (G.). Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

were observed on liver CD8 T cells from mice injected with $3.3x10^9$. Consequently, it was determined the best dose of rAAV-Pb-1 for prime-and-trap to be between $2.5x10^9$ and 10^9 viral particles per mouse.

3.2.4 Analysis of sterile protection against sporozoite infection by prime-and-trap vaccination using the Pb-1 antigen:

Given the significant increase in liver T_{RM} cells induced by the introduction of the rAAV-Pb-1 component, the capacity of prime-and-trap with the Pb-1 epitope to induce sterilizing immunity in B6 mice challenged with PbA sporozoites was assessed.

On previous experiments, the dose of Ab was 2 µg of αClec9-Pb-1 mAb per mouse and the optimal dose of rAAV-Pb-1 ranged between 1-2.5 x 10^9 viral particles. For a first set of experiments we sought to investigate if higher doses of Ab could revert the upregulation of PD-1 and Tim-3 at high rAAV doses. For this, five different B6 mice groups were analysed. Two groups received 2 µg of αClec9-Pb-1 mAb plus CpG plus either 2.5 X 10^9 or 10^{10} of rAAV- Pb-1. The other two groups received 8 µg of αClec9-Pb-1 mAb plus CpG plus either 2.5 X 10^9 or 10^{10} of rAAV- Pb-1. The last group remained naive. On day 35, 3 mice per group were used for the enumeration of memory T cells populations in liver and spleen. The remaining mice (n=6) were challenged intravenously with 200 sporozoites and sterile protection was determined on day 18 post infection.

While there was a tendency towards higher numbers, there was no significant increase in liver T_{RM} cells in mice vaccinated with the higher dose of αClec9-Pb-1 mAb (Fig. 3.10 C). On the other hand, the percentage of Pb-1 specific, circulating CD8 T cells on day 7 post immunisation was lower in mice injected with high doses of the viral particles compared to the mice injected with 2.5 x 10^9 rAAV- Pb-1 (Fig. 3.10 B). A similar tendency was found on the number of liver T_{RM} cells (Fig. 3.10 C), though this did not reach significance. PD-1 and Tim-3 expression was significantly higher on memory CD8 T cells of mice injected

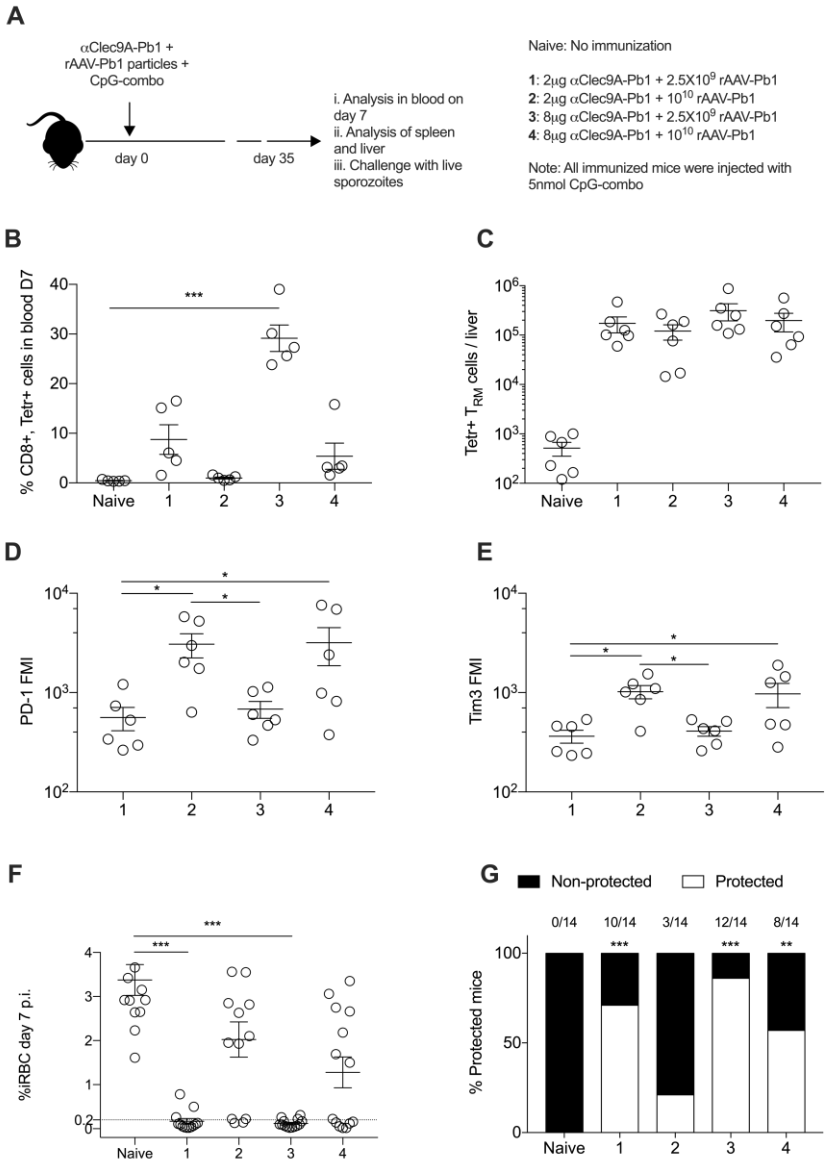

Figure 3.10 Effect of high doses of rAAV-Pb-1 on the induction of protection in mice vaccinated with Prime-and-trap-Pb-1. B6 mice were immunised with two different doses of αClec9-Pb-1, 75 μg of CpG and two different doses of rAAV-Pb-1. One group of mice remained naive as a control for the viability of the sporozoites. Spleens and livers from 4 mice were collected on day 35 or 46. Single cell suspensions were generated. The remaining mice (n=6) were challenged intravenously with 200 living PbA sporozoites on day 35. A. Schematic of the mice immunisation B. Percentage of tetramer-positive cells in blood on day 7. C. Number of T_{RM} cells per liver D. and E. Fluorescence mean intensity for PD-1 and Tim-3. F. Percentage of infected red blood cells (iRBC) in blood on day 7 post challenge. G. Percentage of sterile protection determined by day 18 after challenge. Results are a pool of two independent experiments. Data were compared using Kruskal- Wallis test with Dunn's multiple comparison post-test in (B. and F.), log-transformed and compared using one-way ANOVA and Tukey's multiple comparisons test in (D. and E.) or using Fisher's exact test in (G.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

with 10^{10} rAAV- Pb-1, compared to the lower dose of viral particles (Fig. 3.10 D, E). Protection was clearly affected by the use of high doses of the viral particles (Fig. 3.10 F, G). Mice injected with the lowest dose of viral particles showed a lower percentage of infected red blood cells on day 7 post infection and superior sterile protection. Importantly, approximately 80% sterile protection was achieved in mice vaccinated with 8 µg of αClec9- Pb-1 + 2.5 X 10^9 rAAV- Pb-1 + 75 µg CpG (Fig. 3.10 G).

These results together with the observation that memory cells from mice injected with 3.3x10^9 rAAV-Pb-1 expressed significantly higher levels of PD-1 compared to those injected with 10^9, led to the speculation that the optimal dose of viral particles for the for prime-and-trap-Pb-1 is 10^9 rAAV-Pb-1.

During the first round of experiments (Fig. 3.1 B and 3.6 B), it was observed that 16 µg of αClec9-Pb-1 mAb induced the highest number of liver T_{RM} cells when used alone, i.e. without rAAV-Pb-1 particles. Consequently, the role of this high dose of αClec9-Pb-1 mAb was investigated in the context of a malaria infection in prime-and-trap vaccinated mice.

B6 were vaccinated with either 2 µg or 16 µg of αClec9-Pb-1 mAb, CpG and 10^9 rAAV-Pb-1 (some mice were transferred with PbT-I cells as an experimental control). On day 35, when the virus has been cleared and thus memory cells have formed, some mice were used for the numeration of memory cells in spleen and liver, while the remaining mice were infected intravenously with 200 sporozoites as indicated on figure 3.11 A. The average number of liver T_{RM} cells generated on day 35 ranged between 2.3 to 3.3 x 10^5 cells per liver among all the different groups (Fig. 3.11 B). Surprisingly, mice transferred with PbT-I cells prior vaccination did not form more liver T_{RM} cells than mice with a normal endogenous T cell repertoire. Furthermore, increasing the dose of αClec9-Pb-1 from 2 µg up to 16 µg had no effect on the formation of T_{RM} cells. The number of effector memory cells in spleen and liver were similar among the

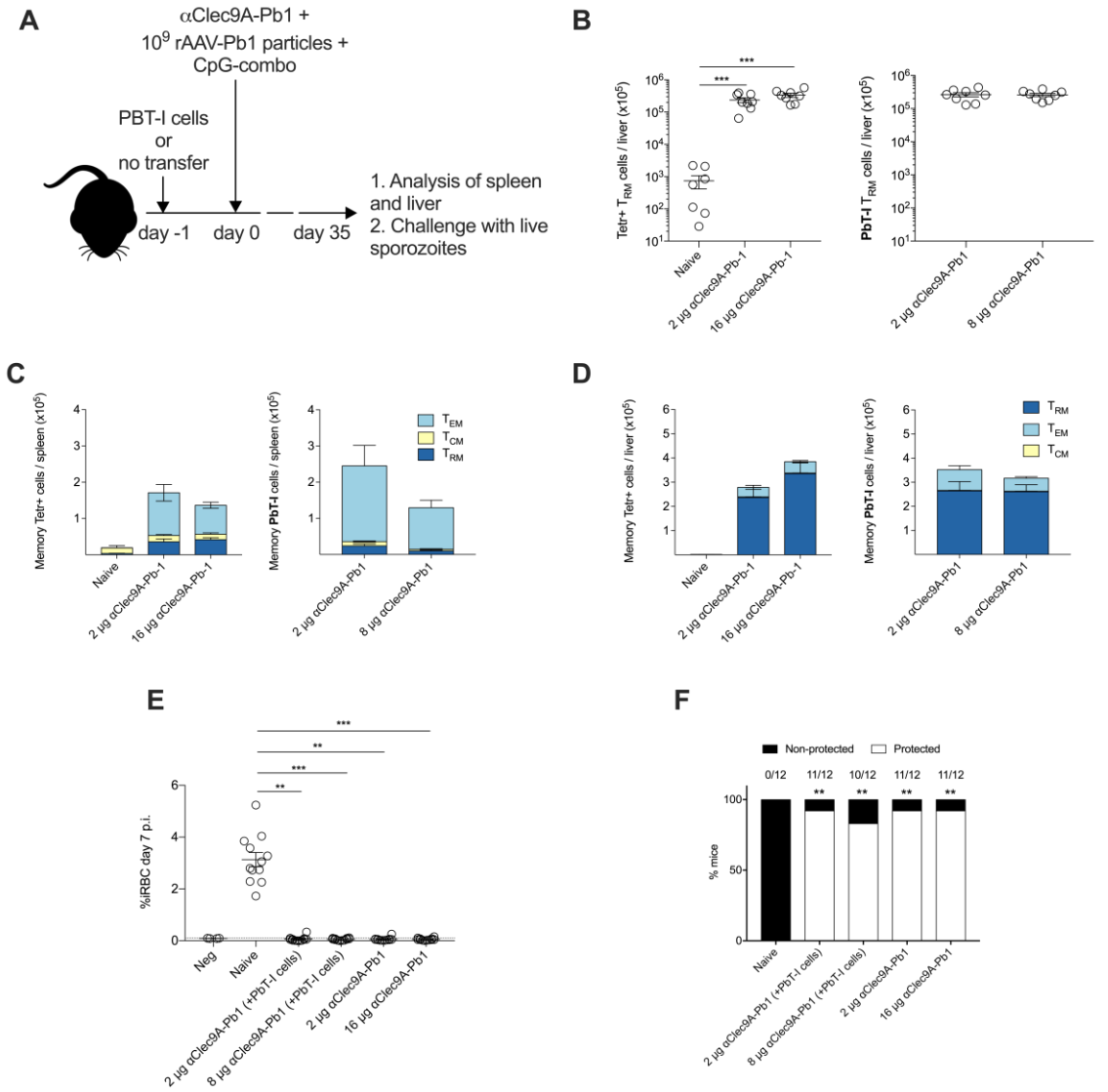

Figure 3.11 Prime-and-trap-Pb-1 induces more than 90% protection in B6 mice against sporozoite challenge. B6 mice were transferred with 50,000 PbT-I cells or no cells one day prior vaccination. Next day, mice immunised with different doses of αClec9-Pb-1, 75 µg of CpG and 10^9 viral particles of rAAV-Pb-1. Spleens and livers from 4 mice were collected on day 35. Single cell suspensions were generated. The remaining mice (n=6) were challenged intravenously with 200 living PbA sporozoites on day 35. A. Schematic of the mice immunisation B. Number of specific liver T_{RM} cells C. Number of memory CD8 T cells in spleen and livers of mice transferred with PbT-I cells D. Number of memory CD8 T cells in spleen and livers of mice with normal endogenous T cell repertoire E. Percentage of infected red blood cells (iRBC) in blood on day 7 post challenge. F. Percentage of sterile protection determined by day 18 after challenge. Results are a pool of two independent experiments. Data were log-transformed and compared using one-way ANOVA and Tukey's multiple comparisons test in (B.), using Kruskal- Wallis test with Dunn's multiple comparison post-test in (E.), or using Fisher's exact test in (F.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

different groups (Fig. 3.11 C and D). The levels of parasitemia on day 7 post infection were very low; additionally, more than 90% of the mice with a normal endogenous T cell repertoire or those transferred with PbT-I cells were sterilely protected against liver stage malaria (Fig. 3.11E and F).

In 2018 Gola and collaborators published a study using a similar vaccination principle to prime-and-trap, to protect B6 mice from *P. berghei* sporozoite infection, which they termed prime-and-target. For this strategy, they first primed the mice by injecting them intramuscularly with a modified adenovirus expressing an antigen of interest; and then two weeks later, they target the circulating specific CD8 T cells to the liver by injecting intravenously nanoparticles containing the specific peptide (Gola et al., 2018). With this technique they achieved high numbers of OVA-specific liver-associated CD8 T cells conferring 100% protection against transgenic *P. berghei* parasites expressing the OVA peptide (Gola et al., 2018).

These findings from Gola and collaborators motivated the following experiments, in which the effect of a “delayed trapping” for the generation of liver T_{RM} cells was tested: B6 mice were immunised with αClec9-Pb-1 mAb plus CpG and 14 days later they were injected with 10^9 rAAV-Pb-1 viral particles. On day 35 after the last injection the number of liver T_{RM} cells was determined and mice were challenged with sporozoites.

The number of liver T_{RM} cells was not affected by the “delayed trapping” of two weeks, as similarly high numbers of these cells were obtained in mice vaccinated with the original prime-and-trap or the “delayed trapping” immunisation (Fig. 3.12 B and D). This was reflected on the high levels of protection (i.e. 100%) observed for all vaccinated groups (Fig. 3.12 E and F).

In summary, these data suggest that vaccination with prime-and-trap using the Pb-1 epitope is able to confer high levels of sterile protection in B6 mice either transferred with PbT-I cells or through their normal endogenous T cell repertoire.

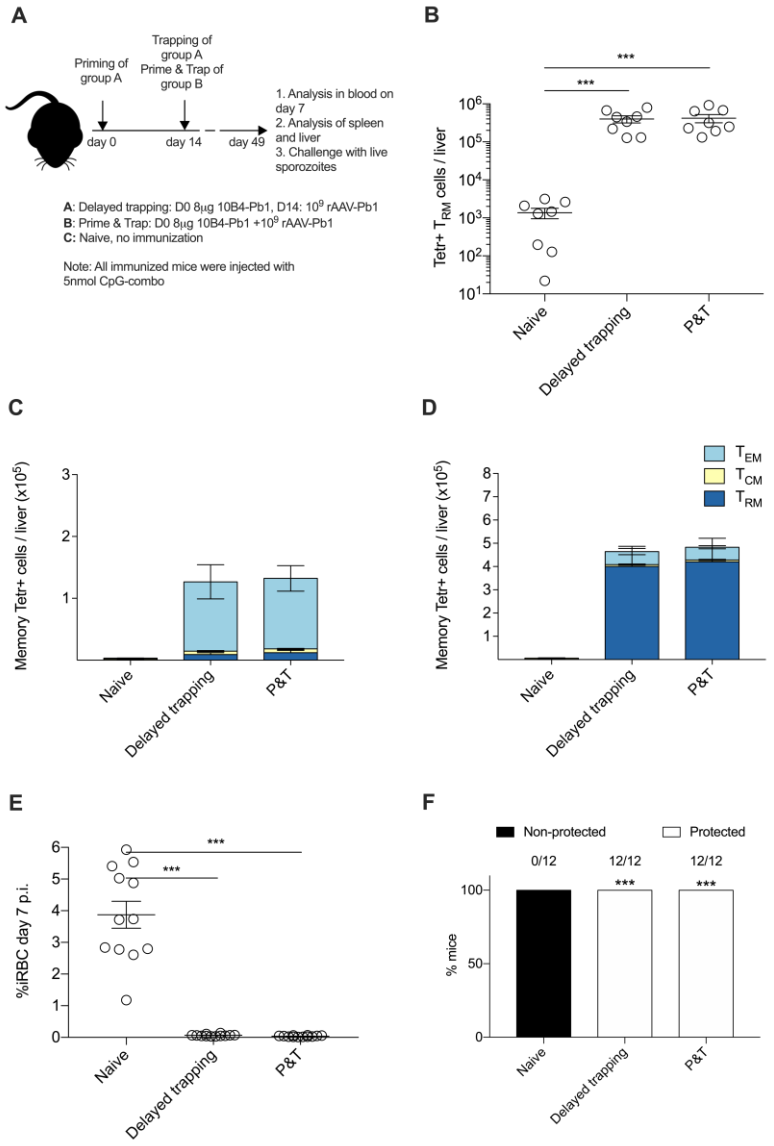

Figure 3.12 Delayed trapping with prime-and-trap-Pb-1. On day 0 a group of mice were injected with 8 µg of αClec9- Pb-1 + 75 µg CpG, 14 days later same group of mice were injected with 10^9 rAAV- Pb-1, while a second group of mice was vaccinated with 8 µg of αClec9- Pb-1 + 10^9 rAAV- Pb-1 + 75 µg CpG. Spleens and livers from 4 mice were collected on day 35. Single cell suspensions were generated. The remaining mice (n=6) were challenge intravenously with 200 living PbA sporozoites on day 35. A. Schematic of the mice immunisation B. Number of specific liver T_{RM} cells C. and D. Number of memory CD8 T cells in spleen and liver E. Percentage of infected red blood cells (iRBC) in blood on day 7 post challenge. F. Percentage of sterile protection determined by day 18 after challenge. Results are a pool of two independent experiments. Data were log-transformed and compared using one-way ANOVA and Tukey's multiple comparisons test in (B.), using Kruskal- Wallis test with Dunn's multiple comparison post-test in (E.), or using Fisher's exact test in (F.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001..

Additionally, these results suggest that the optimal dose of prime-and-trap for the generation of liver T_{RM} cells and induction of sterile protection ranged from 2 µg to 16 µg of αClec9- Pb-1 + 10^9 rAAV- Pb-1 + 75 µg CpG.

3.3 Discussion:

In this study, a series of different immunisation strategies targeting the Pb-1 epitope were implemented and specific CD8 T cell responses were assessed in B6 mice. Results demonstrated a large initial expansion of PbT-I cells in the spleen and the formation of relevant memory cell subpopulations in spleen and liver. Remarkably, analysis of the T cell repertoire of naive B6 mice revealed that the number of specific CD8 T cell precursors for the Pb-1 epitope was very large. These cells were successfully expanded using prime-and-trap with Pb-1 and large numbers of specific T_{RM} cells were formed in the livers of immunised mice, which were efficiently protected from infection. In summary, the data presented in this chapter demonstrate the highly immunogenic nature of the Pb-1 epitope and its ability to confer high levels of sterile protection B6 mice with a normal endogenous T cell repertoire.

3.3.1 Novel MHC-I restricted epitope derived from P. berghei ANKA:

The critical role of CD8 T cell in protection against liver-stage malaria has been long confirmed by depletion and adoptive-transfer experiments in murine models (Jobe et al., 2007; Rodrigues et al., 1991; Schofield et al., 1987; Weiss et al., 1988). Despite many years of investigation, the quantitative and qualitative features of the protection and the effector functions mediated by CD8 T cells remain largely elusive. Only recently, liver-resident memory CD8 T cells have been implicated as an effective memory subpopulation for the induction of sterile protection (Fernandez-Ruiz et al., 2016; Tse et al., 2014). Other studies have also shown that γδ T cells play an important role in the memory response against liver-stage malaria (Ishizuka et al., 2016; Zaidi et al., 2017). The modest progress on revealing the nature of the of this CD8 T cell response, is partially explained

by the complexity of the *Plasmodium* genome and the limited number of defined MHC-I epitopes derived from the *Plasmodium* parasites (Schmidt et al., 2010). The identification and characterization of liver-stage derived antigens able to induce CD8 T cells responses through immunisation is critical for the development of new vaccine candidates (Van Braeckel-Budimir and Harty, 2014).

To date only two MHC-I restricted epitopes from PbANKA have been described to any level of protection against liver stage infection in B6 mice, a mouse strain that has been considered difficult to protect (Doolan and Hoffman, 2000; Schmidt et al., 2010). The first characterized epitope was the $PbTRAP_{130-138}$. Hafalla and collaborators found that B6 mice vaccinated with a modified adenovirus expressing $PbTRAP_{130-138}$ showed 95% reduction in liver parasite load, but this vaccine did not induced sterile protection (Hafalla et al., 2013). The second epitope, $PBANKA\_1031000_{631-639}$ (Kb-17) is contained within the MIF4G-like protein of unknown function (Pichugin et al., 2018; Speake et al., 2016). Pichugin and colleagues showed that B6 mice primed intramuscularly with Kb-17plasmid DNA and boosted intravenously with a recombinant adenovirus expressing Kb-17 had 7-fold less liver parasite burden compared to control mice (Pichugin et al., 2018). Nonetheless, this vaccination strategy using the Kb-17 epitope also failed to confer sterile protection in B6 mice.

In 2014, members of our group developed a B6 TCR transgenic line able to produce CD8 T cells for PbA. These cells were shown to respond to blood stage infection with different *Plasmodium*-species, to liver-stage parasites (PbA RAS) and to confer sterile protection against PbA sporozoite challenge (Lau et al., 2014). Very recently the authentic cognate epitope for the PbT-I cells, here termed Pb-1, was described. This MHC-I epitope is part of the ribosomal protein RPL6 of PbANKA (PBANKA_1351900) with orthologs in *P. yoelli* (PY02722), and *P. chabaudi* (PCHAS_1356500); and it is expressed during the liver and blood stage of the infection (Valencia-Hernandez et al., 2020).

In this chapter, the suitability of the Pb-1 epitope to evoke memory and induce protection mediated by two DC-targeted vaccination strategies, was investigated.

3.3.2 PbT-I cell expansion in response to immunisation targeting the Pb-1 epitope:

Clec9A is DC surface protein specifically expressed by XCR1 and cDC1 subpopulations (Guilliams et al., 2014; Kroczek and Henn, 2012; Shortman and Heath, 2010). Correspondingly, Clec9A is also selectively expressed on human BDCA3+ DC (Huysamen et al., 2008; Kerrigan and Brown, 2010; Poulin et al., 2010). Moreover, studies have demonstrated the important role of the cDC1 as the main antigen presenting cell stimulating T cells during blood-stage malaria infections (Fernandez-Ruiz et al., 2017; Lau et al., 2014).

Antigen targeting DC via Clec9A can enhance immune responses of CD8 T cell, CD4 T cell and B cells (Caminschi et al., 2008; Lahoud et al., 2011; Park et al., 2013). Other evidence, also showed that antigen targeting Clec9A in the absence of adjuvant, drives the development of long lasting follicular helper T cells and a potent antibody response (Kato et al., 2015; Park et al., 2013, 2017).

The nature of the CD8 T cell immune response induced by delivering peptides to DC via Clec9A has been investigated using model antigens such as OVA (Caminschi et al., 2008; Park et al., 2013; Sancho et al., 2008). These studies revealed the efficient formation of OVA-specific CTL responses in mice immunised with anti-Clec9A mAb together with an adjuvant. Furthermore, the induction of this specific CTL response at a memory time point accounted for the reduction of OVA-expressing lung tumour cells after or prior challenge (Sancho et al., 2008). These studies suggested that in principle delivering Ag via Clec9A could be exploited as an immunisation strategy to mediated CD8 T cell memory responses against intracellular infections.

For most of the experiments described in this chapter, the response of PbT-I cells was examined first, to test the effectiveness of priming reagents such as Clec9A mAb and the rAAV. Accordingly, mice adoptively transferred with PbA transgenic-specific CD8 T cells (i.e. PbT-I cells) and immunised with anti-Clec9A mAb targeting the Pb-1 epitope revealed a superior expansion of the PbT-I cells compared to mice immunised with the NVY mimic epitope. Relevant memory subpopulations, including T_{CM}, T_{EM} and T_{RM}, were also formed in spleen and liver after immunisation with anti-Clec9A-Pb-1 mAb. A similar level of expansion has been achieved in mice adoptively transferred with OT-I cells and immunised with anti-Clec9A mAb targeting an OVA peptide (Holz et al., 2018). Supporting the idea that Pb-1 is the authentic cognate epitope for the PbT-I cells.

3.3.3 Endogenous response and protection induced by immunisation with the Pb-1 antigen:

The magnitude and expansion of a given T cells response is partially determined by the frequency of the naive T cell precursor for a specific peptide (Jenkins and Moon, 2012; Moon et al., 2007; Obar et al., 2008). Therefore, to learn about the potential magnitude of the response to the Pb-1 epitope, the frequency of the naive T cell precursors for this epitope was determined. Results showed that the number of Pb-1 naive CD8 T cell precursors is extraordinarily high in naive B6 mice. In average, a female naive B6 mouse harbors approximately 2300 Pb-1 specific naive cells per mouse. On the other hand, the frequency of naive T cell precursor for $PbTRAP_{130-138}$ was less than 10 cells per mouse. Other well-known specificities like $OVA_{257-264}$ or LCMV GP_{33-41} only report 183 ± 33 and 358 ± 40 cells per mouse, respectively (Van Braeckel-Budimir et al., 2017; Jenkins and Moon, 2012). It is not unprecedented to find large frequencies in B6 mice. In fact, a previous study reported that another PbA epitope, $GAP50_{40-48}$, had an extremely large naive CD8 T cell repertoire of approximately 3000 cells per mouse (Van Braeckel-Budimir et al., 2017). In this study, they determined that more than 98% of the $GAP50_{40-48}$-specific CD8 T cells express Vβ8.1 TCR and suggested that this extreme TCR bias is driven by

a strong thymic selection mediated by the peptide itself rather than MHC reactivity (Van Braeckel-Budimir et al., 2017). Future research investigating the expression of specific Vα and Vβ TCR genes on Pb-1 specific CD8 T cells as well as structural analysis of the TCR:Pb-1 epitope interaction can provide evidence for the mechanisms underlying the high frequency of Pb-1 naive CD8 T cell precursors.

The large number of Pb-1 naive CD8 T cell supports the idea that the magnitude of the endogenous CD8 T cell response to the Pb-1 epitope would be greater than the one for the PbTRAP epitope. Nonetheless, it has been suggested that epitopes with low precursor frequencies could be good vaccination targets if effective boosting strategies are applied (Doll et al., 2016). Hypothetically, the high frequency of Pb-1 naive CD8 T cell precursors could provide for a superior initial expansion and induction of memory formation mediated by DC-targeted vaccination. In fact, large memory populations, especially liver T_{RM} cells, were formed in B6 mice immunised with anti-Clec9A mAb targeting the Pb-1 epitope in absence of PbT-I cells. In this regard, earlier studies have demonstrated the importance of the liver T_{RM} cells for the protection against malaria liver-stage (Fernandez-Ruiz et al., 2016; Lau et al., 2014). Remarkably, 50% of B6 mice immunised with anti-Clec9A-Pb-1 were protected against liver-stage parasites in the absence of the transgenic PbT-I cells. Several murine studies have shown that immunisations with Clec9A targeting antigens drives potent humoral responses using model antigens such as OVA (Caminschi et al., 2008; Lahoud et al., 2011); and very recently in the context of infectious diseases, using antigens derived from pathogens like influenza and enterovirus 71 (Park et al., 2017). Other studies have demonstrated the CTL antitumor capacity of antigen targeting Clec9A using OVA-expressing tumour cells (Sancho et al., 2008). This is the first time that in the context of an infectious disease, like malaria, high levels of protection were achieved by inducing the formation of endogenous-specific memory CD8 T cells through Clec9A targeted vaccination.

3.3.4 Sterile protection against malaria induced by prime-and-trap vaccination using the Pb-1 epitope:

There are approximately 140 million hepatocytes/g liver in mice and humans (Sohlenius-Sternbeck, 2006) and the number of infected hepatocytes under a normal course of infection has been estimated to be only 1 in one million and one in 100 million cells in mice and humans, respectively (Van Braeckel-Budimir and Harty, 2014). Furthermore, the liver stage occurs in a very limited period of time, 2 days with *P. berghei* and 6–8 days with *P. falciparum* in mice and humans, respectively (Sturm et al., 2006; Todryk and Hill, 2007). Accordingly, the number of memory CD8 T cells required for the surveillance of the whole liver in that very small time window in order to induce sterile protection is massive. In the context of other infectious diseases, such as *L. monocytogenes*, the transfer of specific memory CD8 T cells induced a significant reduction on the bacterial load in the liver, leading to a sublethal infection that can be cleared from all the mice (Badovinac et al., 2002). In the case of B6 mice infected with *P. berghei* all infected hepatocytes have to be cleared before any parasite progresses to blood stage and the mouse succumb to a lethal malaria.

Early studies published in 2008 and 2010 by Schmidt and collaborators found that sterilizing immunity induced after several i.v. injections with RAS in mice was correlated with the number of specific CD8 T cells in the blood and liver (Schmidt et al., 2010, 2008). They estimated that the threshold of specific cells is one million in the spleen and 20,000 in the liver of BALB/c mice to achieve sterile protection against *P. berghei* (Schmidt et al., 2008). This threshold is even higher when protecting B6 mice from *P. berghei* sporozoites (Schmidt et al., 2010). These studies addressed mainly the requirement of circulating memory CD8 T cells.

Data have revealed that liver T_{RM} cells are very effective at inducing sterilizing immunity against *P. berghei* in B6 mice. These cells constantly survey

for liver infection by patrolling through the liver sinusoids (Fernandez-Ruiz et al., 2016). The main advantage of T_{RM} cells over conventional circulating memory CD8 T cells is that they are already present and resident in the liver when parasites start infecting hepatocytes; and no extra time is invested in recruiting these cells. Liver T_{RM} cells, as effector memory cells, are also equipped for the elimination of malaria infected hepatocytes (Fernandez-Ruiz et al., 2016). Given the high efficacy of the liver T_{RM} cells at inducing sterile protection, the next goal was to maximize the formation of endogenous-specific liver T_{RM} cells able to confer protection.

Previous reports have shown that the presence of local antigen and inflammatory stimuli provide by adjuvants enhanced the liver T_{RM} cell formation through vaccination strategies like prime-and-trap (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Data here produced revealed that more than 90% of the mice vaccinated with prime-and-trap using the Pb-1 epitope were sterile protected against infection with *P. berghei* sporozoites.

During the optimisation experiments for the different doses of the prime-and-trap components, it was observed that slight changes in the dose of the recombinant adenovirus (rAAV-Pb-1) led to dramatic changes in the number of liver T_{RM} cells. In fact, beyond the optimal dose of the virus, significant reduction of liver T_{RM} cells was observed, accompanied by a T cell exhaustion phenotype, which translated in poor sterile protection. Elegant studies have demonstrated that CD8 T cell exhaustion, in the context of chronic viral infections, develops in a hierarchy manner (McLane et al., 2019; Wherry, 2011). The model suggest that initially during a chronic infection, CD8 T cells are highly functional and they progressively lose function and their ability to produce proinflammatory cytokines if they are constantly exposed to specific antigen. In this hierarchical model, it was also demonstrated that ultimately specific-CD8 T cell populations were physical deleted when exposed to high antigen loads, as oppose to other specific populations exposed lower antigen loads (Wherry et al., 2013). For the prime-and-trap system, high doses of rAAV-Pb-1 appeared to induce loss of function by

specific-CD8 T cells inducing to poor control of infection; and may have even led to the physical deletion of some specific-CD8 T cells.

In summary, this data revealed the highly immunogenic nature of the Pb-1 epitope; and the capacity of the prime-and-trap vaccine using Pb-1 to induce the formation of endogenous-specific liver T_{RM} cells which were effective at conferring high levels of sterile protection. This study also demonstrate that vaccination strategies that achieve the formation of T_{RM} cells have the potential to induce effective protection, even if they stimulate cells specific for a single antigen.

The development of sub-unit vaccines aiming for the formation of liver T_{RM} cells represents a novel and potentially effective strategy to be implemented in clinical trials. One of the biggest limitations for the development of effective malaria vaccines mediated by CD8 T cell immunity is the lack of tools that allow the monitoring of liver T_{RM} cell formation. Currently, clinical trials study circulating T cell responses through cytokine production assays performed under *ex vivo* settings and the local immune response in the tissue remain unknown. Therefore, there is an urgent need for the identification of biomarkers in blood that could effectively predict the formation of T_{RM} cells. Moreover, there is high demand for the discovery of immunogenic epitopes derived from human malaria parasites expressed during liver-stage infection. The major challenge for the identification of these antigens is the lack of a suitable and sufficient source of infected hepatocytes. These concepts together with other translational approaches will be further discussed on Chapter 5.

CHAPTER 4

Analysis of nucleic acid-based adjuvants for liver T_{RM} cell formation induced by Clec9A targeted immunisation.

4.1 Introduction:

Tissue-resident memory T cells (T_{RM} cells) are part of a memory T cell subpopulation that are often localised to the tissue where the initial infection occurred. In case of secondary infections, the strategic location of the T_{RM} cells allows them to mediate effector responses more rapidly than their counterparts, the circulating memory cells (Mueller and Mackay, 2016). T_{RM} cells exert effective killing of infected cells through many effector mechanisms, including the expression of granzyme B (Casey et al., 2012; Mackay et al., 2012b; Schenkel et al., 2014) and the production of proinflammatory cytokines such as IFN-γ (Glennie et al., 2015; Schenkel et al., 2013), the latter of which also promotes the recruitment of circulating memory T cells. A detailed description of the T_{RM} cell effector functions is found in Chapter 1.

Several studies have revealed the critical role of T_{RM} cell-mediated protection in the context of different pathogen models, such as herpes simplex, vaccinia and influenza virus infections (Ariotti et al., 2012; Gebhardt et al., 2009; Jiang et al., 2012; Mackay et al., 2012a; Shin and Iwasaki, 2012; Teijaro et al., 2011). Other studies have analysed the role of T_{RM} cells in the context of viral hepatitis in humans. Pallet *et al*. described that T_{RM} cells are enriched in samples of liver tissues from patients with chronic hepatitis B virus (HBV) infection as compared with healthy controls. Importantly, they observed an inverse correlation between T_{RM} frequency and HBV viral load, and was associated with the *ex vivo* capacity of these cells to produce IFNγ and TNFα in response to antigen stimulation (Pallett et al., 2017). Liver T_{RM} cells have also been reported to be effective at inducing sterile immunity against *P. berghei* infection in mice (Fernandez-Ruiz et al., 2016). These T_{RM} cells patrol the liver sinusoids, scanning for infected hepatocytes. Antibody depletion of liver T_{RM} cells from vaccinated mice abrogated protection against sporozoite challenge, indicating the critical role of these cells in vaccine-mediated protection (Fernandez-Ruiz et al., 2016).

Previous reports and data collected in Chapter 3 revealed that the number of liver T_{RM} cells correlates with the level of sterile protection against malaria infection (Fernandez-Ruiz et al., 2016). Given the importance of liver T_{RM} cells in the context of protection against malaria and other liver-associated pathogens (Fernandez-Ruiz et al., 2016; Pallett et al., 2017; Stelma et al., 2017), it may be valuable to study how to enhance their formation.

It has been recently reported that liver T_{RM} cell formation is increased by TLR agonists, used as adjuvants, in the absence of antigenic stimuli *in vivo* (Holz et al., 2018). Moreover, studies applying the prime-and-trap vaccination strategy provide evidence that the nature of the adjuvant influences liver T_{RM} cell formation; justifying a greater investigation of adjuvant options (Fernandez-Ruiz et al., 2016; Chapter 3 in this thesis). It is the aim of this chapter to assess various nucleic acid agonists as adjuvants for liver T_{RM} cell formation induced by Clec9A targeted immunisation *in vivo*.

4.1.1 The adjuvants

Historically, TLR9 agonists, such as synthetic CpG oligonucleotides (ODN), are used as vaccine adjuvants (Bode et al., 2011). Moreover, model antigen studies have reported the use of synthetic CpG ODN, as the preferred adjuvant, for the induction of CD8 T cell and cytotoxic responses mediated by immunisations with mAb targeting antigen to Clec9A (Caminschi et al., 2008; Lahoud et al., 2011).

There are four major types of synthetic CpG ODN, (A-, B-, C- and P-Class) each with distinct base pair composition and biological properties (Bode et al., 2011). Detailed properties for the different CpG ODN were reviewed in Chapter 1.

B-class ODN have been shown to be required for optimal cytokine production and DC activation by binding to DEC-205, a surface molecule highly expressed on cross-presenting DC (Lahoud et al., 2012). Also, it has been

reported that signalling via TLR9 with B-class ODN induces the formation of intrahepatic myeloid cell aggregates (iMATEs) that facilitate the accumulation of CD8 T cells in the liver (Huang et al. 2013). These two factors might explain why CpG is a suitable adjuvant for the generation of effective liver TRM cells mediated by prime-and-trap vaccination (Fernandez-Ruiz et al. 2016).

Polyinosinic–polycytidylic acid (p I:C), a TLR3 agonist, has also been used as an adjuvant to induce CTL responses mediated by targeting antigen to Clec9A *in vivo* (Caminschi et al., 2012b, 2008; Lahoud et al., 2011). Recently, it has been shown in vaccinated mice and cell transfer experiments that stimulation of TLR3 trough p I:C increases the formation of liver T_{RM} cells and yet is less potent than CpG (Fernandez-Ruiz et al., 2016; Holz et al., 2018; Chapter 3 in this thesis).

Both synthetic CpG ODN and p I:C mediate type I IFN responses through TLR engagement. Other adjuvants investigated in this chapter also mediate type I IFN responses by binding to specific nucleic acid receptors. Based on location, there are two groups of nucleic acid receptors described at the moment; the endosomal and the cytosolic sensors (Junt and Barchet, 2015). While those members of the TLR family that recognise nucleic acids, which are generally localised in the endosome, are primarily expressed by cell types of the innate immune system, cytosolic receptors are broadly expressed by most cell types (Roers et al., 2016). The fact that TLR and cytosolic receptors are differentially expressed, suggests that their ligands might be used as strategic adjuvants to specifically activate different cell types depending on the desired immune response.

To cover a range of different receptors, two endosomal and two cytosolic receptors were selected and their agonists studied as adjuvants for the induction of liver T_{RM} cells. Agonists were selected for TLR7 and TLR9, receptors located in the endosome, and for RIG-I and cGAS, located in the cytoplasm.

TLR7 detects ssRNA, short dsRNA and short synthetic GU-rich ODN (Zhang et al., 2016). Retinoic acid-inducible gene I (RIG-I), recognizes short double stranded RNA bearing 5′ triphosphorylated or 5′ diphosphorylated ends (Goubau et al., 2014; Hornung et al., 2006). In a recent study, it was observed that RIG-I ligands promoted cross-presentation enhancing CTL responses in the context of an antiviral vaccine (Hochheiser et al., 2016). Cyclic GMP–AMP synthase (cGAS) recognizes dsDNA, which upon activation produces a second messenger that signals through the stimulator of IFN genes (STING) (Sun et al., 2013). The activation of endosomal and cytosolic sensors leads to the production of type-I IFN and NF-κB-dependent pro-inflammatory cytokines, the signature of innate immune activation during infection (Junt and Barchet, 2015). Further information about nucleic acid receptors, their agonists and mediated immune responses was given in Chapter 1.

4.2 Results:

4.2.1 TLR9 ligands are potent at inducing liver T_{RM} cell formation

In Chapter 3, the highly immunogenic nature of the novel Pb-1 epitope derived from PbA was revealed. It was demonstrated that vaccinations targeting Pb-1 to Clec9A induced the formation of effective liver T_{RM} cells able to confer high levels of sterilizing protection in B6 mice.

In this chapter, immunisation targeting the novel Pb-1 epitope to Clec9A was used as a platform for the assessment of the different agonists as adjuvants for the formation of liver T_{RM} cells.

For the first round of experiments, only TLR agonists were assessed. The TLR4 agonist lipopolysaccharide (LPS) was included as a non-nucleic acid ligand. To control for the initial number of responding cells and to facilitate the analysis of these experiments, mice were transferred with PbT-I GFP expressing cells one day prior to the priming. The next day, mice were injected with 2 μg of αClec9A-Pb-1 mAb – which is a dose that induces significant expansion of PbT-I cells (see Chapter 3) – plus one of the selected adjuvants in a single injection. A small blood sample was taken at day 7 as these data provide a rapid indication of the effectiveness of the priming as well as an approximation of the proportion of memory cells that could be formed at a later time point. At day 28, post immunisation spleens and livers were collected, and the formation of memory cells was investigated (Figure 4.1A).

Table 4.1. Characteristics of the adjuvants tested during the first round of experiments:

Name	Receptor	Class	Dose per mouse
CpG-combo	TLR9	CpG class B (ODN2006)	75 μg

		linked to a CpG class P (21798)	
CpG 1668	TLR9	CpG class B	75 μg
Poly I:C (p I:C)	TLR3, RIG-I / MDA5	Synthetic analog of dsRNA	50 μg
Lipopolysaccharide (LPS)	TLR4	Non-nucleic acid	1 μg

On day 7 post immunization, CpG-combo showed a significant superior capacity at inducing expansion of PbT-I cells compared to the naive control mice, though p I:C was capable of inducing similar PbT-I cell expansion (Fig. 4.1 B). On day 28, the four adjuvants, CpG-combo, CpG-1668, p I:C and LPS induced significantly more liver T_{RM} cells compared to naive control mice, with CpG-combo the most potent adjuvant for the induction of liver T_{RM} cells (Fig. 4.1 C and E). Mice primed with αClec9A-Pb-1 mAb plus CpG-combo formed on average 1.1 x 10^5 liver T_{RM} cells, while CpG-1668 induced 5 times less cells. P I:C and LPS were poor at inducing T_{RM} cell formation compared to CpG-combo. Interestingly, CpG-1668 and p I:C induced approximately 1.4 x 10^5 T_{EM} cells in spleen while CpG-combo induced approximately 7.7 x 10^4 cells (Fig. 4.1 D).

It is important to mention that the doses of the adjuvants differed somewhat. LPS was used at a relatively low mass as higher doses cause toxicity. The doses of CpG-combo, CpG-1668 and p I:C corresponded to values used previously in murine studies were they were combined with αClec9A mAb to provide adjuvant activity (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Results from these studies revealed that these doses are well tolerated and effective at inducing memory T cell formation (Fernandez-Ruiz et al., 2016; Holz et al., 2018).

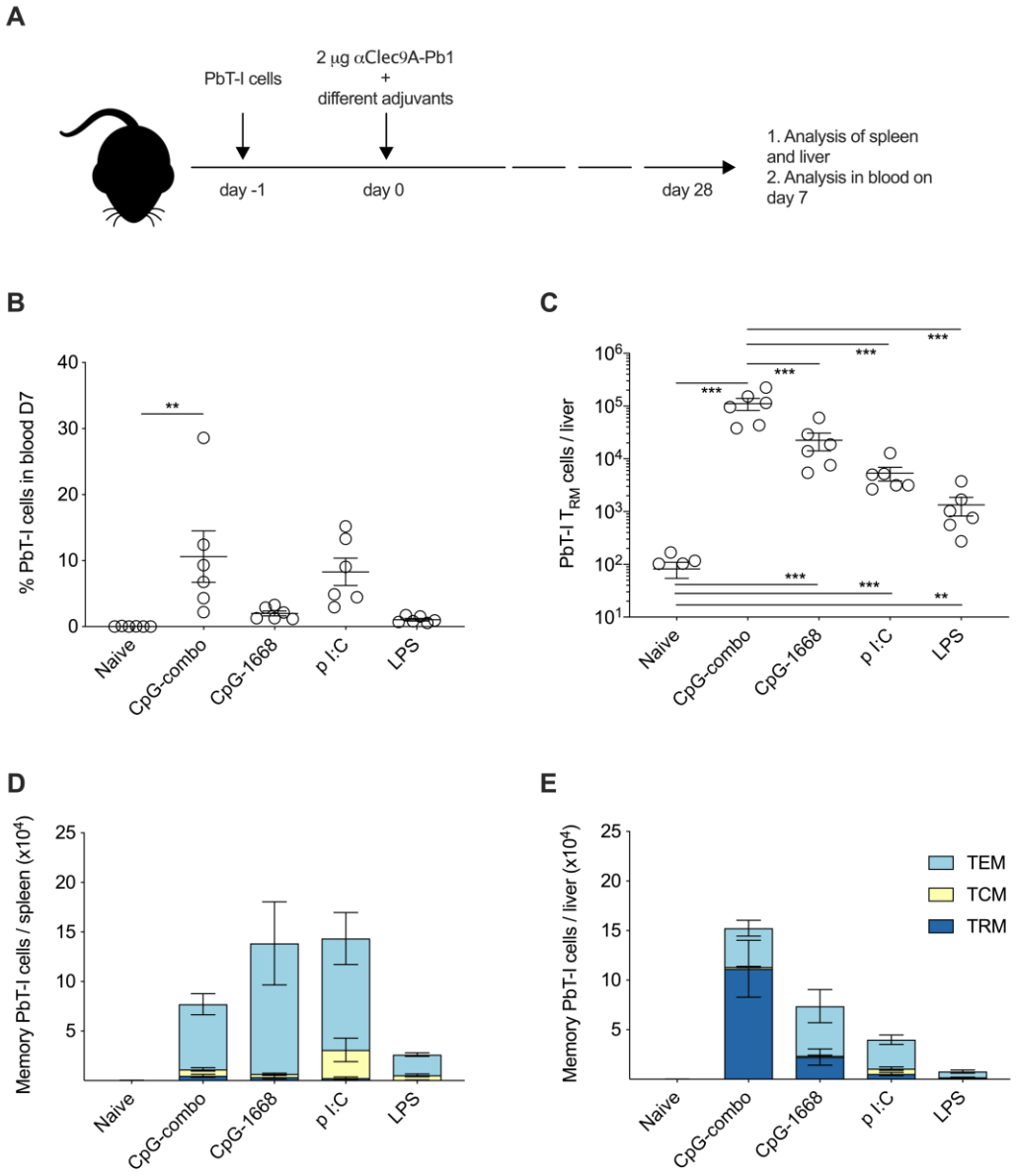

Figure 4.1 CpG-combo is a potent adjuvant for the induction of liver memory cells. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells. The next day, mice were immunized with 2 μg of αClec9-Pb-1 plus 75 μg of CpG-combo, 75 μg of CpG-1668, 50 μg of p I:C or 1 μg of LPS. One group of mice group remain naive. Spleens and livers were collected on day 28. Single cell suspensions were generated, and the number of PbT-I cells and memory status were determined by flow cytometry. A. Schematic for the protection experiment. B. Percentage of PbT-I cells (CD8+, GFP + cells) in blood on day 7 post immunization. C Number of T_{RM} cells per liver determined as CD8+, GFP+, CD44Hi, CD62L-, CD69+. D. and E. Total number of PbT-I cells in spleen and live, respectively. Results are a pool of two independent experiments. Results are a pool of two independent experiments. Data were compared using a Kruskal-Wallis test with Dunn's multiple comparison post-test in (B.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (C.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001..

For a second series of experiments, agonists for the cytosolic receptors cGAS and RIG-I, as well as ligands of the endosomal receptors TLR7 and TLR9, were included on the assessment and compared to CpG-combo. Moreover, these additional ligands were mixed and complexed in liposomal transfection reagents, which protects them from extracellular degradation and promotes the cellular uptake of nucleic acids (Smyth Templeton, 2002).

CpG-ODN 2006 (CpG-2006) and a GU-rich ssRNA TLR7 ligand (TLR7-L) were complexed in DOTAP, which is a cationic lipid that mediates the delivery of CpG-ODN and TLR7 ligands to the endosomal compartment (Heil et al., 2004; Honda et al., 2005; Yasuda et al., 2005; Yotsumoto et al., 2008). RIG-I ligand (RIG-I-L) and cGAS ligand (cGAS-L) were complexed in in vivo-jetPEI®, a polyethyleneimine that enhances *in vivo* delivery of both DNA and RNA (Yin et al., 2014). The nucleic acid sequences of each agonist were listed in Chapter 2, as well as the protocol used to complex them with the liposomal transfection reagents.

Table 4.2. Characteristics of the adjuvants that were complexed in transfection reagents:

Name	Receptor	Class	Transfection reagent	Dose per mouse
CpG-combo	TLR9	CpG class B (ODN2006) linked to a CpG class P (21798)	No	20 μg or 75 μg
CpG-2006	TLR9	CpG class B (ODN2006)	DOTAP	20 μg
RIG-I-L	RIG-I	5’ 3p dsRNA cytosol ligand	jet-PEI®	20 μg
TLR7-L	TLR7	ssRNA endosome ligand	DOTAP	30 μg

cGAS-L	cGAS	dsDNA cytosol ligand	jet-PEI®	20 μg

The doses for the different adjuvants were selected based on previous research performed at the Institute for Clinical Chemistry and Clinical Pharmacology at the University Hospital Bonn, Germany (unpublished).

B6 mice were transferred with PbT-I cells and primed the next day with αClec9A-Pb-1 plus one of the selected adjuvants. Two groups of mice received the CpG-combo, one with a lower dose than usual (20 μg instead of 75 μg per mouse). Analysis from these results allowed for the direct comparison of a potential dose impact on the expansion at the effector phase and the formation of memory cells. Another group of mice were only injected with the αClec9A-Pb-1 as a control to account only for the adjuvant effect. Similar to the previous experiment, a small blood sample was taken on day 7, and on day 28 memory cell formation was assessed in the spleen and liver.

Analysis on day 7 revealed that RIG-I-L induced the highest percentage of PbT-I cells in blood (26%) as compared to the other agonists including CpG-combo-high dose (10%) (Fig. 4.2 B). Mice injected with a low dose of CpG-combo (i.e. 20 μg) expanded less PbT-I cells than mice primed with the high dose. Additionally, 20 μg of CpG-2006 complexed in DOTAP induced a significantly higher percentage of PbT-I cells in the blood as compared to mice primed only with αClec9A-Pb-1. TLR7-L and cGAS-L did not induce significant expansion.

On day 28 post priming, stimulation with 75 μg of CpG-combo per mouse induced the largest number of T_{RM} cells, reaching approximately 10^5 cells per liver (Fig. 4.3 C and E). Mice that were treated with 20 μg of CpG-combo generated significantly lower numbers of liver T_{RM} cells (9.3×10^3 cells). CpG-2006 and RIG-I ligand induced significantly higher numbers of T_{RM} cells compared to prime no-adjuvant treated mice, whereas TLR7-L and cGAS-L failed to induce liver T_{RM} cell formation (Fig. 4.3 C and E), consistent with their poor

responses on day 7. In the spleen, CpG-2006 prompted the formation of large numbers of T_{EM} cells, closely followed by RIG-I-L and CpG-combo-high dose with similar numbers (Fig. 4.3 D).

All together, these results suggest that CpG-combo is the best adjuvant, given that mice treated with αClec9A-Pb-1 mAb plus 75 μg of CpG-combo generated the highest liver T_{RM} cell numbers, with an average of 10^5 T_{RM} cells per liver. CpG-1668 and CpG-2006, the last complexed in DOTAP, also induced high numbers of liver memory cells. For most of the group treatments, the memory data from day 28 mirrored the results of the early expansion on day 7. RIG-I-L induced a large number of spleen T_{EM} cells, in similar proportion to CpG-combo and CpG-2006, and low numbers of liver T_{RM} cells. An important finding is the dramatic dose effect observed in mice that received 20 μg instead of 75 μg of CpG-combo. Mice from the last group formed on average 10 times more liver T_{RM} cells than the mice in the group of the lower dose of CpG-combo. In a later section on this chapter the comparison between liver T_{RM} cells and circulating memory T cells will be evaluated.

4.2.2 High dose of agonists enhanced liver T_{RM} cells formation

Given that the number of liver T_{RM} cells was significantly influenced by the dose of CpG-combo, it was important to determine whether a similar effect could be observed for the other adjuvants. For this, the dose of adjuvants was increased to 75 μg per mouse, which corresponds to the dose of CpG-combo used in a previous published study (Fernandez-Ruiz et al., 2016) and for the experiments carried out in Chapter 3.

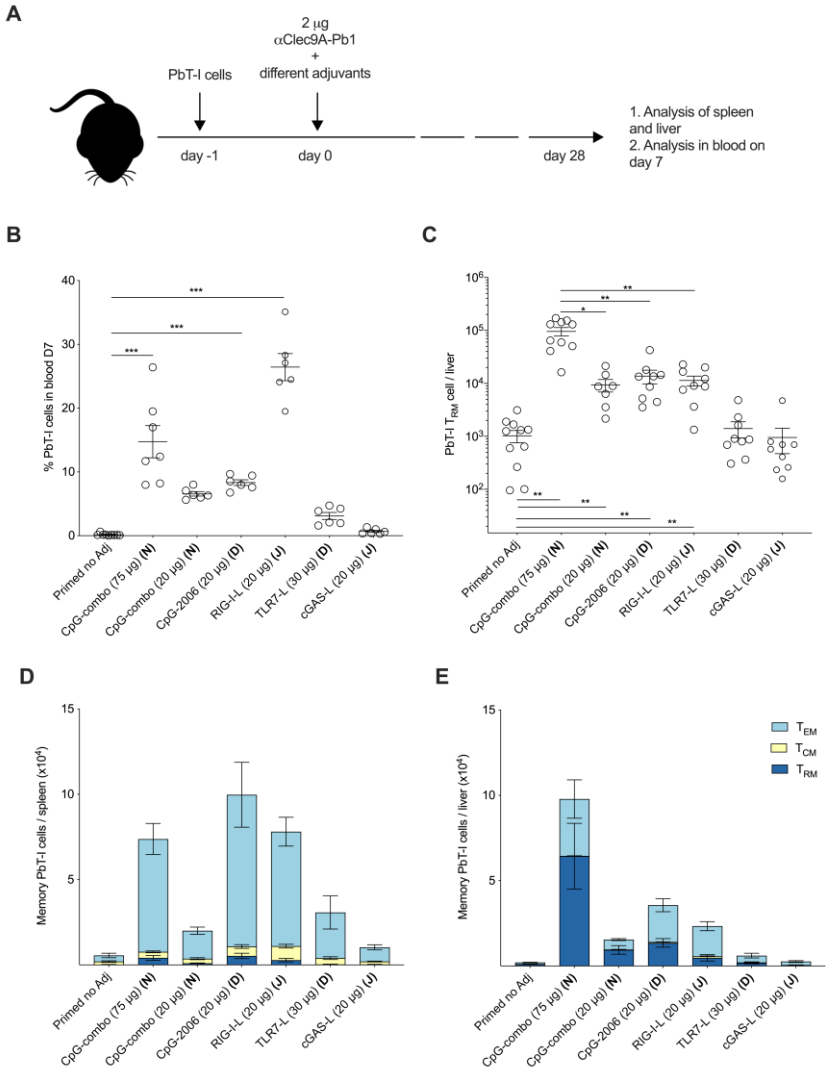

Figure 4.2 RIG-I ligand and CpG 2006 also induce great numbers of memory cells in liver. A. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells. The next day, mice were immunized with 2 μg of αClec9A-Pb-1 plus: 75 μg or 20 μg of CpG-combo, 20 μg of CpG-2006 complexed in DOTAP, 20 μg of RIG-I-L complexed in *in vivo* jet-PEI®, 30 μg of TLR7-L complexed in DOTAP or 20 μg of cGAS-L complexed in *in vivo* jet-PEI®. On day 7, mouse blood was analyzed by flow cytometry. Spleens and livers were collected at day 28, single cell suspensions were generated, and the number of PbT-I cells and memory status were determined by flow cytometry. B. Percentage of PbT-I cells (CD8+, GFP + cells) in blood on day 7 post immunization. C Number of T_{RM} cells per liver determined as CD8+, GFP+, CD44Hi, CD62L-, CD69+. D. and E. Total number of PbT-I cells in spleen and live, respectively. Results are a pool of two independent experiments. Data were compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (B.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (C.). (N) refers to naked or uncomplexed adjuvants, (D) for the ligands complexed in DOTAP and (J) for the agonists complexed in *in vivo* jet-PEI®. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

Mice were transferred with PbT-I cells and primed the next day with αClec9A-Pb-1 mAb plus a high dose of one of the selected adjuvants. High doses of CpG-2006, TLR7-L, RIG-I-L and cGAS-L were assessed and compared to the CpG-combo. To control for toxicity, during the first two days, mice were monitored twice per day and their body condition was assessed. Body weight was recorded daily for 7 days. If body condition did not comply to healthy standards or 15% body weight was lost, mice were euthanized.

Records of the body weight are presented in figure 4.3. Each plot shows the weight fluctuation over 7 days after stimulation with each individual adjuvant. The dash line represents 15% of body weight loss of the lightest mouse at day 0. In general, a high dose of the adjuvants was well tolerated. The exception was for the RIG-I-L, which was not tolerated at 75 μg per mouse and was changed for a tolerable dose of 40 μg per mouse (Fig. 4.3). In addition, in the group of mice injected with 75 μg of TLR7-L, one mouse registered a weight loss of 15% and was euthanized. As expected, no fluctuation was evident in mice that received no adjuvant. Minor body weight loss was observed in mice primed with CpG-combo, CpG-2006 and 40 μg of RIG-I-L but this weight was recovered by day 7 (Fig. 4.3). Mice immunised with 75 μg of TLR7-L or cGAS-L did not experience fluctuations in their weight (Fig. 4.3).

After testing the mouse tolerability to a high dose of the adjuvants, agonists were assessed for their capacity to induce PbT-I cell expansion and liver T_{RM} cell formation. Blood samples from the surviving mice were taken on day 7 after immunisation and on day 28 spleen and liver were harvested and analysed for the formation of memory T cells.

CpG-combo, CpG-2006 and RIG-I-L promoted significant expansion of PbT-I cells on day 7, while no expansion was evident in mice stimulated with TLR7-L and cGAS-L, compared to mice that received no adjuvant (Fig. 4.4 B).

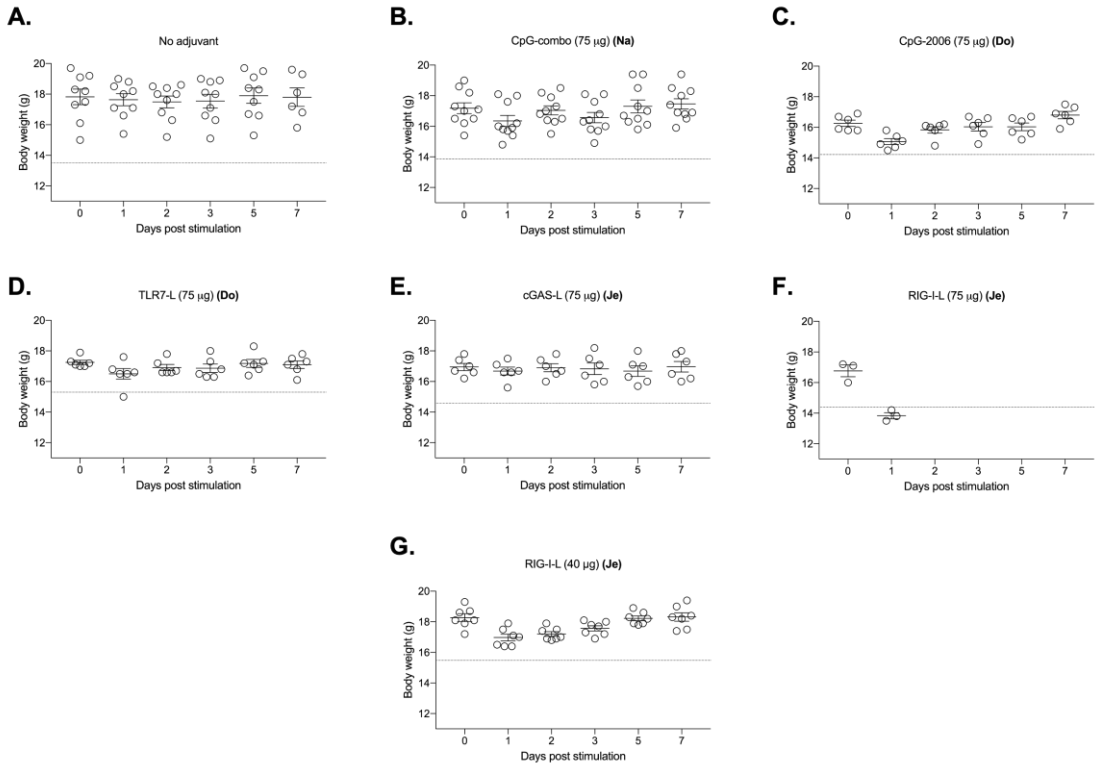

Figure 4.3 Mice body weight after priming with high dose of adjuvants. Female B6 mice, ranging from 6 to 8 weeks of age, were transferred intravenously with 50,000 PbT-I/uGFP cells. The next day, body weight was recorded and mice were immunized with 2 μg of αClec9A-Pb-1 plus: 75 μg of CpG-combo (B.), 75 μg of CpG-2006 complexed in DOTAP (C.), 75 μg of TLR7-L complexed in DOTAP (D.), 75 μg of cGAS-L complexed in *in vivo* jet-PEI® (E.), 75 μg (F.) or 40 μg of RIG-I-L complexed in *in vivo* jet-PEI® (G.). (Na) refers to naked or uncomplexed adjuvants, (Do) for the ligands complexed in DOTAP and (Je) for the agonists complexed in *in vivo* jet-PEI®.During the first two days, mice were monitored twice per day and the body condition was assessed. Body weight was recorded daily for 7 days. If body condition did not comply to healthy standards or 15% body weight was lost, mice were euthanized. Open circles represent each individual mouse and the dash line represent 15% body weight loss relative to day 0 per group. Data were pooled from 2 to 3 independent experiments.

Analysis of memory cells on day 28 revealed that CpG-combo, CpG-2006, TLR7-L and RIG-I-L induced significantly high numbers of liver T_{RM} cells compared to mice that did not receive an adjuvant. cGAS-L was poor at inducing liver T_{RM} cell formation (Fig. 4.4 C). CpG-combo, CpG-2006 and RIG-I-L induced significantly higher numbers of liver T_{RM} cells compared to TLR7-L and cGAS-L. Moreover, CpG-combo and CpG-2006 also induced higher number of cells compared to RIG-I-L (Fig. 4.4 C). CpG-2006 (75 μg) in DOTAP induced twice the number of liver T_{RM} cells compared to uncomplexed CpG-combo, although non-significant difference was observed. Mice that received CpG-2006 (75 μg) formed in average 234,344 liver T_{RM} cells, while CpG-combo (75 μg) induced 125,508 cells (Fig. 4.4 E). CpG-2006 also induced a slightly higher number of T_{EM} cells in the spleen as compared to CpG-combo but this was not a significant difference (Fig. 4.4 D).

When comparing with previous results described on Fig. 4.2, mice that received 75 μg of CpG-combo formed 13 times more liver T_{RM} cells than mice receiving 20 μg. Similarly, though not directly compared in the same experiment, 75 μg of CpG-2006 induced a 17-fold increase in liver T_{RM} cells compared to 20 μg of CpG-2006; for RIG-I-L and TLR7-L the increment was 2- and 1.5-fold, respectively (Fig. 4.4 C and Fig. 4.2 C).

These data indicate that the liver T_{RM} cell formation is influenced by the dose of the adjuvant for most of the cases, except for cGAS-L agonist which seems to fail to provide an adjuvant effect in this system.

4.2.3 CpG-ligands preferentially induce liver T_{RM} cell formation

As discussed in Chapter 1, the agonists investigated here stimulate a broad range of different cell types by activating diverse pathways expressed differentially on the target cells (Junt and Barchet, 2015; Roers et al., 2016). Additionally, results depicted in this chapter demonstrated that the dose of the adjuvants influenced the formation of liver T_{RM} cells (Fig. 4.2).

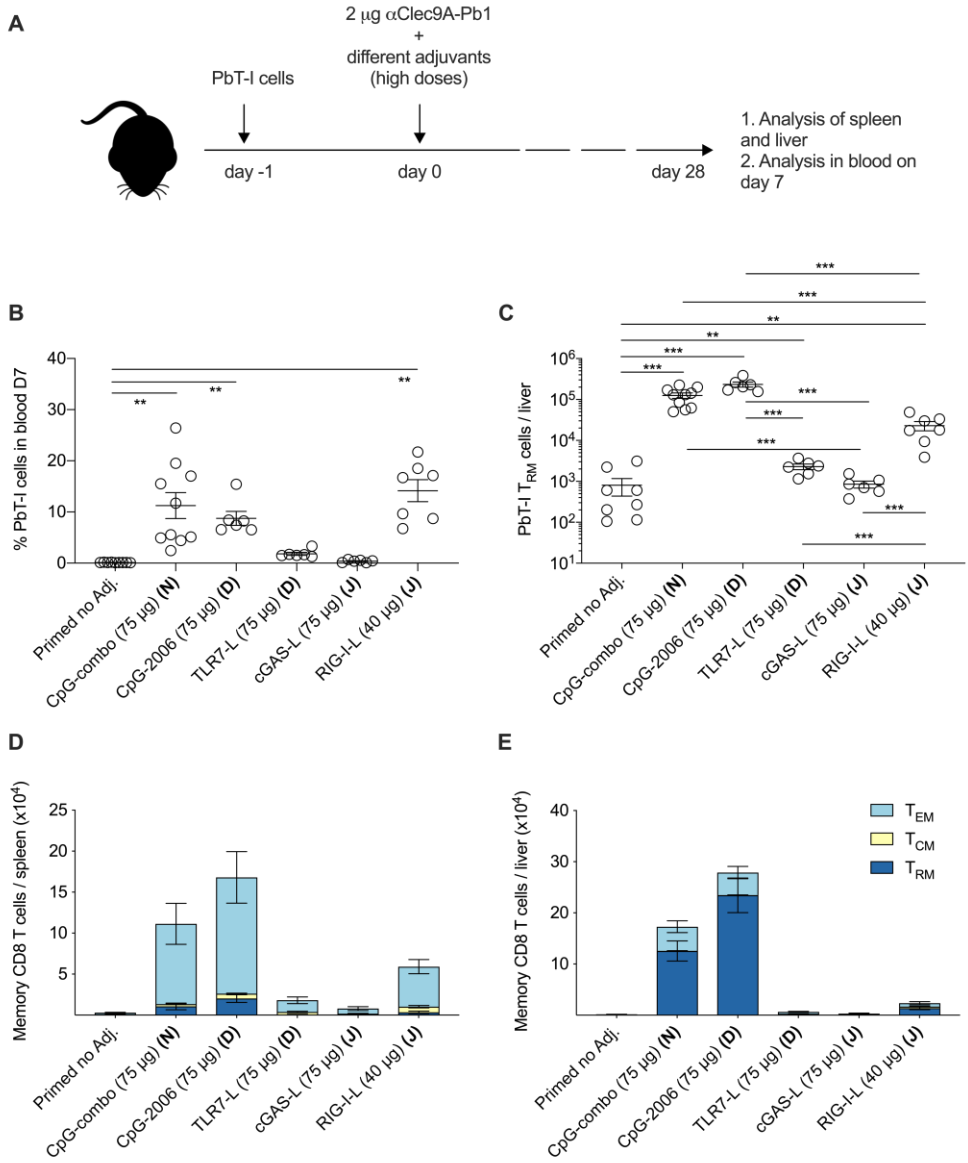

Figure 4.4 High dose of CpG-2006 is very potent at inducing liver T_{RM} cell formation. A. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and primed the next day with 2 μg of αClec9A-Pb-1 plus: 75 μg of CpG-combo, 75 μg of CpG-2006 complexed in DOTAP, 75 μg of TLR7-L complexed in DOTAP, 75 μg of cGAS-L complexed in *in vivo* jet-PEI®, or 40 μg of RIG-I-L complexed in *in vivo* jet-PEI®. B. Percentage of PbT-I cells (CD8+, GFP + cells) in blood on day 7 post immunization. C Number of T_{RM} cells per liver determined as CD8+, GFP+, CD44Hi, CD62L-, CD69+. D. and E. Total number of PbT-I cells in spleen and live, respectively. Results are a pool of two independent experiments. Data were compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (B.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (C.). (N) refers to naked or uncomplexed adjuvants, (D) for the ligands complexed in DOTAP and (J) for the agonists complexed in *in vivo* jet-PEI®. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001..

Since the aim of this study was to identify an adjuvant that favoured the induction of liver T_{RM} cells, the data collected from previous experiments were assessed for the ability to induce T_{RM} cells relative to the induction of circulating memory cells. To do this in a simple way, the ratio of liver T_{RM} cells over circulating memory cells – T_{EM} plus T_{CM} cells from the spleen – was calculated. Analysis of this ratio allows a comparison of the capacity of different vaccination strategies (e.g. different adjuvants) to promote T_{RM} cell differentiation from a pool of circulating memory T cells (Holz et al., 2018).

Examination of the ratio of T_{RM} cells to circulating memory T cells revealed that a high dose of CpG-2006 and CpG-combo combo (e.g. 75 μg instead of 20 μg per mouse) had the best adjuvant effect for the induction of liver T_{RM} cell differentiation, as compared to the primed no-adjuvanted mice (Fig. 4.5). There was a tendency for lower doses to generate less liver T_{RM} cells as compared to higher doses of same adjuvant, although this difference was not significant (Fig. 4.5). Remarkably, all other adjuvants, including CpG-1668 (uncomplexed) and high doses of RIG-I-L, TLR7-L and cGAS-L had similarly poor capacity to preferentially induce liver T_{RM} cells in this particular system (Fig. 4.5).

All together, these data support previous evidence demonstrating that CpG ODN enhance the formation of resident memory T cells in the liver (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Potential mechanisms behind this effect will be explore in the discussion of this chapter.

4.2.4 CpG-combo has no significant effect on the liver T_{RM} cell formation induced by RAS

Published studies have demonstrated that vaccination with radiation-attenuated sporozoites (RAS) induces liver T_{RM} cells and provides a certain level of protection against sporozoite challenge

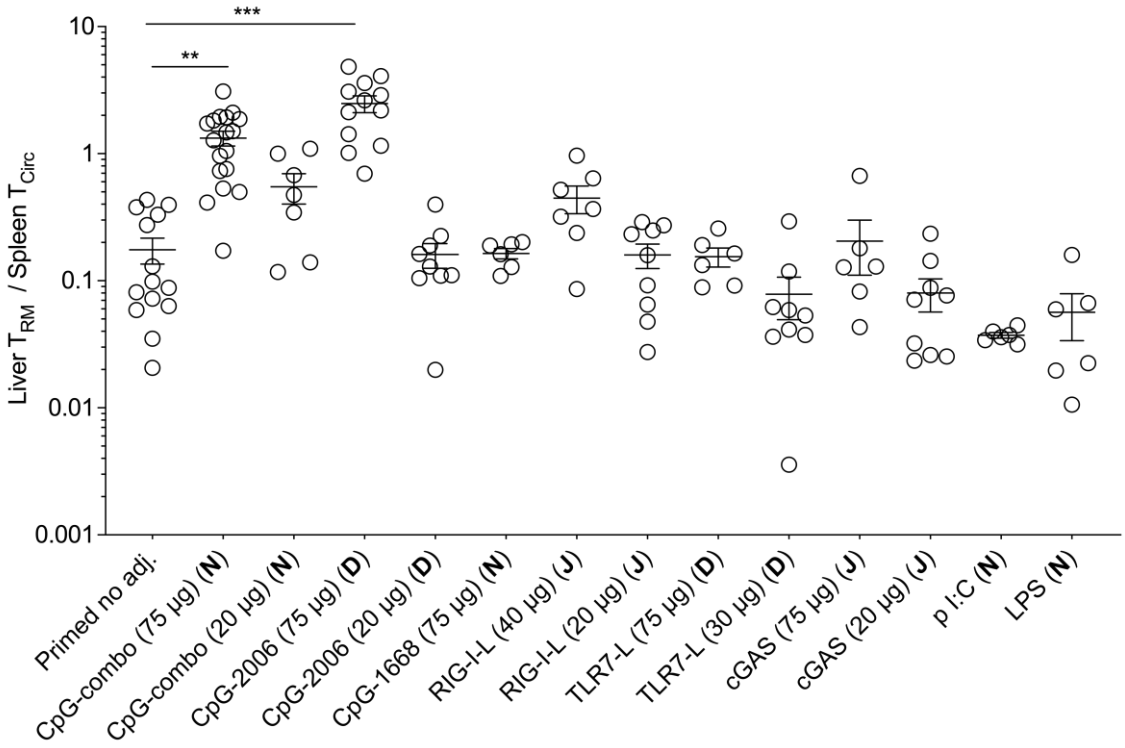

Figure 4.5 CpG-ligands are the best at inducing T_{RM} cell formation. The number of liver T_{RM} cells was divided by the number of circulating T cells (Tcirc) in the spleen (T_{EM} cells plus T_{CM} cells) to calculate the relative proportion of T_{RM} cells in the liver. This figure compiles results from figures 4.1, 4.2 and 4.4, which are results pooled from 2 to 4 independent experiments. (N) refers to naked or uncomplexed adjuvants, (D) for the ligands complexed in DOTAP and (J) for the agonists complexed in *in vivo* jet-PEI®. Groups were compared using a Kruskal-Wallis test with Dunn's multiple comparison post- test.

(Epstein et al., 2011; Fernandez-Ruiz et al., 2016). As CpG-based adjuvants were the best adjuvants for inducing liver T_{RM} cells in the context of Clec9A targeted immunisation, the role of CpG as adjuvant for RAS vaccination was investigated. For this experiment, mice were adoptively transferred with PbT-I cells, as these cells are known to expand in response to RAS vaccination and can be easily monitored (Fernandez-Ruiz et al., 2016; Lau et al., 2014). The next day, mice were vaccinated with RAS or RAS plus CpG-combo. On day 28, when memory cells had been formed, spleens and livers were harvested from 4 mice per group and the memory T cell profile was assessed by flow cytometry. Remaining mice (n=6) were infected with live sporozoites and monitored for the occurrence of parasites in blood.

Data from day 28 determined that CpG-combo had no significant effect on the formation of liver T_{RM} cells (Fig. 4.6 B, D). Mice vaccinated with RAS generated on average 1.8 +/- 0.1 x 10^5 T_{RM} cells per liver, and mice that received RAS plus CpG-combo formed a similar number of 1.3 +/- 0.1 x 10^5 cells (Fig. 4.6 B, D). Likewise, other memory populations in the spleen or liver were unaffected by the addition of CpG-comb, although mice that received only RAS formed slightly higher numbers of T_{EM} cells in the spleen and liver compared to RAS plus CpG-combo vaccinated mice (Fig. 4.6 C, D). It was previously reported in Chapter 3 and in a recent publication, that relatively high numbers of PbT-I T_{RM} cells in the liver effectively protect against sporozoite challenge (Fernandez-Ruiz et al., 2016). As a consequence, mice vaccinated here with both, RAS or RAS plus CpG-combo had significantly less parasitemia as compared to naive mice (Fig. 4.6 E). Additionally, 78% of the mice were sterilely protected for both treatment groups (Fig. 4.6 F).

These data indicate that CpG-combo does not enhance the formation of liver T_{RM} cell induced by one dose of RAS vaccination in B6 mice and has no effect on the protection induced by this immunisation.

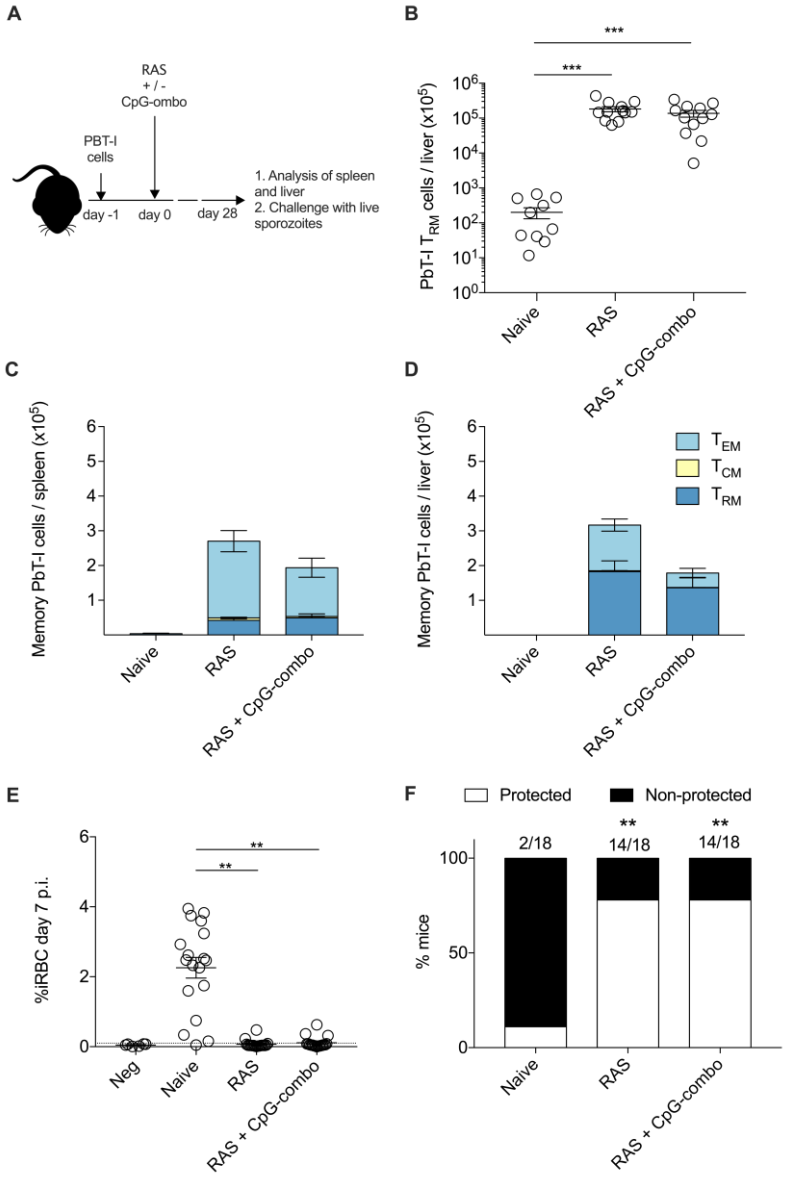

Figure 4.6 CpG-combo has no significant effect on the liver T_{RM} cell formation induced by RAS vaccination. A. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and vaccinated the next day with 50,000 RAS with or without 75 μg of CpG-combo. Spleens and livers from 4 mice were collected on day 28, single cell suspensions were prepared, and memory T cell formation was assessed by flow cytometry. The remaining mice (n=6) were challenged intravenously with 200 live sporozoites. Parasitemia was monitored in blood until day 10 post infection. B. Number of T_{RM} cells per liver on day 28. D. and E. Total number of PbT-I cells in spleen and live, respectively. E. Percentage of infected red blood cells (iRBC) in blood on day 7 post F. Percentage of sterile protection. Results are a pool of two or three independent experiments. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (E.), compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (E.) or using Fisher's exact test (F.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

4.2.5 DOTAP enhances the effect of the CpG-ligands on the formation of liver T_{RM} cells

The above studies revealed that TLR9 ligands were the best adjuvants at inducing liver T_{RM} cell formation and yet each one induced different numbers of cells. In fact, CpG-2006 induced twice as many T_{RM} cells as CpG-combo (though this was not a significant difference; Fig. 4.4 C). As previously mentioned, some of the adjuvants were mixed and complexed in transfection reagents to enhance the differential delivery of agonists within the cell to the endosome or cytosol. Importantly, CpG-2006 was complexed in the transfection reagent DOTAP, whereas CpG-combo was used naked, i.e. without a transfection reagent. To understand the role of a transfection reagent on the induction of T_{RM} cells, the effect of DOTAP on the three CpG tested here (CpG-2006, CpG-combo and CpG-1668) was investigated.

For this, mice were transferred with PbT-I cells and primed the next day with αClec9A-Pb-1 mAb plus CpG-2006, CpG-combo or CpG-1668 naked or complexed in DOTAP (Figure 4A). On day 7, a small blood sample was taken to investigate early cell expansion, and on day 28 the analysis on the memory cell formation in the spleen and liver was assessed.

Results from day 7 bleeds revealed that mice injected with CpG-2006 or CpG-1668 complexed in DOTAP expanded significantly more PbT-I cells as compared to mice injected with the same CpG in absence of transfection reagent (Fig. 4.7B). CpG-combo complexed in DOTAP induced slightly more PbT-I cells in blood as compared to naked CpG-combo, but a significant difference was not evident. Data from day 28 mirrored the results from day 7. CpG-2006 complexed in DOTAP induced a 14-fold increase liver T_{RM} cells as compared to CpG-2006 in absence of DOTAP. In a similar fashion, CpG-1668 in DOTAP prompted the formation of 7-fold more T_{RM} cells as compared to naked CpG-1668 (Fig. 4.7C, E). CpG-combo in DOTAP induced 3 times more T_{RM} cells compared to CpG-combo in the absence of a transfection reagent, although this difference was not

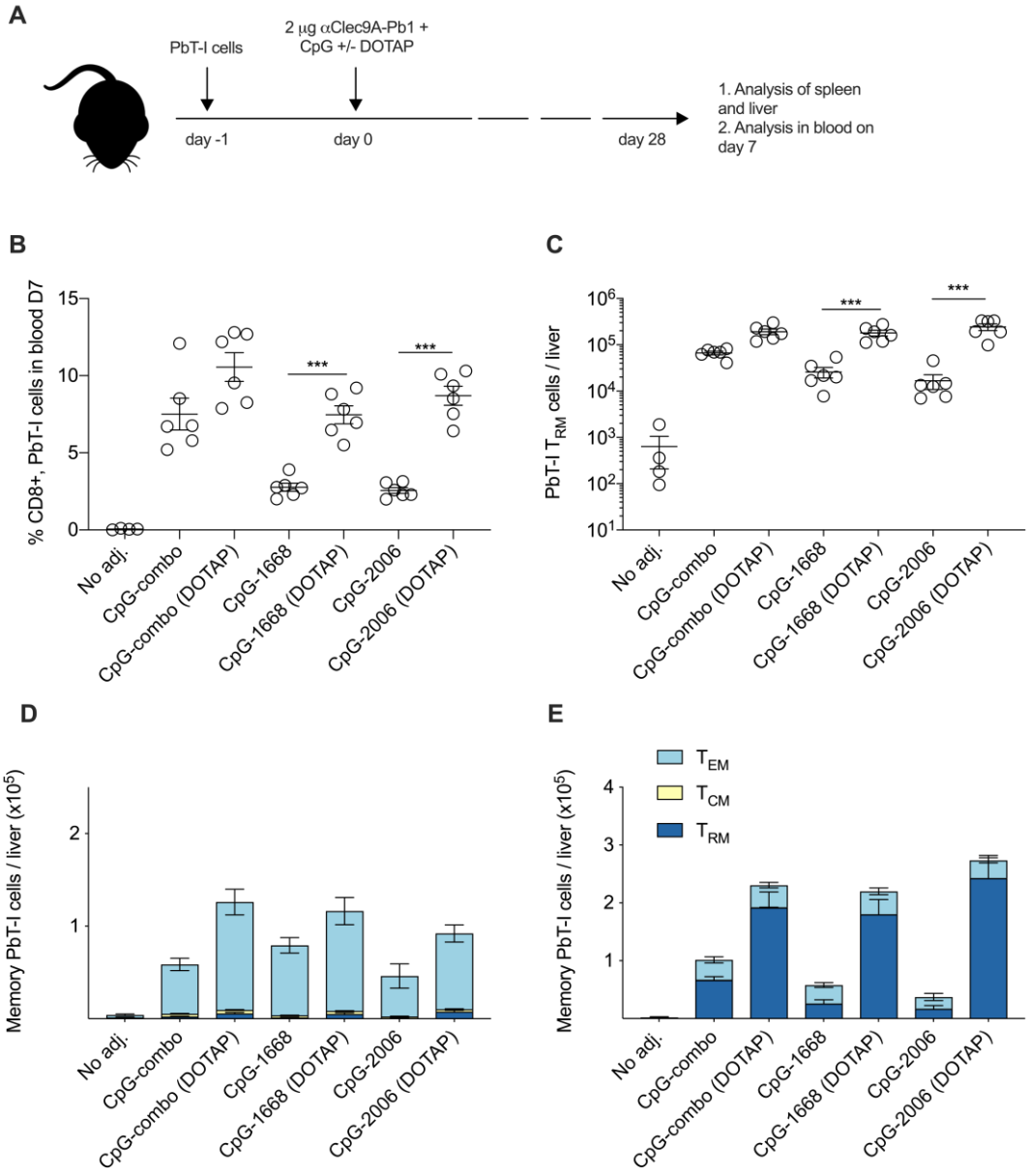

Figure 4.7 DOTAP enhances the effect of the CpG-ligands on the formation of liver T_{RM} cells. A. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and primed next day with 2 μg of αClec9A-Pb-1 plus: 75 μg of CpG-combo, 75 μg of CpG-2006, 75 μg of CpG-1668 complexed or not in DOTAP. On day 7 mice blood was analyzed by flow cytometry. Spleens and livers were collected at day 28, single cell suspensions were generated, and the number of PbT-I cells and memory status were determined by flow cytometry. B. Percentage of PbT-I cells (CD8+, GFP + cells) in blood on day 7 post immunization. C Number of T_{RM} cells per liver determined as CD8+, GFP+, CD44Hi, CD62L-, CD69+. D. and E. Total number of PbT-I cells in spleen and live, respectively. Results are a pool of two independent experiments. Data were compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (B.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (C.) P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

significant (Fig. 4.7C, E). A similar pattern was observed for T_{EM} cells in the spleen, as DOTAP enhances the number of these cells compared to CpG in absence of the transfection reagent (Fig. 4.7D).

After demonstrating that DOTAP enhances the ability of CpG to induce liver T_{RM} cells during Clec9A vaccination, the role of this transfection reagent was investigated in the context of RAS vaccination (Fig. 4.8). For this experiment, mice were transferred with PbT-I cells, then on the next day were vaccinated with RAS plus CpG-combo complexed or not in DOTAP (Fig. 4A). Blood was collected on day 7 to measure the percentage of the PbT-I cells and monitor their expansion (Fig. 4B). On day 28, spleens and livers were harvested, and the memory T cell profile was assessed (Fig. 4C). Remaining mice were infected with live sporozoites and monitored for sterile immunity (Fig. 4D, E). Of note, only one experiment was performed due to time constraints. Analysis of bloods on day 7 showed PbT-I cell expansion in mice that were vaccinated with RAS or RAS + naked CpG-combo, while PbT-I cells expanded poorly in mice receiving RAS + CpG-combo complexed in DOTAP (Fig. 4.8 B). Similarly, on day 28, mice generated high numbers of liver T_{RM} cells when vaccinated with RAS or RAS + naked CpG-combo, but not in those immunised with RAS + CpG complexed in DOTAP formed (Fig. 4.8 C). As a consequence of the poor induction of liver T_{RM} cell formation, mice vaccinated with RAS + CpG-combo in DOTAP developed levels of parasitemia comparable to naïve untreated mice after 7 days of challenge (Fig. 4.8 D) and none were sterilely protected (Fig. 4.8 E). These preliminary data suggest that CpG-combo complexed in DOTAP has an adverse effect on the formation of liver T_{RM} cells mediated by RAS.

While complexing CpG-combo in DOTAP had an adverse effect on T_{RM} cell formation induced by RAS, it was still possible that when used for prime-and-trap vaccination, memory cell formation might be boosted. To test this possibility, mice were vaccinated with prime-and-trap using the Pb-1 epitope. One group received CpG-combo complexed in DOTAP, while another group received

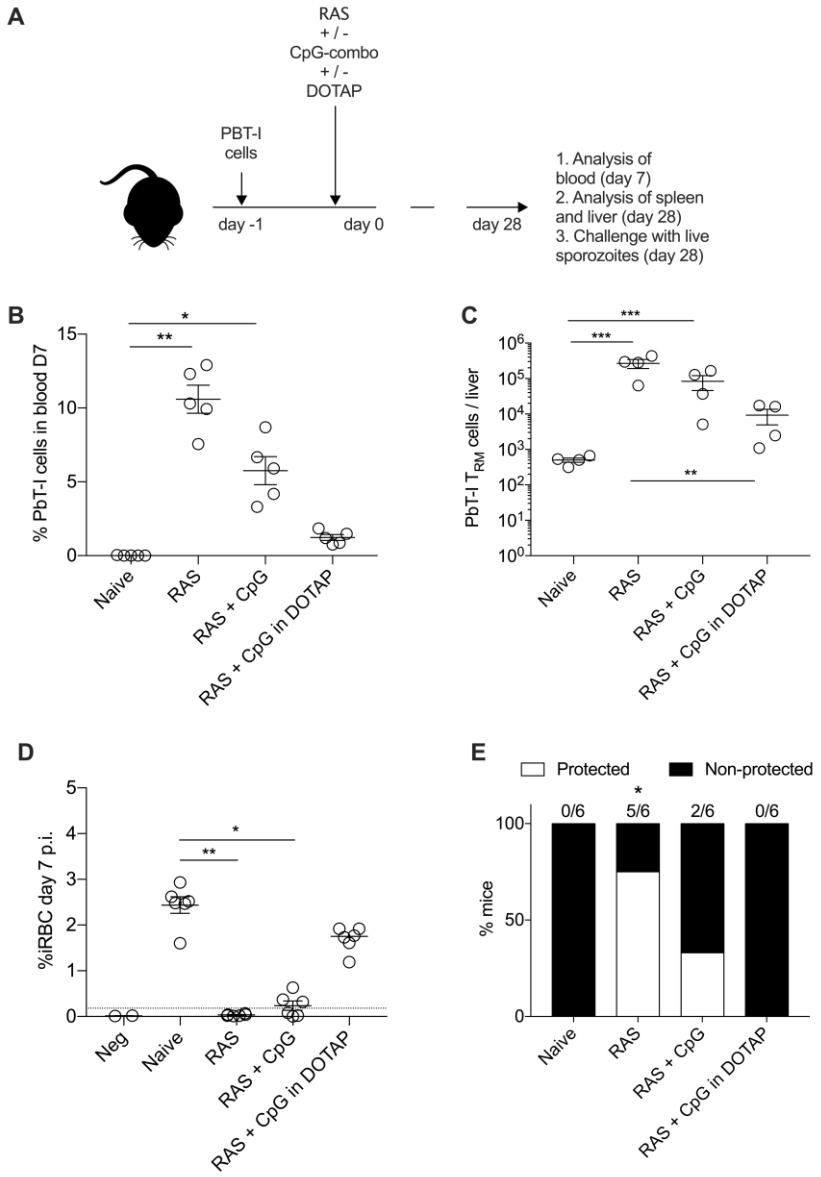

Figure 4.8 DOTAP has an adverse effect on the formation of T_{RM} cells induced by RAS (preliminary results). A. B6 mice were transferred intravenously with 50,000 PbT-I/uGFP cells and vaccinated the next day with 50,000 RAS with or without 75 μg of CpG-combo complexed or not in DOTAP. On day 7, mice blood was analyzed by flow cytometry. Spleens and livers from 4 mice were collected on day 28 and memory T cell formation was assessed by flow cytometry. The remaining mice (n=6) were challenged intravenously with 200 live sporozoites. Parasitemia was monitored in blood until day 10 post infection. B. Percentage of PbT-I cells in blood on day 7 post immunization. C Number of T_{RM} cells per liver. D. Percentage of infected red blood cells (iRBC) in blood on day 7 post F. Percentage of sterile protection. Results are from a single experiment. Data were compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (B. and D.) or were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (C.) or compared by using Fisher's exact test (E.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

CpG-combo in the absence of DOTAP. On day 35, 4 mice per group were used for the enumeration of memory T cells populations in the liver and spleen. The remaining mice (n=6) were challenged intravenously with 200 sporozoites and sterile protection was determined. Surviving mice were challenged a second time on day 104 after immunisation, since this data provides insights for the role of DOTAP in a long-term set up.

Results from day 35 indicated that the number of liver T_{RM} cells induced in mice vaccinated with prime-and-trap was not significantly altered by the presence of the transfection reagent DOTAP, as similar high numbers of T_{RM} cells were formed in mice from both groups (Fig. 4.9B). As previously reported in Chapter 3, mice challenged on day 35 that have been vaccinated with prime-and-trap targeting Pb-1 developed significantly less parasitemia as compared to naive mice, which was reflected on the induction of high levels of sterile protection (Fig. 4.9 C, D). These observations were also true for mice vaccinated with prime-and-trap using CpG-combo complexed in DOTAP; in fact, the level of sterile protection was the same as compared to the original prime-and-trap vaccine.

It is important to mention that data from day 104 were based on a single experiment due to time constraints. On day 104, some of the surviving mice from both the prime-and-trap vaccine with or without DOTAP were infected with 200 live sporozoites. Results on day 7 p.i. indicated a tendency in both groups of vaccinated mice to control parasitemia as compared to naive mice, although this difference was not significant (Fig. 4.9 E). A similar tendency was also observed in the level of protection (Fig. 4.9 F).

Together, these data indicate that even though DOTAP was able to enhance liver T_{RM} cell formation mediated by CpG when vaccinating with Clec9A-targeted antigen, it did not have a significant effect in the context of prime-and-trap vaccination.

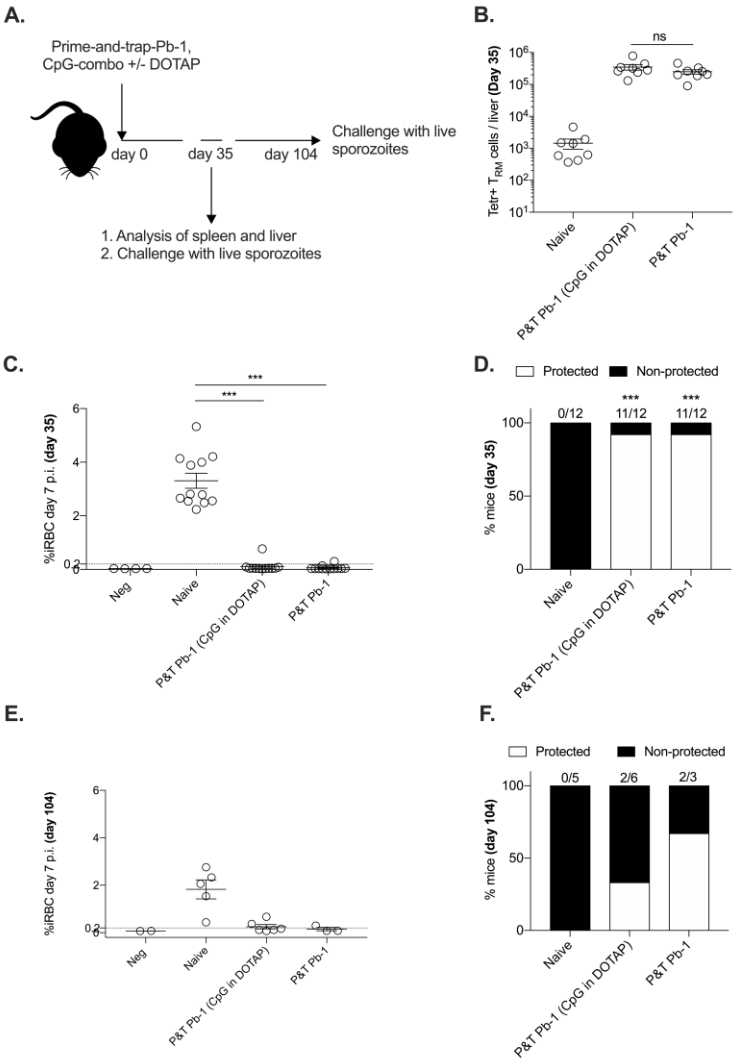

Figure 4.9 DOTAP has no significant effect on the sterile protection induced by prime-and-trap targeting the Pb-1 epitope. A. B6 mice were vaccinated with 8 μg of αClec9A-Pb-1, 10^9 rAAV-Pb-1 and 75 μg of CpG-combo complexed or not in DOTAP. Spleens and livers from 4 mice were collected on day 35 and single cell suspensions were prepared, and memory T cell formation was assessed by flow cytometry. The remaining mice (n=6) were challenged intravenously with 200 live sporozoites. Parasitemia was monitored in the blood up to day 10 post infection. Sterilely protected mice were challenged a second time with 200 living PbA sporozoites on day 104 B. Number of T_{RM} cells per liver on day 35. C. Percentage of infected red blood cells (iRBC) in blood on day 7 post challenge in mice infected on day 35. D. Percentage of sterile protection in mice challenged on day 35. E. Percentage of iRBC on day 7 post challenge in mice infected on day 104. F. Percentage of sterile protection in mice challenged on day 104. Results are a pool of two independent experiments, for data of day 35 and a single experiment for data of day 104. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test in (B.) or compared using a Kruskal- Wallis test with Dunn's multiple comparison post-test in (C.) and (E.), or compared by using Fisher's exact test (D.) and (F.). P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

4.2.6 Assessment of adjuvants for type I IFN production: Control experiments

In the above studies, CpG ODN performed best for induction of T_{RM} cells while most other adjuvants favoured induction of circulating T cells. Two adjuvants, TLR7-L and cGAS-L had particular poor capacity to induce the formation of memory cells in these Clec9A priming studies, prompting the question the whether or not they were functional.

In preliminary studies prior to the assessment with the Clec9A Ab, adjuvants were tested for the stimulation of type I IFN response. These experiments were carried out in Germany before undertaking the Clec9A priming studies in Australia. For this, the synthetically produced CpG-2006, TLR7-L, cGAS-L and RIG-I-L were tested *in vivo* for the production of IFN-α, a well-known type I IFN cytokine. Additionally, the expression of several interferon stimulated genes (ISG), a hallmark for type I IFN response (Schneider et al., 2014), was investigated in the spleen and liver. Since these experiments were performed before assessing the adjuvants for the formation of liver T_{RM} cells, only low doses of the adjuvants were assessed.

Mice were grouped into independent experiments for endosomal delivery and cytosolic delivery. The first group was stimulated with CpG-2006 and TLR7-L complexed in DOTAP, while the second group received cGAS-L and RIG-I-L complexed in Jet-PEI. A few control mice received a non-stimulating nucleic acid (NA) complexed in DOTAP or Jet-PEI for the endosomal and cytosolic delivery, respectively. Four h after stimulation, mice were cheek bled and serum was collected. At 6 h, mice were sacrificed and blood, the spleen and the liver were collected. The serum was stored and RNA was isolated from the homogenized organs. Production of IFNα was measured in serum by ELISA and the expression of several ISG was investigated by qPCR.

Results from these preliminary experiments revealed extensive variation between experiments. Importantly, substantial background for the production of IFN-α was observed in mice that received non-stimulating NA, potentially masking the stimulation induced by the adjuvants. There was a tendency towards higher production of IFN-α induced by CpG-2006 and TLR7-L as compared to control mice (Fig. 4.10 B). A similar tendency was found in mice stimulated with cGAS-L and RIG-I-L (Fig. 4.10 C).

The relative gene expression of representative ISG *Irf 7* and *Ifi 44* is shown in Fig. 4.10 D, E. Cytosolic stimulation of cGAS and RIG-I leads to the activation of the transcription factor IRF7, which promotes the production of IFN and other ISG (Schneider et al., 2014; Schoggins and Rice, 2012). Preliminary results from two mice revealed that while cGAS and RIG-I induced higher expression of *Irf 7* and *Ifi 44* in the spleen and liver compared to CpG-2006 and TLR7-L, these last two also induced a 10-fold increase of *Irf 7* expression in liver (Fig. 4.10 D, E). Data from these pilot experiments suggested these adjuvants were functional in their capacity to induce type IFN.

CpG-2006, TLR7-L, cGAS-L and RIG-I-L were shipped from Germany to Australia prior the Clec9A priming studies. Given the limited effect of some of those adjuvants, it was necessary to confirm their function once again in Australia. For this purpose, mice were stimulated with the different adjuvants and the production of IFNα was assessed in sera. Mice were grouped in two different functional analysis: i) they received high or low doses of TLR7-L, cGAS-L or RIG-I-L and ii) they were stimulated with different CpG in the presence or absence of DOTAP. Control mice received only DOTAP or remained naive. Four and 10 h later, mice were bled and serum collected.

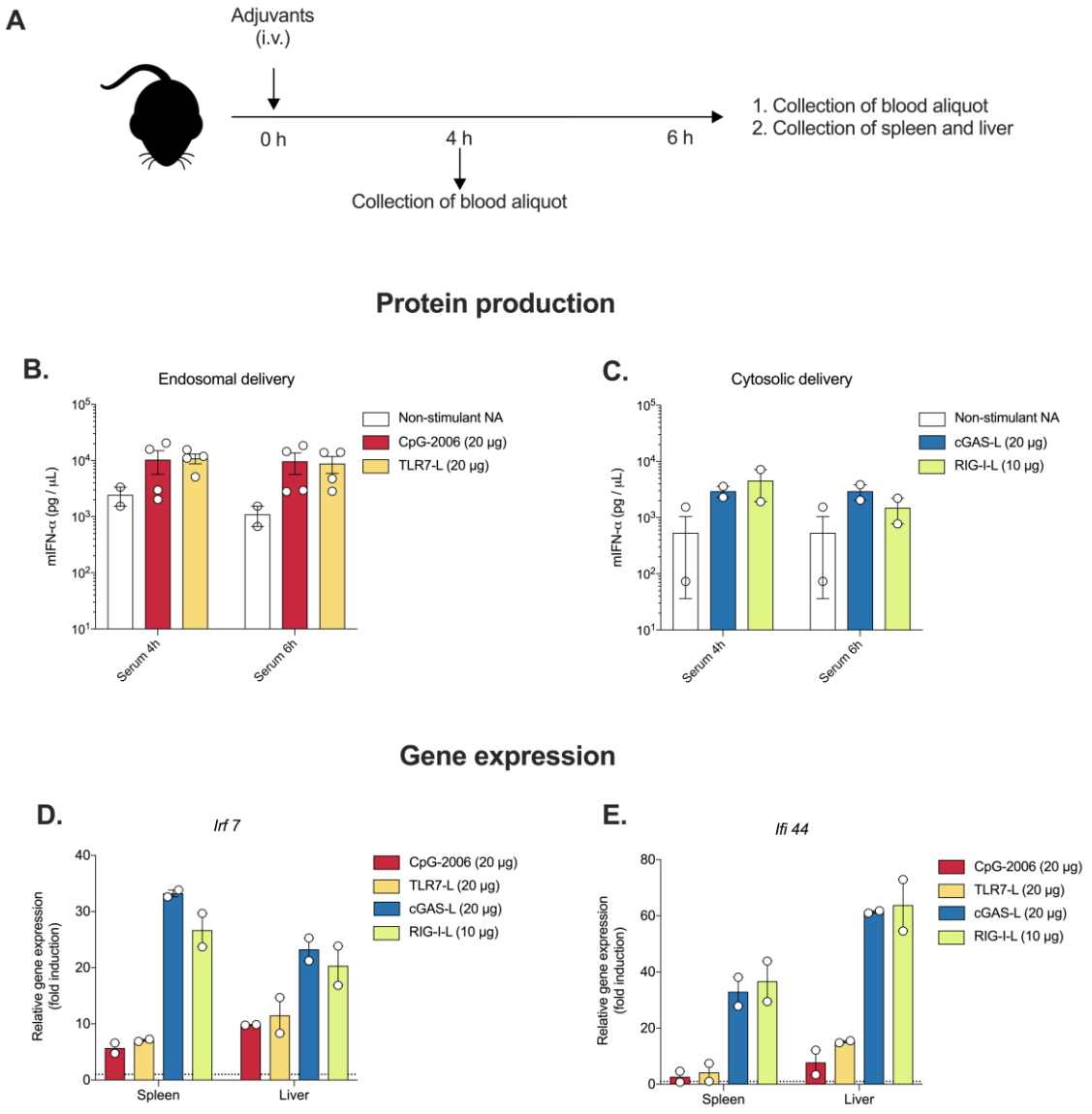

Figure 4.10 Adjuvants induce type I IFN production, preliminary experiment. A. B6 mice were stimulated i.v. with 20 µg of CpG-2006, 20 µg of TLR7-L or 20 µg of non-stimulating DNA complexed in DOTAP; or with 20 µg of cGAS-L, 10 µg of RIG-I-L or 10 µg of non-stimulating RNA complexed in Jet-PEI. 4 h later mice were cheek bled and serum collected. 6 h later mice were sacrificed, blood, spleen and liver were collected. Serum was collected and RNA was isolated from the homogenized organs. Production of mIFN-α was measured in serum by ELISA and the expression of several ISG was investigated in spleen and liver by qPCR. Production of mIFN-α in sera of mice stimulated with CpG-2006 or TLR7-L (B.) and cGAS-L or RIG-I-L (C.). Relative gene expression of *Irf 7* (D.) and *Ifi 44* (E.) expressed by fold induction in the spleen and liver.

These data are from a single experiment and require confirmation. After 4 h, DOTAP substantially enhances the early production of IFNα induced by CpG-combo and CpG-2006 and it had no effect when complexed with CpG-1668 (Fig. 4.11 A). Despite the limited production of IFNα induced by CpG-1668, this adjuvant was clearly functional as it induced the formation of liver T_{RM} cells in the absence or presence of DOTAP (Fig. 4.1 and Fig. 4.7). All three CpG in the absence of DOTAP failed to induce the production of this cytokine as compared to control mice (Fig. 4.11 A). After 10 h of stimulation, the levels of IFN-α in sera were all similar to control mice (Fig. 4.11 B).

One of the major reasons driving this experiment was the poor performance of the cGAS-L and TLR7-L to induce expansion and formation of liver T_{RM} cells. Importantly, results from this experiment showed that these two adjuvants (at both high and low doses) had the capacity to induce the early production of IFNα (Fig. 4.11 C). Observations indicated that there was no dose effect for RIG-I-L, TLR7-L or cGAS-L. Interestingly, high levels of the cytokine were still detected after 10 h in mice stimulated with RIG-I-L independently of the dose (Fig. 4.10 D). Preliminary data from this experiment suggested that all adjuvants transported from Germany to Australia were still active and able to induce the production of IFN-α *in vivo*.

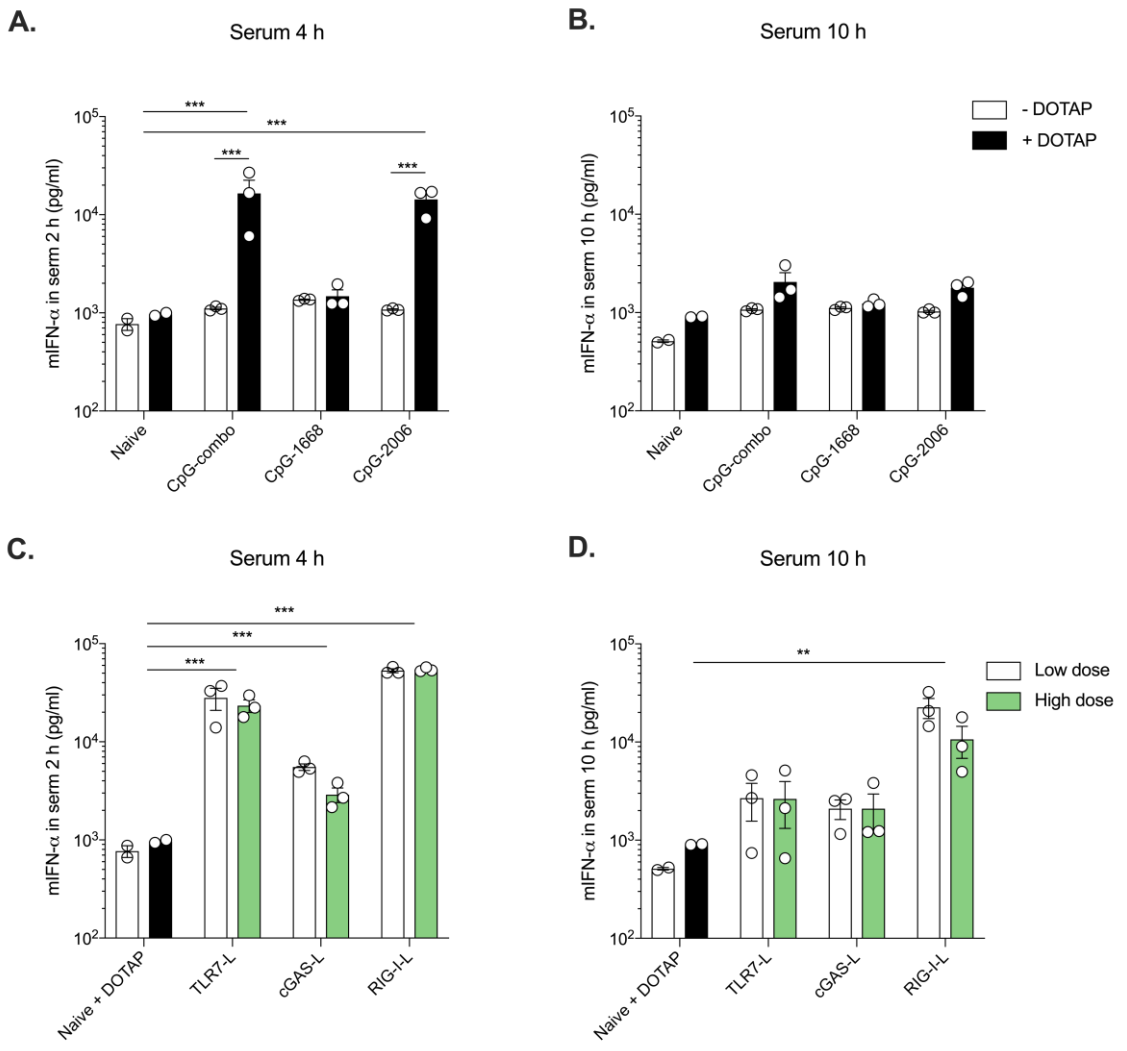

Figure 4.11 Adjuvants induce type I IFN production, late control experiment. A. B6 mice were grouped in 2. The first group received 75 μg of CpG-combo, or CpG-1668 or CpG-combo complexed or not in DOTAP. The second group were stimulated with 30 or 75 μg of TLR7-L complexed in DOTAP, or 20 or 75 μg of cGAS-L complexed in Jet-PEI®, or 20 or 40 μg of RIG-I-L complexed in Jet-PEI®. Control mice received only DOTAP or remained naive. Three mice were stimulated per each treatment. Later, at 4 and 10 h mice were bled and serum collected. Production of mIFNα was measured in serum by ELISA. Production of IFNα in mice stimulated with three different CpG at 4 h (A.) and 10 h (B.). Level of IFNα in mice receiving different doses of cGAS-L or RIG-I-L or TLR7-L at 4 h (C.) and 10 h (D.). Results are from a single experiment. Data were log-transformed and compared by one-way ANOVA and Tukey's multiple comparisons test. P>0.05, *, P<0.05; **, P<0.01 ***, P<0.001.

4.3 Discussion:

In this study, a series of different nucleic acids were assessed as novel adjuvants for the induction of liver T_{RM} cell formation induced by Clec9A targeted immunisation. The agonists investigated here included 3 types of CpG ODN, which are TLR9 ligands; p I:C, a TLR3 ligand; LPS, a TLR4 ligand, a synthetically produced ssRNA as TLR7 ligand, a dsDNA for cGAS stimulation and a 5' 3p dsRNA as RIG-I agonist. Results demonstrated that CpG ODN were the best adjuvant to promote formation of T_{RM} cells in the liver, as well as large numbers of T_{EM} cells in the spleen. Importantly, the number of liver T_{RM} cells was heavily influenced by the amount of the adjuvant per mouse, as a high dose induced the formation of significantly and higher proportions of memory T cells in the liver. The results also showed that the transfection reagent DOTAP enhanced the adjuvant effect mediated by several CpG ODN, at least in their ability to form liver T_{RM} cells; however, it did not improve immunity mediated by the prime-and-trap vaccine. Moreover, CpG ODN had no significant effect on the formation of liver T_{RM} cells induced by RAS vaccination nor on the protection mediated by this vaccine. In summary, the data presented in this chapter demonstrate the superior capacity of CpG ODN to promote liver T_{RM} cell formation in B6 mice mediated by Clec9A targeted immunisation.

4.3.1 CpG ODN as an adjuvant for CD8 T cell expansion

The specific mechanisms that govern the early expansion and the formation of memory CD8 T cells mediated by CpG ODN remain to be fully elucidated. Some of the factors that may influence these effects will be discussed below.

Intracellularly, the stimulation of TLR9 in APC by CpG ODN activates the adaptor protein myeloid differentiation primary response protein 88 (MYD88) (Häcker et al., 2000); which ultimately leads to the expression of type I IFN stimulated genes (ISG) or the production of proinflammatory cytokines via the

NF-κB pathway (Junt and Barchet, 2015). There are 4 types of CpG ODN described, and although all of them bind to TLR9, they have different immunological effects (Bode et al., 2011).

A-class CpG ODN induces strong production of type I IFN by pDC, leading to the activation of cellular antiviral state in human primary cells and murine splenic DC (Guiducci et al., 2006; Hemmi et al., 2003). B-class ODN, triggers pDC differentiation and the production of TNF-α, which in turn leads to T cell activation. Additionally, B-class ODN induces B cells to proliferate and secrete IgM (Hartmann and Krieg, 2000; Hemmi et al., 2003; Rothenfusser et al., 2001; Verthelyi et al., 2001). Studies have demonstrated that the distinct effects mediated by A-class versus B-class relies on differences in the retention time in early or late endosomes (Guiducci et al., 2006; Honda et al., 2005). A-class ODN are able to form complexed structures, allowing for a longer retention into early endosomes. This promotes the signalling through the transcription factor IRF7, which leads to IFN-α expression (Bode et al., 2011; Guiducci et al., 2006; Honda et al., 2005). In contrast, the B-class ODN single strand structure enables rapid transport to late endosomes for signalling via NF-κB, for the production of proinflammatory cytokines, such as TNF-α (Bode et al., 2011; Guiducci et al., 2006; Honda et al., 2005). C-class combines features from both A and B-class, as it stimulates B cells to secrete IL-6 and pDC to produce IFN-α (Hartmann et al., 2003; Marshall et al., 2003). P-class ODN is the most recently described ODN, shown to produce more IFN-α, IL-6 and the CXCR3 ligand CXCL10 than B- or C-class ODN (Samulowitz et al., 2010).

The diversity of immune responses mediated by CpG ODN and their relatively simple structure have triggered investigation of these compounds as adjuvants. In fact, several vaccine clinical trials have been performed using CpG ODN for the generation of antibodies for hepatitis B (Cooper et al., 2004) and malaria (Mullen et al., 2008); and for the induction of CD8 T cell responses against melanoma, breast and ovarian cancer (Valmori et al., 2007).

In this chapter and previous published studies, data revealed that CpG-combo, which is a B-class ODN linked to a P-class ODN, was one of the best adjuvants at inducing early expansion of CD8 T cells and liver T_{RM} cell formation (Fernandez-Ruiz et al., 2016; Holz et al., 2018). B-class ODN not only promotes pDC differentiation and production of TNF-α, but also binds to DEC205, a molecule highly expressed on CD8+ DC, improving their maturation, production of IL-12 and stimulation of CTL (Caminschi et al., 2013; Lahoud et al., 2012). Therefore, by linking a B-class to a P-class ODN, the superior capacity for cytokine induction of the P-class ODN is targeted to CD8+ DC. Importantly, the Clec9A mAb used in this study for the antigen immunisation also target CD8+ DC, thus both the antigenic peptide and this potent adjuvant could converge in the same DC. These features make CpG-combo a superior adjuvant for priming of CD8 T cells mediated by Clec9A targeted immunisation.

In the current study, it was demonstrated that as long as CpG-2006 and CpG-1668 were complexed in DOTAP, they were as potent as CpG-combo for the induction of T_{RM} cells in the liver. CpG-2006 and CpG-1668 are both B-class ODN characterised by a simple structure that makes them susceptible to the activity of DNases present in sera (Hanagata, 2012). If they successfully reach the endosome, they are rapidly transported to lysosomes where they signal TLR9 through the NF-κB pathway (Honda et al., 2005) before being degraded. The P-class ODN, present in CpG-combo, contains palindromic sequences that enable the formation of complex structures, protecting them from extracellular degradation (Samulowitz et al., 2010). Potentially, this feature also promotes the retention of the ODN within early endosomes for the activation of type I IFN. Forming a complex of B-class ODN with a cationic lipid such as DOTAP provides several advantages. First, encapsulation within a cationic lipid protects DNA from extracellular degradation (Capaccioli et al., 1993). Second, the now positively charged DNA-lipid complex enhances uptake by the negatively charged cellular membrane (Chesnoy and Huang, 2000). Finally, this complex is more likely to be retained into both the early and late endosome, as compared to a “naked” B-class

ODN, triggering type I IFN as well as proinflammatory cytokines mediated by NF-κB (de Jong et al., 2010; Khalil et al., 2006).

The superior capacity of liposomes to enhance CpG-mediated CD8 T cell responses has been previously demonstrated by several studies. Vaccination studies of a breast cancer model, using a HER-2/neu epitope, revealed that encapsulation of the peptide together with CpG ODN in liposomes substantially enhanced the peptide-specific CD8 T cell response as measured by IFN-γ production (Li et al., 2003). In a B16 melanoma model, Jérôme and collaborators showed that co-administration of liposome-encapsulated antigen and a B-class CpG-ODN elicited a potent anti-tumor CTL response (Jérôme et al., 2006). For the OVA antigen model, it was also described that delivery by liposomal-nanoparticles of CpG-ODN in the presence of OVA peptide, enhanced the specific killing mediated by CD8 T cells (Chikh et al., 2009).

Despite the potent adjuvant effect of CpG-combo, it failed to enhance the formation of liver T_{RM} cells mediated by RAS vaccination. Similarly, it was previously reported that B-class CpG ODN do not enhance the expansion of malaria specific-CD8 T cells (i.e. PbT-I cells) mediated by RAS immunisation (Lau et al., 2014). Interestingly, a preliminary observation reported in this chapter indicated that mice vaccinated with RAS plus CpG-combo complexed in DOTAP generated less liver T_{RM} cells than mice receiving only RAS and failed to control infection. This may be explained by the fact that non-specific-protection against sporozoite challenge is induced by CpG-combo alone, at least for a short time (Gramzinski et al., 2002, unpublished data Heath lab). It is possible that interference with hepatocyte invasion by RAS reduces it capacity to induce the formation of memory cells. Further experiments are required to unveil the mechanisms that govern this effect. For example, if the adjuvant CpG is administered 24 h after RAS vaccination, sporozoites may have more time to infect hepatocytes to ensure local antigen presentation and liver T_{RM} cell formation.

In summary, the data presented here are in line with several previous publications, demonstrating the superior capacity of CpG to induce CD8 T cell expansion and for the cationic liposome DOTAP to enhance this adjuvant effect.

4.3.2 Other agonists

In addition to early CD8 T cell expansion, CpG ODN also enhanced the formation of liver T_{RM} cells mediated by Clec9A targeted immunisation. All other adjuvants tested, i.e. p I:C, LPS, a TLR7, cGAS and RIG-I ligand; failed to promote the formation of liver T_{RM} cells as effectively as CpG ODN.

As briefly mentioned before, CpG ODN has the capacity to induce inflammation in the liver and the formation of iMATEs, which in turn, supports proliferation and accumulation of CD8 T cells in the liver (Huang et al. 2013). This feature is of special interest for the design of strategies designed to induce CD8 T cell responses in the liver. Importantly, Huang and collaborators also tested MPLA a TLR4 ligand less toxic than LPS, p I:C and a TLR7 ligand. They showed that MPLA induced a moderate number of CTL in the liver and that neither p I:C nor TLR7 ligand had the capacity to induce the formation of iMATEs. Consequently, the proliferation of CD8 T cells was reduced (Huang et al., 2013). Data generated here support the idea that LPS, p I:C and TLR7-L are poor adjuvants for the formation of memory CD8 T cells in the liver.

Whereas, TRL4 activation enhances Th1 immune responses and ligands of TLR4 have been exploited as potential adjuvants (Steinhagen et al., 2011), LPS, a well-known TLR4 agonist, is also known to induce tolerance. Excessive MyD88 activation by large amounts of free LPS causes the production of pro-inflammatory cytokines that can lead to endotoxin shock when overproduced systemically (Biswas and Lopez-Collazo, 2009). Endotoxin tolerance is a host protection mechanism intended to counteract the effects of extreme inflammation. In fact, mice exposed to non-lethal doses of LPS are protected from a secondary otherwise lethal dose of LPS (Cavaillon and Adib-Conquy, 2006;

Freudenberg and Galanos, 1988). In the clinic, patients who survive sepsis and have undergone “cytokine storm”, usually exhibit an immunocompromised phase correlated with high risk of secondary infection and mortality (Biswas and Lopez-Collazo, 2009). *In vitro* studies in mouse macrophages and human monocytes have demonstrated a reduced ability to produce pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 after a second administration of LPS (Akira and Takeda, 2004). Importantly, *in vivo* stimulation with LPS leads to a substantial loss of DC and further impairment in IL-12 production (Wysocka et al., 2001) – possibly influencing subsequent priming of CD8 T cells and their recruitment to the liver. Novel biosynthetically engineered TLR4 ligands have been designed to overcome the induction of toxicity and currently are being tested as adjuvants for vaccines against bacterial infections, such as *Bordetella pertussis*, *Shigella flexneri* and *Vibrio cholerae* (Zariri and Van Der Ley, 2015).

TLR3 signalling induces strong type I IFN response and promotes cross-presentation leading to potent CD8 T cell responses (Schulz et al., 2005; Sistigu et al., 2014). In this regard, poly I:C, a synthetic TLR3 agonist has been shown to have substantial adjuvant capacity for an influenza vaccine and strong antitumor activity in murine tumour models (Currie et al., 2008; Ichinohe et al., 2005; Pulko et al., 2009; Salem et al., 2005). These studies differ from the experiments described in this chapter by two major aspects. First, the route of administration is different. While mice here received p I:C systemically through intravenous injection, for the influenza vaccine and the solid tumour studies, p I:C was locally administered, respectively, by intranasal or intra-tumoral application (Currie et al., 2008; Ichinohe et al., 2005). Second, the time frame of the CD8 T cell response is different. These published studies investigated the therapeutic properties of effector CD8 T cells mediated by p I:C stimulation (Pulko et al., 2009; Salem et al., 2005) rather than the formation of memory CD8 T cells, which is the main topic of investigation in this chapter.

Studies have demonstrated the capacity of TLR7 agonists as adjuvants for the induction of CTL antitumor responses in murine models (Drobits et al.,

2012; J. Wu et al., 2017). Particularly, for these two studies, the TLR7 ligands were applied locally either topically in the case of the melanoma model, or intra-tumorally in case of the breast cancer model (Drobits et al., 2012; J. Wu et al., 2017). Similarly, pulmonary administration of a synthetically modified TLR7 ligand, termed 1V270, was shown to increase resistance to lethal pulmonary viral infection in mice (Wu et al., 2014). These data may indicate that the cell-mediated immunity induced by stimulation with TLR7 agonists is better achieved by a local rather than the systemic administration route used in this chapter. In fact, some studies have reported *in vivo* tolerance induced by the systemic and repeated administration of TLR7 agonists (Bourquin et al., 2011; Gunzer et al., 2005; Hayashi et al., 2009). Pre-treatment of pDC and myeloid DC with TLR7 ligands leads to a reduced production of the proinflammatory cytokines IFN-α, IL-6 and IL-12p70 after TLR restimulation (Bourquin et al., 2011). Moreover, *in vitro* cultures of pDC with TLR7 ligand showed increased secretion of the anti-inflammatory cytokine IL-10 after a second stimulation with the ligand (Bourquin et al., 2011), and high expression of inhibitory molecule PD-L1 after a single TLR7 stimulation (Wu et al., 2019).

Despite promising data suggesting RIG-I agonists have the potential to be used as pan-antivirals, vaccine adjuvants or mediators of antitumor activity (Y. Wu et al., 2017; Yong and Luo, 2018), only a few studies have demonstrated the effect of RIG-I ligands as vaccine adjuvants (Kasumba and Grandvaux, 2019). For a lethal influenza murine model, a sendai virus-derived RIG-I ligand was shown to increase specific IgG titres when co-administered with inactivated virus vaccine, leading to reduced morbidity and mortality after challenge (Martínez-Gil et al., 2013). Moreover, the effect of a 3pRNA RIG-I agonist as an adjuvant was confirmed for an OVA murine model, in which mice vaccinated with OVA peptide plus 3pRNA exhibited high specific cytotoxicity corelated to viral clearance after challenge with a modified adenovirus expressing OVA (Hochheiser et al., 2016). This last study supports the idea that RIG-I agonists could be exploited as adjuvants for hepatic infections, like hepatitis and malaria liver-stage. However, in this chapter, the RIG-I ligand used as adjuvant had a limited effect on the

induction of liver T_{RM} cells. Importantly, Hochheiser *et. al.* complexed the adjuvant together with the antigen, ensuring simultaneous delivery of adjuvant and antigen to the same APC (Hochheiser et al., 2016). For the experiments described here, Clec9A mAb coupled to the antigen was co-administered in the same injection as the complexed RIG-I-L, but this does not ensure simultaneous delivery to the same cell. This factor may explain why RIG-I-L and other agonists failed to induce the formation of substantial liver T_{RM} cells.

Recent studies have identified cGAS as a sensor of cytosolic DNA able to induce interferons and cytokines. Upon activation, cGAS produces a cyclic dinucleotide GMP-AMP (cGAMP) (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013). cGAMP functions as a second messenger and activates the adaptor protein STING (Burdette et al., 2011; Burdette and Vance, 2013; Danilchanka and Mekalanos, 2013). In turn, STING activates the transcription factors NF-κB and IRF3 to induce type I IFN and cytokines (Tanaka and Chen, 2012; Wu et al., 2013). In recent reports, cGAMP rather than ligands for cGAS has been used as an antiviral or adjuvant in different murine models. Li and collaborators demonstrated that cGAMP functions as an adjuvant for T cell activation and antibody production in an OVA *in vivo* model (Li et al., 2013). Additionally, a study in healthy volunteers confirmed that cGAMP enhances the antigen-specific expansion, activation and killing of melanoma cell lines mediated by CD8 T cells isolated from PBMC (Gutjahr et al., 2019). Two important factors may influence the fact that cGAS ligand had a limited effect for the induction of liver T_{RM} cells. First and as already mentioned, the adjuvant may not reach the same APC as the antigen, failing to provide the licensing signals for an adequate CD8 T cell priming. Second, if the cGAS ligand successfully reaches the cytoplasm, this does not ensure the production of cGAMP and further activation of the STING pathway.

The last observation in this chapter showed early production of IFN-α detected in serum of mice stimulated with TLR-7-L, cGAS-L and RIG-I-L. Importantly, it was also stablished that these three agonists did not promote

substantial formation of liver T_{RM} cells. Further experiments are required to confirm these findings, but as it stands this may indicate that level of IFN-α in serum does not correlate with T_{RM} cell formation in liver.

Studies have demonstrated that licensing of DC for optimal stimulation of CD8 T cells requires cis-activation by danger signals rather than indirect stimulation provide by inflammatory cytokines produced by adjacent cells (Desch et al., 2014). Accordingly, vaccine formulations that link antigen and danger signals, which act as adjuvants showed an enhanced capacity to induce humoral and cellular immunity (Demento et al., 2010; Hou et al., 2011; Moon et al., 2011; Steers et al., 2009). Thus, further experiments exploring the possibility of linking the here tested adjuvants to the antigen represent a strategic variation to enhance the expansion of CD8 T cells and the potential formation of liver T_{RM} cells. Translational implications will be further discussed on Chapter 5.

CHAPTER 5

General discussion

5.1 Context

In 2014, members of our group developed a B6 TCR transgenic line able to produce CD8 T cells specific for PbA. This TCR transgenic line, termed PbT-I, produced cells that responded to blood-stage PbA, *P. yoelii* XNL and *P. chabaudi*, and to liver stage PbA parasites (RAS) (Lau et al., 2014). Soon after, these cells were used to develop the novel vaccination strategy “prime-and-trap” and generate liver PbT-I T_{RM} cells, which were shown to be crucial for protection against PbA sporozoite challenge (Fernandez-Ruiz et al., 2016). T_{RM} cells are part of a memory T cell subpopulation that are localised in tissues and provide potent immune surveillance. The strategic location of T_{RM} cells allows these cells to mediate effector responses against secondary infections more rapidly than their counterparts, the circulating memory cells, which need to be recruited to the infected organ (Mueller and Mackay, 2016). Given the importance of liver T_{RM} cells in the context of protection against malaria and other liver-associated pathogens (Fernandez-Ruiz et al., 2016; Pallett et al., 2017; Stelma et al., 2017), studies on how to enhance their formation are highly relevant, and thus the experiments conducted and presented in this thesis aimed to investigate this goal. Recently, the cognate epitope for the PbT-I cells, called the Pb-1 epitope in this thesis, has been identified as part of the RPL6 protein of PbA (Valencia-Hernandez et al., 2020). Here we addressed the suitability of this epitope for the induction of endogenous liver T_{RM} cells that confer sterilizing protection through vaccination. Additionally, it is known that inflammation favours the formation T_{RM} cells (Mackay et al., 2012a) and that adjuvants can affect their numbers (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Recent data showed that CpG induced liver inflammation (Huang et al., 2013) and supports the formation of T_{RM} cells in this organ (Fernandez-Ruiz et al., 2016; Holz et al., 2018). Based on these findings, in this thesis, nucleic acid sensor agonists were assessed as novel adjuvants for the formation of liver T_{RM} cells.

5.2 Summary of main findings

In chapter 3, a series of different immunisation strategies targeting the Pb-1 epitope were implemented and specific CD8 T cell responses were assessed in B6 mice. Analysis of the T cell repertoire of naive B6 mice revealed that the number of specific CD8 T cell precursors for the Pb-1 epitope was very large. These cells were successfully expanded by two different immunisation strategies: i) Single injection with Clec9A mAb coupled to Pb-1 plus adjuvant (vaccine 1) and ii) Prime-and-trap targeting the Pb-1 epitope. Large numbers of specific T_{RM} cells were formed in the livers of vaccinated mice. While mice developed a certain level of protection when vaccinated with vaccine 1, higher levels of protection were achieved in mice vaccinated with prime-and-trap.

In Chapter 4, various pattern recognition receptor (PRR) agonists were assessed as novel adjuvants for the induction of liver T_{RM} cell formation induced by Clec9A targeted immunisation. The agonists investigated included: i) LPS, a TLR4 ligand, which is a plasma membrane receptor; ii) agonists of the endosomal receptors TLR3, TLR7 and 3 types of TLR9 ligands; and iii) ligands of the cytosolic receptors cGAS and RIG-I. The data revealed that CpG ODN was the best adjuvant to promote formation of T_{RM} cells in the liver. Also, the number of liver T_{RM} cells was influenced by the amount of adjuvant administered; that is, a high dose induced the formation of a higher proportion of memory T cells in the liver. It was also determined that the transfection reagent DOTAP enhanced the adjuvant effect mediated by several CpG ODN, at least in their ability to form liver T_{RM} cells. However, use of DOTAP with the prime-and-trap vaccine did not improve protection. Finally, CpG ODN did not enhance formation of liver T_{RM} cells when used in combination with RAS, nor improve protection mediated by this vaccine.

5.3 A protective malaria epitope

Malaria still claims more than 400,000 lives per year, mostly among African children (WHO, 2019). Naturally acquired immunity (NAI) to clinical malaria only develops with age in areas with high parasite transmission. In adults, NAI protects against clinical malaria by maintaining low parasitemia levels (Baird et al., 2003; Owusu-Agyei et al., 2001). Sterilizing immunity, i.e. complete absence of blood stage parasites, never develops and clinical immunity is not long lasting (Doolan et al., 2009; Keegan and Dushoff, 2013). Hence, there is an urgent need for the development of an effective vaccine. However, despite decades of intense research, an effective malaria vaccine for application in endemic areas remains elusive (Coelho et al., 2017; Draper et al., 2018). Vaccines targeting pre-erythrocytic (PE) parasite stages are ideal because they avoid the occurrence of symptoms and block transmission. PE vaccines based on whole-sporozoite administration have proven to be effective at conferring protection in murine models and in malaria naïve humans (Clyde, 1990; Mordmüller et al., 2017; Nussenzweig et al., 1967; Seder et al., 2013). However, these approaches face major limitations that question their suitability for routine use in endemic areas (Draper et al., 2018). Some of those challenges are associated with technical and logistical issues of large scale sporozoite production (mosquito breeding and manual dissection) and distribution in endemic areas (maintenance of the cold chain). However, a greater concern is that while RAS vaccination induces protection in malaria naïve humans, it has failed to protect with a comparable efficacy in malaria-experienced adults in Mali (Sissoko et al., 2017).

It is known that the sterilizing immunity induced by PE vaccines is substantially mediated by CD8 T cells (Cockburn et al., 2014; Doll et al., 2016; Epstein et al., 2011; Ishizuka et al., 2016; Nganou-Makamdop et al., 2012; Schofield et al., 1987; Weiss et al., 1988), with liver-associated memory cells having been recently shown to play a prominent role (Fernandez-Ruiz et al., 2016; Tse et al., 2014). In this regard, vaccine strategies aimed at generating

CD8 T cell responses against specific PE epitopes have been developed recently. These liver-stage subunit vaccines overcome the manufacturing challenges of whole-parasite approaches, but also come with the limitation that highly protective *Plasmodium* epitopes are largely unknown.

Prime and boost immunisations with heterologous viral vectors encoding for PE antigens have been exploited for their capacity to elicit CD8 T cell responses (Draper et al., 2018). Murine studies demonstrated high levels of protection associated with CD8 T cells induced by prime and boost immunisations with viral vectors encoding several PE antigens – contained in a construct termed ME-TRAP (Gilbert et al., 2002; Li et al., 1993; Reyes-Sandoval et al., 2010; Schneider et al., 1998). In contrast, mixed results in vaccine efficacy were obtained in humans depending on malaria pre-exposure. That is, in malaria naïve volunteers, vaccine efficacy (determined as sterilizing immunity or delay in the onset of parasitemia) was partial ranging from 46-57%, whereas clinical trials in malaria endemic countries revealed negligible efficacy (Bliss et al., 2017; Ewer et al., 2013; Hodgson et al., 2015; Mensah et al., 2016; Ogwang et al., 2015; Tiono et al., 2018).

Given these mixed results, efforts have been made to improve the immunogenicity and improve protection levels of liver-stage subunit vaccines. Viral vectored immunisations with other PE antigens not included in ME-TRAP have failed to protect with a comparable efficacy to those induced by the ME-TRAP construct (Hodgson et al., 2015). Immunisations with viral vectors encoding for blood-stage antigens failed to confer protection against sporozoite challenge in malaria naïve volunteers (Sheehy et al., 2012). Even the co-administration of viral vectors encoding for ME-TRAP together with the blood-stage epitope MSP1 did not improve vaccine efficacy (Sheehy et al., 2012). In another attempt to increase the efficacy of the viral vectored ME-TRAP vaccine, malaria naïve volunteers were immunised with RTS,S, which induces Ab against CSP, plus viral vectors encoding for ME-TRAP simultaneously. The groups who received RTS,S plus the viral vectors had lower protection levels compared to

the groups immunised with RTS,S only, although differences were not significant due to sample size (Rampling et al., 2018).

To date, CSP and TRAP have been extensively targeted by several vaccination strategies, since they have been considered important immunogenic antigens found in *P. falciparum* (Müller et al., 1993; Rogers et al., 1992), because both are essential for sporozoite motility and liver-stage infection (Nussenzweig and Nussenzweig, 1985; Sultan et al., 1997). However, as mentioned before, these strategies have failed to provide robust protection. Therefore, there is need for the discovery of novel and highly immunogenic CD8 T cell epitopes. One of the major challenges for the design of liver-stage sub-unit vaccines is the limited number of known protective MHC-I epitopes derived from liver stage *Plasmodium* parasites (Draper et al., 2018; Schmidt et al., 2010). The lack of a suitable and sufficient source of epitopes eluted from MHC molecules of infected hepatocytes hampers the efforts to identify immunogenic CD8 T cell epitopes. In fact, the identification of the Pb-1 epitope, described in chapter 3, took more than 6 years. Several strategies have been recently developed to tackle this problem.

Transcriptomic studies revising gene expression patterns across different parasite stages and *Plasmodium* species provided valuable information on proteins differentially expressed during liver-stage infection (Howick et al., 2019; Sacci et al., 2005; Speake et al., 2016; Tarun et al., 2008). This information together with epitope prediction software (Vita et al., 2019) support the identification of immunogenic peptides that can be potentially targeted by liver-stage subunit vaccines (Hafalla et al., 2013). Furthermore, humanized liver-chimeric mouse models and new *in vitro* liver-stage cell culture methods are currently exploited as a way to yield sufficient epitopes from infected hepatocytes for analysis by mass spectrometry (Longley et al., 2015). In a recent study, human liver chimera FAH-/- NOD Rag1-/- IL2Rγ^{NULL} (FNRG) mice were infected with *P. falciparum* sporozoites and then peptides from the liver were isolated for sequencing. The collected peptides were derived from the proteasomal degradation of proteins and potentially have the right size to be presented by

MHC molecules (Winer et al., 2020). Deviating from several other studies, the authors used a top down mass spectrometry approach, a technique that avoids the digestion of peptides and the loss of post-translational modifications. This approach allows for the identification of peptides in their native form (Cui et al., 2011). In this preliminary study, a series of CD8 T cell *P. falciparum*-derived antigens were identified as potential targets for a malaria vaccines (Winer et al., 2020).

As well as identifying potential immunogenic epitopes, it is important to understand the features of protective antigens to allow application of this knowledge to the selection of new antigenic targets for use in subunits vaccines. Some factors that may influence immunogenicity of CD8 T cell epitopes include: i) the number of naïve CD8 T cell precursors (Jenkins and Moon, 2012), ii) the expression pattern during liver-stage infection (Butler et al., 2011; Speake et al., 2016), iii) the presence of the protein in the hepatocyte cytosol for presentation on MHC I (Boddey et al., 2016; Cockburn et al., 2011; Montagna et al., 2014), and iv) protein polymorphism.

As mentioned before, the TRAP protein has been an antigen of choice for liver-stage subunit vaccines and, as such, has been tested in several clinical trials. However, robust immunity has not been achieved (Bliss et al., 2017; Ewer et al., 2013; Hodgson et al., 2015; Mensah et al., 2016; Ogwang et al., 2015; Tiono et al., 2018). Moreover, in B6 mice – a strain that is considered difficult to protect (Doolan and Hoffman, 2000; Schmidt et al., 2010) – vaccination targeting TRAP induced a reduction in liver parasite load, but failed to induce sterile protection (Hafalla et al., 2013). Recently published data generated by members of our group revealed that B6 mice vaccinated with prime-and-trap targeting a TRAP epitope generated large numbers of specific liver T_{RM} cells, but only low levels of sterile protection (Valencia-Hernandez et al., 2020). On the contrary, results from chapter 3 demonstrated that B6 mice immunised with prime-and-trap targeting the RPL6 protein developed both, high numbers of endogenous liver T_{RM} cells as well as high levels of sterile protection.

We hypothesize that two major factors may contribute to the different immunogenicity of RPL6 and TRAP in B6 mice. The first is the relatively high number of Pb-1-specific precursors compared to the small number specific for the TRAP epitope (Chapter 3). It has been demonstrated that the frequency of peptide-specific naive T cell precursor determines to a certain extent the magnitude and expansion of the subsequent T cell response after exposure to the specific peptide (Jenkins and Moon, 2012; Moon et al., 2007; Joshua J Obar et al., 2008). The second factor is the differences in the expression patterns of the proteins. While TRAP is mainly expressed by sporozoites and not liver-stage parasites (Amino et al., 2006), RPL6 is expressed throughout the liver and blood stages of infection by *P. berghei* and *P. yoelii* (Howick et al., 2019; Sacci et al., 2005; Tarun et al., 2008). This feature of RPL6 increases its possibility to be presented by hepatocytes and mediate CD8 T cell recognition and activation of effector functions.

An important characteristic of RPL6 that might be beneficial for future translation of a vaccine that targets this protein is its highly conserved nature (Valencia-Hernandez et al., 2020). Analysis on a dataset of more than 2,000 *P. falciparum* genomes from 15 countries determine that more than 99% of the sequences share same haplotype for PfRPL6 (Valencia-Hernandez et al., 2020). By contrast, TRAP, which is a surface protein and likely subjected to Ab selection pressure, is highly polymorphic, as confirmed by sequencing of isolates from endemic areas (Kosuwin et al., 2014; Ohashi et al., 2014; Robson et al., 1990; Srisutham et al., 2018).

When compared to a single injection of RAS, prime-and-trap targeting PbRPL6 induced higher levels of protection in B6 mice (data reported in Chapter 3 and 4). Data presented here indicated that T_{RM} cell-based subunit vaccines have the potential to induce sterile protection in B6 mice, even when using a single epitope. Moreover, the fact that *P. falciparum* RPL6 is highly conserved, makes of this protein a potential target for the development of a human malaria vaccine. Notably, our research group has also now identified an immunogenic

HLA*A02:01-restricted epitope in *P. falciparum* RPL6 (Valencia-Hernandez et al., 2020).

5.4 Adjuvants for liver T_{RM} cell formation

T_{RM} cells provide effective tissue surveillance and can respond rapidly to infection by providing local effector functions and the recruitment of immune cells leading to the elimination of pathogens (Schenkel et al., 2014, 2013). Mouse studies have revealed that T_{RM} cells play important roles in fighting infections against several types of pathogens, including viruses (Gebhardt et al., 2009; Teijaro et al., 2011), bacteria (Benoun et al., 2018; Sheridan et al., 2014; Wilk et al., 2017), fungi (Park et al., 2018), and parasites (Fernandez-Ruiz et al., 2016; Glennie et al., 2015).

Recently, efforts to develop vaccination strategies aimed at generating T_{RM} cells have been increasing. Animal vaccine studies have shown promising results targeting T_{RM} responses at barrier sites such as lung (Perdomo et al., 2016; Wakim et al., 2015; Zens et al., 2016), female reproductive tract (Çuburu et al., 2014; Shin et al., 2016; Tan et al., 2018), and liver (Fernandez-Ruiz et al., 2016; Gola et al., 2018). However, only a few studies have investigated the effect of adjuvants on the formation of T_{RM} cells induced by those vaccination strategies.

In an influenza virus model, mice receiving *in vitro* activated effector T cells were immunised intranasally with DC-targeted mAb coupled to OVA plus LPS as adjuvant (Wakim et al., 2015). These mice generated lung T_{RM} cells that contributed to high levels of protection against a lethal influenza virus challenge, but the effect of immunisation with the mAb in absence of LPS was not examined (Wakim et al., 2015).

Fernandez-Ruiz and collaborators explored the role of adjuvants on the induction of liver T_{RM} cells in vaccinated mice. Using prime-and-trap vaccination, they observed that the adjuvants poly I:C and CpG both enabled the formation of

liver T_{RM} cells, but CpG was superior and, in contrast to poly I:C, was able to induce strong protection against malaria liver-stage infection (Fernandez-Ruiz et al., 2016). A later study also showed that in the absence of antigen presentation, *in vitro* activated CD8 T cells formed liver T_{RM} cells and that their formation was increased by poly I:C and CpG (Holz et al., 2018). This effect may be in part explained by the fact that i.v. injection of CpG induces intrahepatic myeloid cell aggregates that supports the accumulation of CD8 T cells in the livers of stimulated mice (Huang et al., 2013). Another group also investigated the role of adjuvants on the formation of liver T_{RM} cells mediated by vaccination (Gola et al., 2018). For this study, liver T_{RM} cells were generated in mice by priming with a modified adenovirus and boosting with nanoparticles both containing OVA antigen. They showed that the presence of R848-MPL (ligand for TLR7 and TLR4, respectively) on nanoparticles that also contained OVA antigen did not enhance the formation of liver T_{RM} cells (Gola et al., 2018).

Given that inflammation favours T_{RM} cell formation (Mackay et al., 2012b), adjuvants that induce inflammation in particular organs can be used to enhance numbers of T_{RM} cells in those organs. In this thesis, a wide variety of adjuvants were assessed for the induction of liver T_{RM} cells in the context of antigen targeted immunisation. The studied agonists included 3 types of CpG ODN, ligands for TLR3, TLR4, TLR7, as well as agonists for cGAS and RIG-I. The above 8 ligands bind a diverse range of pattern recognition receptors located in the plasma membrane, the endosomal compartment or the cytoplasm, and have the ability to induce the release of proinflammatory cytokines and the upregulation genes involve in type I IFN response. We hypothesized that some of the proinflammatory signals triggered by this type of adjuvant could promote the formation of liver T_{RM} cells. However, the liver has particular features that may pose a challenge for the generation of a certain type of inflammation that could enhance T_{RM} cell formation without causing tissue damage. These features will be discussed in the following paragraphs.

5.5 Challenges for the induction of liver inflammation

The liver receives blood from the gastrointestinal track through the portal vein, meaning it is constantly exposed to PAMPS derived from microbes present in the gut lumen. While the liver is constantly exposed to molecules from microorganisms and diet, it maintains homeostasis via tolerogenic immune mechanisms (Bigorgne and Crispe, 2010; Kesar and Odin, 2014; Schwabe et al., 2006). Liver tolerance has been associated with a tight regulation of the expression levels of pattern recognition receptors. For instance, the expression levels of TLR, as well as the signalling adaptor molecule MyD88 in liver cells are lower as compared to the levels observed in cells from other organs (De Creus et al., 2005; Faure-Dupuy et al., 2018; Lichtman et al., 1998; Zarember and Godowski, 2002).

Hepatic immune regulation can also be promoted by liver resident immune cells. Upon activation, human and murine Kupfer cells (hepatic macrophages) release proinflammatory cytokines such as IL-6, TNFα, as well as the anti-inflammatory cytokine IL-10 (Frink et al., 2007; Knolle et al., 1995; Overland et al., 2005; Seki et al., 2001; Wu et al., 2009). It has been shown by *in vitro* assays that murine freshly isolated Kupffer cells respond less strongly to LPS if they have been pre-exposed to LPS, whereas pre-exposure to CpG does not affect the response of Kupffer cells to LPS stimulation (Schuchmann et al., 2004). Moreover, one study suggests that hepatic DC express lower mRNA levels of TLR4 relative to their splenic counterparts and that this phenotype may contribute to the tolerogenic environment in the liver (De Creus et al., 2005).

Dysregulation of the liver tolerance environment, in particular of the TLR stimulation pathway, has been associated with inflammatory liver diseases, such as alcoholic liver disease and hepatocellular carcinoma (Kiziltas, 2016; Mencin et al., 2009; Pimentel-Nunes et al., 2010; Soares et al., 2010). For example, it has been shown *in vivo* that chronic alcohol intake leads to the upregulation of the mRNA levels of TLR2, TLR4, TLR7, TLR8 and TLR9 in the liver, which is

associated with hepatitis (Gustot et al., 2006). Remarkably, in a murine model of alcoholic liver disease, TLR4 deficient mice were protected from the liver tissue damage induced by the prolonged alcohol intake (Hritz et al., 2008; Uesugi et al., 2001).

In a model of chemical-induced liver cancer, the LPS levels in sera of treated mice were higher compared to healthy control mice; and additionally, animals lacking TLR4 or microbiota developed significantly less hepatocellular carcinoma (Dapito et al., 2012; Yu et al., 2010). In liver samples of patients with non-alcoholic fatty liver disease the protein levels of TLR4 and TLR9 were higher as compared to healthy volunteers (Mridha et al., 2017). In the same study, authors showed in a murine model of non-alcoholic fatty liver disease that mice lacking TLR9 had reduced chronic inflammation and consequently, were protected from developing the disease (Mridha et al., 2017).

Based on the information described above, the liver is an organ known for its immunotolerance microenvironment that upon dysregulation can lead to severe chronic diseases. Under these circumstances, generating beneficial inflammation, one that could enhance T_{RM} cell formation (Mackay et al., 2012a) without inducing liver tissue damage, is a difficult task to achieve. On the one hand, the threshold for TLR activation in the liver is higher given the downregulation of TLR expression compared to other organs (De Creus et al., 2005; Faure-Dupuy et al., 2018; Lichtman et al., 1998; Zarember and Godowski, 2002), thus potent immune stimulators should be used to generate intrahepatic inflammation. On the other hand, hyperstimulation of TLR could lead hepatocyte apoptosis, to tissue damage (Dapito et al., 2012; Yu et al., 2010), and as a consequence, poor liver T cell accumulation.

In this thesis, mice were immunised with anti-Clec9A antibodies targeting the RPL6 *P. berghei* protein, in the presence of several nucleic acid-based adjuvants. The hypothesized mechanism is that these antibodies target cDC1 which when activated by the adjuvant, prime naïve CD8 T cells in secondary

lymphoid organs (Caminschi et al., 2008; Sancho et al., 2008). After priming, CD8 T cells would then migrate to the liver recruited by the inflammation induced locally by the adjuvant and ultimately generate T_{RM} cells. Results from experiments performed in this thesis showed that mice receiving CpG-based adjuvants developed substantially more liver T_{RM} cells, when compared to mice that received the other agonists (TLR3, -4, -7, RIG-I and cGAS ligands). These results support previous findings showing that CpG ODN are better at promoting T cell liver residency, when compared to TLR3 and TLR4 ligands (Fernandez-Ruiz et al., 2016; Holz et al., 2018). CpG ODN has the capacity to induce the formation of intrahepatic myeloid cell aggregates that promote proliferation and accumulation of CD8 T cells in the liver (Huang et al. 2013); and possibly, this is the mechanism by which CpG favours the formation of liver T_{RM} cells. Huang and collaborators also showed in this study that TLR3, TLR4 and TLR7 ligands failed to induce the formation of such myeloid cell aggregates and subsequently, the proliferation of CD8 T cells was reduced (Huang et al. 2013). Accordingly, data generated in this thesis showed that TLR3, TLR4 and TLR7 ligands are poor adjuvants for the formation of memory CD8 T cells in the liver.

TLR3 synthetic ligands, such as poly I:C and TLR7 agonists have been used as adjuvants to promote potent CD8 T cells responses in the context of vaccines against viral infection and tumour therapy (Currie et al., 2008; Drobits et al., 2012; Ichinohe et al., 2005; Pulko et al., 2009; Salem et al., 2005; J. Wu et al., 2017). These published studies differ from the experiments described in this thesis by two major features, the route of administration and the triggered CD8 T cell response.

In this thesis, mice received the adjuvants systemically through intravenous injection, whereas on the published studies the TLR3 and TLR7 ligands were administered locally (intranasal, intrapulmonary, topically or intra-tumoral) depending on the model (Currie et al., 2008; Drobits et al., 2012; Ichinohe et al., 2005; J. Wu et al., 2017). These data may indicate that the cell-mediated immunity induced by stimulation with TLR3 and TLR7 agonists is better

achieved by a local rather than a systemic administration. Importantly, the systemic and repeated administration of TLR7 ligands has been shown to induce tolerance in *in vivo* studies (Bourquin et al., 2011; Gunzer et al., 2005; Hayashi et al., 2009). Moreover, the above published reports investigated the therapeutic effects of effector CD8 T cells rather than differentiated memory T cells (Currie et al., 2008; Drobits et al., 2012; Ichinohe et al., 2005; Pulko et al., 2009; Salem et al., 2005; J. Wu et al., 2017), thus, information about these adjuvants for the generation of potent CD8 T cell memory responses, particularly liver Trm cells, is missing from the published data.

Activation of TRL4 enhances Th1 immune responses and accordingly, ligands of TLR4 have been exploited as potential adjuvants (Steinhagen et al., 2011). However, not all TLR4 agonists can be used as adjuvants. In fact, LPS, a well-known TLR4 agonist, induce excessive activation and can generate tolerance and endotoxin shock (Biswas and Lopez-Collazo, 2009). Therefore, alternative TLR4 ligands have been designed to avoid toxicity and tolerance while keeping the benefits of TLR4 activation.

Monophosphoryl lipid A (MPLA) is a detoxified derivate of LPS isolated from *Salmonella minnesota* that activates TLR4 (Baldridge et al., 2004; Casella and Mitchell, 2008; Ribi et al., 1979). Inspection of the literature indicates in most cases MPLA, and not LPS, has been used in combination with other adjuvants in animal vaccination studies and clinical trials. For example, MacLeod and colleagues (2011) conducted an influenza vaccine study in which mice that received antigen plus MPL together with alum (a well-known adjuvant in human vaccines) generated higher CTL differentiation and long-lasting memory CD8 T cells, as compared with mice that received antigen plus only one of the two adjuvants. In the same study, mice vaccinated with influenza antigen with MPL plus alum developed higher levels of protection against challenge as compared to mice vaccinated with antigen plus only one adjuvant (MacLeod et al., 2011). These data indicate that signals from both adjuvants (MPL and alum) are required to enhance vaccine efficacy and drive potent CD8 T cell responses. To date, at

least two adjuvants based on TLR4 ligands (AS01 and AS04) are used in human licensed vaccines against virus, including herpes zoster, hepatitis B and human papillomavirus (Del Giudice et al., 2018). Both AS01 and AS04 are composed of MPL plus a second adjuvant, saponin QS-21 and alum hydroxide, respectively (Didierlaurent et al., 2017, 2009).

As mentioned, data generated in this thesis revealed the poor adjuvant capacity of LPS to induce liver T_{RM} cell formation mediated by Clec9A targeted immunisation (Chapter 4). Future studies investigating the adjuvant effects on T_{RM} cells would benefit from exploring MPL instead of LPS together with other adjuvants such alum salts and the saponin SQ-21.

Another ligand that showed a poor adjuvant capacity for the induction of liver memory CD8 T cells in experiments performed in this thesis was the cGAS ligand (Chapter 4). Upon activation of cGAS by cytoplasmic DNA, it produces a messenger molecule, known as cGAMP, which in turn activates STING that ultimately leads to the production of pro-inflammatory cytokines and type I IFN (Ablasser et al., 2013; Burdette et al., 2011; Diner et al., 2013; Sun et al., 2013; Tanaka and Chen, 2012). STING agonists have been extensively investigated in murine models as vaccine adjuvants against bacterial and viral infections and tumour malignancies (Blaauboer et al., 2015; Corrales et al., 2015; Ebensen et al., 2007; Karaolis et al., 2007b; Ogunniyi et al., 2008; Wang et al., 2016). Interestingly, published data suggests that WT murine hepatocytes express undetectable levels of STING and cGAS when compared to spleenocytes (Thomsen et al., 2016). This has important implications for hepatotropic infections with DNA viruses including hepatitis B virus (HBV) and cytomegalovirus (CMV). In fact, two recent studies have shown that ablation of STING or cGAS has no effect on the *in vivo* immune response to HBV and MCMV as compared to WT mice (Tegtmeyer et al., 2019; Thomsen et al., 2016). Importantly, upon induction of interferon stimulated genes by poly I:C (TLR3 ligand) or by forcing the expression of STING in hepatocytes, the STING deficient mice gained the ability to reduce the HBV viral load after infection (Thomsen et al., 2016). These findings

suggest that cGAS or STING ligands are probably poor inducers of inflammation mediated by hepatocytes because of the low levels of expression in these cells. In this sense, combining cGAS ligands with other adjuvants might provide signals important for CD8 T cell memory formation in the liver.

RIG-I was first discovered as a host sensor for viral RNA and upon activation leads to type I IFN response (Rothenfusser et al., 2005; Yoneyama et al., 2004). Since then, RIG-I ligands have been effectively used in animal models to induce antiviral and antitumoral responses (Chiang et al., 2015; Goulet et al., 2013; Kasumba and Grandvaux, 2019; Linehan et al., 2018; Y. Wu et al., 2017; Yong and Luo, 2018). Moreover, two studies have confirmed the use of RIG-I agonists as adjuvant in murine vaccines against infections with influenza virus and an adenovirus expressing OVA (Hochheiser et al., 2016; Martínez-Gil et al., 2013). This last study supports the idea that RIG-I agonists could be exploited as adjuvants for hepatotropic infections, like hepatitis and malaria liver-stage. However, in this thesis, the RIG-I ligand had a limited effect on the induction of liver T_{RM} cells. Importantly, Hochheiser and colleagues used a transfection reagent to complexed the adjuvant together with the antigen, aiming for a simultaneous delivery of adjuvant and antigen to the same APC (Hochheiser et al., 2016). For the experiments described in this thesis, Clec9A mAb coupled to the antigen was co-administered in the same injection as the RIG-I ligand, but this does not ensure simultaneous delivery to the same cell. This factor may explain why RIG-I ligand and other agonists failed to induce an adequate initial priming of the CD8 T cells.

5.6 CpG favours liver T_{RM} cell formation

Data generated in this thesis demonstrate the superior capacity of CpG (compared to the other tested adjuvants) to promote liver T_{RM} cell formation mediated by Clec9A targeted immunisation. As mentioned before, the superiority of CpG may be related to the capacity of CpG to generate intrahepatic myeloid cell aggregates that support proliferation of CD8 T cells in the liver (Huang et al.,

2013). Remarkably, the transfection reagent DOTAP enhanced the formation of liver T_{RM} cells mediated by different types of CpG (Chapter 4). DOTAP is a cationic lipid that mediates intracellular delivery and enhances DC activation (Honda et al., 2005; Yasuda et al., 2005; Yotsumoto et al., 2008).

In the current study, two B-class CpG and a special CpG, named CpG-combo (a B-class linked to a P-class CpG), were assessed for the induction of liver T_{RM} cell formation. B-class CpG are known to promote pDC differentiation and the production of TNF-α (Hartmann and Krieg, 2000; Hemmi et al., 2003; Rothenfusser et al., 2001; Verthelyi et al., 2001), which in turn leads to T cell activation. P-class CpG, on the other hand, is known to promote a strong production of IFN-α, IL-6 and the CXCR3 ligand CXCL10 *in vivo* (Samulowitz et al., 2010).

Results from chapter 4 showed that mice receiving CpG-combo developed much higher numbers of liver T_{RM} cells compared to mice that received B-class CpG. However, when B-class CpG were complexed in DOTAP they induced as many T_{RM} cells as CpG-combo, regardless of whether or not CpG-combo was also complexed in DOTAP. Structural differences between the two CpG types might explain this finding. B-class CpG are composed by single strand structures, which are susceptible to degradation by DNases present in sera (Hanagata, 2012). On the contrary, the P-class ODN, present in the CpG-combo, contains palindromic sequences that enable the formation of complex structures (Samulowitz et al., 2010), protecting it from extracellular degradation. It is possible that B-class CpG and DOTAP complexes are more stable than free B-class CpG and therefore more effective at inducing DC activation and/or inflammation in the liver. These complexes are less prone to extracellular degradation (Capaccioli et al., 1993) and as they are positively charged, they could be easily drawn-in by the negatively charged cellular membrane (Chesnoy and Huang, 2000). Existing evidence from vaccination studies suggest that transfection reagents based on liposomes enhance the CpG-mediated CD8 T cells responses. In fact, the encapsulation of relevant antigen together with CpG

in liposomes, increases CTL responses *in vivo* for the OVA antigen model and tumour models for breast cancer and melanoma (Chikh et al., 2009; Jérôme et al., 2006; Li et al., 2003).

5.7 Too much of a good thing?

Pre-erythrocytic malaria vaccines based on the administration of whole sporozoites have demonstrated high efficacy against infection in mice and humans. From these approaches, immunisation with radiation-attenuated sporozoites (RAS) is the most clinically tested strategy, and it has been demonstrated to induce sterile protection against sporozoite challenge in animal models and humans (Clyde, 1990; Gwadz et al., 1979; Ishizuka et al., 2016; Nussenzweig et al., 1967; Seder et al., 2013). Recently, murine studies showed that vaccination with RAS induces liver T_{RM} cells providing protection against sporozoite challenge (Epstein et al., 2011; Fernandez-Ruiz et al., 2016).

Given the superior capacity of CpG-combo to favour the formation of liver T_{RM} cells mediated by Clec9A immunisation (chapter 4), this compound was assessed as an adjuvant for RAS vaccination. Surprisingly, CpG-combo had no enhancing effect on the generation of liver T_{RM} cells nor on the protection induced by RAS. Additionally, preliminary results indicate that mice receiving RAS together with CpG-combo complexed in the transfection reagent DOTAP generated less T_{RM} cells than mice immunised with RAS alone and therefore failed to control infection (chapter 4). These findings question the capacity of CpG-combo to adjuvant RAS vaccination, but it will be important to repeat these last experiments to confirm our findings.

Data from chapter 4 also indicate that CpG not only failed to enhance the formation of memory cells, it also failed to promote the early expansion of circulating CD8 T cells 7 days after priming with RAS. Similarly, published data show that CpG does not enhance early expansion of malaria transgenic-CD8 T cells (i.e. PbT-I cells) mediated by RAS immunisation (Lau et al., 2014). Notably,

preliminary data suggest that mice vaccinated with RAS and CpG in DOTAP had fewer specific cells in blood at day 7 post priming than mice receiving only RAS (chapter 4). Altogether, these findings indicate that administration of CpG, in particular CpG in DOTAP, reduces not only the initial CD8 T cell expansion and the later formation of liver memory cells, but also the protection against sporozoite challenge normally induced by immunisation with RAS.

It is known that insufficient early expansion of CD8 T cells may result from an inadequate priming process. For instance, it has been recently reported that suboptimal protection induced by intradermal RAS vaccination results from insufficient early CD8 T cell activation, which in turn is a consequence of a suboptimal priming by cDC1 (Haeberlein et al., 2017). In light of the results from chapter 4, it is possible that the administration of CpG (in the presence or absence of DOTAP) somehow alters the CD8 T cell priming induced by RAS.

Early studies have shown that immunisation with non-viable sporozoites, e.g. heat-killed and freeze-thawed parasites, provide much less protection against sporozoite challenge as compared to RAS (Alger and Harant, 1976; Hafalla et al., 2006). This indicates that certain characteristics of viable parasites, in this case of RAS, are important for the induction of effective protection. Indeed, the protective immunity induced by RAS is abrogated by treatment of hosts with primaquine to eliminate the remaining liver parasites (Klotz et al., 1995; Scheller and Azad, 1995). It is possible that if attenuated parasites fail to establish a hepatocyte infection, the probability of antigen acquisition and presentation reduces and with that the chances to prime T cell responses.

We and others have observed that CpG alone induces a short (1-2 days) non-specific-protection against sporozoite infection (Gramzinski et al., 2002, unpublished data Heath lab). Importantly, these authors have also shown that this effect mediated by CpG is abrogated by treatment with monoclonal antibodies against IL-12 or IFN-γ (Gramzinski et al., 2002). In line with these findings, early studies in mice and primates have demonstrated that pre-

treatment (1-2 days prior infection) with recombinant IFN-γ or IL-12 inhibits the development of liver-stage infection by both human and mouse *Plasmodium* species (Ferreira et al., 1986; Hoffman et al., 1997; Sedegah et al., 1994). Additionally, the administration of IFN-γ induces the production of nitric oxide and reactive nitrogen intermediates inside hepatocytes, which in turn mediates the elimination of intracellular parasites (Klotz et al., 1995; Nahrevanian and Dascombe, 2001).

Shortly after systemic administration, CpG complexed in DOTAP induces significantly higher amounts of IL-12 and IFN-γ than CpG alone (Suzuki et al., 2004; Yotsumoto et al., 2008). Based on these previous studies describing the role of IL-12 and IFN-γ on the elimination of intra-hepatic parasites, we hypothesize that CpG in DOTAP induces “too much of a good thing” and hinders the protective immunity induced by RAS. Hypothetically, high levels of IL-12 and IFN-γ induced by the systemic administration of CpG in DOTAP contribute to the early elimination of intrahepatic RAS parasites, reducing the availability of liver-stage antigens for the priming and activation of CD8 T cells. Ultimately, this results in a reduction of liver T_{RM} cells and therefore, a loss of protection against sporozoite infection. Further experiments are required to unveil the mechanisms that govern this effect. For example, if the adjuvant CpG is administered days later after RAS vaccination, sporozoites may have more time to infect hepatocytes to ensure antigen presentation and liver T_{RM} cell formation.

5.8 The translational potential of ‘prime-and-trap’

Prime-and-trap vaccination successfully induces the formation of liver T_{RM} cells that protect against sporozoite challenge in B6 mice transferred with *Plasmodium*-specific transgenic cells (Fernandez-Ruiz et al., 2016), or in mice with a normal endogenous T cell repertoire (chapter 3) (Valencia-Hernandez et al., 2020). Ideally, this vaccine could be engineered for human use, but prime-and-trap is a complex 3-component vaccine that currently faces some challenges before translation is actually feasible. These complications are not necessarily

insurmountable and in principle a vaccine like prime-and-trap can be produced for clinical trials.

The first component of the vaccine consists of a monoclonal antibody anti-Clec9A (a cDC1 receptor) coupled to a highly immunogenic liver-stage epitope (Fernandez-Ruiz et al., 2016; Valencia-Hernandez et al., 2020), designed to achieve epitope delivery to cDC1 (Caminschi et al., 2008; Lahoud et al., 2011). Antigen targeting using anti-Clec9A antibodies together with adjuvants has shown to be well tolerated and to induce effective humoral responses in non-human primates (Li et al., 2015). To date, no clinical studies have demonstrated the safety of anti-Clec9A antibodies administration. However, the first human studies using antibodies targeting another DC receptor, the DEC-205 (also expressed by cCDC1), coupled to a tumour antigen, have demonstrated the feasibility, safety and biological activity of this vaccine (Dhodapkar et al., 2014; Griffiths et al., 2018). Hence, in principle, protein DC-targeted vaccines are safe for use in humans, as well in non-human primates. Moreover, current advances in technologies for large-scale production of plant-derived antibodies for immunotherapy (Buyel et al., 2017), can lower the cost of production increasing feasibility.

The second component is a recombinant adeno-associated virus (rAAV) engineered to selectively infect hepatocytes (Tay et al., 2014), which expresses the targeted antigen under the control of a hepatocyte-specific promoter, to induce the trapping of the T cells to the liver (Fernandez-Ruiz et al., 2016; Valencia-Hernandez et al., 2020). Clinical trials based on adenovirus vector-vaccines expressing antigens against several infectious diseases including malaria, HIV, HCV and Ebola have increased rapidly during the last decade (Ewer et al., 2016; Lauer et al., 2017; Naso et al., 2017; Rauch et al., 2018). These studies not only demonstrate the safety of this approach but also report promising vaccine efficacies (Hodgson et al., 2015; Mensah et al., 2016; Ogwang et al., 2015; Tiono et al., 2018). Currently, human malaria vaccine studies use a chimpanzee adenovirus (ChAd63) and a modified vaccinia virus (MVA) to

express different *P. falciparum* peptides (Ewer et al., 2016). In mice, intravenous administration of ChAd63 or MVA induces liver-tropism and hepatic expression of antigen, facilitating the formation of liver T_{RM} cells (Gola et al., 2018). Given the existing clinical data for ChAd64 administration, it will be beneficial to design a ChAd63 viral vector expressing the RPL6 epitope and use it for the prime-and-trap vaccine instead of the original adeno-associated virus.

The third and last component is a CpG adjuvant used for the licensing of DC involve in CD8 T cell priming (Lahoud et al., 2011) and to induce liver inflammation (Huang et al., 2013), known to support T_{RM} cell formation (Holz et al., 2018; Mackay et al., 2012a). CpG has been used extensively in clinical trials as an adjuvant for vaccines against several infection diseases and tumours and was recently approved as part of a licensed vaccine against HBV (Food and Drug Administration, 2020; Del Giudice et al., 2018; Scheiermann and Klinman, 2014). This indicates that CpG is well tolerated and actually improves vaccine efficacy in humans. Tolerability to different types of CpG, in particular of “CpG-combo” and administration in liposomal transfection reagents, such as DOTAP, are yet to be determined.

In summary, these clinical studies indicate that the development of the prime-and-trap components for human use is feasible in the near future. Some components could be changed, for example, instead of using anti-Clec9A to target DC, anti-DEC205 antibodies could be used, given their ability to induce potent CD8 T cell responses (Lahoud et al., 2011) and safety administration in humans (Dhodapkar et al., 2014; Griffiths et al., 2018). To induce the trapping of primed T cells to the liver, the ChAd63 viral vector could be used instead of adeno-associated virus, since the former has been tested in clinical trials and it is known to favour the formation of liver T_{RM} cells (Gola et al., 2018; Hodgson et al., 2015; Mensah et al., 2016). Despite the need to simplify and modify the actual components, the main idea behind this vaccination strategy, to achieve formation of liver T_{RM} cells, should remain.

Currently, all our experimental data for the prime-and-trap vaccine are based on intravenous administration in mice and no other application routes have been investigated (Fernandez-Ruiz et al., 2016; Valencia-Hernandez et al., 2020). Conventional vaccination uses oral and parental administration, which includes intradermal, intramuscular and subcutaneous delivery (WHO, 2020; Zhang et al., 2015). In spite of the reluctance to employ intravenous vaccines, recent work suggests they are safe and immunogenic. A recent study in macaques showed that intravenous vaccination with BCG increased the number of T cells in lung expressing T_{RM} markers and that these animals developed higher levels of protection against *Mycobacterium tuberculosis* as compared to macaques that received the BCG intradermally (Darrah et al., 2020). RAS immunisation, which protects against malaria, is also delivered through intravenous injections in humans (Seder et al., 2013). Importantly, a small phase I clinical trial revealed that intravenous injection with a viral vector expressing malaria epitopes (ChAd63-ME-TRAP) caused only moderate adverse events and induced immunogenicity measured by *ex vivo* assays (Gola et al., 2018). Altogether, these studies point to promising translational prospects and an increase feasibility of the prime-and-trap vaccine and similar strategies.

5.9 T_{RM}-based vaccines for humans

An important limitation of the approaches favouring the generation of T_{RM} cells is a lack of tools to monitor T_{RM} cell formation. The identification of bona fide human T_{RM} cells is controversial because gold standard tests (e.g. parabiosis experiments and intravascular cell labelling) cannot be performed in humans. Currently, clinical trials assess vaccine immunogenicity of T cell responses through cytokine production assays performed on circulating cells and hence, the local immune response in the tissue remains unknown. Data presented in this thesis suggests that, for most cases, the initial expansion of specific CD8 T cells measured in blood at day 7 post immunisation reflected the numbers of liver T_{RM} cells at a later time point. However, this did not hold true for all treatments e.g. for mice receiving RIG-I-L as adjuvant. Therefore, there is an urgent need for the

identification of biomarkers in blood that could effectively predict the formation of T_{RM} cells.

Efforts have been made to characterize human liver T_{RM} cells, by several means including extensive studies of protein expression and gene transcriptional profiling. These studies have identified a unique CD8 T cell subpopulation in the liver, absent in PBMC, of healthy and HBV infected humans that matched core signature features of T_{RM} cells (Pallett et al., 2017; Swadling et al., 2020). These recent studies realized there are important differences in how human and murine liver T_{RM} cells are formed and maintained. For example, while murine liver T_{RM} cells lack the expression of CD103 (Fernandez-Ruiz et al., 2016; McNamara et al., 2017), an integrin expressed by T_{RM} cells located in epithelial and neuronal tissues (Mueller and Mackay, 2016), this molecule is apparently found in the liver T_{RM} cells of healthy and HBV infected humans (Pallett et al., 2017). At the transcriptional level, whereas Blimp-1 and Hobit regulate the maintenance of murine liver T_{RM} cells (Mackay et al., 2016), it has been suggested that human T_{RM} cells express low levels of Blimp-1 and Hobit (Pallett et al., 2017). Functionally, liver T_{RM} cells have been found to express high and low levels of granzyme B in mice and humans, respectively (Fernandez-Ruiz et al., 2016; Pallett et al., 2017). There are also shared features between the human and murine liver T_{RM} cells. For instance, for both cases, liver T_{RM} cells express high levels of CD69, CXCR6, CXCR3 and require IL-15 exposure (Fernandez-Ruiz et al., 2016; Holz et al., 2018; Pallett et al., 2017). Additionally, data presented in this thesis support previous findings suggesting that the generation of large numbers of cells is boosted by hepatocyte antigen presentation, even though local antigen exposure is not required for the formation of liver T_{RM} cells in mice and humans (Fernandez-Ruiz et al., 2016; Holz et al., 2018; Pallett et al., 2017).

Differences similar to those described between human and mice T_{RM} cells in the liver (see above) have also been found for other tissues, including the lung, intestine and skin (Kumar et al., 2017; Szabo et al., 2019). This situation raises concerns about the translational potential of inbred mouse models to investigate

human T_{RM} cell responses. Therefore, there is a necessity for developing models that better recapitulate the human immunology within tissues. It has been recently proposed that outbred mice from pet stores, which have been exposed to pathogens and have an enriched microbiota compared to the “clean” lab mice, have T_{RM} cells that more closely resemble those from humans (Beura et al., 2018, 2016; Kumar et al., 2017). Extensive investigation on “dirty” mice is still required to determine their translational potential as compared to inbred mice.

In the context of vaccines for infectious diseases, several mouse studies have demonstrated that protective T_{RM} responses can be generated through different immunisation strategies. For instance, administration of live-attenuated pathogens locally or intravenously in mice generates T_{RM} cells that mediate protection against influenza (Zens et al., 2016), tuberculosis (Perdomo et al., 2016) and malaria (Fernandez-Ruiz et al., 2016; Tse et al., 2013). Intravaginal administration of viral vectors to deliver targeted antigens to the vaginal mucosa of mice successfully induces protective T_{RM} cells against HIV (Tan et al., 2018) and genital herpex virus 2 (Çuburu et al., 2014). Another particularly successful vaccination strategy for the induction of protective T_{RM} cells is known as “prime and pull”. Those vaccines and their variations incorporate a priming step, with a relevant antigen, together with a pull or target step for the recruitment of T_{RM} precursors to a specific tissue. With this approach, protective T_{RM} responses have been proven against HSV-2 challenge (Bernstein et al., 2019; Shin et al., 2016; Shin and Iwasaki, 2012) and *Plasmodium* sporozoite infection (Fernandez-Ruiz et al., 2016; Gola et al., 2018; Valencia-Hernandez et al., 2020). Altogether, these studies provide evidence of the potential of T_{RM}-based vaccines to protect against infections. Whether or not these strategies can be applied to humans will likely be determined in the coming years.

5.10 Concluding remarks

The data presented in this thesis demonstrated the highly immunogenic nature of an epitope within the RPL6 protein of PbA and its ability to evoke high

levels of sterile protection against liver stage infection in vaccinated mice with a normal endogenous T cell repertoire. Additionally, the results showed the superior capacity of CpG ODN to promote liver T_{RM} cell formation in mice vaccinated with antigen targeted immunisation. These findings provide insights into elements that favour the generation of protective liver T_{RM} cells; information that can be used for the design of T_{RM} cell-based subunit vaccines against *Plasmodium* infection. While the 'prime-and-trap' vaccine currently faces challenges, they are not insurmountable and cutting-edge scientific and technological developments are increasing the translational prospects of T_{RM}-based vaccines.

REFERENCES

Ablasser, A., Goldeck, M., Cavlar, T., Deimling, T., Witte, G., Röhl, I., Hopfner, K.-P. P., Ludwig, J., Hornung, V., & Hournung, V. (2013). CGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. *Nature*, *498*(7454), 380–384. https://doi.org/10.1038/nature12306

Adams, S., O'Neill, D. W., Nonaka, D., Hardin, E., Chiriboga, L., Siu, K., Cruz, C. M., Angiulli, A., Angiulli, F., Ritter, E., Holman, R. M., Shapiro, R. L., Berman, R. S., Berner, N., Shao, Y., Manches, O., Pan, L., Venhaus, R. R., Hoffman, E. W., … Bhardwaj, N. (2008). Immunization of Malignant Melanoma Patients with Full-Length NY-ESO-1 Protein Using TLR7 Agonist Imiquimod as Vaccine Adjuvant. *The Journal of Immunology*, *181*(1), 776–784. https://doi.org/10.4049/jimmunol.181.1.776

Adepoju, P. (2019). RTS,S malaria vaccine pilots in three African countries. *Lancet (London, England)*, *393*(10182), 1685. https://doi.org/10.1016/S0140-6736(19)30937-7

Ahrens, S., Zelenay, S., Sancho, D., Hanč, P., Kjær, S., Feest, C., Fletcher, G., Durkin, C., Postigo, A., Skehel, M., Batista, F., Thompson, B., Way, M., Reis e Sousa, C., & Schulz, O. (2012). F-Actin Is an Evolutionarily Conserved Damage-Associated Molecular Pattern Recognized by DNGR-1, a Receptor for Dead Cells. *Immunity*, *36*(4), 635–645. https://doi.org/10.1016/j.immuni.2012.03.008

Akhouri, R. R., Sharma, A., Malhotra, P., & Sharma, A. (2008). Role of Plasmodium falciparum thrombospondin-related anonymous protein in host-cell interactions. *Malaria Journal*, *7*, 1–11. https://doi.org/10.1186/1475-2875-7-63

Akira, S., & Takeda, K. (2004). Functions of toll-like receptors: Lessons from KO mice. *Comptes Rendus - Biologies*, *327*(6), 581–589. https://doi.org/10.1016/j.crvi.2004.04.002

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*, *124*(4), 783–801. https://doi.org/10.1016/j.cell.2006.02.015

Alger, N. E., & Harant, J. (1976). Plasmodium berghei: Heat-treated sporozoite vaccination of mice. *Experimental Parasitology*, *40*(2), 261–268. https://doi.org/10.1016/0014-4894(76)90089-8

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., & Ménard, R. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. *Nature Medicine*, *12*(2), 220–224. https://doi.org/10.1038/nm1350

Ammi, R., De Waele, J., Willemen, Y., Van Brussel, I., Schrijvers, D. M., Lion, E., & Smits, E. L. J. (2015). Poly(I:C) as cancer vaccine adjuvant: Knocking on the door of medical breakthroughs. *Pharmacology and Therapeutics*, *146*, 120–131. https://doi.org/10.1016/j.pharmthera.2014.09.010

Anders, R. F., Crewther, P. E., Edwards, S., Margetts, M., Matthew, M. L. S. M., Pollock, B., & Pye, D. (1998). Immunisation with recombinant AMA-1 protects mice against infection with Plasmodium chabaudi. *Vaccine*, *16*(2–3), 240–247. https://doi.org/10.1016/S0264-410X(97)88331-4

Anderson, K. G., Sung, H., Skon, C. N., Lefrancois, L., Deisinger, A., Vezys, V., & Masopust, D. (2012). Cutting Edge: Intravascular Staining Redefines Lung CD8 T Cell Responses. *The Journal of Immunology*, *189*(6), 2702–2706. https://doi.org/10.4049/jimmunol.1201682

Apostólico, J. D. S., Alves, V., Lunardelli, S., Coirada, F. C., Boscardin, S. B., & Rosa, D. S. (2016). Adjuvants : Classification , Modus Operandi , and Licensing. *Journal of Immunology Research*, *2016*.

Ariotti, S., Beltman, J. B., Chodaczek, G., Hoekstra, M. E., Van Beek, A. E., Gomez-Eerland, R., Ritsma, L., Van Rheenen, J., Marée, A. F. M., Zal, T., De Boer, R. J., Haanen, J. B. A. G., & Schumacher, T. N. (2012). Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly recognize local antigen. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(48), 19739–19744. https://doi.org/10.1073/pnas.1208927109

Awate, S., Babiuk, L. A., & Mutwiri, G. (2013). Mechanisms of action of adjuvants. *Frontiers in Immunology*, *4*(MAY), 1–10. https://doi.org/10.3389/fimmu.2013.00114

Badovinac, V. P., Porter, B. B., & Harty, J. T. (2002). Programmed contraction of cd8+ t cells after infection. *Nature Immunology*, *3*(7), 619–626. https://doi.org/10.1038/ni804

Baer, K., Klotz, C., Kappe, S. H. I., Schnieder, T., & Frevert, U. (2007). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. *PLoS Pathogens*, *3*(11), 1651–1668. https://doi.org/10.1371/journal.ppat.0030171

Baird, J. K. (1995). Host age as determinant of naturrally acquired immunity to Plasmodium falciparum. *Parasitology Today*, *11*(3), 105–111. https://doi.org/10.1055/s-2001-14479

Baird, J. K., Basri, H., Weina, P., Maguire, J. D., Barcus, M. J., Picarema, H., Elyazar, I. R. F., Ayomi, E., & Sekartuti, A. (2003). Adult Javanese migrants to Indonesian Papua at high risk of severe disease caused by malaria. *Epidemiology and Infection*, *131*(1), 791–797. https://doi.org/10.1017/S0950268803008422

Baird, J. K., Masbar, S., Basri, H., Tirtokusumo, S., Subianto, B., & Hoffman, S. L. (1998). Age-Dependent Susceptibility to Severe Disease with Primary Exposure to Plasmodium falciparum. *Journal of Infectious Diseases*, *178*(2), 592–595. https://doi.org/10.1086/517482

Baker, R. P., Wijetilaka, R., & Urban, S. (2006). Two Plasmodium rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathogens*, *2*(10), 0922–0932. https://doi.org/10.1371/journal.ppat.0020113

Balam, S., Romero, J. F., Bongfen, S. E., Guillaume, P., & Corradin, G. (2012). CSP-A Model for In Vivo Presentation of Plasmodium berghei Sporozoite Antigens by Hepatocytes. *PLoS ONE*, *7*(12), 8–15. https://doi.org/10.1371/journal.pone.0051875

Baldridge, J. R., McGowan, P., Evans, J. T., Cluff, C., Mossman, S., Johnson, D., & Persing, D. (2004). Taking a toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. *Expert Opinion on Biological Therapy*, *4*(7), 1129–1138. https://doi.org/10.1517/14712598.4.7.1129

Bartoloni, A., & Zammarchi, L. (2012). Clinical aspects of uncomplicated and severe malaria. *Mediterranean Journal of Hematology and Infectious Diseases*, *4*(1). https://doi.org/10.4084/MJHID.2012.026

Bauer, M., Heeg, K., Wagner, H., & Lipford, G. B. (1999). DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology*, *97*(4), 699–705. https://doi.org/10.1046/j.1365-2567.1999.00811.x

Bauer, S., Kirschning, C. J., Häcker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., & Lipford, G. B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(16), 9237–9242. https://doi.org/10.1073/pnas.161293498

Bayik, D., Gursel, I., & Klinman, D. M. (2016). Structure, mechanism and therapeutic utility of immunosuppressive oligonucleotides. *Pharmacological Research*, *105*, 216–225. https://doi.org/10.1016/j.phrs.2015.11.010

Beaudette, T. T., Bachelder, E. M., Cohen, J. A., Obermeyer, A. C., Broaders, K. E., Fréchet, J. M. J., Kang, E. S., Mende, I., Tseng, W. W., Davidson, M. G., & Engleman, E. G. (2009). In vivo studies on the effect of co-encapsulation of CpG DNA and antigen in acid-degradable microparticle vaccines. *Molecular Pharmaceutics*, *6*(4), 1160–1169. https://doi.org/10.1021/mp900038e

Beignon, A. S., Briand, J. P., Muller, S., & Partidos, C. D. (2002). Immunization onto bare skin with synthetic peptides: Immunomodulation with a CpG-

containing oligodeoxynucleotide and effective priming of influenza virus-specific CD4+ T cells. *Immunology*, *105*(2), 204–212. https://doi.org/10.1046/j.0019-2805.2001.01357.x

Belnoue, E., Costa, F. T. M., Frankenberg, T., Vigário, A. M., Voza, T., Leroy, N., Rodrigues, M. M., Landau, I., Snounou, G., & Rénia, L. (2004). Protective T Cell Immunity against Malaria Liver Stage after Vaccination with Live Sporozoites under Chloroquine Treatment. *The Journal of Immunology*, *172*(4), 2487–2495. https://doi.org/10.4049/jimmunol.172.4.2487

Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J.-C., Rooijen, N. van, Viguier, M., Snounou, G., & Rénia, L. (2002). On the Pathogenic Role of Brain-Sequestered αβ CD8 + T Cells in Experimental Cerebral Malaria . *The Journal of Immunology*, *169*(11), 6369–6375. https://doi.org/10.4049/jimmunol.169.11.6369

Belnoue, E., Potter, S. M., Rosa, D. S., Mauduit, M., Grüner, A. C., Kayibanda, M., Mitchell, A. J., Hunt, N. H., & Rénia, L. (2008). Control of pathogenic CD8+ T cell migration to the brain by IFN-γ during experimental cerebral malaria. *Parasite Immunology*, *30*(10), 544–553. https://doi.org/10.1111/j.1365-3024.2008.01053.x

Bendelac, A., Rivera, M. N., Park, S.-H., & Roark, J. H. (1997). MOUSE CD1-SPECIFIC NK1 T CELLS: Development, Specificity, and Function. *Annual Review of Immunology*, *15*(1), 535–562. https://doi.org/10.1146/annurev.immunol.15.1.535

Benedict, M. Q. (1997). Care and maintenance of anopheline mosquito colonies. In L. C. Crampton JM, Beard CB (Ed.), *The Molecular Biology of Insect Disease Vectors.* Chapman & Hall.

Benoun, J. M., Peres, N. G., Wang, N., Pham, O. H., Rudisill, V. L., Fogassy, Z. N., Whitney, P. G., Fernandez-Ruiz, D., Gebhardt, T., Pham, Q. M., Puddington, L., Bedoui, S., Strugnell, R. A., & McSorley, S. J. (2018). Optimal protection against Salmonella infection requires noncirculating memory. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(41), 10416–10421. https://doi.org/10.1073/pnas.1808339115

Berenzon, D., Schwenk, R. J., Letellier, L., Guebre-Xabier, M., Williams, J., & Krzych, U. (2003). Protracted Protection to Plasmodium berghei Malaria Is Linked to Functionally and Phenotypically Heterogeneous Liver Memory CD8 + T Cells . *The Journal of Immunology*, *171*(4), 2024–2034. https://doi.org/10.4049/jimmunol.171.4.2024

Bergsbaken, T., & Bevan, M. J. (2015). Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident CD8+ T cells responding to infection. *Nature Immunology*, *16*(4), 406–414. https://doi.org/10.1038/ni.3108

Berman, B., & Caperton. (2011). Safety, efficacy, and patient acceptability of imiquimod for topical treatment of actinic keratoses. *Clinical, Cosmetic and Investigational Dermatology*, 35. https://doi.org/10.2147/ccid.s14109

Bernstein, D. I., Cardin, R. D., Bravo, F. J., Awasthi, S., Lu, P., Pullum, D. A., Dixon, D. A., Iwasaki, A., & Friedman, H. M. (2019). Successful application of prime and pull strategy for a therapeutic HSV vaccine. *Npj Vaccines*, *4*(1). https://doi.org/10.1038/s41541-019-0129-1

Bertolino, P., Bowen, D. G., McCaughan, G. W., & Fazekas de St. Groth, B. (2001). Antigen-Specific Primary Activation of CD8 + T Cells Within the Liver . *The Journal of Immunology*, *166*(9), 5430–5438. https://doi.org/10.4049/jimmunol.166.9.5430

Beura, L. K., Hamilton, S. E., Bi, K., Schenkel, J. M., Odumade, O. A., Casey, K. A., Thompson, E. A., Fraser, K. A., Rosato, P. C., Filali-Mouhim, A., Sekaly, R. P., Jenkins, M. K., Vezys, V., Nicholas Haining, W., Jameson, S. C., & Masopust, D. (2016). Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature*, *532*(7600), 512–516. https://doi.org/10.1038/nature17655

Beura, L. K., Wijeyesinghe, S., Thompson, E. A., Macchietto, M. G., Rosato, P. C., Pierson, M. J., Schenkel, J. M., Mitchell, J. S., Vezys, V., Fife, B. T., Shen, S., & Masopust, D. (2018). T Cells in Nonlymphoid Tissues Give Rise to Lymph-Node-Resident Memory T Cells. *Immunity*, *48*(2), 327-338.e5. https://doi.org/10.1016/j.immuni.2018.01.015

Bigorgne, A. E., & Crispe, I. N. (2010). TLRs in hepatic cellular crosstalk. *Gastroenterology Research and Practice*, *2010*. https://doi.org/10.1155/2010/618260

Biswas, S. K., & Lopez-Collazo, E. (2009). Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends in Immunology*, *30*(10), 475–487. https://doi.org/10.1016/j.it.2009.07.009

Blaauboer, S. M., Mansouri, S., Tucker, H. R., Wang, H. L., Gabrielle, V. D., & Jin, L. (2015). The mucosal adjuvant cyclic di-GMP enhances antigen uptake and selectively activates pinocytosis-efficient cells in vivo. *ELife*, *2015*(4), 1–25. https://doi.org/10.7554/eLife.06670

Bliss, C. M., Drammeh, A., Bowyer, G., Sanou, G. S., Jagne, Y. J., Ouedraogo, O., Edwards, N. J., Tarama, C., Ouedraogo, N., Ouedraogo, M., Njie-Jobe, J., Diarra, A., Afolabi, M. O., Tiono, A. B., Yaro, J. B., Adetifa, U. J., Hodgson, S. H., Anagnostou, N. A., Roberts, R., … Ewer, K. J. (2017). Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants. *Molecular Therapy*, *25*(2), 547–559. https://doi.org/10.1016/j.ymthe.2016.11.003

Boddey, J. A., & Cowman, A. F. (2013). Plasmodium Nesting: Remaking the

Erythrocyte from the Inside Out . *Annual Review of Microbiology*, *67*(1), 243–269. https://doi.org/10.1146/annurev-micro-092412-155730

Boddey, J. A., O'Neill, M. T., Lopaticki, S., Carvalho, T. G., Hodder, A. N., Nebl, T., Wawra, S., Van West, P., Ebrahimzadeh, Z., Richard, D., Flemming, S., Spielmann, T., Przyborski, J., Babon, J. J., & Cowman, A. F. (2016). Export of malaria proteins requires co-translational processing of the PEXEL motif independent of phosphatidylinositol-3-phosphate binding. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms10470

Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., & Klinman, D. M. (2011). CpG DNA as a vaccine adjuvant (author manuscript). *Expert Reviews Vaccines*, *10*(4), 499–511. https://doi.org/10.1586/erv.10.174.CpG

Bongfen, S. E., Torgler, R., Romero, J. F., Renia, L., & Corradin, G. (2007). Plasmodium berghei -Infected Primary Hepatocytes Process and Present the Circumsporozoite Protein to Specific CD8 + T Cells In Vitro . *The Journal of Immunology*, *178*(11), 7054–7063. https://doi.org/10.4049/jimmunol.178.11.7054

Bosio, C. M., Moore, B. D., Warfield, K. L., Ruthel, G., Mohamadzadeh, M., Aman, M. J., & Bavari, S. (2004). Ebola and Marburg virus-like particles activate human myeloid dendritic cells. *Virology*, *326*(2), 280–287. https://doi.org/10.1016/j.virol.2004.05.025

Bourquin, C., Hotz, C., Noerenberg, D., Voelkl, A., Heidegger, S., Roetzer, L. C., Storch, B., Sandholzer, N., Wurzenberger, C., Anz, D., & Endres, S. (2011). Systemic cancer therapy with a small molecule agonist of toll-like receptor 7 can be improved by circumventing TLR tolerance. *Cancer Research*, *71*(15), 5123–5133. https://doi.org/10.1158/0008-5472.CAN-10-3903

Bruce-chwatt, L. J. (1963). A Longitudinal Survey of Natural Malaria Infection in a Group of West African Adults. Part II. *West African Medical Journal*, *5*, 199–217.

Buffet, P. A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G. D., & Mercereau-Puijalon, O. (2011). The pathogenesis of Plasmodium falciparum malaria in humans: Insights from splenic physiology. *Blood*, *117*(2), 381–392. https://doi.org/10.1182/blood-2010-04-202911

Buonaguro, L., Racioppi, L., Tornesello, M. L., Arra, C., Visciano, M. L., Biryahwaho, B., Sempala, S. D. K., Giraldo, G., & Buonaguro, F. M. (2002). Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. *Antiviral Research*, *54*(3), 189–201. https://doi.org/10.1016/S0166-3542(02)00004-9

Bürckstümmer, T., Baumann, C., Blüml, S., Dixit, E., Dürnberger, G., Jahn, H., Planyavsky, M., Bilban, M., Colinge, J., Bennett, K. L., & Superti-Furga, G. (2009). An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nature Immunology*, *10*(3), 266–272. https://doi.org/10.1038/ni.1702

Burdette, D. L., Monroe, K. M., Sotelo-Troha, K., Iwig, J. S., Eckert, B., Hyodo, M., Hayakawa, Y., & Vance, R. E. (2011). STING is a direct innate immune sensor of cyclic di-GMP. *Nature*, *478*(7370), 515–518. https://doi.org/10.1038/nature10429

Burdette, D. L., & Vance, R. E. (2013). STING and the innate immune response to nucleic acids in the cytosol. *Nature Immunology*, *14*(1), 19–26. https://doi.org/10.1038/ni.2491

Butler, N. S., Schmidt, N. W., Vaughan, A. M., Aly, A. S., Kappe, S. H. I., & Harty, J. T. (2011). Superior antimalarial immunity after vaccination with late liver stage-arresting genetically attenuated parasites. *Cell Host and Microbe*, *9*(6), 451–462. https://doi.org/10.1016/j.chom.2011.05.008

Buyel, J. F., Twyman, R. M., & Fischer, R. (2017). Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnology Advances*, *35*(4), 458–465. https://doi.org/10.1016/j.biotechadv.2017.03.011

Caminschi, I., Lahoud, M. H., Pizzolla, A., & Wakim, L. M. (2019). Zymosan by-passes the requirement for pulmonary antigen encounter in lung tissue-resident memory CD8 + T cell development. *Mucosal Immunology*, *12*(2), 403–412. https://doi.org/10.1038/s41385-018-0124-2

Caminschi, I., Maraskovsky, E., & Heath, W. R. (2012). Targeting dendritic cells in vivo for cancer therapy. *Frontiers in Immunology*, *3*(FEB), 1–13. https://doi.org/10.3389/fimmu.2012.00013

Caminschi, I., Meuter, S., & Heath, W. R. (2013). DEC-205 is a cell surface receptor for CpG oligonucleotides. *Oncoimmunology*, *2*(3), e23128. https://doi.org/10.4161/onci.23128

Caminschi, I., Proietto, A. I., Ahmet, F., Kitsoulis, S., Teh, J. S., Lo, J. C. Y., Rizzitelli, A., Wu, L., Vremec, D., Van Dommelen, S. L. H., Campbell, I. K., Maraskovsky, E., Braley, H., Davey, G. M., Mottram, P., Van De Velde, N., Jensen, K., Lew, A. M., Wright, M. D., … Lahoud, M. H. (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood*, *112*(8), 3264–3273. https://doi.org/10.1182/blood-2008-05-155176

Caminschi, I., Vremec, D., Ahmet, F., Lahoud, M. H., Villadangos, J. A., Murphy, K. M., Heath, W. R., & Shortman, K. (2012). Antibody responses initiated by Clec9A-bearing dendritic cells in normal and Batf3 -/- mice. *Molecular*

Immunology, *50*(1–2), 9–17. https://doi.org/10.1016/j.molimm.2011.11.008

Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T., & Quattrone, A. (1993). Cationic lipids improve antisense oligonucleitide uptake and prevent degratadion in cultured cells and in human serum. *Biochemical and Biophysical Research Communications*, *197*(2), 818–825.

Carbone, F. R. (2015). Tissue-Resident Memory T Cells and Fixed Immune Surveillance in Nonlymphoid Organs. *The Journal of Immunology*, *195*(1), 17–22. https://doi.org/10.4049/jimmunol.1500515

Carding, S. R., & Egan, P. J. (2002). γδ T cells: Functional plasticity and heterogeneity. *Nature Reviews Immunology*, *2*(5), 336–345. https://doi.org/10.1038/nri797

Carneiro, I., Roca-Feltrer, A., Griffin, J. T., Smith, L., Tanner, M., Schellenberg, J. A., Greenwood, B., & Schellenberg, D. (2010). Age-patterns of malaria vary with severity, transmission intensity and seasonality in sub-Saharan Africa: A systematic review and pooled analysis. *PLoS ONE*, *5*(2). https://doi.org/10.1371/journal.pone.0008988

Carvalho, L. H., Sano, G. I., Hafalla, J. C. R., Morrot, A., De Lafaille, M. A. C., & Zavala, F. (2002). IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nature Medicine*, *8*(2), 166–170. https://doi.org/10.1038/nm0202-166

Casella, C. R., & Mitchell, T. C. (2008). Putting endotoxin to work for us: Monophosphoryl lipid a as a safe and effective vaccine adjuvant. *Cellular and Molecular Life Sciences*, *65*(20), 3231–3240. https://doi.org/10.1007/s00018-008-8228-6

Casey, K. A., Fraser, K. A., Schenkel, J. M., Moran, A., Abt, M. C., Beura, L. K., Lucas, P. J., Artis, D., Wherry, E. J., Hogquist, K., Vezys, V., & Masopust, D. (2012). Antigen-Independent Differentiation and Maintenance of Effector-like Resident Memory T Cells in Tissues. *The Journal of Immunology*, *188*(10), 4866–4875. https://doi.org/10.4049/jimmunol.1200402

Cavaillon, J. M., & Adib-Conquy, M. (2006). Bench-to-bedside review: Endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Critical Care*, *10*(5), 1–8. https://doi.org/10.1186/cc5055

Cha, S. J., Kim, M. S., Pandey, A., & Jacobs-Lorena, M. (2016). Identification of GAP DH on the surface of Plasmodium sporozoites as a new candidate for targeting malaria liver invasion. *Journal of Experimental Medicine*, *213*(10), 2099–2112. https://doi.org/10.1084/jem.20160059

Chakravarty, S., Cockburn, I. A., Kuk, S., Overstreet, M. G., Sacci, J. B., &

Zavala, F. (2007). CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. *Nature Medicine*, *13*(9), 1035–1041. https://doi.org/10.1038/nm1628

Chandele, A., Mukerjee, P., Das, G., Ahmed, R., & Chauhan, V. S. (2011). Phenotypic and functional profiling of malaria-induced CD8 and CD4 T cells during blood-stage infection with Plasmodium yoelii. *Immunology*, *132*(2), 273–286. https://doi.org/10.1111/j.1365-2567.2010.03363.x

Chandra, D., Quispe-Tintaya, W., Jahangir, A., Asafu-Adjei, D., Ramos, I., Sintim, H. O., Zhou, J., Hayakawa, Y., Karaolis, D. K. R., & Gravekamp, C. (2014). STING Ligand c-di-GMP Improves Cancer Vaccination against Metastatic Breast Cancer. *Cancer Immunology Research*, *2*(9), 901–910. https://doi.org/10.1158/2326-6066.CIR-13-0123

Charoenpan, P., Indraprasit, S., Kiatboonsri, S., Suvachittanont, O., & Tanomsup, S. (1990). Pulmonary edema in severe falciparum malaria. Hemodynamic study and clinicophysiologic correlation. *Chest*, *97*(5), 1190–1197. https://doi.org/10.1378/chest.97.5.1190

Charoenvit, Y., Mellouk, S., Sedegah, M., Toyoshima, T., Leef, M. F., De la Vega, P., Beaudoin, R. L., Aikawa, M., Fallarme, V., & Hoffman, S. L. (1995). Plasmoium yoelli: 17-KDa Hepatic and Erythrocyte Stage protein is the target of an inhibitory monoclonal antibody. *Experimental Parasitology*, *80*, 419–429. https://doi.org/10.1006/expr.1995.1054

Charpian, S., & Przyborski, J. M. (2008). Protein transport across the parasitophorous vacuole of Plasmodium falciparum: Into the great wide open. *Traffic*, *9*(2), 157–165. https://doi.org/10.1111/j.1600-0854.2007.00648.x

Chesnoy, S., & Huang, L. (2000). Structure and function of lipid-DNA complexed for gene delivery. *Annual Review of Biophysics and Biomolecular Structure*, *29*(1), 27–47. http://www.annualreviews.org/doi/full/10.1146/annurev.biochem.69.1.651

Chiang, C., Beljanski, V., Yin, K., Olagnier, D., Ben Yebdri, F., Steel, C., Goulet, M.-L., DeFilippis, V. R., Streblow, D. N., Haddad, E. K., Trautmann, L., Ross, T., Lin, R., & Hiscott, J. (2015). Sequence-Specific Modifications Enhance the Broad-Spectrum Antiviral Response Activated by RIG-I Agonists. *Journal of Virology*, *89*(15), 8011–8025. https://doi.org/10.1128/jvi.00845-15

Chikh, G., De Jong, S. D., Sekirov, L., Raney, S. G., Kazem, M., Wilson, K. D., Cullis, P. R., Dutz, J. P., & Tam, Y. K. (2009). Synthetic methylated CpG ODNs are potent in vivo adjuvants when delivered in liposomal nanoparticles. *International Immunology*, *21*(7), 757–767. https://doi.org/10.1093/intimm/dxp044

Choi, J. P., Kim, Y. S., Kim, O. Y., Kim, Y. M., Jeon, S. G., Roh, T. Y., Park, J. S., Gho, Y. S., & Kim, Y. K. (2012). TNF-alpha is a key mediator in the development of Th2 cell response to inhaled allergens induced by a viral PAMP double-stranded RNA. *Allergy: European Journal of Allergy and Clinical Immunology*, *67*(9), 1138–1148. https://doi.org/10.1111/j.1398-9995.2012.02871.x

Chuang, I., Sedegah, M., Cicatelli, S., Spring, M., Polhemus, M., Tamminga, C., Patterson, N., Guerrero, M., Bennett, J. W., McGrath, S., Ganeshan, H., Belmonte, M., Farooq, F., Abot, E., Banania, J. G., Huang, J., Newcomer, R., Rein, L., Litilit, D., … Richie, T. L. (2013). DNA Prime/Adenovirus Boost Malaria Vaccine Encoding P. falciparum CSP and AMA1 Induces Sterile Protection Associated with Cell-Mediated Immunity. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0055571

Cluff, C. W. (2013). *Monophosphoryl Lipid A (MPL) as an Adjuvant for Anti-Cancer Vaccines: Clinical Results*. Madame Curie Bioscience Database [Internet].

Clyde, D. F. (1990). Immunity to falciparum and vivax malaria induced by irradiated sporozoites: A review of the University of Maryland studies, 1971-75. *Bulletin of the World Health Organization*, *68*(SUPPL.), 9–12.

Coban, C., Ishii, K. J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S., Yamamoto, M., Takeuchi, O., Itagaki, S., Kumar, N., Horii, T., & Akira, S. (2005). Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *Journal of Experimental Medicine*, *201*(1), 19–25. https://doi.org/10.1084/jem.20041836

Cockburn, I. A., Amino, R., Kelemen, R. K., Kuo, S. C., Tse, S. W., Radtke, A., Mac-Daniel, L., Ganusov, V. V., Zavala, F., & Ménard, R. (2013). In vivo imaging of CD8+ T cell-mediated elimination of malaria liver stages. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(22), 9090–9095. https://doi.org/10.1073/pnas.1303858110

Cockburn, I. A., Tse, S. W., Radtke, A. J., Srinivasan, P., Chen, Y. C., Sinnis, P., & Zavala, F. (2011). Dendritic cells and hepatocytes use distinct pathways to process protective antigen from Plasmodium in vivo. *PLoS Pathogens*, *7*(3), 2–11. https://doi.org/10.1371/journal.ppat.1001318

Cockburn, I. A., Tse, S. W., & Zavala, F. (2014). CD8+ T cells eliminate liver-stage Plasmodium berghei parasites without detectable bystander effect. *Infection and Immunity*, *82*(4), 1460–1464. https://doi.org/10.1128/IAI.01500-13

Coelho, C. H., Doritchamou, J. Y. A., Zaidi, I., & Duffy, P. E. (2017). Advances in malaria vaccine development: Report from the 2017 malaria vaccine symposium. *Npj Vaccines*, *2*(1). https://doi.org/10.1038/s41541-017-0035-3

Coffman, R. L., Sher, A., & Seder, R. A. (2010). Vaccine adjuvants: Putting innate immunity to work. *Immunity*, *33*(4), 492–503. https://doi.org/10.1016/j.immuni.2010.10.002

Cooper, C. L., Davis, H. L., Morris, M. L., Efler, S. M., Al Adhami, M., Krieg, A. M., Cameron, D. W., & Heathcote, J. (2004). CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B® HBV vaccine in healthy adults: A double-blind phase I/II study. *Journal of Clinical Immunology*, *24*(6), 693–701. https://doi.org/10.1007/s10875-004-6244-3

Coppi, A., Natarajan, R., Pradel, G., Bennett, B. L., James, E. R., Roggero, M. A., Corradin, G., Persson, C., Tewari, R., & Sinnis, P. (2011). The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *Journal of Experimental Medicine*, *208*(2), 341–356. https://doi.org/10.1084/jem.20101488

Coppi, A., Tewari, R., Bishop, J. R., Bennett, B. L., Lawrence, R., Esko, J. D., Billker, O., & Sinnis, P. (2007). Heparan Sulfate Proteoglycans Provide a Signal to Plasmodium Sporozoites to Stop Migrating and Productively Invade Host Cells. *Cell Host and Microbe*, *2*(5), 316–327. https://doi.org/10.1016/j.chom.2007.10.002

Corbett, C. E., Duarte, M., Lancellotti, C., Silva, M. A., & Andrade Júnior, H. F. (1989). Cytoadherence in human falciparum malaria as a cause of respiratory distress. *Journal of Tropical Medicine and Hygiene*, *92*(2), 112–120.

Coronado, L. M., Nadovich, C. T., & Spadafora, C. (2014). Malarial Hemozoin: From target to tool. *Biochim Biophys Acta*, *1840*(6), 2032–2041. https://doi.org/10.1038/jid.2014.371

Corrales, L., Glickman, L. H., McWhirter, S. M., Kanne, D. B., Sivick, K. E., Katibah, G. E., Woo, S. R., Lemmens, E., Banda, T., Leong, J. J., Metchette, K., Dubensky, T. W., & Gajewski, T. F. (2015). Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Reports*, *11*(7), 1018–1030. https://doi.org/10.1016/j.celrep.2015.04.031

Cowman, A. F., Healer, J., Marapana, D., & Marsh, K. (2016). Malaria: Biology and Disease. *Cell*, *167*(3), 610–624. https://doi.org/10.1016/j.cell.2016.07.055

Çuburu, N., Wang, K., Goodman, K. N., Pang, Y. Y., Thompson, C. D., Lowy, D. R., Cohen, J. I., & Schiller, J. T. (2014). Topical Herpes Simplex Virus 2 (HSV-2) Vaccination with Human Papillomavirus Vectors Expressing gB/gD Ectodomains Induces Genital-Tissue-Resident Memory CD8 + T Cells and Reduces Genital Disease and Viral Shedding after HSV-2

Challenge. *Journal of Virology*, *89*(1), 83–96. https://doi.org/10.1128/jvi.02380-14

Cui, W., Rohrs, H. W., & Gross, M. L. (2011). Top-down mass spectrometry: Recent developments, applications and perspectives. *Analyst*, *136*(19), 3854–3864. https://doi.org/10.1039/c1an15286f

Currie, A. J., van der Most, R. G., Broomfield, S. A., Prosser, A. C., Tovey, M. G., & Robinson, B. W. S. (2008). Targeting the Effector Site with IFN-αβ-Inducing TLR Ligands Reactivates Tumor-Resident CD8 T Cell Responses to Eradicate Established Solid Tumors. *The Journal of Immunology*, *180*(3), 1535–1544. https://doi.org/10.4049/jimmunol.180.3.1535

Da Silva, D. M., Fausch, S. C., Verbeek, J. S., & Kast, W. M. (2007). Uptake of Human Papillomavirus Virus-Like Particles by Dendritic Cells Is Mediated by Fcγ Receptors and Contributes to Acquisition of T Cell Immunity. *The Journal of Immunology*, *178*(12), 7587–7597. https://doi.org/10.4049/jimmunol.178.12.7587

Dalba, C., Bellier, B., Kasahara, N., & Klatzmann, D. (2007). Replication-competent vectors and empty virus-like particles: New retroviral vector designs for cancer gene therapy or vaccines. *Molecular Therapy*, *15*(3), 457–466. https://doi.org/10.1038/sj.mt.6300054

Danhier, F., Ansorena, E., Silva, J. M., Coco, R., Le Breton, A., & Préat, V. (2012). PLGA-based nanoparticles: An overview of biomedical applications. *Journal of Controlled Release*, *161*(2), 505–522. https://doi.org/10.1016/j.jconrel.2012.01.043

Danilchanka, O., & Mekalanos, J. J. (2013). Cyclic dinucleotides and the innate immune response. *Cell*, *154*(5), 962–970. https://doi.org/10.1016/j.cell.2013.08.014

Dapito, D. H., Mencin, A., Gwak, G. Y., Pradere, J. P., Jang, M. K., Mederacke, I., Caviglia, J. M., Khiabanian, H., Adeyemi, A., Bataller, R., Lefkowitch, J. H., Bower, M., Friedman, R., Sartor, R. B., Rabadan, R., & Schwabe, R. F. (2012). Promotion of Hepatocellular Carcinoma by the Intestinal Microbiota and TLR4. *Cancer Cell*, *21*(4), 504–516. https://doi.org/10.1016/j.ccr.2012.02.007

Darrah, P. A., Zeppa, J. J., Maiello, P., Hackney, J. A., Wadsworth, M. H., Hughes, T. K., Pokkali, S., Swanson, P. A., Grant, N. L., Rodgers, M. A., Kamath, M., Causgrove, C. M., Laddy, D. J., Bonavia, A., Casimiro, D., Lin, P. L., Klein, E., White, A. G., Scanga, C. A., … Seder, R. A. (2020). Prevention of tuberculosis in macaques after intravenous BCG immunization. *Nature*, *577*(7788), 95–102. https://doi.org/10.1038/s41586-019-1817-8

Davey, G. M., Wojtasiak, M., Proietto, A. I., Carbone, F. R., Heath, W. R., & Bedoui, S. (2010). Cutting Edge: Priming of CD8 T Cell Immunity to Herpes Simplex Virus Type 1 Requires Cognate TLR3 Expression In Vivo . *The Journal of Immunology*, *184*(5), 2243–2246. https://doi.org/10.4049/jimmunol.0903013

Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., & Krieg, A. M. (1998). Immunized with Recombinant Hepatitis B Surface Antigen 1. *Journal of Immunology*, *160*, 870–876.

De Creus, A., Abe, M., Lau, A. H., Hackstein, H., Raimondi, G., & Thomson, A. W. (2005). Low TLR4 Expression by Liver Dendritic Cells Correlates with Reduced Capacity to Activate Allogeneic T Cells in Response to Endotoxin. *The Journal of Immunology*, *174*(4), 2037–2045. https://doi.org/10.4049/jimmunol.174.4.2037

de Jong, S. D., Basha, G., Wilson, K. D., Kazem, M., Cullis, P., Jefferies, W., & Tam, Y. (2010). The Immunostimulatory Activity of Unmethylated and Methylated CpG Oligodeoxynucleotide Is Dependent on Their Ability To Colocalize with TLR9 in Late Endosomes. *The Journal of Immunology*, *184*(11), 6092–6102. https://doi.org/10.4049/jimmunol.0802442

De, S. L., Stanisic, D. I., van Breda, K., Bellete, B., Harris, I., McCallum, F., Edstein, M. D., & Good, M. F. (2016). Persistence and immunogenicity of chemically attenuated blood stage Plasmodium falciparum in Aotus monkeys. *International Journal for Parasitology*, *46*(9), 581–591. https://doi.org/10.1016/j.ijpara.2016.05.002

Del Giudice, G., Rappuoli, R., & Didierlaurent, A. M. (2018). Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. *Seminars in Immunology*, *39*(May), 14–21. https://doi.org/10.1016/j.smim.2018.05.001

Demento, S. L., Bonafé, N., Cui, W., Kaech, S. M., Caplan, M. J., Fikrig, E., Ledizet, M., & Fahmy, T. M. (2010). TLR9-Targeted Biodegradable Nanoparticles as Immunization Vectors Protect against West Nile Encephalitis. *The Journal of Immunology*, *185*(5), 2989–2997. https://doi.org/10.4049/jimmunol.1000768

Desch, A. N., Gibbings, S. L., Clambey, E. T., Janssen, W. J., Slansky, J. E., Kedl, R. M., Henson, P. M., & Jakubzick, C. (2014). Dendritic cell subsets require cis-activation for cytotoxic CD8 T-cell induction. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms5674

Desch, A. N., Randolph, G. J., Murphy, K., Gautier, E. L., Kedl, R. M., Lahoud, M. H., Caminschi, I., Shortman, K., Henson, P. M., & Jakubzick, C. V. (2011). CD103 + pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *Journal of Experimental Medicine*, *208*(9), 1789–1797. https://doi.org/10.1084/jem.20110538

Dhodapkar, M. V., Sznol, M., Zhao, B., Wang, D., Carvajal, R. D., Keohan, M. L., Chuang, E., Sanborn, R. E., Lutzky, J., Powderly, J., Kluger, H., Tejwani, S., Green, J., Ramakrishna, V., Crocker, A., Vitale, L., Yellin, M., Davis, T., & Keler, T. (2014). Induction of antigen-specific immunity with a vaccine targeting NY-ESO-1 to the dendritic cell receptor DEC-205. *Science Translational Medicine*, *6*(232). https://doi.org/10.1126/scitranslmed.3008068

Di Pasquale, A., Preiss, S., Da Silva, F. T., & Garçon, N. (2015). Vaccine adjuvants: From 1920 to 2015 and beyond. *Vaccines*, *3*(2), 320–343. https://doi.org/10.3390/vaccines3020320

Didierlaurent, A. M., Laupèze, B., Di Pasquale, A., Hergli, N., Collignon, C., & Garçon, N. (2017). Adjuvant system AS01: helping to overcome the challenges of modern vaccines. *Expert Review of Vaccines*, *16*(1), 55–63. https://doi.org/10.1080/14760584.2016.1213632

Didierlaurent, A. M., Morel, S., Lockman, L., Giannini, S. L., Bisteau, M., Carlsen, H., Kielland, A., Vosters, O., Vanderheyde, N., Schiavetti, F., Larocque, D., Van Mechelen, M., & Garçon, N. (2009). AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity. *The Journal of Immunology*, *183*(10), 6186–6197. https://doi.org/10.4049/jimmunol.0901474

Díez-Domingo, J., Baldó, J. M., Planelles-Catarino, M. V., Garcés-Sánchez, M., Ubeda, I., Jubert-Rosich, A., Marès, J., Garcia-Corbeira, P., Moris, P., Teko, M., Vanden Abeele, C., & Gillard, P. (2015). Phase II, randomized, open, controlled study of AS03-adjuvanted H5N1 pre-pandemic influenza vaccine in children aged 3 to 9 years: Follow-up of safety and immunogenicity persistence at 24 months post-vaccination. *Influenza and Other Respiratory Viruses*, *9*(2), 68–77. https://doi.org/10.1111/irv.12295

Diner, E. J., Burdette, D. L., Wilson, S. C., Monroe, K. M., Kellenberger, C. A., Hyodo, M., Hayakawa, Y., Hammond, M. C., & Vance, R. E. (2013). The Innate Immune DNA Sensor cGAS Produces a Noncanonical Cyclic Dinucleotide that Activates Human STING. *Cell Reports*, *3*(5), 1355–1361. https://doi.org/10.1016/j.celrep.2013.05.009

Dodoo, D., Staalsoe, T., Giha, H., Kurtzhals, J. A. L., Akanmori, B. D., Koram, K., Dunyo, S., Nkrumah, F. K., Hviid, L., & Theander, T. G. (2001). Antibodies to variant antigens on the surfaces of infected erythrocytes are associated with protection from malaria in Ghanaian children. *Infection and Immunity*, *69*(6), 3713–3718. https://doi.org/10.1128/IAI.69.6.3713-3718.2001

Doll, K. L., Pewe, L. L., Kurup, S. P., & Harty, J. T. (2016). Cell Responses T +-Specific CD8 Plasmodium Nonprotective Discriminating Protective from. *J Immunol References*, *196*, 4253–4262. https://doi.org/10.4049/jimmunol.1600155

Dondorp, A. M., Lee, S. J., Faiz, M. A., Mishra, S., Price, R., Tjitra, E., Than, M., Htut, Y., Mohanty, S., Yunus, E. B., Rahman, R., Nosten, F., Anstey, N. M., Day, N. P. J., & White, N. J. (2008). The Relationship between Age and the Manifestations of and Mortality Associated with Severe Malaria. *Clinical Infectious Diseases*, *47*(2), 151–157. https://doi.org/10.1086/589287

Doolan, D. L., Dobaño, C., & Baird, J. K. (2009). Acquired immunity to Malaria. *Clinical Microbiology Reviews*, *22*(1), 13–36. https://doi.org/10.1128/CMR.00025-08

Doolan, D. L., Hedstrom, R. C., Rogers, W. O., Charoenvit, Y., Rogers, M., De La Vegar, P., & Hoffman, S. L. (1996). Identification and characterization of the protective hepatocyte erythrocyte protein 17 dDa gene of Plasmodium yoelii, homolog of Plasmodium falciparum exported protein 1. *Journal of Biological Chemistry*, *271*(30), 17861–17868. https://doi.org/10.1074/jbc.271.30.17861

Doolan, D. L., & Hoffman, S. L. (2000). The Complexity of Protective Immunity Against Liver-Stage Malaria. *The Journal of Immunology*, *165*(3), 1453–1462. https://doi.org/10.4049/jimmunol.165.3.1453

Dörmer, P., Dietrich, M., Kern, P., & Horstmann, R. D. (1983). Ineffective erythropoiesis in acute human P. falciparum malaria. *Blut*, *46*(5), 279–288. https://doi.org/10.1007/BF00319868

Dorovini-Zis, K., Schmidt, K., Huynh, H., Fu, W., Whitten, R. O., Milner, D., Kamiza, S., Molyneux, M., & Taylor, T. E. (2011). The neuropathology of fatal cerebral malaria in Malawian children. *American Journal of Pathology*, *178*(5), 2146–2158. https://doi.org/10.1016/j.ajpath.2011.01.016

Douglas, A. D., Baldeviano, G. C., Lucas, C. M., Lugo-Roman, L. A., Crosnier, C., Bartholdson, S. J., Diouf, A., Miura, K., Lambert, L. E., Ventocilla, J. A., Leiva, K. P., Milne, K. H., Illingworth, J. J., Spencer, A. J., Hjerrild, K. A., Alanine, D. G. W., Turner, A. V., Moorhead, J. T., Edgel, K. A., … Draper, S. J. (2015). A PfRH5-based vaccine is efficacious against heterologous strain blood-stage plasmodium falciparum infection in Aotus monkeys. *Cell Host and Microbe*, *17*(1), 130–139. https://doi.org/10.1016/j.chom.2014.11.017

Douglas, A. D., Williams, A. R., Illingworth, J. J., Kamuyu, G., Biswas, S., Goodman, A. L., Wyllie, D. H., Crosnier, C., Miura, K., Wright, G. J., Long, C. A., Osier, F. H., Marsh, K., Turner, A. V., Hill, A. V. S., & Draper, S. J. (2011). The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nature Communications*, *2*(1). https://doi.org/10.1038/ncomms1615

Draper, S. J., Sack, B. K., King, C. R., Nielsen, C. M., Rayner, J. C., Higgins, M.

K., Long, C. A., & Seder, R. A. (2018). Malaria Vaccines: Recent Advances and New Horizons. *Cell Host and Microbe*, *24*(1), 43–56. https://doi.org/10.1016/j.chom.2018.06.008

Drobits, B., Holcmann, M., Amberg, N., Swiecki, M., Grundtner, R., Hammer, M., Colonna, M., & Sibilia, M. (2012). Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *The Journal of Clinical Investigation*, *122*(2), 575–585. https://doi.org/10.1172/JCI61034DS1

Ebensen, T., Schulze, K., Riese, P., Morr, M., & Guzmán, C. A. (2007). The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. *Clinical and Vaccine Immunology*, *14*(8), 952–958. https://doi.org/10.1128/CVI.00119-07

Edwards, A. D., Diebold, S. S., Slack, E. M. C., Tomizawa, H., Hemmi, H., Kaisho, T., Akira, S., & Reis e Sousa, C. (2003). Toll-like receptor expression in murine DC subsets: Lack of TLR7 expresion of CD8α+ DC correlates with unresponsiveness to imidazoquinolines. *European Journal of Immunology*, *33*(4), 827–833. https://doi.org/10.1002/eji.200323797

Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S., & Flavell, R. A. (2008). Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*, *453*(7198), 1122–1126. https://doi.org/10.1038/nature06939

Ejigiri, I., Ragheb, D. R. T., Pino, P., Coppi, A., Bennett, B. L., Soldati-Favre, D., & Sinnis, P. (2012). Shedding of TRAP by a rhomboid protease from the malaria sporozoite surface is essential for gliding motility and sporozoite infectivity. *PLoS Pathogens*, *8*(7), 7. https://doi.org/10.1371/journal.ppat.1002725

Epiphanio, S., Mikolajczak, S. A., Gonçalves, L. A., Pamplona, A., Portugal, S., Albuquerque, S., Goldberg, M., Rebelo, S., Anderson, D. G., Akinc, A., Vornlocher, H. P., Kappe, S. H. I., Soares, M. P., & Mota, M. M. (2008). Heme Oxygenase-1 Is an Anti-Inflammatory Host Factor that Promotes Murine Plasmodium Liver Infection. *Cell Host and Microbe*, *3*(5), 331–338. https://doi.org/10.1016/j.chom.2008.04.003

Epstein, J. E., Tewari, K., Lyke, K. E., Sim, B. K. L., Billingsley, P. F., Laurens, M. B., Gunasekera, A., Chakravarty, S., James, E. R., Sedegah, M., Richman, A., Velmurugan, S., Reyes, S., Li, M., Tucker, K., Ahumada, A., Ruben, A. J., Li, T., Stafford, R., … Hoffman, S. L. (2011). Live attenuated malaria vaccine designed to protect through hepatic CD8 + T cell immunity. *Science*, *334*(6055), 475–480. https://doi.org/10.1126/science.1211548

Epstein, Judith E., Paolino, K. M., Richie, T. L., Sedegah, M., Singer, A., Ruben, A. J., Chakravarty, S., Stafford, A., Ruck, R. C., Eappen, A. G., Li, T.,

Billingsley, P. F., Manoj, A., Silva, J. C., Moser, K., Nielsen, R., Tosh, D., Cicatelli, S., Ganeshan, H., … Hoffman, S. L. (2017). Protection against Plasmodium falciparum malaria by PfSPZ Vaccine. *JCI Insight*, *2*(1), 1–14. https://doi.org/10.1172/jci.insight.89154

Ewer, K. J., Lambe, T., Rollier, C. S., Spencer, A. J., Hill, A. V. S., & Dorrell, L. (2016). Viral vectors as vaccine platforms: From immunogenicity to impact. *Current Opinion in Immunology*, *41*(Figure 1), 47–54. https://doi.org/10.1016/j.coi.2016.05.014

Ewer, K. J., O'Hara, G. A., Duncan, C. J. A., Collins, K. A., Sheehy, S. H., Reyes-Sandoval, A., Goodman, A. L., Edwards, N. J., Elias, S. C., Halstead, F. D., Longley, R. J., Rowland, R., Poulton, I. D., Draper, S. J., Blagborough, A. M., Berrie, E., Moyle, S., Williams, N., Siani, L., … Hill, A. V. S. (2013). Protective CD8 + T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. *Nature Communications*, *4*, 4–13. https://doi.org/10.1038/ncomms3836

Ewer, K. J., Sierra-Davidson, K., Salman, A. M., Illingworth, J. J., Draper, S. J., Biswas, S., & Hill, A. V. S. (2015). Progress with viral vectored malaria vaccines: A multi-stage approach involving "unnatural immunity." *Vaccine*, *33*(52), 7444–7451. https://doi.org/10.1016/j.vaccine.2015.09.094

Faure-Dupuy, S., Vegna, S., Aillot, L., Dimier, L., Esser, K., Broxtermann, M., Bonnin, M., Bendriss-Vermare, N., Rivoire, M., Passot, G., Lesurtel, M., Mabrut, J. Y., Ducerf, C., Salvetti, A., Protzer, U., Zoulim, F., Durantel, D., & Lucifora, J. (2018). Characterization of Pattern Recognition Receptor Expression and Functionality in Liver Primary Cells and Derived Cell Lines. *Journal of Innate Immunity*, *10*(4), 339–348. https://doi.org/10.1159/000489966

FDA. (2018). *Common Ingredients in U.S. Licensed Vaccines*. U.S. Food and Drug Administration.

Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., & Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, *458*(7237), 509–513. https://doi.org/10.1038/nature07710

Fernandes-Alnemri, T., Yu, J. W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., Eisenlohr, L., Landel, C. P., & Alnemri, E. S. (2010). The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nature Immunology*, *11*(5), 385–393. https://doi.org/10.1038/ni.1859

Fernandez-Ruiz, D., Lau, L. S., Ghazanfari, N., Jones, C. M., Ng, W. Y., Davey, G. M., Berthold, D., Holz, L., Kato, Y., Enders, M. H., Bayarsaikhan, G., Hendriks, S. H., Lansink, L. I. M., Engel, J. A., Soon, M. S. F., James, K. R., Cozijnsen, A., Mollard, V., Uboldi, A. D., … Heath, W. R. (2017). Development of a Novel CD4 + TCR Transgenic Line That Reveals a

Dominant Role for CD8 + Dendritic Cells and CD40 Signaling in the Generation of Helper and CTL Responses to Blood-Stage Malaria . *The Journal of Immunology*, *199*(12), 4165–4179. https://doi.org/10.4049/jimmunol.1700186

Fernandez-Ruiz, D., Ng, W. Y., Holz, L. E., Ma, J. Z., Zaid, A., Wong, Y. C., Lau, L. S., Mollard, V., Cozijnsen, A., Collins, N., Li, J., Davey, G. M., Kato, Y., Devi, S., Skandari, R., Pauley, M., Manton, J. H., Godfrey, D. I., Braun, A., … Heath, W. R. (2016). Liver-Resident Memory CD8+ T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity*, *45*(4), 889–902. https://doi.org/10.1016/j.immuni.2016.08.011

Ferreira, A., Schofield, L., Enea, V., Schellekens, H., van der Meide, P., Collins, W. E., Nussenzweig, R. S., & Nussenzweig, V. (1986). Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. *Science*, *232*(4752), 881–884. https://doi.org/10.1126/science.3085218

Ferreira, M. U., Da Silva Nunes, M., & Wunderlich, G. (2004). Antigenic diversity and immune evasion by malaria parasites. *Clinical and Diagnostic Laboratory Immunology*, *11*(6), 987–995. https://doi.org/10.1128/CDLI.11.6.987-995.2004

Food and Drug Administration. (2020). *Heplisav-B*.

Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., Van Der Keur, M., Van Der Linden, R., Sinden, R. E., Waters, A. P., & Janse, C. J. (2004). A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Molecular and Biochemical Parasitology*, *137*(1), 23–33. https://doi.org/10.1016/j.molbiopara.2004.04.007

Freudenberg, M. A., & Galanos, C. (1988). Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. *Infection and Immunity*, *56*(5), 1352–1357.

Frink, M., Hsieh, Y. C., Thobe, B. M., Choudhry, M. A., Schwacha, M. G., Bland, K. I., & Chaudry, I. H. (2007). TLR4 regulates Kupffer cell chemokine production, systemic inflammation and lung neutrophil infiltration following trauma-hemorrhage. *Molecular Immunology*, *44*(10), 2625–2630. https://doi.org/10.1016/j.molimm.2006.12.009

Gao, P., Ascano, M., Wu, Y., Barchet, W., Gaffney, B. L., Zillinger, T., Serganov, A. A., Liu, Y., Jones, R. A., Hartmann, G., Tuschl, T., & Patel, D. J. (2013). Cyclic [G(2′,5′)pA(3′,5′)p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell*, *153*(5), 1094–1107. https://doi.org/10.1016/j.cell.2013.04.046

Garçon, N., Vaughn, D. W., & Didierlaurent, A. M. (2012). Development and evaluation of AS03, an Adjuvant System containing α-tocopherol and squalene in an oil-in-water emulsion. *Expert Review of Vaccines*, *11*(3), 349–366. https://doi.org/10.1586/erv.11.192

Gebhardt, T., Wakim, L. M., Eidsmo, L., Reading, P. C., Heath, W. R., & Carbone, F. R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nature Immunology*, *10*(5), 524–530. https://doi.org/10.1038/ni.1718

Geissmann, F., Cameron, T. O., Sidobre, S., Manlongat, N., Kronenberg, M., Briskin, M. J., Dustin, M. L., & Littman, D. R. (2005). Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biology*, *3*(4), 0650–0661. https://doi.org/10.1371/journal.pbio.0030113

Gething, P. W., Patil, A. P., Smith, D. L., Guerra, C. A., Elyazar, I. R. F., Johnston, G. L., Tatem, A. J., & Hay, S. I. (2011). A new world malaria map: Plasmodium falciparum endemicity in 2010. *Malaria Journal*, *10*, 1–16. https://doi.org/10.1186/1475-2875-10-378

Ghazanfari, N., Mueller, S. N., & Heath, W. R. (2018). Cerebral malaria in mouse and man. *Frontiers in Immunology*, *9*(SEP), 1–11. https://doi.org/10.3389/fimmu.2018.02016

Giannini, S. L., Hanon, E., Moris, P., Van Mechelen, M., Morel, S., Dessy, F., Fourneau, M. A., Colau, B., Suzich, J., Losonksy, G., Martin, M. T., Dubin, G., & Wettendorff, M. A. (2006). Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine*, *24*(33–34), 5937–5949. https://doi.org/10.1016/j.vaccine.2006.06.005

Gil, M. P., Bohn, E., O'Guin, A. K., Ramana, C. V., Levine, B., Stark, G. R., Virgin, H. W., & Schreiber, R. D. (2001). Biologic consequences of Stat1-independent IFN signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(12), 6680–6685. https://doi.org/10.1073/pnas.111163898

Gilbert, S. C., Schneider, J., Hannan, C. M., Hu, J. T., Plebanski, M., Sinden, R., & Hill, A. V. S. (2002). Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. *Vaccine*, *20*(7–8), 1039–1045. https://doi.org/10.1016/S0264-410X(01)00450-9

Gilbert, S. C., Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J., Harley, C. B., & Morin, G. B. (1997). A protein particle vaccine containing multiple malaria epitopes. *Nature Biotechnology*, *15*(November), 498–502.

Giovannini, D., Späth, S., Lacroix, C., Perazzi, A., Bargieri, D., Lagal, V., Lebugle, C., Combe, A., Thiberge, S., Baldacci, P., Tardieux, I., & Ménard, R. (2011). Independent roles of apical membrane antigen 1 and rhoptry neck proteins during host cell invasion by apicomplexa. *Cell Host and Microbe*, *10*(6), 591–602. https://doi.org/10.1016/j.chom.2011.10.012

GlaxoSmithKline. (2012). *Engerix-B®. Prescribing information* (pp. 1–16).

GlaxoSmithKline. (2015). *CERVARIX® PRODUCT INFORMATION* (Vol. 18, pp. 1–25).

Glennie, N. D., Yeramilli, V. A., Beiting, D. P., Volk, S. W., Weaver, C. T., & Scott, P. (2015). Skin-resident memory CD4+ T cells enhance protection against Leishmania major infection. *Journal of Experimental Medicine*, *212*(9), 1405–1414. https://doi.org/10.1084/jem.20142101

Gola, A., Silman, D., Walters, A. A., Sridhar, S., Uderhardt, S., Salman, A. M., Halbroth, B. R., Bellamy, D., Bowyer, G., Powlson, J., Baker, M., Venkatraman, N., Poulton, I., Berrie, E., Roberts, R., Lawrie, A. M., Angus, B., Khan, S. M., Janse, C. J., … Hill, A. V. S. (2018). Prime and target immunization protects against liver-stage malaria in mice. *Science Translational Medicine*, *10*(460), 1–12. https://doi.org/10.1126/scitranslmed.aap9128

Golden-Mason, L., Palmer, B., Klarquist, J., Mengshol, J. A., Castelblanco, N., & Rosen, H. R. (2007). Upregulation of PD-1 Expression on Circulating and Intrahepatic Hepatitis C Virus-Specific CD8+ T Cells Associated with Reversible Immune Dysfunction. *Journal of Virology*, *81*(17), 9249–9258. https://doi.org/10.1128/jvi.00409-07

González-Amaro, R., Cortés, J. R., Sánchez-Madrid, F., & Martín, P. (2013). Is CD69 an effective brake to control inflammatory diseases? *Trends in Molecular Medicine*, *19*(10), 625–632. https://doi.org/10.1016/j.molmed.2013.07.006

Gonzalez-Aseguinolaza, G., De Oliveira, C., Tomaska, M., Hong, S., Bruna-Romero, O., Nakayama, T., Taniguchi, M., Bendelac, A., Van Kaer, L., Koezuka, Y., & Tsuji, M. (2000). α-Galactosylceramide-activated Vα14 natural killer T cells mediate protection against murine malaria. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(15), 8461–8466. https://doi.org/10.1073/pnas.97.15.8461

Gonzalez-Aseguinolaza, G., Van Kaer, L., Bergmann, C. C., Wilson, J. M., Schmieg, J., Kronenberg, M., Nakayama, T., Taniguchi, M., Koezuka, Y., & Tsuji, M. (2002). Natural killer T cell ligand α-galactosylceramide enhances protective immunity induced by malaria vaccines. *Journal of Experimental Medicine*, *195*(5), 617–624. https://doi.org/10.1084/jem.20011889

Good, M. F., Reiman, J. M., Rodriguez, I. B., Ito, K., Yanow, S. K., El-Deeb, I. M., Batzloff, M. R., Stanisic, D. I., Engwerda, C., Spithill, T., Hoffman, S. L., Lee, M., & McPhun, V. (2013). Cross-species malaria immunity induced by chemically attenuated parasites. *Journal of Clinical Investigation*, *123*(8), 3353–3362. https://doi.org/10.1172/JCI66634

Goubau, D., Schlee, M., Deddouche, S., Pruijssers, A. J., Zillinger, T., Goldeck, M., Schuberth, C., Van Der Veen, A. G., Fujimura, T., Rehwinkel, J., Iskarpatyoti, J. A., Barchet, W., Ludwig, J., Dermody, T. S., Hartmann, G., & Reis E Sousa, C. (2014). Antiviral immunity via RIG-I-mediated recognition of RNA bearing 59-diphosphates. *Nature*, *514*(7522), 372–375. https://doi.org/10.1038/nature13590

Goulet, M. L., Olagnier, D., Xu, Z., Paz, S., Belgnaoui, S. M., Lafferty, E. I., Janelle, V., Arguello, M., Paquet, M., Ghneim, K., Richards, S., Smith, A., Wilkinson, P., Cameron, M., Kalinke, U., Qureshi, S., Lamarre, A., Haddad, E. K., Sekaly, R. P., … Hiscott, J. (2013). Systems Analysis of a RIG-I Agonist Inducing Broad Spectrum Inhibition of Virus Infectivity. *PLoS Pathogens*, *9*(4). https://doi.org/10.1371/journal.ppat.1003298

Gramzinski, R. A., Doolan, D. L., Sedegah, M., Davis, H. L., Krieg, A. M., & Hoffman, S. L. (2002). Erratum: Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice (Infection and Immunity (2001) 69:3 (1643-1649)). *Infection and Immunity*, *70*(9), 5338. https://doi.org/10.1128/IAI.70.9.5338.2002

Grau, G. E., Mackenzie, C. D., Carr, R. A., Redard, M., Pizzolato, G., Allasia, C., Cataldo, C., Taylor, T. E., & Molyneux, M. E. (2003). Platelet Accumulation in Brain Microvessels in Fatal Pediatric Cerebral Malaria. *The Journal of Infectious Diseases*, *187*(3), 461–466. https://doi.org/10.1086/367960

Greiner, J., Dorovini-Zis, K., Taylor, T. E., Molyneux, M. E., Beare, N. A. V., Kamiza, S., & White, V. A. (2015). Correlation of hemorrhage, axonal damage and blood-tissue barrier disruption in brain and retina of Malawian children with fatal cerebral malaria. *Frontiers in Cellular and Infection Microbiology*, *5*(FEB), 1–8. https://doi.org/10.3389/fcimb.2015.00018

Griffin, J. T., Déirdre Hollingsworth, T., Reyburn, H., Drakeley, C. J., Riley, E. M., & Ghani, A. C. (2015). Gradual acquisition of immunity to severe malaria with increasing exposure. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1801). https://doi.org/10.1098/rspb.2014.2657

Griffiths, E. A., Srivastava, P., Matsuzaki, J., Brumberger, Z., Wang, E. S., Kocent, J., Miller, A., Roloff, G. W., Wong, H. Y., Paluch, B. E., Lutgen-Dunckley, L. G., Martens, B. L., Odunsi, K., Karpf, A. R., Hourigan, C. S., & Nemeth, M. J. (2018). NY-ESO-1 vaccination in combination with decitabine induces antigen-specific T-lymphocyte responses in patients with myelodysplastic syndrome. *Clinical Cancer Research*, *24*(5), 1019–

1029. https://doi.org/10.1158/1078-0432.CCR-17-1792

Guiducci, C., Ott, G., Chan, J. H., Damon, E., Calacsan, C., Matray, T., Lee, K. D., Coffman, R. L., & Barrat, F. J. (2006). Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *Journal of Experimental Medicine*, *203*(8), 1999–2008. https://doi.org/10.1084/jem.20060401

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S. H., Onai, N., Schraml, B. U., Segura, E., Tussiwand, R., & Yona, S. (2014). Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny. *Nature Reviews Immunology*, *14*(8), 571–578. https://doi.org/10.1038/nri3712

Gunzer, M., Riemann, H., Basoglu, Y., Hillmer, A., Weishaupt, C., Balkow, S., Benninghoff, B., Ernst, B., Steinert, M., Scholzen, T., Sunderkötter, C., & Grabbe, S. (2005). Systemic administration of a TLR7 ligand leads to transient immune incompetence due to peripheral-blood leukocyte depletion. *Blood*, *106*(7), 2424–2432. https://doi.org/10.1182/blood-2005-01-0342

Gupta, R. K., Rost, B. E., Relyveld, E., & Siber, G. R. (1995). Adjuvant properties of aluminum and calcium compounds. *Pharmaceutical Biotechnology*, *6*, 229–248. https://doi.org/10.1007/978-1-4615-1823-5_8

Gustot, T., Lemmers, A., Moreno, C., Nagy, N., Quertinmont, E., Nicaise, C., Franchimont, D., Louis, H., Devière, J., & Le Moine, O. (2006). Differential liver sensitization to toll-like receptor pathways in mice with alcoholic fatty liver. *Hepatology*, *43*(5), 989–1000. https://doi.org/10.1002/hep.21138

Gutjahr, A., Papagno, L., Nicoli, F., Kanuma, T., Kuse, N., Cabral-Piccin, M. P., Rochereau, N., Gostick, E., Lioux, T., Perouzel, E., Price, D. A., Takiguchi, M., Verrier, B., Yamamoto, T., Paul, S., & Appay, V. (2019). The STING ligand cGAMP potentiates the efficacy of vaccine-induced CD8+ T cells. *JCI Insight*, *4*(7), 1–11. https://doi.org/10.1172/jci.insight.125107

Gwadz, R. W., Cochrane, A. H., Nussenzweig, V., & Nussenzweig, R. S. (1979). Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of Plasmodium knowlesi and characterization of surface antigens of these parasites. *Bulletin of the World Health Organization*, *57*(Suppl. 1), 165–173.

Häcker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., & Wagner, H. (2000). Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *Journal of Experimental Medicine*, *192*(4), 595–600. https://doi.org/10.1084/jem.192.4.595

Haeberlein, S., Chevalley-Maurel, S., Ozir-Fazalalikhan, A., Koppejan, H., Winkel, B. M. F., Ramesar, J., Khan, S. M., Sauerwein, R. W.,

Roestenberg, M., Janse, C. J., Smits, H. H., & Franke-Fayard, B. (2017). Protective immunity differs between routes of administration of attenuated malaria parasites independent of parasite liver load. *Scientific Reports*, *7*(1), 1–19. https://doi.org/10.1038/s41598-017-10480-1

Hafalla, Julius C.R., Rai, U., Morrot, A., Bernal-Rubio, D., Zavala, F., & Rodriguez, A. (2006). Priming of CD8 T cell responses following immunization with heat-killed Plasmodium sporozoites. *European Journal of Immunology*, *36*(5), 1179–1186. https://doi.org/10.1002/eji.200535712

Hafalla, Julius Clemence R., Bauza, K., Friesen, J., Gonzalez-Aseguinolaza, G., Hill, A. V. S., & Matuschewski, K. (2013). Identification of Targets of CD8+ T Cell Responses to Malaria Liver Stages by Genome-wide Epitope Profiling. *PLoS Pathogens*, *9*(5). https://doi.org/10.1371/journal.ppat.1003303

Hanagata, N. (2012). Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system. *International Journal of Nanomedicine*, *7*, 2181–2195. https://doi.org/10.2147/IJN.S30197

Hansen, D. S., Bernard, N. J., Nie, C. Q., & Schofield, L. (2007). NK Cells Stimulate Recruitment of CXCR3 + T Cells to the Brain during Plasmodium berghei -Mediated Cerebral Malaria . *The Journal of Immunology*, *178*(9), 5779–5788. https://doi.org/10.4049/jimmunol.178.9.5779

Hanson, J., Lee, S. J., Mohanty, S., Faiz, M. A., Anstey, N. M., Charunwatthana, P., Yunus, E. Bin, Mishra, S. K., Tjitra, E., Ric, N., Rahman, R., Nosten, F., Htut, Y., Hoque, G., Thi, T., Chau, H., Phu, N. H., Hien, T. T., White, N. J., … Dondorp, A. M. (2010). A Simple Score to Predict the Outcome of Severe Malaria in Adults. *Clinical Infectious Diseases*, *50*(5), 679–685. https://doi.org/10.1086/649928.A

Harrison, C. J., Miller, R. L., & Bernstein, D. I. (1994). Posttherapy suppression of genital herpes simplex virus (HSV) recurrences and enhancement of HSV-specific T-cell memory by imiquimod in guinea pigs. *Antimicrobial Agents and Chemotherapy*, *38*(9), 2059–2064. https://doi.org/10.1128/AAC.38.9.2059

Hartmann, G., Weiner, G. J., & Krieg, A. M. (1999). CpG DNA: A potent signal for growth, activation, and maturation of human dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(16), 9305–9310. https://doi.org/10.1073/pnas.96.16.9305

Hartmann, Gunther, Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S., & Endres, S. (2003). Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-α induction in plasmacytoid dendritic cells. *European Journal of Immunology*, *33*(6), 1633–1641. https://doi.org/10.1002/eji.200323813

Hartmann, Gunther, & Krieg, A. M. (2000). Mechanism and Function of a Newly Identified CpG DNA Motif in Human Primary B Cells. *The Journal of Immunology*, *164*(2), 944–953. https://doi.org/10.4049/jimmunol.164.2.944

Hayashi, T., Gray, C. S., Chan, M., Tawatao, R. I., Ronacher, L., McGargill, M. A., Datta, S. K., Carson, D. A., & Corr, M. (2009). Prevention of autoimmune disease by induction of tolerance to toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(8), 2764–2769. https://doi.org/10.1073/pnas.0813037106

Hayday, A. C. (2000). γδ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection. *Annual Review of Immunology*, *18*(1), 975–1026. https://doi.org/10.1146/annurev.immunol.18.1.975

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Akira, S., Lipford, G., Wagner, H., & Bauer, S. (2004). Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science (New York, N.Y.)*, *303*(5663), 1526–1529. papers3://publication/doi/10.1126/science.1093620

Hemmi, H., Kaisho, T., Takeda, K., & Akira, S. (2003). The Roles of Toll-Like Receptor 9, MyD88, and DNA-Dependent Protein Kinase Catalytic Subunit in the Effects of Two Distinct CpG DNAs on Dendritic Cell Subsets. *The Journal of Immunology*, *170*(6), 3059–3064. https://doi.org/10.4049/jimmunol.170.6.3059

Henriques, H. R., Rampazo, E. V., Gonçalves, A. J. S., Vicentin, E. C. M., Amorim, J. H., Panatieri, R. H., Amorim, K. N. S., Yamamoto, M. M., Ferreira, L. C. S., Alves, A. M. B., & Boscardin, S. B. (2013). Targeting the non-structural protein 1 from dengue virus to a dendritic cell population confers protective immunity to lethal virus challenge. *PLoS Neglected Tropical Diseases*, *7*(7). https://doi.org/10.1371/journal.pntd.0002330

Herrera, R., Anderson, C., Kumar, K., Molina-Cruz, A., Nguyen, V., Burkhardt, M., Reiter, K., Shimp, R., Howard, R. F., Srinivasan, P., Nold, M. J., Ragheb, D., Shi, L., DeCotiis, M., Aebig, J., Lambert, L., Rausch, K. M., Muratova, O., Jin, A., … Naruma, D. L. (2015). Reversible conformational change in the plasmodium falciparum circumsporozoite protein masks Its adhesion domains. *Infection and Immunity*, *83*(10), 3771–3780. https://doi.org/10.1128/IAI.02676-14

Hjerrild, K. A., Jin, J., Wright, K. E., Brown, R. E., Marshall, J. M., Labbé, G. M., Silk, S. E., Cherry, C. J., Clemmensen, S. B., Jørgensen, T., Illingworth, J. J., Alanine, D. G. W., Milne, K. H., Ashfield, R., De Jongh, W. A., Douglas, A. D., Higgins, M. K., & Draper, S. J. (2016). Production of full-length soluble Plasmodium falciparum RH5 protein vaccine using a Drosophila melanogaster Schneider 2 stable cell line system. *Scientific Reports*, *6*(July), 1–15. https://doi.org/10.1038/srep30357

Hochheiser, K., Klein, M., Gottschalk, C., Hoss, F., Scheu, S., Coch, C., Hartmann, G., & Kurts, C. (2016). Cutting Edge: The RIG-I Ligand 3pRNA Potently Improves CTL Cross-Priming and Facilitates Antiviral Vaccination. *The Journal of Immunology*, *196*(6), 2439–2443. https://doi.org/10.4049/jimmunol.1501958

Hochman, S., Madaline, T., Seydel, K., Taylor, T., Milner, D., S., L., & K., K. (2013). Fatal pediatric cerebral malaria is associated with intravascular inflammation and coagulation that is exacerbated by HIV-1 co-infection. *American Journal of Tropical Medicine and Hygiene*, *89*(5), 457. https://doi.org/10.1128/mBio.01390-15.Editor

Hodgson, S. H., Ewer, K. J., Bliss, C. M., Edwards, N. J., Rampling, T., Anagnostou, N. A., De Barra, E., Havelock, T., Bowyer, G., Poulton, I. D., De Cassan, S., Longley, R., Illingworth, J. J., Douglas, A. D., Mange, P. B., Collins, K. A., Roberts, R., Gerry, S., Berrie, E., … Hill, A. V. S. (2015). Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals. *Journal of Infectious Diseases*, *211*(7), 1076–1086. https://doi.org/10.1093/infdis/jiu579

Hoffman, S L, Crutcher, J. M., Puri, S. K., Ansari, A. A., Villinger, F., Franke, E. D., Singh, P. P., Finkelman, F., Gately, M. K., Dutta, G. P., & Sedegah, M. (1997). Sterile protection of monkeys against malaria after administration of interleukin-12. *Nature Medicine*, *3*(1), 80–83. https://doi.org/10.1038/nm0197-80

Hoffman, Stephen L., Billingsley, P. F., James, E., Richman, A., Loyevsky, M., Li, T., Chakravarty, S., Gunasekera, A., Chattopadhyay, R., Li, M., Stafford, R., Ahumada, A., Epstein, J. E., Sedegah, M., Reyes, S., Richie, T. L., Lyke, K. E., Edelman, R., Laurens, M. B., … Sim, B. K. L. (2010). Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. *Human Vaccines*, *6*(1). https://doi.org/10.4161/hv.6.1.10396

Hoffman, Stephen L., Oster, C. N., Plowe, C. V., Woollett, G. R., Beier, J. C., Chulay, J. D., Wirtz, R. A., Hollingdale, M. R., & Mugambi, M. (1987). Naturally acquired antibodies to sporozoites do not prevent malaria: Vaccine development implications. *Science*, *237*(4815), 639–642. https://doi.org/10.1126/science.3299709

Hofmann, M., Oschowitzer, A., Kurzhals, S. R., Krüger, C. C., & Pircher, H. (2013). Thymus-resident memory CD8+ T cells mediate local immunity. *European Journal of Immunology*, *43*(9), 2295–2304. https://doi.org/10.1002/eji.201343519

Hofmann, M., & Pircher, H. (2011). E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proceedings of the National Academy of Sciences of the United States of*

America, *108*(40), 16741–16746. https://doi.org/10.1073/pnas.1107200108

Holtmeier, W., & Kabelitz, D. (2005). γδ T cells link innate and adaptive. *Chem Immunol Allergy*, *86*, 151–183.

Holz, L. E., Fernandez-Ruiz, D., & Heath, W. R. (2016). Protective immunity to liver-stage malaria. *Clinical & Translational Immunology*, *5*(10), e105. https://doi.org/10.1038/cti.2016.60

Holz, L. E., Prier, J. E., Freestone, D., Steiner, T. M., English, K., Johnson, D. N., Mollard, V., Cozijnsen, A., Davey, G. M., Godfrey, D. I., Yui, K., Mackay, L. K., Lahoud, M. H., Caminschi, I., McFadden, G. I., Bertolino, P., Fernandez-Ruiz, D., & Heath, W. R. (2018). CD8+ T Cell Activation Leads to Constitutive Formation of Liver Tissue-Resident Memory T Cells that Seed a Large and Flexible Niche in the Liver. *Cell Reports*, *25*(1), 68-79.e4. https://doi.org/10.1016/j.celrep.2018.08.094

Holz, L. E., Warren, A., Le Couteur, D. G., Bowen, D. G., & Bertolino, P. (2010). CD8+ T cell tolerance following antigen recognition on hepatocytes. *Journal of Autoimmunity*, *34*(1), 15–22. https://doi.org/10.1016/j.jaut.2009.08.005

Honda, K., Ohba, Y., Yanai, H., Hegishi, H., Mizutani, T., Takaoka, A., Taya, C., & Taniguchi, T. (2005). Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature*, *434*(7036), 1035–1040. https://doi.org/10.1038/nature03547

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D. R., Latz, E., & Fitzgerald, K. A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature*, *458*(7237), 514–518. https://doi.org/10.1038/nature07725

Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., & Hartmann, G. (2006). 5′-Triphosphate RNA is the ligand for RIG-I. *Science*, *314*(5801), 994–997. https://doi.org/10.1126/science.1132505

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S., & Hartmann, G. (2002). Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. *The Journal of Immunology*, *168*(9), 4531–4537. https://doi.org/10.4049/jimmunol.168.9.4531

Hou, B., Saudan, P., Ott, G., Wheeler, M. L., Ji, M., Kuzmich, L., Lee, L. M., Coffman, R. L., Bachmann, M. F., & DeFranco, A. L. (2011). Selective utilization of toll-like receptor and Myd88 signaling in B cells for enhancement of the antiviral germinal center response. *Immunity*, *34*(3),

375–384. https://doi.org/10.1016/j.immuni.2011.01.011

Howick, V. M., Russell, A. J. C., Andrews, T., Heaton, H., Reid, A. J., Natarajan, K., Butungi, H., Metcalf, T., Verzier, L. H., Rayner, J. C., Berriman, M., Herren, J. K., Billker, O., Hemberg, M., Talman, A. M., & Lawniczak, M. K. N. (2019). The malaria cell atlas: Single parasite transcriptomes across the complete Plasmodium life cycle. *Science*, *365*(6455). https://doi.org/10.1126/science.aaw2619

Hritz, I., Mandrekar, P., Velayudham, A., Catalano, D., Dolganiuc, A., Kodys, K., Kurt-Jones, E., & Szabo, G. (2008). The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88. *Hepatology*, *48*(4), 1224–1231. https://doi.org/10.1002/hep.22470

Hu, D. L., Narita, K., Hyodo, M., Hayakawa, Y., Nakane, A., & Karaolis, D. K. R. (2009). c-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant Staphylococcus aureus (MRSA) infection. *Vaccine*, *27*(35), 4867–4873. https://doi.org/10.1016/j.vaccine.2009.04.053

Huang, L. R., Wohlleber, D., Reisinger, F., Jenne, C. N., Cheng, R. L., Abdullah, Z., Schildberg, F. A., Odenthal, M., Dienes, H. P., Van Rooijen, N., Schmitt, E., Garbi, N., Croft, M., Kurts, C., Kubes, P., Protzer, U., Heikenwalder, M., & Knolle, P. A. (2013). Intrahepatic myeloid-cell aggregates enable local proliferation of CD8 + T cells and successful immunotherapy against chronic viral liver infection. *Nature Immunology*, *14*(6), 574–583. https://doi.org/10.1038/ni.2573

Huret, C., Desjardins, D., Miyalou, M., Levacher, B., Amadoudji Zin, M., Bonduelle, O., Combadière, B., Dalba, C., Klatzmann, D., & Bellier, B. (2013). Recombinant retrovirus-derived virus-like particle-based vaccines induce hepatitis C virus-specific cellular and neutralizing immune responses in mice. *Vaccine*, *31*(11), 1540–1547. https://doi.org/10.1016/j.vaccine.2012.05.025

Huysamen, C., Willment, J. A., Dennehy, K. M., & Brown, G. D. (2008). CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3+ dendritic cells and a subset of monocytes. *Journal of Biological Chemistry*, *283*(24), 16693–16701. https://doi.org/10.1074/jbc.M709923200

Hviid, L., & Staalsoe, T. (2004). Malaria immunity in infants: A special case of a general phenomenon? *Trends in Parasitology*, *20*(2), 66–72. https://doi.org/10.1016/j.pt.2003.11.009

Iborra, S., Izquierdo, H. M., Martinez-Lopez, M., Blanco-Menendez, N., Reis e Sousa, C., & Sancho, D. (2012). *The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice*. *122*(5), 1628–1643. https://doi.org/10.1172/JCI60660DS1

Ichinohe, T., Watanabe, I., Ito, S., Fujii, H., Moriyama, M., Tamura, S. -i., Takahashi, H., Sawa, H., Chiba, J., Kurata, T., Sata, T., & Hasegawa, H. (2005). Synthetic Double-Stranded RNA Poly(I:C) Combined with Mucosal Vaccine Protects against Influenza Virus Infection. *Journal of Virology*, *79*(5), 2910–2919. https://doi.org/10.1128/jvi.79.5.2910-2919.2005

Illingworth, J. J., Alanine, D. G., Brown, R., Marshall, J. M., Bartlett, H. E., Silk, S. E., Labbé, G. M., Quinkert, D., Cho, J. S., Wendler, J. P., Pattinson, D. J., Barfod, L., Douglas, A. D., Shea, M. W., Wright, K. E., De Cassan, S. C., Higgins, M. K., & Draper, S. J. (2019). Functional comparison of blood-stage plasmodium falciparummalaria vaccine candidate antigens. *Frontiers in Immunology*, *10*(JUN), 1–17. https://doi.org/10.3389/fimmu.2019.01254

Ishino, T., Boisson, B., Orito, B. B., Lacroix, C., Bischoff, E., Loussert, C., Janse, C., Ménard, R., Yuda, M., & Baldacci, P. (2009). LISP1 is important for the egress of Plasmodium berghei parasites from liver cellsc. *Cellular Microbiology*, *11*(9), 1329–1339. https://doi.org/10.1111/j.1462-5822.2009.01333.x

Ishino, T., Chinzei, Y., & Yuda, M. (2005). Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Molecular Microbiology*, *58*(5), 1264–1275. https://doi.org/10.1111/j.1365-2958.2005.04801.x

Ishino, T., Yano, K., Chinzei, Y., & Yuda, M. (2004). Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biology*, *2*(1), 77–84. https://doi.org/10.1371/journal.pbio.0020004

Ishizuka, A. S., Lyke, K. E., DeZure, A., Berry, A. A., Richie, T. L., Mendoza, F. H., Enama, M. E., Gordon, I. J., Chang, L. J., Sarwar, U. N., Zephir, K. L., Holman, L. A., James, E. R., Billingsley, P. F., Gunasekera, A., Chakravarty, S., Manoj, A., Li, M., Ruben, A. J., … Seder, R. A. (2016). Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nature Medicine*, *22*(6), 614–623. https://doi.org/10.1038/nm.4110

Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F., & Lanzavecchia, A. (2001). Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *European Journal of Immunology*, *31*(11), 3388–3393. https://doi.org/10.1002/1521-4141(200111)31:11<3388::AID-IMMU3388>3.0.CO;2-Q

Jayabalasingham, B., Bano, N., & Coppens, I. (2010). Metamorphosis of the malaria parasite in the liver is associated with organelle clearance. *Cell Research*, *20*(9), 1043–1059. https://doi.org/10.1038/cr.2010.88

Jenkins, M. K., & Moon, J. J. (2012). The role of naïve T cell precursor frequency and recruitment in dictating immune response magnitude. *J Immunol*,

9(188), 4135–4140. https://doi.org/10.1016/j.immuni.2010.12.017.Two-stage

Jérôme, V., Graser, A., Müller, R., Kontermann, R. E., & Konur, A. (2006). Cytotoxic T lymphocytes responding to low dose TRP2 antigen are induced against B16 melanoma by liposome-encapsulated TRP2 peptide and CpG DNA adjuvant. *Journal of Immunotherapy*, *29*(3), 294–305. https://doi.org/10.1097/01.cji.0000199195.97845.18

Jiang, X., Clark, R. A., Liu, L., Wagers, A. J., Fuhlbrigge, R. C., & Kupper, T. S. (2012). Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. *Nature Communications*, *483*(7388), 227–231. https://doi.org/10.1038/nature10851

Jobe, O., Lumsden, J., Mueller, A., Williams, J., Silva-Rivera, H., Kappe, S. H. I., Schwenk, R. J., Matuschewski, K., & Krzych, U. (2007). Genetically Attenuated Plasmodium berghei Liver Stages Induce Sterile Protracted Protection That Is Mediated by Major Histocompatibility Complex Class I–Dependent Interferon-γ–Producing CD8 + T Cells . *The Journal of Infectious Diseases*, *196*(4), 599–607. https://doi.org/10.1086/519743

Jones, R. B., Ndhlovu, L. C., Barbour, J. D., Sheth, P. M., Jha, A. R., Long, B. R., Wong, J. C., Satkunarajah, M., Schweneker, M., Chapman, J. M., Gyenes, G., Vali, B., Hyrcza, M. D., Yue, F. Y., Kovacs, C., Sassi, A., Loutfy, M., Halpenny, R., Persad, D., … Ostrowski, M. A. (2008). Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *Journal of Experimental Medicine*, *205*(12), 2763–2779. https://doi.org/10.1084/jem.20081398

Joshi, N. S., Cui, W., Chandele, A., Lee, H. K., Urso, D. R., Hagman, J., Gapin, L., & Kaech, S. M. (2007). Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity*, *27*(2), 281–295. https://doi.org/10.1016/j.immuni.2007.07.010

Josling, G. A., & Llinás, M. (2015). Sexual development in Plasmodium parasites: Knowing when it's time to commit. *Nature Reviews Microbiology*, *13*(9), 573–587. https://doi.org/10.1038/nrmicro3519

Junt, T., & Barchet, W. (2015). Translating nucleic acid-sensing pathways into therapies. *Nature Reviews Immunology*, *15*(9), 529–544. https://doi.org/10.1038/nri3875

Kadowaki, N., Ho, S., Antonenko, S., De Waal Malefyt, R., Kastelein, R. A., Bazan, F., & Liu, Y. J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine*, *194*(6), 863–869. https://doi.org/10.1084/jem.194.6.863

Kaiser, K., Matuschewski, K., Camargo, N., Ross, J., & Kappe, S. H. I. (2004). Differential transcriptome profiling identifies Plasmodium genes encoding pre-erythrocytic stage-specific proteins. *Molecular Microbiology*, *51*(5), 1221–1232. https://doi.org/10.1046/j.1365-2958.2003.03909.x

Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, V., & Ménard, R. (1999). Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. *Journal of Cell Biology*, *147*(5), 937–943. https://doi.org/10.1083/jcb.147.5.937

Karaolis, D. K. R., Means, T. K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., Philpott, D., Schroeder, J. T., Hyodo, M., Hayakawa, Y., Talbot, B. G., Brouillette, E., & Malouin, F. (2007). Bacterial c-di-GMP Is an Immunostimulatory Molecule. *The Journal of Immunology*, *178*(4), 2171–2181. https://doi.org/10.4049/jimmunol.178.4.2171

Karaolis, D. K. R., Newstead, M. W., Zeng, X., Hyodo, M., Hayakawa, Y., Bhan, U., Liang, H., & Standiford, T. J. (2007). Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infection and Immunity*, *75*(10), 4942–4950. https://doi.org/10.1128/IAI.01762-06

Kariu, T., Ishino, T., Yano, K., Chinzei, Y., & Yuda, M. (2006). CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Molecular Microbiology*, *59*(5), 1369–1379. https://doi.org/10.1111/j.1365-2958.2005.05024.x

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin IV, H. W. (2003). STAT1-dependent innate immunity to a norwalk-like virus. *Science*, *299*(5612), 1575–1578. https://doi.org/10.1126/science.1077905

Karunaweera, N. D., Grau, G. E., Gamage, P., Carter, R., & Mendis, K. N. (1992). Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(8), 3200–3203. https://doi.org/10.1073/pnas.89.8.3200

Kassim, O. O., Ako-Anai, K. A., Torimiro, S. E., Hollowell, G. P., Okoye, V. C., & Martin, S. K. (2000). Inhibitory factors in breastmilk, maternal and infant sera against in vitro growth of Plasmodium falciparum malaria parasite. *Journal of Tropical Pediatrics*, *46*(2), 92–96. https://doi.org/10.1093/tropej/46.2.92

Kasumba, D. M., & Grandvaux, N. (2019). Therapeutic Targeting of RIG-I and MDA5 Might Not Lead to the Same Rome. *Trends in Pharmacological Sciences*, *40*(2), 116–127. https://doi.org/10.1016/j.tips.2018.12.003

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T. S., Fujita, T., & Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic

acid-inducible gene-I and melanoma differentiation-associated gene 5. *Journal of Experimental Medicine*, *205*(7), 1601–1610. https://doi.org/10.1084/jem.20080091

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Reis E Sousa, C., Matsuura, Y., Fujita, T., & Akira, S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*, *441*(1), 101–105. https://doi.org/10.1038/nature04734

Kato, Y., Zaid, A., Davey, G. M., Mueller, S. N., Nutt, S. L., Zotos, D., Tarlinton, D. M., Shortman, K., Lahoud, M. H., Heath, W. R., & Caminschi, I. (2015). Targeting Antigen to Clec9A Primes Follicular Th Cell Memory Responses Capable of Robust Recall. *The Journal of Immunology*, *195*(3), 1006–1014. https://doi.org/10.4049/jimmunol.1500767

Kaushansky, A., Douglass, A. N., Arang, N., Vigdorovich, V., Dambrauskas, N., Kain, H. S., Austin, L. S., Sather, D. N., & Kappe, S. H. I. (2015). Malaria parasites target the hepatocyte receptor EphA2 for successful host infection. *Science*, *350*(6264), 1089–1092. https://doi.org/10.1126/science.aad3318

Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. *Trends in Molecular Medicine*, *13*(11), 460–469. https://doi.org/10.1016/j.molmed.2007.09.002

Kebaier, C., Voza, T., & Vanderberg, J. (2009). Kinetics of mosquito-injected plasmodium sporozoites in mice: Fewer sporozoites are injected into sporozoite-immunized mice. *PLoS Pathogens*, *5*(4). https://doi.org/10.1371/journal.ppat.1000399

Keegan, L. T., & Dushoff, J. (2013). Population-level effects of clinical immunity to malaria. *BMC Infectious Diseases*, *13*(1). https://doi.org/10.1186/1471-2334-13-428

Kerrigan, A. M., & Brown, G. D. (2010). Syk-coupled C-type lectin receptors that mediate cellular activation via single tyrosine based activation motifs. *Immunological Reviews*, *234*(1), 335–352. https://doi.org/10.1111/j.0105-2896.2009.00882.x

Kesar, V., & Odin, J. A. (2014). Toll-like receptors and liver disease. *Liver International*, *34*(2), 184–196. https://doi.org/10.1111/liv.12315

Khalil, I. A., Kogure, K., Akita, H., & Harashima, H. (2006). Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *The American Society for Pharmacology and Experimental Therapeutics*, *58*(1), 32–45. https://doi.org/10.1124/pr.58.1.8.32

Kimura, T., Nakayama, K., Penninger, J., Kitagawa, M., Harada, H., Matsuyama, T., Tanaka, N., Kamijo, R., Vilček, J., Mak, T. W., & Taniguchi, T. (1994). Involvement of the IRF-1 transcription factor in antiviral responses to interferons. *Science*, *264*(5167), 1921–1924. https://doi.org/10.1126/science.8009222

Kiziltas, S. (2016). Toll-like receptors in pathophysiology of liver diseases. *World Journal of Hepatology*, *8*(32), 1354–1369. https://doi.org/10.4254/wjh.v8.i32.1354

Klinman, D. M. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Reviews Immunology*, *4*(4), 249–258. https://doi.org/10.1038/nri1329

Klonowski, K. D., Williams, K. J., Marzo, A. L., Blair, D. A., Lingenheld, E. G., & Lefrançois, L. (2004). Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity*, *20*(5), 551–562. https://doi.org/10.1016/S1074-7613(04)00103-7

Klotz, F. W., Scheller, L. F., Seguin, M. C., Kumar, N., Marletta, M. A., Green, S. J., & Azad, A. F. (1995). Co-localization of inducible-nitric oxide synthase and Plasmodium berghei in hepatocytes from rats immunized with irradiated sporozoites. *Journal of Immunology*, *154*(7), 3391–3395.

Knolle, P., Schlaak, J., Uhrig, A., Kempf, P., zum Büschenfelde, K. H. M., & Gerken, G. (1995). Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *Journal of Hepatology*, *22*(2), 226–229. https://doi.org/10.1016/0168-8278(95)80433-1

Koning-Ward, T. F., Sperança, M. A., Waters, A. P., & Janse, C. J. (1999). Analysis of stage specificity of promoters in Plasmodium berghei using luciferase as a reporter. *Molecular and Biochemical Parasitology*, *100*(1), 141–146. https://doi.org/10.1016/S0166-6851(99)00042-0

Kool, M., Soullié, T., Van Nimwegen, M., Willart, M. A. M., Muskens, F., Jung, S., Hoogsteden, H. C., Hammad, H., & Lambrecht, B. N. (2008). Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *Journal of Experimental Medicine*, *205*(4), 869–882. https://doi.org/10.1084/jem.20071087

Korbel, D. S., Finney, O. C., & Riley, E. M. (2004). Natural killer cells and innate immunity to protozoan pathogens. *International Journal for Parasitology*, *34*(13–14), 1517–1528. https://doi.org/10.1016/j.ijpara.2004.10.006

Korten, S., Anderson, R. J., Hannan, C. M., Sheu, E. G., Sinden, R., Gadola, S., Taniguchi, M., & Hil, A. V. S. (2005). Invariant Vα14 chain NKT cells promote Plasmodium berghei circumsporozoite protein-specific gamma interferon- and tumor necrosis factor alpha-producing CD8+ T cells in the liver after poxvirus vaccination of mice. *Infection and Immunity*, *73*(2),

849–858. https://doi.org/10.1128/IAI.73.2.849-858.2005

Kosuwin, R., Putaporntip, C., Tachibana, H., & Jongwutiwes, S. (2014). Spatial variation in genetic diversity and natural selection on the thrombospondin-related adhesive protein locus of Plasmodium vivax(PvTRAP). *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0110463

Krieg, A. M., Yi, A., Matson, S., Waldschmldtt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., & Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*, *374*, 546–549. https://doi.org/10.1038/374546a0

Kroczek, R. A., & Henn, V. (2012). The role of XCR1 and its ligand XCL1 in antigen cross-presentation by murine and human dendritic cells. *Frontiers in Immunology*, *3*(FEB), 1–5. https://doi.org/10.3389/fimmu.2012.00014

Krotoski, W. A., Collins, W. E., Bray, R. S., Garnham, P. C., Cogswell, F. B., Gwadz, R. W., Killick-Kendrick, R., Wolf, R., Sinden, R., Koontz, L. C., & Stanfill, P. . (1982). Demonstration of hypnozoites in sporozoite-transmitted Plasmodium vivax infection. *The American Journal of Tropical Medicine and Hygiene*, *31*(6), 1291–1293. https://doi.org/10.4269/ajtmh.1982.31.1291

Kublin, J. G., Mikolajczak, S. A., Sack, B. K., Fishbaugher, M. E., Seilie, A., Shelton, L., VonGoedert, T., Firat, M., Magee, S., Fritzen, E., Betz, W., Kain, H. S., Dankwa, D. A., Steel, R. W. J., Vaughan, A. M., Sather, D. N., Murphy, S. C., & Kappe, S. H. I. (2017). Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects. *Science Translational Medicine*, *9*(371), 1–12. https://doi.org/10.1126/scitranslmed.aad9099

Kulkarni, R. R., Rasheed, M. A. U., Bhaumik, S. K., Ranjan, P., Cao, W., Davis, C., Marisetti, K., Thomas, S., Gangappa, S., Sambhara, S., & Murali-Krishna, K. (2014). Activation of the RIG-I Pathway during Influenza Vaccination Enhances the Germinal Center Reaction, Promotes T Follicular Helper Cell Induction, and Provides a Dose-Sparing Effect and Protective Immunity. *Journal of Virology*, *88*(24), 13990–14001. https://doi.org/10.1128/jvi.02273-14

Kumar, A., Zhang, J., & Yu, F. S. X. (2006). Toll-like receptor 3 agonist poly(I:C)-induced antiviral response in human corneal epithelial cells. *Immunology*, *117*(1), 11–21. https://doi.org/10.1111/j.1365-2567.2005.02258.x

Kumar, H., Kawai, T., & Akira, S. (2011). Pathogen recognition by the innate immune system. *International Reviews of Immunology*, *30*(1), 16–34. https://doi.org/10.3109/08830185.2010.529976

Kumar, B. V., Ma, W., Miron, M., Granot, T., Guyer, R. S., Carpenter, D. J., Senda, T., Sun, X., Ho, S. H., Lerner, H., Friedman, A. L., Shen, Y., &

Farber, D. L. (2017). Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Reports*, *20*(12), 2921–2934. https://doi.org/10.1016/j.celrep.2017.08.078

Kurtis, J. D., Kurtis, J. D., Onyango, F. K., Onyango, F. K., Duffy, P. E., & Duffy, P. E. (2001). *Human Resistance to*. *69*(1), 123–128. https://doi.org/10.1128/IAI.69.1.123

Kurup, S. P., Anthony, S. M., Hancox, L. S., Vijay, R., Pewe, L. L., Moioffer, S. J., Sompallae, R., Janse, C. J., Khan, S. M., & Harty, J. T. (2019). Monocyte-Derived CD11c + Cells Acquire Plasmodium from Hepatocytes to Prime CD8 T Cell Immunity to Liver-Stage Malaria. *Cell Host and Microbe*, *25*(4), 565-577.e6. https://doi.org/10.1016/j.chom.2019.02.014

La Gruta, N. L., Rothwell, W. T., Cukalac, T., Swan, N. G., Valkenburg, S. A., Kedzierska, K., Thomas, P. G., Doherty, P. C., & Turner, S. J. (2010). Primary CTL response magnitude in mice is determined by the extent of naive T cell recruitment and subsequent clonal expansion. *Journal of Clinical Investigation*, *120*(6), 1885–1894. https://doi.org/10.1172/JCI41538

Labaied, M., Harupa, A., Dumpit, R. F., Coppens, I., Mikolajczak, S. A., & Kappe, S. H. I. (2007). Plasmodium yoelii sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. *Infection and Immunity*, *75*(8), 3758–3768. https://doi.org/10.1128/IAI.00225-07

Lahoud, M. H., Ahmet, F., Kitsoulis, S., Wan, S. S., Vremec, D., Lee, C.-N., Phipson, B., Shi, W., Smyth, G. K., Lew, A. M., Kato, Y., Mueller, S. N., Davey, G. M., Heath, W. R., Shortman, K., & Caminschi, I. (2011). Targeting Antigen to Mouse Dendritic Cells via Clec9A Induces Potent CD4 T Cell Responses Biased toward a Follicular Helper Phenotype. *The Journal of Immunology*, *187*(2), 842–850. https://doi.org/10.4049/jimmunol.1101176

Lahoud, M. H., Ahmet, F., Zhang, J. G., Meuter, S., Policheni, A. N., Kitsoulis, S., Lee, C. N., O'Keeffe, M., Sullivan, L. C., Brooks, A. G., Berry, R., Rossjohn, J., Mintern, J. D., Vega-Ramos, J., Villadangos, J. A., Nicola, N. A., Nussenzweig, M. C., Stacey, K. J., Shortman, K., … Caminschi, I. (2012). DEC-205 is a cell surface receptor for CpG oligonucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(40), 16270–16275. https://doi.org/10.1073/pnas.1208796109

Lai, J. C. Y., Cheng, W. K., Hopkins, P. D., Komba, M., Carlow, D. A., & Dutz, J. P. (2019). Topical Adjuvant Application during Subcutaneous Vaccination Promotes Resident Memory T Cell Generation. *The Journal of Immunology*, *203*(9), 2443–2450.

https://doi.org/10.4049/jimmunol.1900199

Langhorne, J., Ndungu, F. M., Sponaas, A. M., & Marsh, K. (2008). Immunity to malaria: More questions than answers. *Nature Immunology*, *9*(7), 725–732. https://doi.org/10.1038/ni.f.205

Lapuente, D., Storcksdieck Genannt Bonsmann, M., Maaske, A., Stab, V., Heinecke, V., Watzstedt, K., Heß, R., Westendorf, A. M., Bayer, W., Ehrhardt, C., & Tenbusch, M. (2018). IL-1β as mucosal vaccine adjuvant: The specific induction of tissue-resident memory T cells improves the heterosubtypic immunity against influenza A viruses article. *Mucosal Immunology*, *11*(4), 1265–1278. https://doi.org/10.1038/s41385-018-0017-4

Lau, A. H., & Thomson, A. W. (2003). Dendritic cells and immune regulation in the liver. *Gut*, *52*(2), 307–314. https://doi.org/10.1136/gut.52.2.307

Lau, L. S., Fernandez-Ruiz, D., Mollard, V., Sturm, A., Neller, M. A., Cozijnsen, A., Gregory, J. L., Davey, G. M., Jones, C. M., Lin, Y. H., Haque, A., Engwerda, C. R., Nie, C. Q., Hansen, D. S., Murphy, K. M., Papenfuss, A. T., Miles, J. J., Burrows, S. R., de Koning-Ward, T., … Heath, W. R. (2014). CD8+ T Cells from a Novel T Cell Receptor Transgenic Mouse Induce Liver-Stage Immunity That Can Be Boosted by Blood-Stage Infection in Rodent Malaria. *PLoS Pathogens*, *10*(5). https://doi.org/10.1371/journal.ppat.1004135

Lau, L. S., Fernandez Ruiz, D., Davey, G. M., De Koning-Ward, T. F., Papenfuss, A. T., Carbone, F. R., Brooks, A. G., Crabb, B. S., & Heath, W. R. (2011). Blood-stage plasmodium berghei infection generates a potent, specific CD8+ T-cell response despite residence largely in cells lacking MHC i processing machinery. *Journal of Infectious Diseases*, *204*(12), 1989–1996. https://doi.org/10.1093/infdis/jir656

Lauer, K. B., Borrow, R., & Blancharda, T. J. (2017). Multivalent and multipathoven viral vector vaccines. *Clinical and Vaccine Immunology*, *24*(1), 1–15.

Leiriao, P., Mota, M. M., & Rodriguez, A. (2005). Apoptotic Plasmodium- Infected Hepatocytes Provide Antigens to Liver Dendritic Cells . *The Journal of Infectious Diseases*, *191*(10), 1576–1581. https://doi.org/10.1086/429635

Li, J., Ahmet, F., Sullivan, L. C., Brooks, A. G., Kent, S. J., De Rose, R., Salazar, A. M., Reis e Sousa, C., Shortman, K., Lahoud, M. H., Heath, W. R., & Caminschi, I. (2015). Antibodies targeting Clec9A promote strong humoral immunity without adjuvant in mice and non-human primates. *European Journal of Immunology*, *45*(3), 854–864. https://doi.org/10.1002/eji.201445127

Li, S., Rodrigues, M., Rodriguez, D., Rodriguez, J. R., Esteban, M., Palese, P.,

Nussenzweig, R. S., & Zavala, F. (1993). Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(11), 5214–5218. https://doi.org/10.1073/pnas.90.11.5214

Li, W. M., Dragowska, W. H., Bally, M. B., & Schutze-Redelmeier, M. P. (2003). Effective induction of CD8+ T-cell response using CpG oligodeoxynucleotides and HER-2/neu-derived peptide co-encapsulated in liposomes. *Vaccine*, *21*(23), 3319–3329. https://doi.org/10.1016/S0264-410X(03)00172-5

Li, X.-D., Wu, J., Gao, D., Wang, H., Sun, L., & Chen, Z. J. (2013). Pivotal Roles of cGAS-cGAMP Signaling in Antiviral Defense and Immune Adjuvant Effects. *Science*, *341*(6152), 1–7. https://doi.org/10.1038/jid.2014.371

Lichtman, S. N., Wang, J., & Lemasters, J. J. (1998). LPS receptor CD14 participates in release of TNF-α in RAW 264.7 and peritoneal cells but not in Kupffer cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *275*(1 38-1), 39–46. https://doi.org/10.1152/ajpgi.1998.275.1.g39

Lieberman, M. D., Sigal, R. K., Williams, N. N., & Daly, J. M. (1991). Natural killer cell stimulatory factor (NKSF) augments natural killer cell and antibody-dependent tumoricidal response against colon carcinoma cell lines. *Journal of Surgical Research*, *50*(4), 410–415. https://doi.org/10.1016/0022-4804(91)90211-4

Liehl, P., Zuzarte-Luís, V., Chan, J., Zillinger, T., Baptista, F., Carapau, D., Konert, M., Hanson, K. K., Carret, C., Lassnig, C., Müller, M., Kalinke, U., Saeed, M., Chora, A. F., Golenbock, D. T., Strobl, B., Prudêncio, M., Coelho, L. P., Kappe, S. H., … Mota, M. M. (2014). Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection. *Nature Medicine*, *20*(1), 47–53. https://doi.org/10.1038/nm.3424

Lindblad, E. B. (2004). Aluminium compounds for use in vaccines. *Immunology and Cell Biology*, *82*(5), 497–505. https://doi.org/10.1111/j.0818-9641.2004.01286.x

Lindner, S. E., Swearingen, K. E., Harupa, A., Vaughan, A. M., Sinnis, P., Moritz, R. L., & Kappe, S. H. I. (2013). Total and putative surface proteomics of malaria parasite salivary gland sporozoites. *Molecular and Cellular Proteomics*, *12*(5), 1127–1143. https://doi.org/10.1074/mcp.M112.024505

Linehan, M. M., Dickey, T. H., Molinari, E. S., Fitzgerald, M. E., Potapova, O., Iwasaki, A., & Pyle, A. M. (2018). A minimal RNA ligand for potent RIG-I activation in living mice. *Science Advances*, *4*(2), 1–10. https://doi.org/10.1126/sciadv.1701854

Longhi, M. P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A. M., Colonna, M., & Steinman, R. M. (2009). Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *Journal of Experimental Medicine*, *206*(7), 1589–1602. https://doi.org/10.1084/jem.20090247

Longley, R. J., Hill, A. V. S., & Spencer, A. J. (2015). Malaria vaccines: Identifying Plasmodium falciparum liver-stage targets. *Frontiers in Microbiology*, *6*(SEP), 1–8. https://doi.org/10.3389/fmicb.2015.00965

Loo, Y. M., & Gale, M. (2011). Immune Signaling by RIG-I-like Receptors. *Immunity*, *34*(5), 680–692. https://doi.org/10.1016/j.immuni.2011.05.003

Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., & Flavell, R. A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(15), 5598–5603. https://doi.org/10.1073/pnas.0400937101

Mackay, L. K., Minnich, M., Kragten, N. A. M., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F. M., Stark, R., Pellicci, D. G., Godfrey, D. I., Belz, G. T., Pellegrini, M., Gebhardt, T., … Van Gisbergen, K. P. J. M. (2016). Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science*, *352*(6284), 459–463. https://doi.org/10.1126/science.aad2035

Mackay, L. K., Stock, A. T., Ma, J. Z., Jones, C. M., Kent, S. J., Mueller, S. N., Heath, W. R., Carbone, F. R., & Gebhardt, T. (2012). Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(18), 7037–7042. https://doi.org/10.1073/pnas.1202288109

Mackay, L. K., Wakim, L., van Vliet, C. J., Jones, C. M., Mueller, S. N., Bannard, O., Fearon, D. T., Heath, W. R., & Carbone, F. R. (2012). Maintenance of T Cell Function in the Face of Chronic Antigen Stimulation and Repeated Reactivation for a Latent Virus Infection. *The Journal of Immunology*, *188*(5), 2173–2178. https://doi.org/10.4049/jimmunol.1102719

MacKintosh, C. L., Beeson, J. G., & Marsh, K. (2004). Clinical features and pathogenesis of severe malaria. *Trends in Parasitology*, *20*(12), 597–603. https://doi.org/10.1016/j.pt.2004.09.006

MacLeod, M. K. L., McKee, A. S., David, A., Wang, J., Mason, R., Kappler, J. W., & Marrack, P. (2011). Vaccine adjuvants aluminum and monophosphoryl lipid A provide distinct signals to generate protective cytotoxic memory CD8 T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(19), 7914–7919.

https://doi.org/10.1073/pnas.1104588108

MacPherson, G. G., Warrell, M. J., White, N. J., & Looareesuwan, S. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *American Journal of Pathology*, *119*(3), 385–401.

Madhun, A. S., Haaheim, L. R., Nøstbakken, J. K., Ebensen, T., Chichester, J., Yusibov, V., Guzman, C. A., & Cox, R. J. (2011). Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 CD4+ cells and strong mucosal and systemic antibody responses in mice. *Vaccine*, *29*(31), 4973–4982. https://doi.org/10.1016/j.vaccine.2011.04.094

Magistrado, P. A., Lusingu, J., Vestergaard, L. S., Lemnge, M., Lavstsen, T., Turner, L., Hviid, L., Jensen, A. T. R., & Theander, T. G. (2007). Immunoglobulin G antibody reactivity to a group A Plasmodium falciparum erythrocyte membrane protein 1 and protection from P. falciparum malaria. *Infection and Immunity*, *75*(5), 2415–2420. https://doi.org/10.1128/IAI.00951-06

Maier, A. G., Cooke, B. M., Cowman, A. F., & Tilley, L. (2009). Malaria parasite proteins that remodel the host erythrocyte. *Nature Reviews Microbiology*, *7*(5), 341–354. https://doi.org/10.1038/nrmicro2110

Maitland, K., & Newton, C. R. J. C. (2005). Acidosis of severe falciparum malaria: Heading for a shock? *Trends in Parasitology*, *21*(1), 11–16. https://doi.org/10.1016/j.pt.2004.10.010

Malkin, E., Hu, J., Li, Z., Chen, Z., Bi, X., Reed, Z., Dubovsky, F., Liu, J., Wang, Q., Pan, X., Chen, T., Giersing, B., Xu, Y., Kang, X., Gu, J., Shen, Q., Tucker, K., Tierney, E., Pan, W., … Cao, Z. (2008). A Phase 1 trial of PfCP2.9: An AMA1/MSP1 chimeric recombinant protein vaccine for Plasmodium falciparum malaria. *Vaccine*, *26*(52), 6864–6873. https://doi.org/10.1016/j.vaccine.2008.09.081

Manish, M., Rahi, A., Kaur, M., Bhatnagar, R., & Singh, S. (2013). A Single-Dose PLGA Encapsulated Protective Antigen Domain 4 Nanoformulation Protects Mice against Bacillus anthracis Spore Challenge. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0061885

Markus, M. B. (2011). Malaria: Origin of the Term “Hypnozoite.” *Journal of the History of Biology*, *44*(4), 781–786. https://doi.org/10.1007/s10739-010-9239-3

Marsh, K., & Kinyanjui, S. (2006). Immune effector mechanisms in malaria. *Parasite Immunology*, *28*(1–2), 51–60. https://doi.org/10.1111/j.1365-3024.2006.00808.x

Marsh, Kevin, Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P., Peshu, N., Pasvol, G., Snow, R., Marsh, K., Forster, D., Winstanley, P., Warn, P., Pasvol, G., Snow, R., & Newton, C. (1995). Indicators of life-threatening malaria in african children. *New England Journal of Medicine*, *332*(21), 1399–1404. https://doi.org/10.1056/NEJM199505253322102

Marshall, J. D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R. L., & Nest, G. Van. (2003). Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions Abstract : Recent reports have identified two ma- jor classes of CpG motif-containing oligodeoxynu. *Journal of Leukocyte Biology*, *73*(June), 781–792. https://doi.org/10.1189/jlb.1202630.Journal

Martínez-Gil, L., Goff, P. H., Hai, R., García-Sastre, A., Shaw, M. L., & Palese, P. (2013). A Sendai Virus-Derived RNA Agonist of RIG-I as a Virus Vaccine Adjuvant. *Journal of Virology*, *87*(3), 1290–1300. https://doi.org/10.1128/jvi.02338-12

Masopust, D., Choo, D., Vezys, V., Wherry, E. J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K. A., Barber, D. L., Kawamura, K. S., Fraser, K. A., Webby, R. J., Brinkmann, V., Butcher, E. C., Newell, K. A., & Ahmed, R. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. *Journal of Experimental Medicine*, *207*(3), 553–564. https://doi.org/10.1084/jem.20090858

Masopust, D., & Soerens, A. G. (2019). Tissue-Resident T Cells and Other Resident Leukocytes. *Annual Review of Immunology*, *37*(1), 521–546. https://doi.org/10.1146/annurev-immunol-042617-053214

Mata-Haro, V., Cekic, C., Martin, M., Chilton, P. M., Casella, C. R., & Mitchell, T. C. (2007). The Vaccine Adjuvant Monophosphoryl Lipid A as a TRIF-Biased Agonist of TLR4. *Science*, *316*(August), 1628–1633.

Mbogo, C. N. M., Snow, R. W., Kabiru, E. W., Ouma, J. H., Githure, J. I., Marsh, K., & Beier, J. C. (1993). Low-level Plasmodium falciparum transmission and the incidence of severe malaria infections on the kenyan coast. *The American Society of Tropical Medicine and Hygiene*, *49*(2), 245–253.

Mbogo, C. N. M., Snow, R. W., Khamala, C. P. M., Kabiru, E. W., Ouma, J. H., Githure, J. I., Marsh, K., & Beier, J. C. (1995). Relationships between Plasmodium falciparum transmission by vector populations and the incidence of severe disease at nine sites on the Kenyan coast. *The American Society of Tropical Medicine and Hygiene*, *52*(3), 201–206.

McCarthy, J. S., Marjason, J., Elliott, S., Fahey, P., Bang, G., Malkin, E., Tierney, E., Aked-Hurditch, H., Adda, C., Cross, N., Richards, J. S., Fowkes, F. J. I., Boyle, M. J., Long, C., Druilhe, P., Beeson, J. G., & Anders, R. F. (2011). A phase 1 trial of MSP2-C1, a blood-stage malaria vaccine containing 2

isoforms of MSP2 formulated with montanide® ISA 720. *PLoS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0024413

McLane, L. M., Abdel-Hakeem, M. S., & Wherry, E. J. (2019). CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annual Review of Immunology*, *37*(1), 457–495. https://doi.org/10.1146/annurev-immunol-041015-055318

McNamara, H. A., Cai, Y., Wagle, M. V., Sontani, Y., Roots, C. M., Miosge, L. A., O'Connor, J. H., Sutton, H. J., Ganusov, V. V., Heath, W. R., Bertolino, P., Goodnow, C. G., Parish, I. A., Enders, A., & Cockburn, I. A. (2017). Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids. *Science Immunology*, *2*(9). https://doi.org/10.1126/sciimmunol.aaj1996

Meis, J. F. G. M., Verhave, J. P., Jap, P. H. K., Sinden, R. E., & Meuwissen, J. H. E. T. (1983). Malaria parasites - Discovery of the early liver form. *Nature*, *302*(5907), 424–426. https://doi.org/10.1038/302424a0

Mencin, A., Kluwe, J., & Schwabe, R. F. (2009). Toll-like receptors as targets in chronic liver diseases. *Gut*, *58*(5), 704–720. https://doi.org/10.1136/gut.2008.156307

Mensah, V. A., Gueye, A., Ndiaye, M., Edwards, N. J., Wright, D., Anagnostou, N. A., Syll, M., Ndaw, A., Abiola, A., Bliss, C., Gomis, J. F., Petersen, I., Ogwang, C., Dieye, T., Viebig, N. K., Lawrie, A. M., Roberts, R., Nicosia, A., Faye, B., … Cisse, B. (2016). Safety, immunogenicity and efficacy of prime-Boost vaccination with chad63 and mva encoding me-trap against plasmodium falciparum infection in adults in senegal. *PLoS ONE*, *11*(12), 1–16. https://doi.org/10.1371/journal.pone.0167951

Mesén-Ramírez, P., Bergmann, B., Tran, T. T., Garten, M., Stäcker, J., Naranjo-Prado, I., Höhn, K., Zimmerberg, J., & Spielmann, T. (2019). EXP1 is critical for nutrient uptake across the parasitophorous vacuole membrane of malaria parasites. In *PLoS Biology* (Vol. 17, Issue 9). https://doi.org/10.1371/journal.pbio.3000473

Mikolajczak, S. A., Lakshmanan, V., Fishbaugher, M., Camargo, N., Harupa, A., Kaushansky, A., Douglass, A. N., Baldwin, M., Healer, J., O'neill, M., Phuong, T., Cowman, A., & Kappe, S. H. I. (2014). A Next-generation genetically attenuated plasmodium falciparum parasite created by triple gene deletion. *Molecular Therapy*, *22*(9), 1707–1715. https://doi.org/10.1038/mt.2014.85

Mikolajczak, S. A., Vaughan, A. M., Kangwanrangsan, N., Roobsoong, W., Fishbaugher, M., Yimamnuaychok, N., Rezakhani, N., Lakshmanan, V., Singh, N., Kaushansky, A., Camargo, N., Baldwin, M., Lindner, S. E., Adams, J. H., Sattabongkot, J., & Kappe, S. H. I. (2015). Plasmodium vivax liver stage development and hypnozoite persistence in human liver-

chimeric mice. *Cell Host and Microbe*, *17*(4), 526–535. https://doi.org/10.1016/j.chom.2015.02.011

Miller, J. L., Sack, B. K., Baldwin, M., Vaughan, A. M., & Kappe, S. H. I. (2014). Interferon-Mediated Innate Immune Responses against Malaria Parasite Liver Stages. *Cell Reports*, *7*(2), 436–447. https://doi.org/10.1016/j.celrep.2014.03.018

Minang, J. T., Inglefield, J. R., Harris, A. M., Lathey, J. L., Alleva, D. G., Sweeney, D. L., Hopkins, R. J., Lacy, M. J., & Bernton, E. W. (2014). Enhanced early innate and T cell-mediated responses in subjects immunized with Anthrax Vaccine Adsorbed Plus CPG 7909 (AV7909). *Vaccine*, *32*(50), 6847–6854. https://doi.org/10.1016/j.vaccine.2014.01.096

Miura, K. (2016). Progress and prospects for blood-stage malaria vaccines. *Expert Review of Vaccines*, *15*(6), 765–781. https://doi.org/10.1586/14760584.2016.1141680

Miura, K., Keister, D. B., Muratova, O. V., Sattabongkot, J., Long, C. A., & Saul, A. (2007). Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. *Malaria Journal*, *6*, 1–13. https://doi.org/10.1186/1475-2875-6-107

Monney, L., Sabatos, C. A., Gaglia, J. L., Ryu, A., Waldner, H., Chernova, T., Manning, S., Greenfield, E. A., Coyle, A. J., Sobel, R. A., Freeman, G. J., & Kuchroo, V. K. (2002). Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*, *415*(6871), 536–541. https://doi.org/10.1038/415536a

Montagna, G. N., Beigier-Bompadre, M., Becker, M., Kroczek, R. A., Kaufmann, S. H. E., & Matuschewsk, K. (2014). Antigen export during liver Infection of the malaria parasite augments protective Immunity. *MBio*, *5*(4), 1–11. https://doi.org/10.1128/mBio.01321-14

Moon, J. J., Chu, H. H., Pepper, M., McSorley, S. J., Jameson, S. C., Kedl, R. M. M., & Jenkins, M. K. (2007). Naive CD4+ T Cell Frequency Varies for Different Epitopes and Predicts Repertoire Diversity and Response Magnitude. *Immunity*, *27*(2), 203–213. https://doi.org/10.1016/j.immuni.2007.07.007

Moon, J. J., Suh, H., Bershteyn, A., Stephan, M. T., Liu, H., Huang, B., Sohail, M., Luo, S., Ho Um, S., Khant, H., Goodwin, J. T., Ramos, J., Chiu, W., & Irvine, D. J. (2011). Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. *Nat Mater*, *10*(3), 243–251. https://doi.org/10.1016/j.physbeh.2017.03.040

Moon, J. J., Suh, H., Polhemus, M. E., Ockenhouse, C. F., Yadava, A., & Irvine, D. J. (2012). Antigen-displaying lipid-enveloped PLGA nanoparticles as delivery agents for a Plasmodium vivax malaria vaccine. *PLoS ONE*, *7*(2).

https://doi.org/10.1371/journal.pone.0031472

Mordmüller, B., Surat, G., Lagler, H., Chakravarty, S., Ishizuka, A. S., Lalremruata, A., Gmeiner, M., Campo, J. J., Esen, M., Ruben, A. J., Held, J., Calle, C. L., Mengue, J. B., Gebru, T., Ibáñez, J., Sulyok, M., James, E. R., Billingsley, P. F., Natasha, K., … Kremsner, P. G. (2017). Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature*, *542*(7642), 445–449. https://doi.org/10.1038/nature21060

Moreira, C. K., Templeton, T. J., Lavazec, C., Hayward, R. E., Hobbs, C. V., Kroeze, H., Janse, C. J., Waters, A. P., Sinnis, P., & Coppi, A. (2008). The Plasmodium TRAP/MIC2 family member, TRAP-Like Protein (TLP), is involved in tissue traversal by sporozoites. *Cellular Microbiology*, *10*(7), 1505–1516. https://doi.org/10.1111/j.1462-5822.2008.01143.x

Morel, S., Didierlaurent, A., Bourguignon, P., Delhaye, S., Baras, B., Jacob, V., Planty, C., Elouahabi, A., Harvengt, P., Carlsen, H., Kielland, A., Chomez, P., Garçon, N., & Van Mechelen, M. (2011). Adjuvant System AS03 containing α-tocopherol modulates innate immune response and leads to improved adaptive immunity. *Vaccine*, *29*(13), 2461–2473. https://doi.org/10.1016/j.vaccine.2011.01.011

Mota, M. M., Prade, G., Vanderberg Jerome P., Hafalla Julius C. R., Frevert Ute, Nussenzweig Ruth S., Nussenzweig Victor, & Rodriguez., A. (2001). Migration of Plasmodium Sporozoites Through Cells Before Infection. *Science*, *291*(5501), 141. http://search.ebscohost.com/login.aspx?direct=true&db=ehh&AN=4019612&site=ehost-live

Mridha, A. R., Haczeyni, F., Yeh, M. M., Haigh, W. G., Ioannou, G. N., Barn, V., Ajamieh, H., Adams, L., Hamdorf, J. M., Teoh, N. C., & Farrell, G. C. (2017). TLR9 is up-regulated in human and murine NASH: Pivotal role in inflammatory recruitment and cell survival. *Clinical Science*, *131*(16), 2145–2159. https://doi.org/10.1042/CS20160838

Mueller, A.-K., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U., Matuschewski, K., & Kappe, S. H. I. (2005). Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(8), 3022–3027. https://doi.org/10.1073/pnas.0408442102

Mueller, A.-K., Deckert, M., Heiss, K., Goetz, K., Matuschewski, K., & Schlüter, D. (2007). Genetically attenuated Plasmodium berghei liver stages persist and elicit sterile protection primarily via CD8 T cells. *American Journal of Pathology*, *171*(1), 107–115. https://doi.org/10.2353/ajpath.2007.060792

Mueller, S. N., & Mackay, L. K. (2016). Tissue-resident memory T cells: Local specialists in immune defence. *Nature Reviews Immunology*, *16*(2), 79–89. https://doi.org/10.1038/nri.2015.3

Mullen, G. E. D., Ellis, R. D., Miura, K., Malkin, E., Nolan, C., Hay, M., Fay, M. P., Saul, A., Zhu, D., Rausch, K., Moretz, S., Zhou, H., Long, C. A., Miller, L. H., & Treanor, J. (2008). Phase 1 trial of AMA1-C1/Alhydrogel plus CPG 7909: An asexual blood-stage vaccine for Plasmodium falciparum malaria. *PLoS ONE*, *3*(8). https://doi.org/10.1371/journal.pone.0002940

Müller, H. M., Reckmann, I., Hollingdale, M. R., Bujard, H., Robson, K. J., & Crisanti, A. (1993). Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. *The EMBO Journal*, *12*(7), 2881–2889. https://doi.org/10.1002/j.1460-2075.1993.tb05950.x

Muraoka, D., Kato, T., Wang, L., Maeda, Y., Noguchi, T., Harada, N., Takeda, K., Yagita, H., Guillaume, P., Luescher, I., Old, L. J., Shiku, H., & Nishikawa, H. (2010). Peptide Vaccine Induces Enhanced Tumor Growth Associated with Apoptosis Induction in CD8 + T Cells . *The Journal of Immunology*, *185*(6), 3768–3776. https://doi.org/10.4049/jimmunol.0903649

Nahrevanian, H., & Dascombe, M. J. (2001). Nitric oxide and reactive nitrogen intermediates during lethal and nonlethal strains of murine malaria. *Parasite Immunology*, *23*(9), 491–501. https://doi.org/10.1046/j.1365-3024.2001.00406.x

Nakagawa, R., Motoki, K., Ueno, H., Iijima, R., Nakamura, H., Kobayashi, E., Shimosaka, A., & Koezuka, Y. (1998). Treatment of hepatic metastasis of the Colon26 adenocarcinoma with an α-galactosylceramide, KRN7000. *Cancer Research*, *58*(6), 1202–1207.

Nardin, E. H., Oliveira, G. A., Calvo-Calle, J. M., Wetzel, K., Maier, C., Birkett, A. J., Sarpotdar, P., Corado, M. L., Thornton, G. B., & Schmidt, A. (2004). Phase I testing of a malaria vaccine composed of hepatitis B virus core particles expressing Plasmodium falciparum circumsporozoite epitopes. *Infection and Immunity*, *72*(11), 6519–6527. https://doi.org/10.1128/IAI.72.11.6519-6527.2004

Naso, M. F., Tomkowicz, B., Perry, W. L., & Strohl, W. R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs*, *31*(4), 317–334. https://doi.org/10.1007/s40259-017-0234-5

Nganou-Makamdop, K., van Gemert, G. J., Arens, T., Hermsen, C. C., & Sauerwein, R. W. (2012). Long term protection after immunization with p. berghei sporozoites correlates with sustained IFNγ responses of hepatic CD8+ memory t cells. *PLoS ONE*, *7*(5), 1–9. https://doi.org/10.1371/journal.pone.0036508

NIH. (2020). *ClinicalTrials.gov*. National Institutes of Health, U.S. National Library of Medicine. https://clinicaltrials.gov/

Nosten, F., McGready, R., Simpson, J. A., Thwai, K. L., Balkan, S., Cho, T., Hkirijaroen, L., Looareesuwan, S., & White, N. J. (1999). Effects of Plasmodium vivax malaria in pregnancy. *Lancet*, *354*(9178), 546–549. https://doi.org/10.1016/S0140-6736(98)09247-2

Nussenzweig, R. S. S., Vanderberg, J., Most, H., & Orton, C. (1967). Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei. *Nature*, *216*(5111), 160–162. https://doi.org/10.1038/216160a0

Nussenzweig, V., & Nussenzweig, R. S. (1985). Circumsporozoite proteins of malaria parasites. *Cell*, *42*, 401–403.

O'Hagan, D. T., Ott, G. S., Van Nest, G., Rappuoli, R., & Del Giudice, G. (2013). The history of MF59® adjuvant: A phoenix that arose from the ashes. *Expert Review of Vaccines*, *12*(1), 13–30. https://doi.org/10.1586/erv.12.140

Obar, J. J., Khanna, K. M., & Lefrançois, L. (2008). Primary and Memory Responses to Infection. *Immunity*, *28*(6), 859–869. https://doi.org/10.1016/j.immuni.2008.04.010.Endogenous

Obeid, M., Franetich, J. F., Lorthiois, A., Gego, A., Grüner, A. C., Tefit, M., Boucheix, C., Snounou, G., & Mazier, D. (2013). Skin-draining lymph node priming is sufficient to induce sterile immunity against pre-erythrocytic malaria. *EMBO Molecular Medicine*, *5*(2), 250–263. https://doi.org/10.1002/emmm.201201677

Ofori, M. F., Dodoo, 1 Daniel, Staalsoe, T., Kurtzhals, J. A. L., Koram, K., Theander, T. G., Akanmori, B. D., & Hviid, L. (2002). Malaria-induced acquisition of antibodies to Plasmodium falciparum Variant Surface Antigens. *Infection and Immunity*, *70*(6), 2982–2988. https://doi.org/10.1128/IAI.70.6.2982

Ogunniyi, A. D., Paton, J. C., Kirby, A. C., McCullers, J. A., Cook, J., Hyodo, M., Hayakawa, Y., & Karaolis, D. K. R. (2008). c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. *Vaccine*, *26*(36), 4676–4685. https://doi.org/10.1016/j.vaccine.2008.06.099

Ogwang, C., Kimani, D., Edwards, N. J., Roberts, R., Mwacharo, J., Bowyer, G., Bliss, C., Hodgson, S. H., Njuguna, P., Viebig, N. K., Nicosia, A., Gitau, E., Douglas, S., Illingworth, J., Marsh, K., Lawrie, A., Imoukhuede, E. B., Ewer, K., Urban, B. C., … Ogutu, B. (2015). Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against Plasmodium falciparum infection in Kenyan adults. *Science Translational Medicine*, *7*(286), 1–20. https://doi.org/10.1126/scitranslmed.aaa2373

Ohashi, J., Suzuki, Y., Naka, I., Hananantachai, H., & Patarapotikul, J. (2014). Diversifying selection on the thrombospondin-related adhesive protein (TRAP) gene of plasmodium falciparum in Thailand. *PLoS ONE*, *9*(2). https://doi.org/10.1371/journal.pone.0090522

Okazaki, T., & Honjo, T. (2006). The PD-1-PD-L pathway in immunological tolerance. *Trends in Immunology*, *27*(4), 195–201. https://doi.org/10.1016/j.it.2006.02.001

Okiro, E. A., Al-Taiar, A., Reyburn, H., Idro, R., Berkley, J. A., & Snow, R. W. (2009). Age patterns of severe paediatric malaria and their relationship to Plasmodium falciparum transmission intensity. *Malaria Journal*, *8*(1), 1–11. https://doi.org/10.1186/1475-2875-8-4

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Awuondo, K. O., Leach, A., Lievens, M., Leboulleux, D., Njuguna, P., Peshu, N., Marsh, K., & Bejon, P. (2013). Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *New England Journal of Medicine*, *368*(12), 1111–1120. https://doi.org/10.1056/NEJMoa1207564

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., Kaslow, D. C., Njuguna, P., Marsh, K., & Bejon, P. (2016). Seven-year efficacy of RTS, S/AS01 malaria vaccine among young african children. *New England Journal of Medicine*, *374*(26), 2519–2529. https://doi.org/10.1056/NEJMoa1515257

Olotu, A., Urbano, V., Hamad, A., Eka, M., Chemba, M., Nyakarungu, E., Raso, J., Eburi, E., Mandumbi, D. O., Hergott, D., Maas, C. D., Ayekaba, M. O., Milang, D. N., Rivas, M. R., Schindler, T., Embon, O. M., Ruben, A. J., Saverino, E., Abebe, Y., … Hoffman, S. L. (2018). Advancing global health through development and clinical trials partnerships: A randomized, placebo-controlled, double-blind assessment of safety, tolerability, and immunogenicity of pfspz vaccine for malaria in healthy equatoguinean men. *American Journal of Tropical Medicine and Hygiene*, *98*(1), 308–318. https://doi.org/10.4269/ajtmh.17-0449

Orito, Y., Ishino, T., Iwanaga, S., Kaneko, I., Kato, T., Menard, R., Chinzei, Y., & Yuda, M. (2013). Liver-specific protein 2: A Plasmodium protein exported to the hepatocyte cytoplasm and required for merozoite formation. *Molecular Microbiology*, *87*(1), 66–79. https://doi.org/10.1111/mmi.12083

Overland, G., Stuestøl, J. F., Dahle, M. K., Myhre, A. E., Netea, M. G., Verweij, P., Yndestad, A., Aukrust, P., Kullberg, B. J., Warris, A., Wang, J. E., & Aasen, A. O. (2005). Cytokine responses to fungal pathogens in Kupffer cells are toll-like receptor 4 independent and mediated by tyrosine kinases. *Scandinavian Journal of Immunology*, *62*(2), 148–154. https://doi.org/10.1111/j.1365-3083.2005.01653.x

Overstreet, M. G., Chen, Y. C., Cockburn, I. A., Tse, S. W., & Zavala, F. (2011).

CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites. *PLoS ONE*, *6*(1). https://doi.org/10.1371/journal.pone.0015948

Owusu-Agyei, S., Koram, K. A., Baird, J. K., Utz, G. C., Binka, F. N., Nkrumah, F. K., Fryauff, D. J., & Hoffman, S. L. (2001). Incidence of symptomatic and asymptomatic Plasmodium falciparum infection following curative therapy in adult residents of northern Ghana. *American Journal of Tropical Medicine and Hygiene*, *65*(3), 197–203. https://doi.org/10.4269/ajtmh.2001.65.197

Oyen, D., Torres, J. L., Wille-Reece, U., Ockenhouse, C. F., Emerling, D., Glanville, J., Volkmuth, W., Flores-Garcia, Y., Zavala, F., Ward, A. B., Richter King, C., & Wilson, I. A. (2017). Structural basis for antibody recognition of the NANP repeats in Plasmodium falciparum circumsporozoite protein. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(48), E10438–E10445. https://doi.org/10.1073/pnas.1715812114

Pallett, L. J., Davies, J., Colbeck, E. J., Robertson, F., Hansi, N., Easom, N. J. W., Burton, A. R., Stegmann, K. A., Schurich, A., Swadling, L., Gill, U. S., Male, V., Luong, T. V., Gander, A., Davidson, B. R., Kennedy, P. T. F., & Maini, M. K. (2017). IL-2high tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. *Journal of Experimental Medicine*, *214*(6), 1567–1580. https://doi.org/10.1084/jem.20162115

Palucka, K., Banchereau, J., & Mellman, I. (2010). Designing vaccines based on biology of human dendritic cell subsets. *Immunity*, *33*(4), 464–478. https://doi.org/10.1016/j.immuni.2010.10.007

Park, C. O., Fu, X., Jiang, X., Pan, Y., Teague, J. E., Collins, N., Tian, T., O'Malley, J. T., Emerson, R. O., Kim, J. H., Jung, Y., Watanabe, R., Fuhlbrigge, R. C., Carbone, F. R., Gebhardt, T., Clark, R. A., Lin, C. P., & Kupper, T. S. (2018). Staged development of long-lived T-cell receptor αβ TH17 resident memory T-cell population to Candida albicans after skin infection. *Journal of Allergy and Clinical Immunology*, *142*(2), 647–662. https://doi.org/10.1016/j.jaci.2017.09.042

Park, H.-Y., Light, A., Lahoud, M. H., Caminschi, I., Tarlinton, D. M., & Shortman, K. (2013). Evolution of B Cell Responses to Clec9A-Targeted Antigen. *The Journal of Immunology*, *191*(10), 4919–4925. https://doi.org/10.4049/jimmunol.1301947

Park, H. Y., Tan, P. S., Kavishna, R., Ker, A., Lu, J., Chan, C. E. Z., Hanson, B. J., MacAry, P. A., Caminschi, I., Shortman, K., Alonso, S., & Lahoud, M. H. (2017). Enhancing vaccine antibody responses by targeting Clec9A on dendritic cells. *Npj Vaccines*, *2*(1). https://doi.org/10.1038/s41541-017-0033-5

Paul, A. S., Egan, E. S., & Duraisingha, M. T. (2015). Host-parasite interactions that guide red blood cell invasion by malaria parasites. *Current Opinion in Hematology*, *22*(3), 220–226. https://doi.org/10.1097/MOH.0000000000000135.Host-parasite

Payne, R. O., Silk, S. E., Elias, S. C., Miura, K., Diouf, A., Galaway, F., De Graaf, H., Brendish, N. J., Poulton, I. D., Griffiths, O. J., Edwards, N. J., Jin, J., Labbé, G. M., Alanine, D. G. W., Siani, L., Marco, S. Di, Roberts, R., Green, N., Berrie, E., … Draper, S. J. (2017). Human vaccination against RH5 induces neutralizing antimalarial antibodies that inhibit RH5 invasion complex interactions. *JCI Insight*, *2*(21), 1–19. https://doi.org/10.1172/jci.insight.96381

Perdomo, C., Zedler, U., Kühl, A. A., Lozza, L., Saikali, P., Sander, L. E., Vogelzang, A., Kaufmann, S. H. E., & Kupz, A. (2016). Mucosal BCG vaccination induces protective lung-resident memory T cell populations against tuberculosis. *MBio*, *7*(6), 1–11. https://doi.org/10.1128/mBio.01686-16

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B., & Ong'echa, J. M. (2011). Severe malarial anemia: Innate immunity and pathogenesis. *International Journal of Biological Sciences*, *7*(9), 1427–1442. https://doi.org/10.7150/ijbs.7.1427

Petitdemange, C., Kasturi, S. P., Kozlowski, P. A., Nabi, R., Quarnstrom, C. F., Reddy, P. B. J., Derdeyn, C. A., Spicer, L. M., Patel, P., Legere, T., Kovalenkov, Y. O., Labranche, C. C., Villinger, F., Tomai, M., Vasilakos, J., Haynes, B., Kang, C. Y., Gibbs, J. S., Yewdell, J. W., … Pulendran, B. (2019). Vaccine induction of antibodies and tissue-resident CD8+ T cells enhances protection against mucosal SHIV-infection in young macaques. *JCI Insight*, *4*(4), 1–20. https://doi.org/10.1172/jci.insight.126047

Phillips, M. A., Burrows, J. N., Manyando, C., Van Huijsduijnen, R. H., Van Voorhis, W. C., & Wells, T. N. C. (2017). Malaria. *Nature Reviews Disease Primers*, *3*. https://doi.org/10.1038/nrdp.2017.50

Phillips, R. E., Looareesuwan, S., Warrell, D. A., Lee, S. H., Karbwang, J., Warrell, M. J., White, N. J., Swasdichais, C., & Weatherall, D. J. (1986). The importance of anaemia in cerebral and uncomplicated falciparum malaria: Role of complications, dyserythropoiesis and iron sequestration. *Quarterly Journal of Medicin*, *58*(3–4), 305–323. https://doi.org/10.1093/oxfordjournals.qjmed.a067960

Pichugin, A., Zarling, S., Perazzo, L., Duffy, P. E., Ploegh, H. L., & Krzych, U. (2018). Identification of a novel CD8 T cell epitope derived from Plasmodium berghei protective liver-stage antigen. In *Frontiers in Immunology* (Vol. 9, Issue JAN). https://doi.org/10.3389/fimmu.2018.00091

Pierce, M. A., Ellis, R. D., Martin, L. B., Malkin, E., Tierney, E., Miura, K., Fay, M. P., Marjason, J., Elliott, S. L., Mullen, G. E. D., Rausch, K., Zhu, D., Long, C. A., & Miller, L. H. (2010). Phase 1 safety and immunogenicity trial of the Plasmodium falciparum blood-stage malaria vaccine AMA1-C1/ISA 720 in Australian adults. *Vaccine*, *28*(10), 2236–2242. https://doi.org/10.1016/j.vaccine.2009.12.049

Pimentel-Nunes, P., Soares, J. B., Roncon-Albuquerque, R., Dinis-Ribeiro, M., & Leite-Moreira, A. F. (2010). Toll-like receptors as therapeutic targets in gastrointestinal diseases. *Expert Opinion on Therapeutic Targets*, *14*(4), 347–368. https://doi.org/10.1517/14728221003642027

Pinzon-Ortiz, C., Friedman, J., Esko, J., & Sinnis, P. (2001). The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for Plasmodium sporozoite attachment to target cells. *The Journal of Biological Chemistry*, *276*(29), 26784–26791. https://doi.org/10.1038/jid.2014.371

Pongponratn, E., Turner, G. D. H., Day, N. P. J., Phu, N. H., Simpson, J. A., Stepniewska, K., Mai, N. T. H., Viriyavejakul, P., Looareesuwan, S., Hien, T. T., Ferguson, D. J. P., & White, N. J. (2003). An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. *American Journal of Tropical Medicine and Hygiene*, *69*(4), 345–359. https://doi.org/10.4269/ajtmh.2003.69.345

Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., & Reis E Sousa, C. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8α+ dendritic cells. *Journal of Experimental Medicine*, *207*(6), 1261–1271. https://doi.org/10.1084/jem.20092618

Price, R. N., Simpson, J. A., Nosten, F., Luxemburger, C., Hkirjaroen, L., Kuile, F. Ter, Chongsuphajaisiddhi, T., & White, N. J. (2001). Factors contribuiting to anemia after uncomplicated falciparum malaria. *Am J Trop Med Hyg*, *65*(5), 614–622.

Prudêncio, M., Rodriguez, A., & Mota, M. M. (2006). The silent path to thousands of merozoites: The Plasmodium liver stage. *Nature Reviews Microbiology*, *4*(11), 849–856. https://doi.org/10.1038/nrmicro1529

Pulko, V., Liu, X., Krco, C. J., Harris, K. J., Frigola, X., Kwon, E. D., & Dong, H. (2009). TLR3-Stimulated Dendritic Cells Up-regulate B7-H1 Expression and Influence the Magnitude of CD8 T Cell Responses to Tumor Vaccination. *The Journal of Immunology*, *183*(6), 3634–3641. https://doi.org/10.4049/jimmunol.0900974

Radtke, A. J., Anderson, C. F., Riteau, N., Rausch, K., Scaria, P., Kelnhofer, E.

R., Howard, R. F., Sher, A., Germain, R. N., & Duffy, P. (2017). Adjuvant and carrier protein-dependent T-cell priming promotes a robust antibody response against the Plasmodium falciparum Pfs25 vaccine candidate. *Scientific Reports*, *7*(August 2016), 1–12. https://doi.org/10.1038/srep40312

Radtke, A. J., Kastenmüller, W., Espinosa, D. A., Gerner, M. Y., Tse, S. W., Sinnis, P., Germain, R. N., Zavala, F. P., & Cockburn, I. A. (2015). Lymph-Node Resident CD8α+ Dendritic Cells Capture Antigens from Migratory Malaria Sporozoites and Induce CD8+ T Cell Responses. *PLoS Pathogens*, *11*(2), 1–23. https://doi.org/10.1371/journal.ppat.1004637

Raja, A. I., Cai, Y., Reiman, J. M., Groves, P., Chakravarty, S., McPhun, V., Doolan, D. L., Cockburn, I., Hoffman, S. L., Stanisic, D. I., & Good, M. F. (2016). Chemically attenuated blood-stage Plasmodium yoelii parasites induce long-lived and strain-transcending protection. *Infection and Immunity*, *84*(8), 2274–2288. https://doi.org/10.1128/IAI.00157-16

Raja, A. I., Stanisic, D. I., & Good, M. F. (2017). Chemical attenuation in the development of a whole-organism malaria vaccine. *Infection and Immunity*, *85*(7), 1–7. https://doi.org/10.1128/IAI.00062-17

Ramakrishnan, C., Delves, M. J., Lal, K., Blagborough, A. M., Butcher, G., & Baker, K. W. (2012). Laboratory maintenance of rodent malaria parasites. In R. Ménard (Ed.), *Malaria. Methods in Molecular Biology* (Vol. 923, pp. 213–219). Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-26-7_14

Raman, V. S., Duthie, M. S., Fox, C. B., Matlashewski, G., & Reed, S. G. (2012). Adjuvants for Leishmania vaccines: From models to clinical application. *Frontiers in Immunology*, *3*(JUN), 1–15. https://doi.org/10.3389/fimmu.2012.00144

Rampling, T., Ewer, K. J., Bowyer, G., Edwards, N. J., Wright, D., Sridhar, S., Payne, R., Powlson, J., Bliss, C., Venkatraman, N., Poulton, I. D., de Graaf, H., Gbesemete, D., Grobbelaar, A., Davies, H., Roberts, R., Angus, B., Ivinson, K., Weltzin, R., … Hill, A. V. S. (2018). Safety and efficacy of novel malaria vaccine regimens of RTS,S/AS01B alone, or with concomitant ChAd63-MVA-vectored vaccines expressing ME-TRAP. *Npj Vaccines*, *3*(1), 1–9. https://doi.org/10.1038/s41541-018-0084-2

Rathinam, V. A. K., Jiang, Z., Waggoner, S. N., Sharma, S., Cole, L. E., Waggoner, L., Vanaja, S. K., Monks, B. G., Ganesan, S., Latz, E., Hornung, V., Vogel, S. N., Szomolanyi-Tsuda, E., & Fitzgerald, K. A. (2010). The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nature Immunology*, *11*(5), 395–402. https://doi.org/10.1038/ni.1864

Rauch, S., Jasny, E., Schmidt, K. E., & Petsch, B. (2018). New vaccine

technologies to combat outbreak situations. *Frontiers in Immunology*, *9*(SEP). https://doi.org/10.3389/fimmu.2018.01963

Rénia, L., & Goh, Y. S. (2016). Malaria parasites: The great escape. *Frontiers in Immunology*, *7*(NOV), 1–14. https://doi.org/10.3389/fimmu.2016.00463

Reyburn, H., Mbatia, R., Drakeley, C., Bruce, J., Carneiro, I., Olomi, R., Cox, J., Nkya, W. M. M. M., Lemnge, M., Greenwood, B. M., & Riley, E. M. (2005). Association of transmission intensity and age with clinical manifestations and case fatality of severe Plasmodium falciparum malaria. *Journal of the American Medical Association*, *293*(12), 1461–1470. https://doi.org/10.1001/jama.293.12.1461

Reyes-Sandoval, A., Berthoud, T., Alder, N., Siani, L., Gilbert, S. C., Nicosia, A., Colloca, S., Cortese, R., & Hill, A. V. S. (2010). Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. *Infection and Immunity*, *78*(1), 145–153. https://doi.org/10.1128/IAI.00740-09

Ribi, E., Parker, R., Strain, S. M., Mizuno, Y., Nowotny, A., Von Eschen, K. B., Cantrell, J. L., McLaughlin, C. A., Hwang, K. M., & Goren, M. B. (1979). Peptides as requirement for immunotherapy of the guinea-pig line-10 tumor with endotoxins. *Cancer Immunology Immunotherapy*, *7*(1), 43–58. https://doi.org/10.1007/BF00205409

Risco-Castillo, V., Topçu, S., Marinach, C., Manzoni, G., Bigorgne, A. E., Briquet, S., Baudin, X., Lebrun, M., Dubremetz, J. F., & Silvie, O. (2015). Malaria sporozoites traverse host cells within transient vacuoles. *Cell Host and Microbe*, *18*(5), 593–603. https://doi.org/10.1016/j.chom.2015.10.006

Roberts, T. L., Idris, A., Dunn, J. A., Kelly, G. M., Burnton, C. M., Hodgson, S., Hardy, L. L., Garceau, V., Sweet, M. J., Ross, I. L., Hume, D. A., & Stacey, K. J. (2009). HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science*, *323*(5917), 1057–1060. https://doi.org/10.1126/science.1169841

Robson, K. J. H., Hall, J. R. S., Davies, L. C., Crisanti, A., Hill, A. V. S., & Wellems, T. E. (1990). Polymorphism of the TRAP gene of Plasmodium falciparum. *Proceedings of the Royal Society B: Biological Sciences*, *242*(1305), 205–216. https://doi.org/10.1098/rspb.1990.0126

Roca-Feltrer, A., Carneiro, I., Smith, L., Schellenberg, J. R. A., Greenwood, B., & Schellenberg, D. (2010). The age patterns of severe malaria syndromes in sub-Saharan Africa across a range of transmission intensities and seasonality settings. *Malaria Journal*, *9*(1), 1–9. https://doi.org/10.1186/1475-2875-9-282

Rodrigues, C. D., Hannus, M., Prudêncio, M., Martin, C., Gonçalves, L. A.,

Portugal, S., Epiphanio, S., Akinc, A., Hadwiger, P., Jahn-Hofmann, K., Röhl, I., van Gemert, G. J., Franetich, J. F., Luty, A. J. F., Sauerwein, R., Mazier, D., Koteliansky, V., Vornlocher, H. P., Echeverri, C. J., & Mota, M. M. (2008). Host Scavenger Receptor SR-BI Plays a Dual Role in the Establishment of Malaria Parasite Liver Infection. *Cell Host and Microbe*, *4*(3), 271–282. https://doi.org/10.1016/j.chom.2008.07.012

Rodrigues, M. M., Cordey, A. S., Arreaza, G., Corradin, G., Romero, P., Maryanski, J. L., Nussenzweig, R. S., & Zavala, F. (1991). CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria. *International Immunology*, *3*(6), 579–585. https://doi.org/10.1093/intimm/3.6.579

Rodriguez-Barraquer, I., Arinaitwe, E., Jagannathan, P., Kamya, M. R., Rosenthal, P. J., Rek, J., Dorsey, G., Nankabirwa, J., Staedke, S. G., Kilama, M., Drakeley, C., Ssewanyana, I., Smith, D. L., & Greenhouse, B. (2018). Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure. *ELife*, *7*, 1–20. https://doi.org/10.7554/eLife.35832

Roers, A., Hiller, B., & Hornung, V. (2016). Recognition of Endogenous Nucleic Acids by the Innate Immune System. *Immunity*, *44*(4), 739–754. https://doi.org/10.1016/j.immuni.2016.04.002

Rogers, W. O., Malik, A., Mellouk, S., Nakamura, K., Rogers, M. D., Szarfman, A., Gordon, D. M., Nussler, A. K., Aikawa, M., & Hoffman, S. L. (1992). Characterization of Plasmodium falciparum Sporozoite Surface Protein 2 Source. *Proceedings of the National Academy of Sciences*, *89*, 9176–9180. https://doi.org/10.1073/pnas.89.19.9176

Roland, J., Soulard, V., Sellier, C., Drapier, A.-M., Di Santo, J. P., Cazenave, P.-A., & Pied, S. (2006). NK Cell Responses to Plasmodium Infection and Control of Intrahepatic Parasite Development . *The Journal of Immunology*, *177*(2), 1229–1239. https://doi.org/10.4049/jimmunol.177.2.1229

Romero, J. F., Eberl, G., Robson Macdonald, H., & Corradin, G. (2001). CD1d-restricted NK T cells are dispensable for specific antibody responses and protective immunity against liver stage malaria infection in mice. *Parasite Immunology*, *23*(5), 267–269. https://doi.org/10.1046/j.1365-3024.2001.00381.x

Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B. G., Schoenemeyer, A., Yamamoto, M., Akira, S., & Fitzgerald, K. A. (2005). The RNA Helicase Lgp2 Inhibits TLR-Independent Sensing of Viral Replication by Retinoic Acid-Inducible Gene-I. *The Journal of Immunology*, *175*(8), 5260–5268. https://doi.org/10.4049/jimmunol.175.8.5260

Rothenfusser, S., Hornung, V., Krug, A., Towarowski, A., Krieg, A. M., Endres, S., & Hartmann, G. (2001). Distinct CpG oligonucleotide sequences activate human γδ T cells via interferon-α/-β. *European Journal of Immunology*, *31*(12), 3525–3534. https://doi.org/10.1002/1521-4141(200112)31:12<3525::AID-IMMU3525>3.0.CO;2-5

RTSS Clinical Trials Partnership. (2015). 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*, *386*(9988), 31–45. https://doi.org/10.1016/S0140-6736(15)60721-8.Efficacy

Sabatos, C. A., Chakravarti, S., Cha, E., Schubart, A., Sánchez-Fueyo, A., Zheng, X. X., Coyle, A. J., Strom, T. B., Freeman, G. J., & Kuchroo, V. K. (2003). Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nature Immunology*, *4*(11), 1102–1110. https://doi.org/10.1038/ni988

Sacci, J. B., Ribeiro, J. M. C., Huang, F., Alam, U., Russell, J. A., Blair, P. L., Witney, A., Carucci, D. J., Azad, A. F., & Aguiar, J. C. (2005). Transcriptional analysis of in vivo Plasmodium yoelii liver stage gene expression. *Molecular and Biochemical Parasitology*, *142*(2), 177–183. https://doi.org/10.1016/j.molbiopara.2005.03.018

Sakuishi, K., Apetoh, L., Sullivan, J. M., Blazar, B. R., Kuchroo, V. K., & Anderson, A. C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *Journal of Experimental Medicine*, *207*(10), 2187–2194. https://doi.org/10.1084/jem.20100643

Salem, M. L., Kadima, A. N., Cole, D. J., & Gillanders, W. E. (2005). Defining the Antigen-Specific T-Cell Response to Vaccination and Poly(I:C)/TLR3 Signaling. *Journal of Immunotherapy*, *28*(3), 220–228. https://doi.org/10.1097/01.cji.0000156828.75196.0d

Sallusto, F., Lenig, D., Förster, R., Lipp, M., & Lanzavecchia, A. (1999). Two subsets of memory T lymphocyteswith distinct homing potentials and effector functions. *Nature*, *401*, 708. www.nature.com

Samulowitz, U., Weber, M., Weeratna, R., Uhlmann, E., Noll, B., Krieg, A. M., & Vollmer, J. (2010). A novel class of immune-stimulatory cpg oligodeoxynucleotides unifies high potency in type i interferon induction with preferred structural properties. *Oligonucleotides*, *20*(2), 93–101. https://doi.org/10.1089/oli.2009.0210

Sánchez-Fueyo, A., Tian, J., Picarella, D., Domenig, C., Zheng, X. X., Sabatos, C. A., Manlongat, N., Bender, O., Kamradt, T., Kuchroo, V. K., Gutiérrez-Ramos, J. C., Coyle, A. J., & Strom, T. B. (2003). Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nature Immunology*, *4*(11), 1093–1101.

https://doi.org/10.1038/ni987

Sancho, D., Mourão-sá, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R., & Reis e Sousa, C. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest*, *118*(6), 2098–2110. https://doi.org/10.1172/JCI34584DS1

Sano, G. I., Hafalla, J. C. R., Morrot, A., Abe, R., Lafaille, J. J., & Zavala, F. (2001). Swift development of protective effector functions in naive CD8+ T cells against malaria liver stages. *Journal of Experimental Medicine*, *194*(2), 173–179. https://doi.org/10.1084/jem.194.2.173

Sarkar, S., Kalia, V., Haining, W. N., Konieczny, B. T., Subramaniam, S., & Ahmed, R. (2008). Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *Journal of Experimental Medicine*, *205*(3), 625–640. https://doi.org/10.1084/jem.20071641

Sauer, J. D., Witte, C. E., Zemansky, J., Hanson, B., Lauer, P., & Portnoy, D. A. (2010). Listeria monocytogenes triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol. *Cell Host and Microbe*, *7*(5), 412–419. https://doi.org/10.1016/j.chom.2010.04.004

Scheiermann, J., & Klinman, D. M. (2014). Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. *Vaccine*, *32*(48), 6377–6389. https://doi.org/10.1016/j.vaccine.2014.06.065

Scheller, L. F., & Azad, A. F. (1995). Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(9), 4066–4068. https://doi.org/10.1073/pnas.92.9.4066

Schenkel, J. M., Fraser, K. A., Beura, L. K., Pauken, K. E., Vezys, V., & Masopust, D. (2014). Resident memory CD8 t cells trigger protective innate and adaptive immune responses. *Science*, *346*(6205), 98–101. https://doi.org/10.1126/science.1254536

Schenkel, J. M., Fraser, K. A., Vezys, V., & Masopust, D. (2013). Sensing and alarm function of resident memory CD8 + T cells. *Nature Immunology*, *14*(5), 509–513. https://doi.org/10.1038/ni.2568

Schmidt, N. W., Butler, N. S., Badovinac, V. P., & Harty, J. T. (2010). Extreme CD8 t cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites. *PLoS Pathogens*, *6*(7), 1–15. https://doi.org/10.1371/journal.ppat.1000998

Schmidt, N. W., Podyminogin, R. L., Butler, N. S., Badovinac, V. P., Tucker, B. J., Bahjat, K. S., Lauer, P., Reyes-Sandoval, A., Hutchings, C. L., Moore,

A. C., Gilbert, S. C., Hill, A. V., Bartholomay, L. C., & Harty, J. T. (2008). Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(37), 14017–14022. https://doi.org/10.1073/pnas.0805452105

Schneider, J., Gilbert, S. C., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., Becker, M., Sinden, R., Smith, G. L., & Hill, A. V. S. (1998). Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Medicine*, *4*(4), 397–402. https://doi.org/10.1038/nm0498-397

Schneider, W. M., Chevillotte, M. D., & Rice, C. M. (2014). Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annual Review of Immunology*, *32*(1), 513–545. https://doi.org/10.1146/annurev-immunol-032713-120231

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R., & Nussenzweig, V. (1987). γ Interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature*, *330*(6149), 664–666. https://doi.org/10.1038/330664a0

Schoggins, J. W., & Rice, C. M. (2012). Interferon-stimulated genes and their antiviral effector functions. *Current Oplinion Virology*, *1*(6), 519–525. https://doi.org/10.1016/j.coviro.2011.10.008.Interferon-stimulated

Schreibelt, G., Klinkenberg, L. J. J., Cruz, L. J., Tacken, P. J., Tel, J., Kreutz, M., Adema, G. J., Brown, G. D., Figdor, C. G., & De Vries, I. J. M. (2012). The C-type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. *Blood*, *119*(10), 2284–2292. https://doi.org/10.1182/blood-2011-08-373944

Schuchmann, M., Hermann, F., Herkel, J., Van Der Zee, R., Galle, P. R., & Lohse, A. W. (2004). HSP60 and CpG-DNA-oligonucleotides differentially regulate LPS-tolerance of hepatic Kupffer cells. *Immunology Letters*, *93*(2–3), 199–204. https://doi.org/10.1016/j.imlet.2004.03.016

Schulz, O., Diebold, S. S., Chen, M., Näslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljeström, P., & Reis E Sousa, C. (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature*, *433*(7028), 887–892. https://doi.org/10.1038/nature03326

Schwabe, R. F., Seki, E., & Brenner, D. A. (2006). Toll-Like Receptor Signaling in the Liver. *Gastroenterology*, *130*(6), 1886–1900. https://doi.org/10.1053/j.gastro.2006.01.038

Sedegah, M., Finkelman, F., & Hoffman, S. L. (1994). Interleukin 12 induction of interferon γ-dependent protection against malaria. *Proceedings of the*

National Academy of Sciences of the United States of America, *91*(22), 10700–10702. https://doi.org/10.1073/pnas.91.22.10700

Sedegah, M., Tamminga, C., McGrath, S., House, B., Ganeshan, H., Lejano, J., Abot, E., Banania, G. J., Sayo, R., Farooq, F., Belmonte, M., Manohar, N., Richie, N. O., Wood, C., Long, C. A., Regis, D., Williams, F. T., Shi, M., Chuang, I., … Richie, T. L. (2011). Adenovirus 5-vectored p. falciparum vaccine expressing CSP and ama1. part a: Safety and immunogenicity in seronegative adults. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0024586

Seder, R. A., Chang, L. J., Enama, M. E., Zephir, K. L., Sarwar, U. N., Gordon, I. J., Holman, L. S. A., James, E. R., Billingsley, P. F., Gunasekera, A., Richman, A., Chakravarty, S., Manoj, A., Velmurugan, S., Li, M. L., Ruben, A. J., Li, T., Eappen, A. G., Stafford, R. E., … Hoffman, S. L. (2013). Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science*, *341*(6152), 1359–1365. https://doi.org/10.1126/science.1241800

Seki, E., Tsutsui, H., Nakano, H., Tsuji, N. M., Hoshino, K., Adachi, O., Adachi, K., Futatsugi, S., Kuida, K., Takeuchi, O., Okamura, H., Fujimoto, J., Akira, S., & Nakanishi, K. (2001). Lipopolysaccharide-Induced IL-18 Secretion from Murine Kupffer Cells Independently of Myeloid Differentiation Factor 88 That Is Critically Involved in Induction of Production of IL-12 and IL-1β. *The Journal of Immunology*, *166*(4), 2651–2657. https://doi.org/10.4049/jimmunol.166.4.2651

Seubert, A., Monaci, E., Pizza, M., O'Hagan, D. T., & Wack, A. (2008). The Adjuvants Aluminum Hydroxide and MF59 Induce Monocyte and Granulocyte Chemoattractants and Enhance Monocyte Differentiation toward Dendritic Cells. *The Journal of Immunology*, *180*(8), 5402–5412. https://doi.org/10.4049/jimmunol.180.8.5402

Seydel, K. B., Kampondeni, S. D., Valim, C., Potchen, M. J., Milner, D. A., Muwalo, F. W., Birbeck, G. L., Bradley, W. G., Fox, L. L., Glover, S. J., Hammond, C. A., Heyderman, R. S., Chilingulo, C. A., Molyneux, M. E., & Taylor, T. E. (2015). Brain swelling and death in children with cerebral malaria. *New England Journal of Medicine*, *372*(12), 1126–1137. https://doi.org/10.1056/NEJMoa1400116

Shackleton, M., Davis, I. D., Hopkins, W., Jackson, H., Dimopoulos, N., Tai, T., Chen, Q., Parente, P., Jefford, M., Masterman, K. A., Caron, D., Chen, W., Maraskovsky, E., & Cebon, J. (2004). The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. *Cancer Immunity*, *4*(September), 1–11.

Sheehy, S. H., Duncan, C. J., Elias, S. C., Choudhary, P., Biswas, S., Halstead, F. D., Collins, K. A., Edwards, N. J., Douglas, A. D., Anagnostou, N. A.,

Ewer, K. J., Havelock, T., Mahungu, T., Bliss, C. M., Miura, K., Poulton, I. D., Lillie, P. J., Antrobus, R. D., Berrie, E., … Draper, S. J. (2012). ChAd63-MVA-vectored blood-stage Malaria vaccines targeting MSP1 and AMA1: Assessment of efficacy against mosquito bite challenge in humans. *Molecular Therapy*, *20*(12), 2355–2368. https://doi.org/10.1038/mt.2012.223

Shen, H., Ackerman, A. L., Cody, V., Giodini, A., Hinson, E. R., Cresswell, P., Edelson, R. L., Saltzman, W. M., & Hanlon, D. J. (2006). Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. *Immunology*, *117*(1), 78–88. https://doi.org/10.1111/j.1365-2567.2005.02268.x

Sheridan, B. S., Pham, Q. M., Lee, Y. T., Cauley, L. S., Puddington, L., & Lefrançois, L. (2014). Oral infection drives a distinct population of intestinal resident memory cd8+ t cells with enhanced protective function. *Immunity*, *40*(5), 747–757. https://doi.org/10.1016/j.immuni.2014.03.007

Shin, H., & Iwasaki, A. (2012). A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature*, *491*(7424), 463–467. https://doi.org/10.1038/nature11522

Shin, H., Kumamoto, Y., Gopinath, S., & Iwasaki, A. (2016). CD301b+ dendritic cells stimulate tissue-resident memory CD8+ T cells to protect against genital HSV-2. *Nature Communications*, *7*, 1–10. https://doi.org/10.1038/ncomms13346

Shortman, K., & Heath, W. R. (2010). The CD8+ dendritic cell subset. *Immunological Reviews*, *234*(1), 18–31. https://doi.org/10.1111/j.0105-2896.2009.00870.x

Shrestha, B., Wang, T., Samuel, M. A., Whitby, K., Craft, J., Fikrig, E., & Diamond, M. S. (2006). Gamma Interferon Plays a Crucial Early Antiviral Role in Protection against West Nile Virus Infection. *Journal of Virology*, *80*(11), 5338–5348. https://doi.org/10.1128/jvi.00274-06

Shukla, N. M., Malladi, S. S., Mutz, C. A., Balakrishna, R., & Sunil, A. D. (2010). Structure-activity relationships in human Toll-like Receptor 7- active Imidazoquinoline analogues. *J Med Chem*, *53*(11), 4450–4465. https://doi.org/10.1021/jm100358c

Sidjanski, S., & Vanderberg, J. P. (1997). Delayed migration of Plasmodium sporozoites from the mosquito bite site to the blood. *American Journal of Tropical Medicine and Hygiene*, *57*(4), 426–429. https://doi.org/10.4269/ajtmh.1997.57.426

Silamut, K., Phu, N. H., Whitty, C., Turner, G. D. H., Louwrier, K., Mai, N. T. H., Simpson, J. A., Hien, T. T., & White, N. J. (1999). A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain.

American Journal of Pathology, *155*(2), 395–410. https://doi.org/10.1016/S0002-9440(10)65136-X

Silvie, O., Franetich, J. F., Boucheix, C., Rubinstein, E., & Mazier, D. (2007). Alternative invasion pathways for plasmodium berghei sporozoites. *International Journal for Parasitology*, *37*(2), 173–182. https://doi.org/10.1016/j.ijpara.2006.10.005

Silvie, O., Franetich, J. F., Charrin, S., Mueller, M. S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C. H., Thomas, A. W., Van Gemert, G. J., Sauerwein, R. W., Blackman, M. J., Anders, R. F., Pluschke, G., & Mazier, D. (2004). A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by Plasmodium falciparum Sporozoites. *Journal of Biological Chemistry*, *279*(10), 9490–9496. https://doi.org/10.1074/jbc.M311331200

Silvie, O., Rubinstein, E., Franetich, J. F., Prenant, M., Belnoue, E., Rénia, L., Hannoun, L., Elings, W., Levy, S., Boucheix, C., & Mazier, D. (2003). Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. *Nature Medicine*, *9*(1), 93–96. https://doi.org/10.1038/nm808

Simpson, J. A., Aarons, L., Collins, W. E., Jeffery, G. M., & White, N. J. (2002). Population dynamics of untreated Plasmodium falciparum malaria within the adult human host during the expansion phase of the infection. *Parasitology*, *124*(3), 247–263. https://doi.org/10.1017/S0031182001001202

Sinden, R. E. (1983). The Cell Biology of Sexual Development in Plasmodium. *Parasitology*, *86*(4), 7–28. https://doi.org/10.1017/S0031182000050824

Sissoko, M. S., Healy, S. A., Katile, A., Omaswa, F., Zaidi, I., Gabriel, E. E., Kamate, B., Samake, Y., Guindo, M. A., Dolo, A., Niangaly, A., Niaré, K., Zeguime, A., Sissoko, K., Diallo, H., Thera, I., Ding, K., Fay, M. P., O'Connell, E. M., … Duffy, P. E. (2017). 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. *The Lancet Infectious Diseases*, *17*(5), 498–509. https://doi.org/10.1016/S1473-3099(17)30104-4

Sistigu, A., Yamazaki, T., Vacchelli, E., Chaba, K., Enot, D. P., Adam, J., Vitale, I., Goubar, A., Baracco, E. E., Remédios, C., Fend, L., Hannani, D., Aymeric, L., Ma, Y., Niso-Santano, M., Kepp, O., Schultze, J. L., Tüting, T., Belardelli, F., … Zitvogel, L. (2014). Cancer cell–autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nature Medicine*, *20*(11), 1301–1309. https://doi.org/10.1038/nm.3708

Smith, D. L., & McKenzie, F. E. (2004). Statics and dynamics of malaria infection in Anopheles mosquitoes. *Malaria Journal*, *3*.

https://doi.org/10.1186/1475-2875-3-13

Smith, D. M., Simon, J. K., & Baker, J. R. (2013). Applications of nanotechnology for immunology. *Nature Reviews Immunology*, *13*(8), 592–605. https://doi.org/10.1038/nri3488

Smyth Templeton, N. (2002). Liposomal delivery of nucleic acids in vivo. *DNA and Cell Biology*, *21*(12), 857–867. https://doi.org/10.1089/104454902762053828

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., & Hay, S. I. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature*, *434*(7030), 214–217. https://doi.org/10.1038/nature03342

Snow, R. W., Omumbo, J. A., Lowe, B., Molyneux, C. S., Obiero, J. O., Palmer, A., Weber, M. W., Pinder, M., Nahlen, B., Obonyo, C., Newbold, C., Gupta, S., & Marsh, K. (1997). Relation between severe malaria morbidity in children and level of Plasmodium falciparum transmission in Africa. *Lancet*, *349*(9066), 1650–1654. https://doi.org/10.1016/S0140-6736(97)02038-2

Soares, J. B., Pimentel-Nunes, P., Roncon-Albuquerque, R., & Leite-Moreira, A. (2010). The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. *Hepatology International*, *4*(4), 659–672. https://doi.org/10.1007/s12072-010-9219-x

Sohlenius-Sternbeck, A. K. (2006). Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicology in Vitro*, *20*(8), 1582–1586. https://doi.org/10.1016/j.tiv.2006.06.003

Soulard, V., Bosson-Vanga, H., Lorthiois, A., Roucher, C., Franetich, J. F., Zanghi, G., Bordessoulles, M., Tefit, M., Thellier, M., Morosan, S., Le Naour, G., Capron, F., Suemizu, H., Snounou, G., Moreno-Sabater, A., & Mazier, D. (2015). Plasmodium falciparum full life cycle and Plasmodium ovale liver stages in humanized mice. *Nature Communications*, *6*(May). https://doi.org/10.1038/ncomms8690

Speake, C., Pichugin, A., Sahu, T., Malkov, V., Morrison, R., Pei, Y., Juompan, L., Milman, N., Zarling, S., Anderson, C., Wong-Madden, S., Wendler, J., Ishizuka, A., MacMillen, Z. W., Garcia, V., Kappe, S. H. I., Krzych, U., & Duffy, P. E. (2016). Identification of novel pre-erythrocytic malaria antigen candidates for combination vaccines with circumsporozoite protein. *PLoS ONE*, *11*(7), 1–21. https://doi.org/10.1371/journal.pone.0159449

Speiser, D. E., Liénard, D., Rufer, N., Rubio-Godoy, V., Rimoldi, D., Lejeune, F., Krieg, A. M., Cerottini, J.-C., & Romero, P. (2005). Rapid and strong human CD8+ T cell resopnses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *The Journal of Clinical Investigation*, *115*(3),

739–746. https://doi.org/10.1172/JCI200523373

Spillman, N. J., Beck, J. R., & Goldberg, D. E. (2015). Protein Export into Malaria Parasite–Infected Erythrocytes: Mechanisms and Functional Consequences. *Annual Review of Biochemistry*, *84*(1), 813–841. https://doi.org/10.1146/annurev-biochem-060614-034157

Srisutham, S., Saralamba, N., Sriprawat, K., Mayxay, M., Smithuis, F., Nosten, F., Pukrittayakamee, S., Day, N. P. J., Dondorp, A. M., & Imwong, M. (2018). Genetic diversity of three surface protein genes in Plasmodium malariae from three Asian countries. *Malaria Journal*, *17*(1), 1–10. https://doi.org/10.1186/s12936-018-2176-x

Stanisic, D. I., Fink, J., Mayer, J., Coghill, S., Gore, L., Liu, X. Q., El-Deeb, I., Rodriguez, I. B., Powell, J., Willemsen, N. M., De, S. L., Ho, M. F., Hoffman, S. L., Gerrard, J., & Good, M. F. (2018). Vaccination with chemically attenuated Plasmodium falciparum asexual blood-stage parasites induces parasite-specific cellular immune responses in malaria-naïve volunteers: A pilot study. *BMC Medicine*, *16*(1), 1–16. https://doi.org/10.1186/s12916-018-1173-9

Stanley, M. A. (2002). Imiquimod and the imidazoquinolones: Mechanism of action and therapeutic potential. *Clinical and Experimental Dermatology*, *27*(7), 571–577. https://doi.org/10.1046/j.1365-2230.2002.01151.x

Steers, N. J., Peachman, K. K., McClain, S., Alving, C. R., & Rao, M. (2009). Liposome-encapsulated HIV-1 Gag p24 containing lipid A induces effector CD4+ T-cells, memory CD8+ T-cells, and pro-inflammatory cytokines. *Vaccine*, *27*(49), 6939–6949. https://doi.org/10.1016/j.vaccine.2009.08.105

Steinbach, K., Vincenti, I., & Merkler, D. (2018). Resident-Memory T Cells in tissue-restricted immune responses: For better or worse? *Frontiers in Immunology*, *9*(NOV), 1–15. https://doi.org/10.3389/fimmu.2018.02827

Steinert, E. M., Schenkel, J. M., Fraser, K. A., Beura, L. K., Manlove, L. S., Igyártó, B. Z., Southern, P. J., & Masopust, D. (2015). Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell*, *161*(4), 737–749. https://doi.org/10.1016/j.cell.2015.03.031

Steinhagen, F., Takeshi, K., Bode, C., & Klinman, D. M. (2011). TLR-Based Immune Adjuvants. *Vaccine*, *29*(17), 3341–3355. https://doi.org/10.1038/jid.2014.371

Stelma, F., De Niet, A., Sinnige, M. J., Van Dort, K. A., Van Gisbergen, K. P. J. M., Verheij, J., Van Leeuwen, E. M. M., Kootstra, N. A., & Reesink, H. W. (2017). Human intrahepatic CD69 + CD8+ T cells have a tissue resident memory T cell phenotype with reduced cytolytic capacity. *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-06352-3

Stephenson, I., Bugarini, R., Nicholson, K. G., Podda, A., Wood, J. M., Zambon, M. C., & Katz, J. M. (2005). Cross-Reactivity to Highly Pathogenic Avian Influenza H5N1 Viruses after Vaccination with Nonadjuvanted and MF59-Adjuvanted Influenza A/Duck/Singapore/97 (H5N3) Vaccine: A Potential Priming Strategy. *The Journal of Infectious Diseases*, *191*(8), 1210–1215. https://doi.org/10.1086/428948

Stern, B. V., Boehm, B. O., & Tary-Lehmann, M. (2002). Vaccination with Tumor Peptide in CpG Adjuvant Protects Via IFN-γ-Dependent CD4 Cell Immunity. *The Journal of Immunology*, *168*(12), 6099–6105. https://doi.org/10.4049/jimmunol.168.12.6099

Stoute, J. A., Slaoui, M., Heppner, D. G., Momin, P., Kester, K. E., Desmons, P., Wellde, B. T., Garçon, N., Krzych, U., Marchand, M., Ballou, W. R., & Cohen, J. D. (1997). A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. *New England Journal of Medicine*, *336*(2), 86–91. https://doi.org/10.1056/NEJM199701093360202

Sturm, A., Amino, R., Van De Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J. M., Menard, R., & Heussler, V. T. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*, *313*(5791), 1287–1290. https://doi.org/10.1126/science.1129720

Sultan, A. A., Thathy, V., Frevert, U., Robson, K. J. H., Crisanti, A., Nussenzweig, V., Nussenzweig, R. S., & Ménard, R. (1997). TRAP is necessary for gliding motility and infectivity of Plasmodium sporozoites. *Cell*, *90*(3), 511–522. https://doi.org/10.1016/S0092-8674(00)80511-5

Sun, L., Wu, J., Du, F., Chen, X., & Chen, Z. J. (2013). Cyclic GMP-AMP Synthase is a Cytosolic DNA Sensor that Activates the Type-I Interferon Pathway. *Science*, *15*(339), 1–7. https://doi.org/10.1126/science.1232458.

Suzuki, Y., Wakita, D., Chamoto, K., Narita, Y., Tsuji, T., Takeshima, T., Gyobu, H., Kawarada, Y., Kondo, S., Akira, S., Katoh, H., Ikeda, H., & Nishimura, T. (2004). Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. *Cancer Research*, *64*(23), 8754–8760. https://doi.org/10.1158/0008-5472.CAN-04-1691

Swadling, L., Pallett, L. J., Diniz, M. O., Baker, J. M., Amin, O. E., Stegmann, K. A., Burton, A. R., Schmidt, N. M., Jeffery-Smith, A., Zakeri, N., Suveizdyte, K., Froghi, F., Fusai, G., Rosenberg, W. M., Davidson, B. R., Schurich, A., Simon, A. K., & Maini, M. K. (2020). Human Liver Memory CD8+ T Cells Use Autophagy for Tissue Residence. *Cell Reports*, *30*(3), 687-698.e6. https://doi.org/10.1016/j.celrep.2019.12.050

Swetter, S. M., Chen, F. W., Kim, D. D., & Egbert, B. M. (2015). Imiquimod 5%

cream as primary or adjuvant therapy for melanoma in situ, lentigo maligna type. *Journal of the American Academy of Dermatology*, *72*(6), 1047–1053. https://doi.org/10.1016/j.jaad.2015.02.008

Szabo, P. A., Miron, M., & Farber, D. L. (2019). Location, location, location: Tissue resident memory T cells in mice and humans. *Science Immunology*, *4*(34), 1–12. https://doi.org/10.1126/sciimmunol.aas9673

Tacken, P. J., De Vries, I. J. M., Torensma, R., & Figdor, C. G. (2007). Dendritic-cell immunotherapy: From ex vivo loading to in vivo targeting. *Nature Reviews Immunology*, *7*(10), 790–802. https://doi.org/10.1038/nri2173

Talaat, K. R., Ellis, R. D., Hurd, J., Hentrich, A., Gabriel, E., Hynes, N. A., Rausch, K. M., Zhu, D., Muratova, O., Herrera, R., Anderson, C., Jones, D., Aebig, J., Brockley, S., MacDonald, N. J., Wang, X., Fay, M. P., Healy, S. A., Durbin, A. P., … Duffy, P. E. (2016). Safety and immunogenicityof Pfs25-EPA/ alhydrogel1, a transmission blocking vaccine against Plasmodium falciparum: An open label study in malaria naïve adults. *PLoS ONE*, *11*(10), 1–17. https://doi.org/10.1371/journal.pone.0163144

Tamayo, R., Pratt, J. T., & Camilli, A. (2007). Roles of Cyclic Diguanylate in the Regulation of Bacterial Pathogenesis. *Annual Review of Microbiology*, *61*(1), 131–148. https://doi.org/10.1146/annurev.micro.61.080706.093426

Tamminga, C., Sedegah, M., Regis, D., Chuang, I., Epstein, J. E., Spring, M., Mendoza-Silveiras, J., McGrath, S., Maiolatesi, S., Reyes, S., Steinbeiss, V., Fedders, C., Smith, K., House, B., Ganeshan, H., Lejano, J., Abot, E., Banania, G. J., Sayo, R., … Richie, T. L. (2011). Adenovirus-5-vectored P. falciparum vaccine expressing CSP and AMA1. Part B: Safety, immunogenicity and protective efficacy of the CSP component. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0025868

Tan, H. X., Wheatley, A. K., Esterbauer, R., Jegaskanda, S., Glass, J. J., Masopust, D., De Rose, R., & Kent, S. J. (2018). Induction of vaginal-resident HIV-specific CD8 T cells with mucosal prime-boost immunization. *Mucosal Immunology*, *11*(3), 994–1007. https://doi.org/10.1038/mi.2017.89

Tanaka, Y., & Chen, Z. J. (2012). STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Science Signaling*, *5*(214), 1–12. https://doi.org/10.1126/scisignal.2002521

Tarun, A. S., Peng, X., Dumpit, R. F., Ogata, Y., Silva-Rivera, H., Camargo, N., Daly, T. M., Bergman, L. W., & Kappe, S. H. I. (2008). A combined transcriptome and proteome survey of malaria parasite liver stages. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(1), 305–310. https://doi.org/10.1073/pnas.0710780104

Tavares, J., Formaglio, P., Thiberge, S., Mordelet, E., Van Rooijen, N.,

Medvinsky, A., Ménard, R., & Amino, R. (2013). Role of host cell traversal by the malaria sporozoite during liver infection. *Journal of Experimental Medicine*, *210*(5), 905–915. https://doi.org/10.1084/jem.20121130

Tay, S. S., Wong, Y. C., McDonald, D. M., Wood, N. A. W., Roediger, B., Sierro, F., Mcguffog, C., Alexander, I. E., Bishop, G. A., Gamble, J. R., Weninger, W., McCaughan, G. W., Bertolino, P., & Bowen, D. G. (2014). Antigen expression level threshold tunes the fate of CD8 T cells during primary hepatic immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(25). https://doi.org/10.1073/pnas.1406674111

Taylor, W. R. J., Hanson, J., Turner, G. D. H., White, N. J., & Dondorp, A. M. (2012). Respiratory manifestations of malaria. *Chest*, *142*(2), 492–505. https://doi.org/10.1378/chest.11-2655

Tegtmeyer, P. K., Spanier, J., Borst, K., Becker, J., Riedl, A., Hirche, C., Ghita, L., Skerra, J., Baumann, K., Lienenklaus, S., Doering, M., Ruzsics, Z., & Kalinke, U. (2019). STING induces early IFN-β in the liver and constrains myeloid cell-mediated dissemination of murine cytomegalovirus. *Nature Communications*, *10*(1), 1–12. https://doi.org/10.1038/s41467-019-10863-0

Teijaro, J. R., Turner, D., Pham, Q., Wherry, E. J., Lefrançois, L., & Farber, D. L. (2011). Tissue-Retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol*, *187*(11), 5510–5514. https://doi.org/10.4049/jimmunol.1102243

Tewari, K., Flynn, B. J., Boscardin, S. B., Kastenmueller, K., Salazar, A. M., Anderson, C. A., Soundarapandian, V., Ahumada, A., Keler, T., Hoffman, S. L., Nussenzweig, M. C., Steinman, R. M., & Seder, R. A. (2010). Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and αDEC-CSP in non human primates. *Vaccine*, *28*(45), 7256–7266. https://doi.org/10.1016/j.vaccine.2010.08.098

Thomas, A. W., Deans, J. A., Mitchell, G. H., Alderson, T., & Cohen, S. (1984). The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit Plasmodium knowlesi invasion of erythrocytes. *Molecular and Biochemical Parasitology*, *13*(2), 187–199. https://doi.org/10.1016/0166-6851(84)90112-9

Thomas, C., Rawat, A., Hope-Weeks, L., & Ahsan, F. (2011). Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine. *Molecular Pharmaceutics*, *8*(2), 405–415. https://doi.org/10.1021/mp100255c

Thompson, E. A., Ols, S., Miura, K., Rausch, K., Narum, D. L., Spångberg, M., Juraska, M., Wille-Reece, U., Weiner, A., Howard, R. F., Long, C. A.,

Duffy, P. E., Johnston, L., O'Neil, C. P., & Loré, K. (2018). TLR-adjuvanted nanoparticle vaccines differentially influence the quality and longevity of responses to malaria antigen Pfs25. *JCI Insight*, *3*(10). https://doi.org/10.1172/jci.insight.120692

Thomsen, M. K., Nandakumar, R., Stadler, D., Malo, A., Valls, R. M., Wang, F., Reinert, L. S., Dagnæs-Hansen, F., Hollensen, A. K., Mikkelsen, J. G., Protzer, U., & Paludan, S. R. (2016). Lack of immunological DNA sensing in hepatocytes facilitates hepatitis B virus infection. *Hepatology*, *64*(3), 746–759. https://doi.org/10.1002/hep.28685

Thomson, A. W., O'Connell, P. J., Steptoe, R. J., & Lu, L. (2002). Immunobiology of liver dendritic cells. *Immunology and Cell Biology*, *80*(1), 65–73. https://doi.org/10.1046/j.0818-9641.2001.01058.x

Tilley, L., Sougrat, R., Lithgow, T., & Hanssen, E. (2008). The twists and turns of Maurer's cleft trafficking in P. falciparum-infected erythrocytes. *Traffic*, *9*(2), 187–197. https://doi.org/10.1111/j.1600-0854.2007.00684.x

Tiono, A. B., Nébié, I., Anagnostou, N., Coulibaly, A. S., Bowyer, G., Lam, E., Bougouma, E. C., Ouedraogo, A., Yaro, J. B. B., Barry, A., Roberts, R., Rampling, T., Bliss, C., Hodgson, S., Lawrie, A., Ouedraogo, A., Imoukhuede, E. B., Ewer, K. J., Viebig, N. K., … Sirima, S. B. (2018). First field efficacy trial of the ChAd63 MVA ME-TRAP vectored malaria vaccine candidate in 5-17 months old infants and children. *PLoS ONE*, *13*(12), 1–19. https://doi.org/10.1371/journal.pone.0208328

Todryk, S. M., & Hill, A. V. S. (2007). Malaria vaccines: The stage we are at. *Nature Reviews Microbiology*, *5*(7), 487–489. https://doi.org/10.1038/nrmicro1712

Tong, N. K. C., Beran, J., Kee, S. A., Miguel, J. L., Sánchez, C., Bayas, J. M., Vilella, A., De Juanes, J. R., Arrazola, P., Calbo-Torrecillas, F., De Novales, E. L., Hamtiaux, V., Lievens, M., & Stoffel, M. (2005). Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. *Kidney International*, *68*(5), 2298–2303. https://doi.org/10.1111/j.1523-1755.2005.00689.x

Triglia, T., Healer, J., Caruana, S. R., Hodder, A. N., Anders, R. F., Crabb, B. S., & Cowman, A. F. (2000). Apical membrane antigen 1 plays a central role in erythrocyte invasion by Plasmodium species. *Molecular Microbiology*, *38*(4), 706–718. https://doi.org/10.1046/j.1365-2958.2000.02175.x

Trumpfheller, C., Caskey, M., Nchinda, G., Longhi, M. P., Mizenina, O., Huang, Y., Schlesinger, S. J., Colonna, M., & Steinman, R. M. (2008). The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(7), 2574–2579. https://doi.org/10.1073/pnas.0711976105

Tse, S. W., Cockburn, I. A., Zhang, H., Scott, A. L., & Zavala, F. (2013). Unique transcriptional profile of liver-resident memory CD8+ T cells induced by immunization with malaria sporozoites. *Genes and Immunity*, *14*(5), 302–309. https://doi.org/10.1038/gene.2013.20

Tse, Sze Wah, Radtke, A. J., Espinosa, D. A., Cockburn, I. A., & Zavala, F. (2014). The chemokine receptor CXCR6 is required for the maintenance of liver memory CD8+ T cells specific for infectious pathogens. *Journal of Infectious Diseases*, *210*(9), 1508–1516. https://doi.org/10.1093/infdis/jiu281

Uesugi, T., Froh, M., Arteel, G. E., Bradford, B. U., & Thurman, R. G. (2001). Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. *Hepatology*, *34*(1), 101–108. https://doi.org/10.1053/jhep.2001.25350

Valencia-Hernandez, A. M., Ng, W. Y., Ghazanfari, N., Ghilas, S., de Menezes, M. N., Holz, L. E., Huang, C., English, K., Naung, M., Tan, P. S., Tullett, K. M., Steiner, T. M., Enders, M. H., Beattie, L., Chua, Y. C., Jones, C. M., Cozijnsen, A., Mollard, V., Cai, Y., … Fernandez-Ruiz, D. (2020). A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver TRM Cell-Mediated Immunity against Malaria in Mice. *Cell Host & Microbe*, 1–13. https://doi.org/10.1016/j.chom.2020.04.010

Valmori, D., Souleimanian, N. E., Tosello, V., Bhardwaj, N., Adams, S., O'Neill, D., Pavlick, A., Escalon, J. B., Cruz, C. M., Angiulli, A., Angiulli, F., Mears, G., Vogel, S. M., Pan, L., Jungbluth, A. A., Hoffmann, E. W., Venhaus, R., Ritter, G., Old, L. J., & Ayyoub, M. (2007). Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(21), 8947–8952. https://doi.org/10.1073/pnas.0703395104

Van Braeckel-Budimir, N., Gras, S., Ladell, K., Josephs, T. M., Pewe, L., Urban, S. L., Miners, K. L., Farenc, C., Price, D. A., Rossjohn, J., & Harty, J. T. (2017). A T Cell Receptor Locus Harbors a Malaria-Specific Immune Response Gene. *Immunity*, *47*(5), 835-847.e4. https://doi.org/10.1016/j.immuni.2017.10.013

Van Braeckel-Budimir, N., & Harty, J. T. (2014). CD8 T-cell-mediated protection against liver-stage malaria: Lessons from a mouse model. *Frontiers in Microbiology*, *5*(JUN), 1–9. https://doi.org/10.3389/fmicb.2014.00272

Van De Sand, C., Horstmann, S., Schmidt, A., Sturm, A., Bolte, S., Krueger, A., Lütgehetmann, M., Pollok, J. M., Libert, C., & Heussler, V. T. (2005). The liver stage of Plasmodium berghei inhibits host cell apoptosis. *Molecular Microbiology*, *58*(3), 731–742. https://doi.org/10.1111/j.1365-2958.2005.04888.x

Van Dis, E., Sogi, K. M., Rae, C. S., Sivick, K. E., Surh, N. H., Leong, M. L., Kanne, D. B., Metchette, K., Leong, J. J., Bruml, J. R., Chen, V., Heydari, K., Cadieux, N., Evans, T., McWhirter, S. M., Dubensky, T. W., Portnoy, D. A., & Stanley, S. A. (2018). STING-Activating Adjuvants Elicit a Th17 Immune Response and Protect against Mycobacterium tuberculosis Infection. *Cell Reports*, *23*(5), 1435–1447. https://doi.org/10.1016/j.celrep.2018.04.003

van Schaijk, B. C. L., Janse, C. J., van Gemert, G. J., van Dijk, M. R., Gego, A., Franetich, J. F., van de Vegte-Bolmer, M., Yalaoui, S., Silvie, O., Hoffman, S. L., Waters, A. P., Mazier, D., Sauerwein, R. W., & Khan, S. M. (2008). Gene discruption of Plasmodium falcifarum p52 results in attenuation of malaria liver stage development in cultured primary human hepatocytes. *PLoS ONE*, *3*(10). https://doi.org/10.1371/journal.pone.0003549

Vasir, J. K., & Labhasetwar, V. (2007). Biodegradable nanoparticles for cytosolic delivery of therapeutics. *Advanced Drug Delivery Reviews*, *59*(8), 718–728. https://doi.org/10.1016/j.addr.2007.06.003

Vaughan, A. M., Sack, B. K., Dankwa, D., Minkah, N., Nguyen, T., Cardamone, H., & Kappe, S. H. I. (2018). A Plasmodium parasite with complete late liver stage arrest protects against preerythrocytic and erythrocytic stage infection in mice. *Infection and Immunity*, *86*(5), 1–18. https://doi.org/10.1128/IAI.00088-18

Vaure, C., & Liu, Y. (2014). A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Frontiers in Immunology*, *5*(JUL), 1–15. https://doi.org/10.3389/fimmu.2014.00316

Verthelyi, D., Ishii, K. J., Gursel, M., Takeshita, F., & Klinman, D. M. (2001). Human Peripheral Blood Cells Differentially Recognize and Respond to Two Distinct CpG Motifs. *The Journal of Immunology*, *166*(4), 2372–2377. https://doi.org/10.4049/jimmunol.166.4.2372

Verthelyi, D., Kenney, R. T., Seder, R. A., Gam, A. A., Friedag, B., & Klinman, D. M. (2002). CpG Oligodeoxynucleotides as Vaccine Adjuvants in Primates. *The Journal of Immunology*, *168*(4), 1659–1663. https://doi.org/10.4049/jimmunol.168.4.1659

Vita, R., Mahajan, S., Overton, J. A., Dhanda, S. K., Martini, S., Cantrell, J. R., Wheeler, D. K., Sette, A., & Peters, B. (2019). The Immune Epitope Database (IEDB): 2018 update. *Nucleic Acids Research*, *47*(D1), D339–D343. https://doi.org/10.1093/nar/gky1006

Vlachou, D., Schlegelmilch, T., Runn, E., Mendes, A., & Kafatos, F. C. (2006). The developmental migration of Plasmodium in mosquitoes. *Current Opinion in Genetics and Development*, *16*(4), 384–391. https://doi.org/10.1016/j.gde.2006.06.012

Von Seidlein, L., Olaosebikan, R., Hendriksen, I. C. E., Lee, S. J., Adedoyin, O. T., Agbenyega, T., Nguah, S. B., Bojang, K., Deen, J. L., Evans, J., Fanello, C. I., Gomes, E., Pedro, A. J., Kahabuka, C., Karema, C., Kivaya, E., Maitland, K., Mokuolu, O. A., Mtove, G., … Dondorp, A. M. (2012). Predicting the clinical outcome of severe falciparum malaria in African children: Findings from a large randomized trial. *Clinical Infectious Diseases*, *54*(8), 1080–1090. https://doi.org/10.1093/cid/cis034

Wakim, L. M., Smith, J., Caminschi, I., Lahoud, M. H., & Villadangos, J. A. (2015). Antibody-targeted vaccination to lung dendritic cells generates tissue-resident memory CD8 T cells that are highly protective against influenza virus infection. *Mucosal Immunology*, *8*(5), 1060–1071. https://doi.org/10.1038/mi.2014.133

Wakim, Linda M., Woodward-Davis, A., & Bevan, M. J. (2010). Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(42), 17872–17879. https://doi.org/10.1073/pnas.1010201107

Wang, J., Li, P., & Wu, M. X. (2016). Natural STING Agonist as an “Ideal” Adjuvant for Cutaneous Vaccination. *Journal of Investigative Dermatology*, *136*(11), 2183–2191. https://doi.org/10.1016/j.jid.2016.05.105

Wang, R., Epstein, J., Baraceros, F. M., Gorak, E. J., Charoenvit, Y., Carucci, D. J., Hedstrom, R. C., Rahardjo, N., Gay, T., Hobart, P., Stout, R., Jones, T. R., Richie, T. L., Parker, S. E., Doolan, D. L., Norman, J., & Hoffman, S. L. (2001). Induction of CD4+ T cell-dependent CD8+ type 1 responses in humans by a malaria DNA vaccine. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(19), 10817–10822. https://doi.org/10.1073/pnas.181123498

Wassmer, S. C., Taylor, T. E., Rathod, P. K., Mishra, S. K., Mohanty, S., Arevalo-Herrera, M., Duraisingh, M. T., & Smith, J. D. (2015). Investigating the pathogenesis of severe malaria: A multidisciplinary and cross-geographical approach. *American Journal of Tropical Medicine and Hygiene*, *93*(Suppl 3), 42–56. https://doi.org/10.4269/ajtmh.14-0841

Webster, D. P., Dunachie, S., Vuola, J. M., Berthoud, T., Keating, S., Laidlaw, S. M., McConkey, S. J., Poulton, I., Andrews, L., Andersen, R. F., Bejon, P., Butcher, G., Sinden, R., Skinner, M. A., Gilbert, S. C., & Hill, A. V. S. (2005). Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(13), 4836–4841. https://doi.org/10.1073/pnas.0406381102

Weiss, G. E., Gilson, P. R., Taechalertpaisarn, T., Tham, W. H., de Jong, N. W.

M., Harvey, K. L., Fowkes, F. J. I., Barlow, P. N., Rayner, J. C., Wright, G. J., Cowman, A. F., & Crabb, B. S. (2015). Revealing the Sequence and Resulting Cellular Morphology of Receptor-Ligand Interactions during Plasmodium falciparum Invasion of Erythrocytes. *PLoS Pathogens*, *11*(2), 1–25. https://doi.org/10.1371/journal.ppat.1004670

Weiss, W. R., Sedegah, M., Beaudoin, R. L., Miller, L. H., & Good, M. F. (1988). CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(2), 573–576. https://doi.org/10.1073/pnas.85.2.573

Wherry, E. J. (2011). T cell exhaustion. *Nature Immunology*, *12*(6), 492–499. https://doi.org/10.1038/ni.2035

Wherry, J. E., Blattman, J. N., Murali-Krishna, K., van der Most, R., & Ahmed, R. (2013). Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment. *Journal of Virology*, *77*(8), 4911–4927. https://doi.org/10.1128/JVI.77.8.4911

White, M. T., Verity, R., Griffin, J. T., Asante, K. P., Owusu-Agyei, S., Greenwood, B., Drakeley, C., Gesase, S., Lusingu, J., Ansong, D., Adjei, S., Agbenyega, T., Ogutu, B., Otieno, L., Otieno, W., Agnandji, S. T., Lell, B., Kremsner, P., Hoffman, I., … Ghani, A. C. (2015). Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: Secondary analysis of data from a phase 3 randomised controlled trial. *The Lancet Infectious Diseases*, *15*(12), 1450–1458. https://doi.org/10.1016/S1473-3099(15)00239-X

White, N. J., Pukrittayakamee, S., Hien, T. T., Faiz, M. A., Mokuolu, O. A., & Dondorp, A. M. (2014). Malaria. *The Lancet*, *383*(9918), 723–735. https://doi.org/10.1016/S0140-6736(13)60024-0

WHO. (2016). World malaria report 2015. In *World Health Organization*. https://doi.org/10.1002/(SICI)1096-8628(19971128)73:1<1::AID-AJMG1>3.0.CO;2-Y

WHO. (2018). World Malaria Report 2018. In *Geneva: World Health Organization; 2018*. https://doi.org/ISBN 978 92 4 1564403

WHO. (2019). *World Malaria Report 2019.* https://www.who.int/publications-detail/world-malaria-report-2019

WHO. (2020). *Vaccine safety basics*. World Health Organization.

Wijesekera, S. K., Carter, R., Rathnayaka, L., & Mendis, K. N. (1996). A malaria parasite toxin associated with Plasmodium vivax paroxysms. *Clinical and Experimental Immunology*, *104*(2), 221–227.

https://doi.org/10.1046/j.1365-2249.1996.07699.x

Wilk, M. M., Misiak, A., McManus, R. M., Allen, A. C., Lynch, M. A., & Mills, K. H. G. (2017). Lung CD4 Tissue-Resident Memory T Cells Mediate Adaptive Immunity Induced by Previous Infection of Mice with Bordetella pertussis . *The Journal of Immunology*, *199*(1), 233–243. https://doi.org/10.4049/jimmunol.1602051

Wilkins, A. L., Kazmin, D., Napolitani, G., Clutterbuck, E. A., Pulendran, B., Siegrist, C. A., & Pollard, A. J. (2017). AS03- and MF59-adjuvanted influenza vaccines in children. *Frontiers in Immunology*, *8*(DEC). https://doi.org/10.3389/fimmu.2017.01760

Winer, B., Edgel, K. A., Zou, X., Sellau, J., Hadiwidjojo, S., Garver, L. S., McDonough, C. E., Kelleher, N. L., Thomas, P. M., Villasante, E., Ploss, A., & Gerbasi, V. R. (2020). Identification of Plasmodium falciparum proteoforms from liver stage models. *Malaria Journal*, *19*(1), 1–13. https://doi.org/10.1186/s12936-019-3093-3

Wu, C. C. N., Crain, B., Yao, S., Sabet, M., Lao, F. S., Tawatao, R. I., Chan, M., Smee, D. F., Julander, J. G., Cottam, H. B., Guiney, D. G., Corr, M., Carson, D. A., & Hayashi, T. (2014). Innate immune protection against infectious diseases by pulmonary administration of a phospholipid-conjugated TLR7 ligand. *Journal of Innate Immunity*, *6*(3), 315–324. https://doi.org/10.1159/000355217

Wu, Jiaxi, Sun, L., Chen, X., Du, F., Shi, H., Chen, C., & Chen, Z. J. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science*, *339*(6121), 826–830. https://doi.org/10.1126/science.1229963

Wu, Jing, Li, S., Li, T., Lv, X., Zhang, M., Zang, G., Qi, C., Liu, Y. J., Xu, L., & Chen, J. (2019). PDC Activation by TLR7/8 Ligand CL097 Compared to TLR7 Ligand IMQ or TLR9 Ligand CpG. *Journal of Immunology Research*, *2019*. https://doi.org/10.1155/2019/1749803

Wu, Jing, Li, S., Yang, Y., Zhu, S., Zhang, M., Qiao, Y., Liu, Y. J., & Chen, J. (2017). TLR-activated plasmacytoid dendritic cells inhibit breast cancer cell growth in vitro and in vivo. *Oncotarget*, *8*(7), 11708–11718. https://doi.org/10.18632/oncotarget.14315

Wu, Jun, Meng, Z., Jiang, M., Zhang, E., Trippler, M., Broering, R., Bucchi, A., Krux, F., Dittmer, U., Yang, D., Roggendorf, M., Gerken, G., Lu, M., & Schlaak, J. F. (2009). Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. *Immunology*, *129*(3), 363–374. https://doi.org/10.1111/j.1365-2567.2009.03179.x

Wu, Yimin, Ellis, R. D., Shaffer, D., Fontes, E., Malkin, E. M., Mahanty, S., Fay, M. P., Narum, D., Rausch, K., Miles, A. P., Aebig, J., Orcutt, A., Muratova,

O., Song, G., Lambert, L., Zhu, D., Miura, K., Long, C., Saul, A., … Durbin, A. P. (2008). Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs 25 formulated with montanide ISA 51. *PLoS ONE*, *3*(7). https://doi.org/10.1371/journal.pone.0002636

Wu, Yuanbing, Wu, X., Wu, L., Wang, X., & Liu, Z. (2017). The anticancer functions of RIG-I–like receptors, RIG-I and MDA5, and their applications in cancer therapy. *Translational Research*, *190*, 51–60. https://doi.org/10.1016/j.trsl.2017.08.004

Wysocka, M., Robertson, S., Riemann, H., Caamano, J., Hunter, C., Mackiewicz, A., Montaner, L. J., Trinchieri, G., & Karp, C. L. (2001). IL-12 Suppression During Experimental Endotoxin Tolerance: Dendritic Cell Loss and Macrophage Hyporesponsiveness. *The Journal of Immunology*, *166*(12), 7504–7513. https://doi.org/10.4049/jimmunol.166.12.7504

Yamauchi Lucy M., L. M., Coppi, A., Snounou, G., & Sinnis, P. (2007). Plasmodium sporozoites trickle out of the injection site. *Cellular Microbiology*, *9*(5), 1215–1222. https://doi.org/10.1111/j.1462-5822.2006.00861.x

Yan, H., KuoLee, R., Tram, K., Qiu, H., Zhang, J., Patel, G. B., & Chen, W. (2009). 3′,5′-Cyclic diguanylic acid elicits mucosal immunity against bacterial infection. *Biochemical and Biophysical Research Communications*, *387*(3), 581–584. https://doi.org/10.1016/j.bbrc.2009.07.061

Yang, A. S. P., Lopaticki, S., O'Neill, M. T., Erickson, S. M., Douglas, D. N., Kneteman, N. M., & Boddey, J. A. (2017). AMA1 and MAEBL are important for Plasmodium falciparum sporozoite infection of the liver. *Cellular Microbiology*, *19*(9), 1–14. https://doi.org/10.1111/cmi.12745

Yasuda, K., Yu, P., Kirschning, C. J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H., & Wagner, H. (2005). Endosomal Translocation of Vertebrate DNA Activates Dendritic Cells via TLR9-Dependent and -Independent Pathways. *The Journal of Immunology*, *174*(10), 6129–6136. https://doi.org/10.4049/jimmunol.174.10.6129

Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., & Anderson, D. G. (2014). Non-viral vectors for gene-based therapy. *Nature Reviews Genetics*, *15*(8), 541–555. https://doi.org/10.1038/nrg3763

Yone, C. L. R. P., Kremsner, P. G., & Luty, A. J. F. (2005). Immunoglobulin G Isotype Responses to Erythrocyte Surface-Expressed Variant Antigens of. *Society*, *73*(4), 2281–2287. https://doi.org/10.1128/IAI.73.4.2281

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., & Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature Immunology*, *5*(7), 730–737.

https://doi.org/10.1038/ni1087

Yong, H. Y., & Luo, D. (2018). RIG-I-like receptors as novel targets for pan-antivirals and vaccine adjuvants against emerging and re-emerging viral infections. *Frontiers in Immunology*, *9*(JUN), 1–9. https://doi.org/10.3389/fimmu.2018.01379

Yotsumoto, S., Saegusa, K., & Aramaki, Y. (2008). Endosomal Translocation of CpG-Oligodeoxynucleotides Inhibits DNA-PKcs-Dependent IL-10 Production in Macrophages. *The Journal of Immunology*, *180*(2), 809–816. https://doi.org/10.4049/jimmunol.180.2.809

Yu, L.-X., Yan, H.-X., Liu, Q., Yang, W., Wu, H.-P., Dong, W., Tang, L., Lin, Y., He, Y.-Q., Zou, S.-S., Wang, C., Zhang, H.-L., Cao, G.-W., Wu, M.-C., & Wang, H.-Y. (2010). Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. *Hepatology*, *52*(4), 1322–1333. https://doi.org/10.1002/hep.23845

Zaidi, I., Diallo, H., Conteh, S., Robbins, Y., Kolasny, J., Orr-Gonzalez, S., Carter, D., Butler, B., Lambert, L., Brickley, E., Morrison, R., Sissoko, M., Healy, S. A., Sim, B. K. L., Doumbo, O. K., Hoffman, S. L., & Duffy, P. E. (2017). γδ T Cells Are Required for the Induction of Sterile Immunity during Irradiated Sporozoite Vaccinations. *The Journal of Immunology*, *199*(11), 3781–3788. https://doi.org/10.4049/jimmunol.1700314

Zarember, K. A., & Godowski, P. J. (2002). Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines. *The Journal of Immunology*, *168*(2), 554–561. https://doi.org/10.4049/jimmunol.168.2.554

Zariri, A., & Van Der Ley, P. (2015). Biosynthetically engineered lipopolysaccharide as vaccine adjuvant. *Expert Review of Vaccines*, *14*(6), 861–876. https://doi.org/10.1586/14760584.2015.1026808

Zens, K. D., Chen, J. K., & Farber, D. L. (2016). Vaccine-generated lung tissue–resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight*, *1*(10), 1–12. https://doi.org/10.1172/jci.insight.85832

Zhang, L., Wang, W., & Wang, S. (2015). Effect of vaccine administration modality on immunogenicity and efficacy. *Expert Review of Vaccines*, *14*(11), 1509–1523. https://doi.org/10.1586/14760584.2015.1081067

Zhang, S., Cubas, R., Li, M., Chen, C., & Yao, Q. (2009). Virus-like particle vaccine activates conventional B2 cells and promotes B cell differentiation to IgG2a producing plasma cells. *Molecular Immunology*, *46*(10), 1988–2001. https://doi.org/10.1016/j.molimm.2009.03.008

Zhang, Z., Ohto, U., Shibata, T., Krayukhina, E., Taoka, M., Yamauchi, Y., Tanji,

H., Isobe, T., Uchiyama, S., Miyake, K., & Shimizu, T. (2016). Structural Analysis Reveals that Toll-like Receptor 7 Is a Dual Receptor for Guanosine and Single-Stranded RNA. *Immunity*, *45*(4), 737–748. https://doi.org/10.1016/j.immuni.2016.09.011

CURRICULUM VITAE

Key experience

- Experience with mouse *in vivo* experiments: Equivalent course on animal welfare FELASA B1. During my PhD I have been performing mouse *in vivo* experiments on C57BL/6 mice, especially intravenous injections and monitoring of infected mice with experimental cerebral malaria. Processing of livers, lymph nodes, blood and spleens for the generation of single cell solutions and flow cytometry analysis.
- Cellular biology: Cell culture in Bio Safety Level 2; flow cytometry for cell death analysis; immunofluorescence microscopy; electron microscopy; cell culture of cells in suspension; growth curves; transfections in *Trypanosoma brucei* and cell culture of *Plasmodium falciparum* in human erythrocytes.
- Molecular biology: Development of CRISPR-Cas system in a model parasite, chromatin immuno-precipitation (ChIP), phage display assays, preparation of libraries for next generation sequencing, qPCRs, western blots, SDS-PAGE, cloning, PCR, plasmid and genomic DNA isolation, RNA isolation, synthesis of cDNA, ligation, transformation.
- Training in software such as "FlowJo" for data analysis of flow cytometry, "Graphpad Prism 5" for statistical analysis of data and "CLC Workbench" for DNA, RNA, and protein sequence data analysis.
- Analytical skills for problem solving, objectivity, leadership, attention to detail and ability to work in team.

Formal Education

2016 - Present PhD student of joint program in Immunology
The University of Melbourne, Australia & University of Bonn, Germany.
Thesis title: "Characterization of the memory response induce by immunisation with a novel antigen and adjuvants for a dendritic cell-targeted malaria vaccine".
Supervisor (University of Melbourne): Prof Dr William R Heath
Supervisor (University of Bonn): Dr. rer. Nat. Winfried Barchet
Scholarship from: University of Melbourne

2013 - 2015 MSc FOKUS Life Sciences.
University Wuerzburg, Germany
Thesis title: "Role of histone acetylation and bromodomain factors in the targeted deposition of H2AZ in *Trypanosoma brucei*".
Supervisor: Prof Dr Nicolai Siegel

2007 – 2011 BsC in Microbiology
Universidad de los Andes, Bogotá, Colombia.
Thesis title: "Importance of the cell death type in the acquisition of adaptive immunity against *Mycobacterium Tuberculosis*".
Supervisor: Prof. Dr. Carlos Alberto Parra Lopez

Complementary Education

2016 DGFI Autumn School, Current Concepts in Immunology (Hours: 40h)

Professional Experience

2016 – 2019 University Hospital Bonn (Germany)
Institute for Clinical Chemistry and Clinical Pharmacology.
Scientific employee, Position: PhD Student, Working hours: 65% (to full time).

- Coordination of a research collaborative project in Malaria vaccination with the participation of different institutes of the University Hospital Bonn, University Hospital Heidelberg in Germany and The Doherty Institute, Melbourne.
- Investigating the role of nucleic acids as adjuvants for a malaria vaccine against liver stage using the mouse model of C57BL/6 and *Plasmodium berghei*.

2015 - 2016 Fraunhofer IME, Aachen (Germany)
Division of Pharmaceutical product development,
Scientific employee, Position: Assistant research scientist (HIWI)

- Performing phage display assays for a phage library containing Fab fragments of antibodies specific against *P. falciparum*.

2013 – 2014 University Wuerzburg (Germany)
Institute of Molecular Infection Biology (IMIB)
Scientific employee, Position: Assistant research scientist (HIWI)

- Studying the presence of proteins involved in autophagy processes during infection of murine macrophages with *L. major.*
- Embedding electron microscope images of murine infected-dendritic cells with promastigotes of *L. major*.

2012 – 2013 University Wuerzburg (Germany)
Institute of Molecular Infection Biology (IMIB)
Scientific employee, Position: Assistant research scientist (HIWI)

- Investigating the mechanisms of transcription termination in *T. brucei*.

Presentations in Events

1. Valencia-Hernandez, AM., Menezes, MN., Lahoud, M., Cozijinzen, A., McFadden GI., La Gruta, N., Bertolino, P., Caminschi, I., Fernandez-Ruiz, D., Barchet, W., & Heath WR. Subunit vaccination targeting novel malaria liver-stage epitope confers protection in mice with natural endogenous repertoire. Oral presentation at the 48th Annual Scientific Meeting of the Australasian Society for Immunology (ASI), 2019.

2. Valencia-Hernandez, AM., Lahoud, M., Cozijinzen, McFadden GI., La Gruta, N., Caminschi, I., Fernandez-Ruiz, D., Barchet, W., & Heath WR. Effect of nucleic acids as adjuvants for the induction of *P. berghei*-specific liver tissue resident memory CD8 T cells. Poster presentation at the Malaria in Melbourne 2019 conference.

3. Semi-finalist 3 Minutes Thesis (3MT), “An army of Immune cells”. The University of Melbourne, 2019.

4. Valencia-Hernandez, AM., Fernandez-Ruiz, D., Caminschi, I., Lahoud, M., Cozijinzen, A., Mollard, V., Barchet, W., & Heath WR. Effect of adjuvants on the induction

of protective CD8+ T cell immunity against liver stage malaria parasites. Oral presentation (Science communication session) at the 47th Annual Scientific Meeting of the Australasian Society for Immunology (ASI), 2018.

5. Valencia-Hernandez, Ana M., Fernandez-Ruiz, D., Müller, AK., Frank, R., Caminschi, I., Lahoud, M., Barchet, W. & Heath WR. Increasing Plasmodium based vaccine immunogenicity to induce protective CD8+ T cell immunity against Malaria liver stage. Poster presentation at the1st Malaria World Congress, 2018.

6. Valencia-Hernandez, Ana M., Fernandez-Ruiz, D., Müller, AK., Frank, R., Caminschi, I., Lahoud, M., Barchet, W. & Heath WR. Increasing Plasmodium based vaccine immunogenicity to induce protective CD8+ T cell immunity against Malaria liver stage. Poster presentation at the 7th Australasian Vaccines & Immunotherapeutics Development Meeting, 2018.

7. Valencia-Hernandez, Ana M., Fernandez-Ruiz, D., Schumak, B., Müller, A., Hörauf, A., Heath W., Hartmann G. and Barchet, W. Increasing *Plasmodium* based vaccine immunogenicity to induce protective $CD8^+$ T cell immunity against Malaria liver stage. Poster presentation at the Annual Meeting of German Center for Infection Research, 2016.

Awards

Scholarship "Programa Quiero Estudiar" (100%) awarded by the Universidad de Los Andes (Colombia) to students with outstanding results in their applications, it covered the full tuition and other education related expenses along the 4 years of my undergraduate studies.

Articles Published in Scientific Journals

1. Ghilas S., Valencia-Hernandez, A. M., Enders, M. H., Heath, W. R & Fernandez-Ruiz, D. (2020). Resident memory T cells and their role within the liver. *International Journal of Molecular Sciences*, 1-15. https://doi.org/10.3390/ijms21228565

2. Valencia-Hernandez, A. M., Ng, W. Y., Ghazanfari, N., Ghilas, S., de Menezes, M. N., Holz, L. E., Huang, C., English, K., Naung, M., Tan, P. S., Tullett, K. M., Steiner, T. M., Enders, M. H., Beattie, L., Chua, Y. C., Jones, C. M., Cozijnsen, A., Mollard, V., Cai, Y., … Fernandez-Ruiz, D. (2020). A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver TRM Cell-Mediated Immunity against Malaria in Mice. *Cell Host & Microbe*, 1–13. https://doi.org/10.1016/j.chom.2020.04.010

3. Holz, L. E., Chua, Y. C., de Menezes, M. N., Anderson, R. J., Draper, S. L., Compton, B. J., Chan, S. T. S., Mathew, J., Li, J., Kedzierski, L., Wang, Z., Beattie, L., Enders, M. H., Ghilas, S., May, R., Steiner, T. M., Lange, J., Fernandez-Ruiz, D., Valencia-Hernandez, A. M., … Heath, W. R. (2020). Glycolipid-peptide vaccination induces liver-resident memory CD8+ T cells that protect against rodent malaria. *Science Immunology*, *5*(48), 1–14. https://doi.org/10.1126/sciimmunol.aaz8035.

4. Masic, A., Valencia-Hernandez, A. M., Hazra, S., Glaser, J., Holzgrabe, U., Hazra, B., & Schurigt, U. (2015). Cinnamic acid bornyl ester derivatives from valeriana wallichii exhibit antileishmanial in vivo activity in leishmania major-infected BALB/c Mice. *PLoS ONE*, *10*(11), 1–20. https://doi.org/10.1371/journal.pone.0142386

References

Available on request