THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Regulation of $CD4^+$ T cell responses during parasitic infections

Marcela Montes de Oca

Bachelor of Biomedical Science (Hons IA)

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in 2015

UQ School of Medicine – North
QIMR Berghofer Medical Research Institute

Abstract

$CD4^+$ T cells play protective roles against viral, bacterial, fungal and parasitic infections. This protective ability results from the capacity of $CD4^+$ T cells to differentiate into various effector T helper subsets which have been extensively described in recent literature. T helper cells aid pathogen killing either directly, by secreting potent pro-inflammatory cytokines such as tumour necrosis factor (TNF) or indirectly, by providing help to B cells in order to promote class switching or secreting cytokines such as interferon gamma (IFNγ) to activate macrophages to enhance their microbicidal activity. $CD4^+$ T cells have a unique characteristic to be plastic, depending on the tissue environment (cytokines and chemokines), expression of master transcription factors, nature of the invading pathogen and the current immune status of the host (immunocompetent vs immunocompromised). Generation of T-helper 1 (Th1) $CD4^+$ T cells requires expression of the master transcription factor T bet and cytokines interleukin (IL)-12, IFNγ and TNF. Studies have identified the protective ability of Th1 cells in *Plasmodium* and *Leishmania* infections and the more recent self-regulatory Th1 cells, known as Tr1 cells have been shown to limit immunopathology via production of IL-10 in addition to IFNγ. While no single master transcription factor has been identified for Tr1 cells, Blimp1, c-maf, Ahr and Egr2 have been reported to induce IL-10 production in Tr1 cells. In an effort to understand what dictates the emergence of Th1 versus Tr1 cells in various disease settings, the latest approaches have focused on deciphering the transcriptional landscape of these cells. Our studies have focused on the downstream effect of the generation of these responses in the context of parasitic infections, namely *Plasmodium* and *Leishmania* which represent inflammatory environments in the host and can potentially be adapted to gain a better understanding of chronic inflammatory diseases.

Here we identify a dominant type I interferon (IFN) response that appears to suppress $CD4^+$ T cell responses in human volunteers experimentally challenged with blood-stage *Plasmodium falciparum* (*P. falciparum*) infection. Using gene array studies to identify a type I IFN signature and peripheral blood mononuclear cells (PBMC's) for functional validation studies, we found that type I IFN's suppressed $CD4^+$ T cell-derived IFNγ production. Additionally, type I IFN's appeared to be required for IL-10 production and generation of Tr1 cells. Linear regression analysis showed that high IL-10 levels were strongly associated with high parasitemia, as determined by area under the parasitemia curve (AUC).

Collectively, these results indicate that type I IFN's promote the generation of Tr1 cells which may potentially suppress anti-parasitic responses during human malaria.

To study the generation of Tr1 cells in greater detail, we employed a mouse model of *Plasmodium* and *Leishmania* infections with a specific focus on the transcriptional regulator Blimp1. Many studies have shown positive associations between Blimp1 and IL-10 and this was confirmed in our models of experimental *Plasmodium* and *Leishmania* infections, where IL-10^+ $CD4^+$ T cells and Tr1 cells (IL-10^+IFNγ^+) expressed the highest levels of Blimp1. To determine a role for Blimp1 in controlling parasite growth, T cell specific Blimp1 deficient mice infected with *Plasmodium* or *Leishmania* exhibited significantly reduced parasite burdens throughout the course of infection and this was associated with enhanced Th1 responses and severely impaired Tr1 responses in both models. Additionally, we noted that IL-12 was required for the generation of Tr1 cells and Blimp1 expression in these cells, thus supporting the notion that Tr1 cells arise from terminally differentiated Th1 cells. Interestingly, TNF producing $CD4^+$ T cells had a lower Blimp1 expression profile, compared to IL-10 producing $CD4^+$ T cells, potentially indicating a negative association where Blimp1 was suppressing TNF during infection. TNF controls parasite growth in *Leishmania* infections, but has also been shown to mediate splenic pathology via the killing of marginal zone macrophages (MZM's) leading to a breakdown in tissue architecture. Strikingly, we observed a significant increase in TNF producing $CD4^+$ T cells in the T cell specific Blimp1 deficient mice which was associated with extensive splenomegaly, a characteristic feature observed in patients with visceral leishmaniasis (VL). Importantly, Blimp1 transcripts were also found to be up-regulated in VL patients, indicating the clinical relevance of Blimp1 during infection. We sought to determine the mechanism by which Blimp1 was regulating pathology in the spleen. Firstly, we confirmed that the loss of MZM's in the T cell specific Blimp1 deficient mice was due to a lack of IL-10, where T cell-specific IL-10 deficient mice also displayed the same phenotype of MZM loss, and this process appeared to be TNF dependent. In support of IL-10 mediating protection against MZM loss, we were able to show that IL-10 signalling in T cells and myeloid-derived populations was a requirement for protection against TNF-mediated destruction. In summary, our data reveals a novel mechanism where Blimp1 induced IL-10 production by Tr1 cells limits pathology during infection. These findings have wider implications for other inflammatory diseases.

Declaration by Author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted *to qualify for the award of any* other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Marcela Montes de Oca
22nd December, 2015

Publications during Candidature

Peer reviewed papers:

Ashraful Haque, Shannon E. Best, **Marcela Montes de Oca**, Kylie R. James, Anne Ammerdorffer, Chelsea L. Edwards, Fabian de Labastida Rivera, Fiona H. Amante, Patrick T. Bunn, Meru Sheel, Ismail Sebina, Motoko Koyama, Antiopi Varelias, Paul J. Hertzog, Ulrich Kalinke, Sin Yee Gun, Laurent Rénia, Christiane Ruedl, Kelli P.A. MacDonald, Geoffrey R. Hill and Christian R. Engwerda, 2014, **Type I IFN signaling in CD8$^-$ DC's impairs Th1-dependent malaria immunity**, *J Clin Invest. 2014 Jun 2; 124(6):2483-96*

Patrick T. Bunn, Amanda C. Stanley, Fabian de Labastida Rivera, Alexander Mulherin, Meru Sheel, Clare E. Alexander, Rebecca J. Faleiro, Fiona H. Amante, **Marcela Montes De Oca**, Shannon E. Best, Kylie R. James, Paul M. Kaye, Ashraful Haque and Christian R. Engwerda, 2013, **Tissue requirements for establishing long-term CD4$^+$ T cell-mediated immunity following *L. donovani* infection**, *J Immunol. 2014 Apr 15; 192(8):3709-18*

Chelsea L. Edwards, Shannon E. Best, Sin Yee Gun, Carla Claser, Kylie R. James, **Marcela Montes de Oca**, Ismail Sebina, Fabian de Labastida Rivera, Fiona H. Amante, Paul J. Hertzog, Christian R. Engwerda, Laurent Renia and Ashraful Haque, 2014, **Spatio-temporal requirements for IRF7 in mediating Type I IFN-dependent susceptibility to blood-stage *Plasmodium* infection**, *Eur J Immunol. 2014 Oct 16; doi: 10.1002/eji.201444824*

Chapter for book:

Marcela Montes de Oca, Christian R. Engwerda, Ashraful Haque, 2013, **Methods in Molecular Biology Series: "Mouse Models of Innate Immunity". *Plasmodium berghei* ANKA (PbA) Infection of C57BL/6J Mice: A Model of Severe Malaria**. *Methods Mol Biol. 2013; 1031:203-13*

Conference Abstracts:

Marcela Montes de Oca, Meru Sheel, Fabian de Labastida Rivera, Fiona H. Amante, Rebecca J. Faleiro, Patrick T. Bunn, Geoff R. Hill, Axel Kallies, Ashraful Haque and Christian R. Engwerda, Blimp1 mediated regulation of CD4$^+$ T cell responses during blood-stage *Plasmodium* infection, Australasian Society for Immunology Meeting, December 2012, Melbourne, VIC, Australia (oral)

Marcela Montes de Oca, Meru Sheel, Fabian de Labastida Rivera, Fiona H. Amante, Rebecca J. Faleiro, Patrick T. Bunn, Geoff R. Hill, Axel Kallies, Ashraful Haque and Christian R. Engwerda, Blimp1 regulates anti-parasitic $CD4^+$ T cell responses, QIMR Berghofer Student retreat, October 2013, Brisbane, QLD, Australia (poster)

Marcela Montes de Oca, Meru Sheel, Fabian de Labastida Rivera, Fiona H. Amante, Rebecca J. Faleiro, Patrick T. Bunn, Geoff R. Hill, Axel Kallies, Ashraful Haque and Christian R. Engwerda, Blimp1 mediated regulation of $CD4^+$ T cell responses and pathology during parasitic infections, Brisbane Immunology Group Meeting, August 2014, Surfers Paradise, QLD, Australia (oral)

Marcela Montes de Oca, Meru Sheel, Fabian de Labastida Rivera, Fiona H. Amante, Rebecca J. Faleiro, Patrick T. Bunn, Chelsea L. Edwards, Susanna S. Ng, Rajiv Kumar, Geoff R. Hill, Axel Kallies, Ashraful Haque and Christian R. Engwerda, Blimp1 regulates TNF-mediated pathology, Keystone Symposia T cells: Regulation and Effector function, March-April 2015, Snowbird, Utah, USA (poster)

Publications included in this thesis:

No publications included.

Contributions by others to the thesis

My supervisor Professor Christian Engwerda (QIMR Berghofer) contributed significantly to the conception of the projects and experiments described in this thesis. Dr. Rajiv Kumar (Netaji Subhas Institute of Technology, New Delhi) performed the qPCR on human PBMC and $CD4^+$ samples from India. Professor Nicholas Anstey (Menzies School of Health Research, Darwin, NT) provided us with cryopreserved PBMC samples from field studies conducted in Timika, Indonesia). Louise Marquart (QIMR Berghofer) provided advice and assisted with the statistical analysis of human data in Chapter 3. Fabian de Labastida Rivera (QIMR Berghofer) performed all the human flex set studies presented in Chapter 3. Susanna Ng (QIMR Berghofer) drew figures: #7, #10 (Chapter 1), #7d and #17 (Chapter 5).

No other significant contributions were made.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

I am sincerely grateful for the last four years and while my perseverance and sanity was tested numerous times, I am grateful because without those challenges I would not be sitting here, writing this thesis. My supervisor taught me from early on that without the lows, we could not appreciate the highs and in retrospect, he was right.

From the early beginnings in my summer semester project to the final stages of this PhD, Professor Christian Engwerda you have been a truly amazing supervisor and mentor and I am very grateful for all your support. Thank you for welcoming me into your lab and for all your guidance, it is greatly appreciated. Dr. Ashraful Haque and Shannon Best, thank you for providing me with all the technical lab/animal skills and methods for data analysis as this has carried on well into my PhD, you are both great teachers and I thank you for your patience and guidance in those early days, it has made all the difference. Dr. Fiona Amante (Lady *falciparum*) and Fabian de Labastida Rivera, you are both amazing! I will always remember our insightful discussions about results of the human trials (good and not so good) and in particular I will be forever grateful for your continued encouragement, especially on difficult days. It may have been a simple encouraging word, a hug or a smile that told me to keep going and not give up, so thank you. Special thank you to Dr. Meru Sheel for all your helpful advice about lab techniques, data presentations and career opportunities, I cannot honestly thank you enough. You have always been there to guide me and for that I will always be grateful. Dr. Lynette Beattie, while I haven't known you for very long, in the short time that I have, I must say I have enjoyed every minute! I appreciate you taking the time to teach me a bit of imaging on the Zeiss 780 NLO and all your advice, many thanks!

Rebecca Faleiro! thank you for sharing the journey of completing a PhD together, you have been there to share the joyful moments and the not so joyful moments and it was during these times that we were able to encourage one another. I wish you all the best for your future endeavours.

Patrick Bunn, well what can I say? You are brilliant! I have learnt so much from you. Thank you for putting things into perspective for me; it is something I will never forget. Keep going, you can do it and may the future bring you many exciting adventures!

Chelsea Edwards, Susanna Ng and Teija Frame, you ladies brighten up my day and I have thoroughly enjoyed our morning teas together! Melody Cheong, I thank you for all lunchtime discussions we had, especially over the last 2 years and for all your kind, encouraging words *fist bump*.

Professor Geoff Hill and all members of the Bone Marrow and Transplantation lab, thank you for providing us with various strains of knock-out mice and lots and lots of Enbrel! And of course all your helpful advice and feedback at joint lab meetings, it is greatly appreciated. Thank you to the Malaria Immunology Lab for all your input at lab meetings and allowing me to use some of your reagents at the last minute!

Dr. Axel Kallies, Dr. Stephen Nutt and Dr. Erika Cretney from the Walter and Eliza Hall Institute, this project and thesis wouldn't exist without your generosity in supplying us with the Blimp1 GFP, Blimp1flox LckCre and Blimp1flox Foxp3Cre mice, so thank you for the mice and for your helpful feedback with the progression of the project.

Dr. Glen Boyle, thank you for generously providing your expertise and lab space in helping us with the microarray experiments and all the subsequent analyses, I am truly most grateful. Thank you to Dr. Denise Doolan and Julie Burel for all your advice regarding the human malaria trials and Jiang Hu for discussions and about the Fluidigm experiments.

I would like to acknowledge and thank the staff from the QIMR Berghofer Animal, Histology and Flow cytometry facilities! I am very grateful for all your assistance and support. Thank you ☺

A very BIG thank you to the Montes de Oca Family! Armando, Margoth, Alison, Bluebird and Pepito you each hold a very special place in my heart (just because you are at the end, does not mean I am any less grateful....just thought I should clarify that). I have been fortunate enough to have my family around me, supporting me and encouraging me each and every day. I believe you deserve honorary degrees because all I have done, I have not done alone and I could not have done without you. It is those special moments in time that I will always remember and be thankful for. Bluebird for all the many early starts, late nights, the laughter and all the tears, we did this together! To my family overseas in Bolivia, my grandparents, aunts, uncles and cousins, I humbly thank you for all your prayers and encouraging words because despite being on the other side of the world, you have been there cheering me on, the whole time. Bless you all.

Dr. Adrian Knack, you have been an unrelenting source of positivity and encouragement, so thank you, I will always be grateful ☺

To my very close friends, Eriza Secondes, Tam Nguyen and Tiffany Au, you are each very dear and beautiful to me. You have made me laugh to point of tears and I have thoroughly enjoyed all the great moments we were able to share together. I have learnt so much from you and I have enjoyed this adventure we call friendship. Thank you for being there for me, making me laugh and for just letting me just be myself, you are all amazing and I pray for an abundance of happiness, love and peace in your lives.

I would like to thank the NHMRC, UQ and QIMR Berghofer for providing me with a scholarship during the course of my candidature, I am sincerely grateful.

And I would also like to thank my examiners, for taking the time to read this thesis.

Keywords

Plasmodium, *Leishmania*, $CD4^+$ T cells, Blimp-1, regulation, pathology, infection, inflammation, TNF, IL-10

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code, 110704, Cellular Immunology, 70%
ANZSRC code, 110309, Infectious Diseases, 20%
ANZSRC code, 110316, Pathology, 10%

Fields of Research (FoR) Classification

FoR code, 1107 Immunology, 80%
FoR code, 1108 Medical Microbiology, 10%
FoR code, 1116 Medical Physiology, 10%

Table of Contents

List of Tables and Figures

TABLES

FIGURES

Chapter 1 Figures

Chapter 3 Figures

Chapter 4 Figures

Chapter 5 Figures

List of Abbreviations

ACT	Artemisinin-based combination therapy
Ahr	aryl hydrocarbon receptor
AIDS	Acquired immune deficiency syndrome
AP-1	Activator protein-1
APC	Antigen presenting cell
Bcl-6	B-cell cll/lymphoma 6 transcription factor
Blimp1	B-lymphocyte induced maturation protein 1
BSA	Bovine serum albumin
CBA	Cytometric Bead Array
CCL5	Chemokine (C-C motif) ligand 5
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complimentary deoxyribonucleic acid
CL	Cutaneous leishmaniasis
CLP	Cecal ligation puncture
c-Maf	Musculoaponeurotic fibrosarcoma oncogene homolog
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL10	C-X-C motif chemokine 10
CXCR5	C-X-C chemokine receptor type 5
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCL	Diffuse cutaneous leishmaniasis
DMSO	Dimethyl sulfoxide
dsRNA	Double stranded ribonucleic acid
ECM	Experimental cerebral malaria
EDTA	Ethylenediaminetetraacetic acid
Egr2	Early growth response -2
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable
Foxp3	Forkhead box P3 transcription factor

GATA3	"G-A-T-A" sequence binding protein 3
GFP	Green fluorescent protein
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen DR
HPRT	Hypoxanthine phosphoribosyltransferase
HZ	Haemozoin
KC	Kupffer cell
IBSM	Induced blood-stage malaria
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IFNαR1	Interferon alpha receptor 1
IFNγ	Interferon gamma
IL	Interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRF	Interferon regulatory factor
i.v.	Intravenously
IVT	*in vitro* transcription
KO	Knock-out
LAG-3	Lymphocyte-activation gene 3
LOD	Limit of detection
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MCP-1	Monocyte chemoattractant protein -1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
MIP1α/β	Macrophage inflammatory protein 1-alpha/beta
ML	Mucocutaneous leishmaniasis
MMM	Marginal zone metallophilic macrophages
MSP1	Merozoite surface protein 1
MZ	Marginal zone
MZM	Marginal zone macrophages

ND	Not detected
NFκB	Nuclear factor kappa B
NHMRC	National health and medical research council
NIH	National institutes of health
NK	Natural killer
PbA	*Plasmodium berghei* ANKA
PbA-luc	Luciferase-expressing transgenic PbA line
PBMC's	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PcAS	*Plasmodium chabaudi chabaudi* AS
PD-1	Programmed cell death protein 1
PFA	Paraformaldehyde
p.i.	Post infection
pRBC	parasitised red blood cell
Prdm1	PR domain zinc finger protein 1
PS	Penicillin/Streptomycin
RAG1	Recombination activation gene-1
RBC	Red blood cell
RPMI	Roswell park memorial institute medium
RORγT	RAR-related orphan receptor γT transcription factor
ROS	Reactive oxygen species
RT qPCR	Real time quantitative polymerase chain reaction
SA	Streptavidin
SCID	Severe combined immunodeficiency
SIM	Sporozoite-induced malaria
SLAM	Signalling lymphocytic activation molecule
Spp	species (plural)
STA	Specific target amplification
STAT	Signal transducers and activators of transcription
SSG	Sodium stibogluconate
Tbet	T-cell-specific T-box transcription factor
TCR	T cell receptor
TdTomato	Tandem tomato fluorescent protein
TE buffer	Tris-EDTA buffer
TGFβ	Transforming growth factor β

TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	Type I regulatory cell
VCAM-1	Vascular cell adhesion molecule 1
VL	Visceral leishmaniasis
WHO	World health organisation
WT	Wild-type

Chapter 1

Introduction and Literature Review

1.1 INTRODUCTION

Throughout history, humans have evolved to adapt and survive outbreaks and pandemics, including the bubonic plague (1347 - 1351) that resulted in 75 million deaths and the Spanish flu (1918 -1919) which caused an estimated 50 million deaths[1, 2]. More recently, the human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) pandemic which began in 1981 is still a major global health concern, along with other infectious diseases including malaria, tuberculosis and leishmaniasis[3, 4, 5]. Just as our immune system has changed through evolution, pathogens have co-evolved alongside us, and the interplay between hosts and pathogens have shaped the immunological landscape and diseases seen today.

Parasitic diseases such as malaria and leishmaniasis are associated with potent inflammatory responses that attempt to eliminate parasites from the host, but can result in host tissue damage. Parasites have evolved to evade immune surveillance and cause immunosuppression of the host by inducing immunoregulatory networks that contribute to parasite persistence. Over time, chronic infection results in extensive immune dysfunction in the host. During infection, immunoregulatory networks serve to minimise inflammation-induced damage of host tissues, thus preserving the structural integrity of essential organs, including the brain, lungs, liver and spleen. Architectural integrity, particularly in the spleen, is important, since it is a site for the generation of immune responses, filtering of the blood and removal of foreign matter. The spleen is home to specialised cell populations such as macrophages, dendritic cells (DC's), T and B cells which traffic throughout this secondary lymphoid organ. Interactions between these cell populations dictate the type and magnitude of immune responses that emerge during infection.

Histopathological changes in the brain or spleen of individuals with malaria or leishmaniasis, respectively, are examples of the consequences of immune dysfunction as a result of exacerbated inflammatory immune responses to parasites. While there is currently no effective vaccine available against these parasitic diseases, there is an urgent need to develop targeted therapeutic strategies to treat these diseases. A more comprehensive understanding of anti-parasitic immune responses, as well as how immunoregulatory networks are established, is necessary for the design and development of such strategies. Furthermore, the development of therapeutic interventions against parasite-induced inflammation has the potential to be used against other chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, psoriasis, inflammatory bowel disease and ulcerative colitis.

This thesis reports on the establishment of immunoregulatory networks in malaria and leishmaniasis, and how these networks serve to limit host pathology, but also contribute to parasite persistence. The findings presented in this thesis offer insights into mechanisms that underpin inflammation-induced pathology in a broad range of other infectious and non-infectious diseases.

1.2 LITERATURE REVIEW

1.2.1 Malaria

Malaria has pervaded human civilization for at least 4000 years and still remains a global health challenge today[6]. The World Health Organisation (WHO), reported 198 million malaria cases world-wide that resulted in 584,000 deaths in 2013[7]. An estimated 90% of all malaria-related deaths occurred in young children under the age of five in sub-Saharan Africa[7] (**Fig 1**). The geographical distribution of malaria coincides with lower income countries and communities[7], and the global cost of malaria is estimated to be at least $12 billion (USD) each year, according to the Centers for Disease Control and Prevention (CDC)[8].

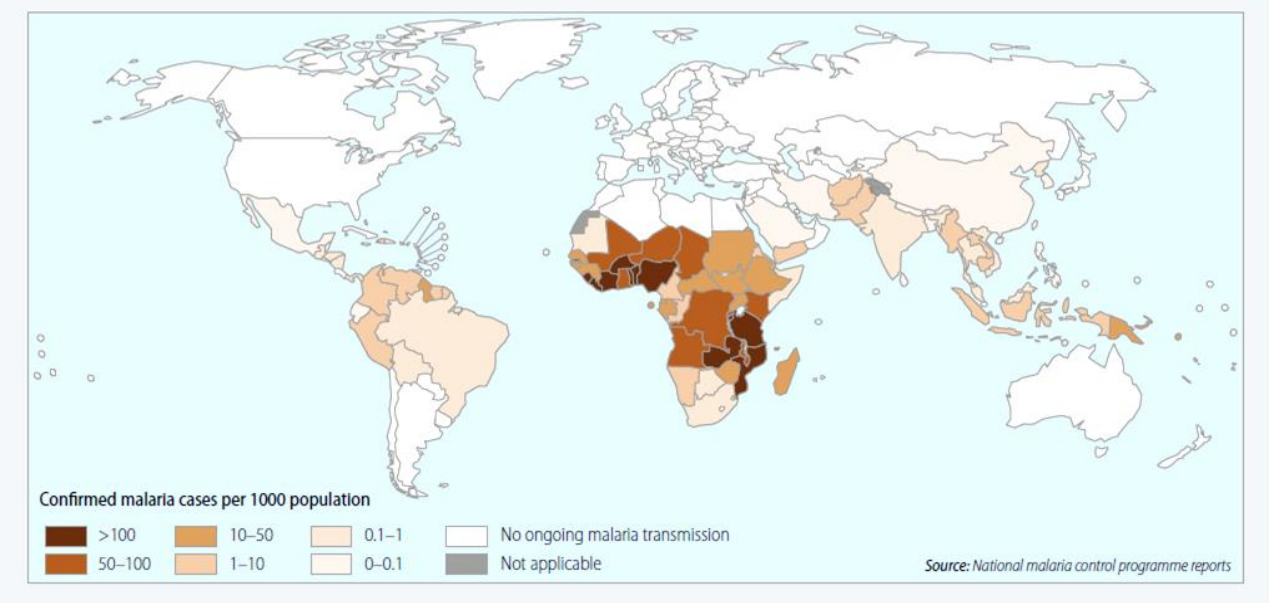


Figure 1: Countries with ongoing transmission of malaria in 2013.

Figure source: Adapted from WHO World Malaria Report 2014[7] from the national malaria control programme reports.

Malaria control and elimination strategies, including vector control (insecticide-treated bed nets or indoor residual spraying), chemoprevention (used on pregnant women and young children), rapid diagnosis and artemisinin-based combination therapy (ACT), have all been shown to contribute to reduced mortality rates[9, 10, 11]. However, the limited efficacy of the recently tested RTS/S/AS01 (**Appendix 1)** vaccine[12], along with increased drug resistance, highlights the urgent need for the development of novel strategies to combat the devastating effects of this disease.

1.2.2 *Plasmodium* species

Malaria-related deaths are caused by protozoan parasites of the genus *Plasmodium*. Six species have been identified to cause disease in humans, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale wallickerie*, *P. ovale curtisii* and a sixth species, *P. knowlesi* which is a zoonotic infection also infecting monkeys in South-East Asia[7]. The most life-threatening of these is *P. falciparum*, which contributes to the majority of all malaria-related deaths due to its prevalence in the African continent[7]. In comparison to *P. falciparum*, *P. vivax* displays a wider geographic spread, due to its ability to survive at higher altitudes, as well as grow in the *Anopheles* mosquito at cooler temperatures and also because it has a dormant liver-stage (hypnozoite) that allows it to remain as a potential reservoir for infection for long periods of time[7].

1.2.3 *Plasmodium* life cycle

Plasmodium spp. are eukaryotic apicomplexan intracellular parasites transmitted by the bite of an infected female *Anopheles* mosquito[13]. Infection of the human host begins when a female *Anopheles* mosquito injects sporozoites during a blood-meal[14] (**Fig 2**). The liver stage of the lifecycle takes place when circulating sporozoites migrate to the liver to invade hepatocytes and develop into schizonts[14, 15]. The liver-stage is transient, causes no pathology and can last between 7-10 days in humans and 2 days in mice[16]. The blood-stage of infection is triggered by the release of 30,000 – 40,000 merozoites (from liver schizonts) that invade red blood cells (RBC's), and then undergo repeated cycles of replication causing RBC's to rupture[17] that can result in the development of clinical symptoms, including: fever, chills, fatigue and nausea. Complications of *P. falciparum* infections result in severe anaemia, respiratory distress syndrome and cerebral malaria[18, 19, 20]. Left untreated, *Plasmodium* infections can lead to multi-organ failure in a matter of days[21, 22].

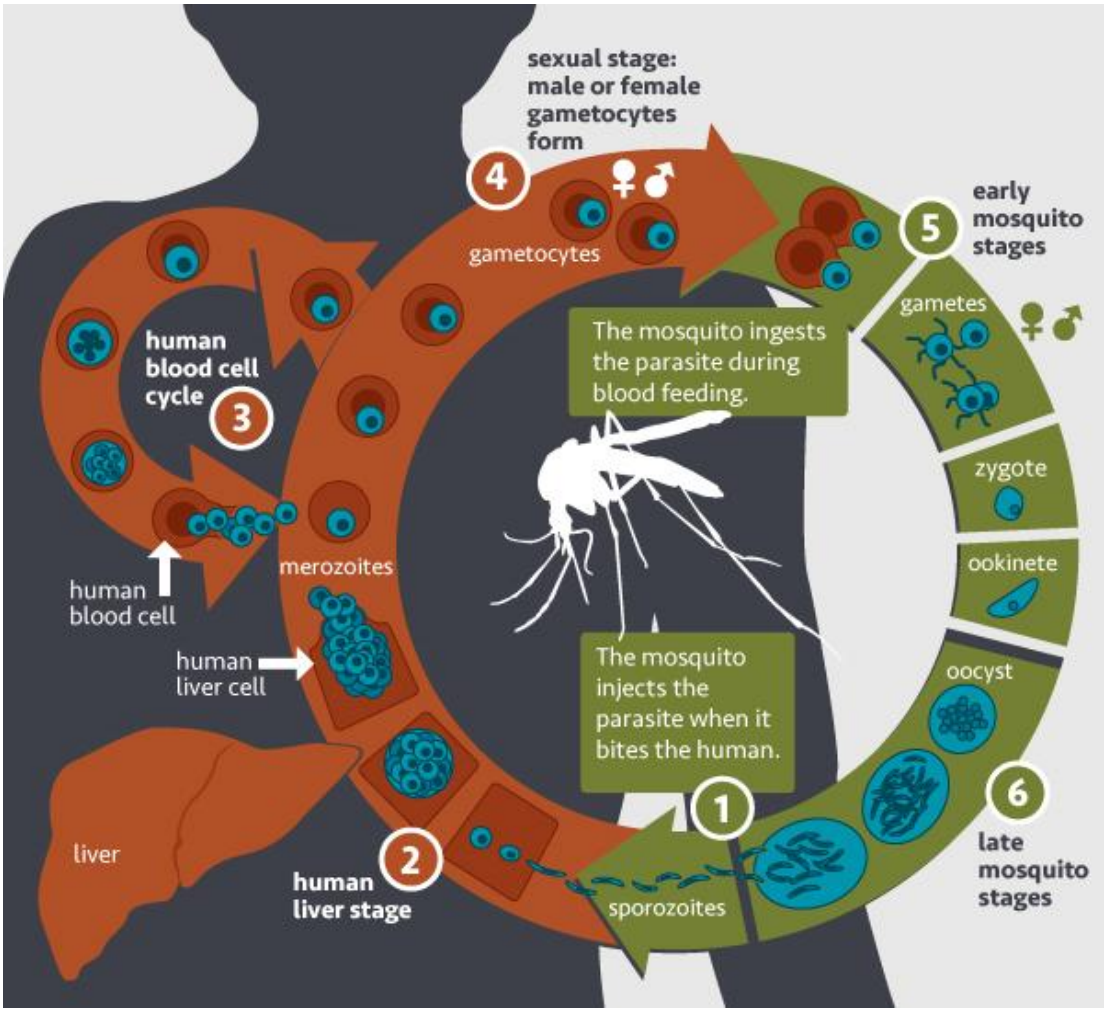


Figure 2: Life cycle of the malaria parasite.

1) A female *Anopheles* mosquito injects sporozoites during a blood meal. Sporozoites travel to the liver to invade hepatocytes. **2)** Liver stage: within 5-16 days in humans, sporozoites divide and release merozoites into the bloodstream **3)** Merozoites go onto invade RBC's and undergo asexual replication, where they then release 30-40,000 merozoites per RBC into the bloodstream to infect new RBC's. This blood-stage of infection gives rise to clinical symptoms and complications if left untreated. **4)** Instead of asexual replication, some merozoite-infected RBC's develop into male and female gametocytes (sexual forms). **5)** Gametocytes are taken up by another mosquito during a blood meal, and within the mosquito gut, gametocytes develop into gametes. Male and female gametes fuse (zygote) and develop into ookinetes that then become oocysts which line the mid-gut of the mosquito. **6)** Each oocyst grows and divides to produce many sporozoites that are released into the mosquito's body cavity which then travel to the salivary glands. The transmission cycle is complete once the mosquito injects sporozoites into another human during a blood meal. Figure source: Adapted from NIAID https://www.niaid.nih.gov/topics/Malaria/Pages/lifecycle.aspx.

1.2.4 Malaria pathogenesis

The WHO defines severe malaria as severe anaemia, cerebral malaria, respiratory distress syndrome, liver dysfunction, renal failure, haemoglobinuria and/or metabolic acidosis[18, 19, 21, 23, 24]. Two processes involved in the pathogenesis of severe malaria include microvascular obstruction leading to hypoxia, as well as immunopathological processes. Haemorrhaging associated with the sequestration of pRBC's, leukocytes and increased blood brain barrier permeability, has been observed in human and mouse cerebral malaria syndromes. The *P. falciparum* membrane protein (PfEMP)-1 is an adhesion protein involved in antigenic variation and cytoadhesion of pRBC's to the microvasculature of the brain, as well as other host cells[25, 26]. PfEMP1 is an important virulence factor, encoded by approximately sixty *var* genes that are regularly switched on, one at a time to generate antigenic variation of pRBC's, thus evading detection[27]. pRBC's sequester to the brain vascular endothelium via PfEMP1-mediated interactions with a number of adhesion molecules including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1[28, 29].

1.2.5 Immunity to malaria

Individuals that are repeatedly exposed to malaria develop natural acquired immunity, also known as premonition, which is characterised by low parasite burden and tolerance to symptoms of severe disease[30, 31]. However, these individuals are only partially protected from disease manifestations, and the little protection that is acquired, rapidly wanes in the absence of re-infection and sterilising immunity is never fully achieved[32, 33, 34]. Immunity to blood-stage parasites is primarily mediated by humoral immune responses[35]. Studies in the early 1960's showed that passive transfer of human serum from naturally immune adults containing parasite-specific immunoglobulin (Ig)-G antibodies could protect children from clinical symptoms[36]. Cellular and innate immune responses play an important role in limiting parasite growth; however, inflammation associated with these responses can also cause disease[37, 38, 39]. Given that the adaptive immune system has evolved with a number of potent regulatory mechanisms, it is not clear why these mechanisms are not sufficient to prevent immune-mediated pathology in the host.

The spleen orchestrates many immune responses against *Plasmodium* infections and usually increases in size, which is initially due to changes in the vasculature[40], but shortly thereafter lymphoid and reticuloendothelial hyperplasia occurs[40]. During *Plasmodium* infections, the spleen removes damaged RBC's and pRBC's from the bloodstream, as well as being a site where *Plasmodium*-specific T and B cell responses are generated within specialised regions of the organ[41, 42, 43, 44].

The protective and pathogenic roles of $CD4^+$ T cells during *Plasmodium* infection have been reported in human and mouse studies[37, 45, 46, 47, 48, 49, 50]. *Plasmodium* induces potent inflammatory responses in the form of cytokine production by various immune cells, including interferon-gamma (IFNγ), tumour necrosis factor (TNF) and interleukin (IL)-12[51, 52, 53]. $CD4^+$ T cell derived IFNγ is critical for controlling parasite growth and the generation of parasite-specific antibody responses are required for protection against re-infection[49, 54, 55, 56]. However, in severe malaria models, $CD4^+$ T cells contribute to immunopathology[37] and the mechanisms that drive this pathological response are poorly understood. $CD4^+$, $CD8^+$ T cells, gamma delta (γδ) T cells and Natural Killer (NK) cells produce IFNγ during *Plasmodium* infections[52, 57, 58, 59]. Despite reports on a protective role for IFNγ during human and mouse *Plasmodium* infections, the exacerbated immunopathology associated with elevated IFNγ levels suggests both protective and pathogenic cellular sources of IFNγ. Studies in mice [60] and humans[61, 62] have shown that the presence of poly-functional $CD4^+$ T cells producing IFNγ, IL-2 and TNF simultaneously, better correlates with protection, compared to single cytokine producing $CD4^+$ T cells. Similar results have been reported during *Leishmania major, Mycobacterium tuberculosis,* and HIV infections[60, 63, 64, 65, 66]. Thus, the quality of the T cell response is critical in determining disease outcome and is of paramount importance in vaccine efficiency. Recently, type I interferons (IFN's) were reported to suppress protective $CD4^+$ T cell-derived IFNγ production via $CD8\alpha^-$ DC's in a severe malaria model[67, 68]. This is but one of many examples of immune regulatory factors that can influence parasite-specific $CD4^+$ T cell development and thus disease outcome in malaria.

1.2.6 Experimental malaria models

Infection of mice with *Plasmodium* parasites have served as experimental malaria models which have made significant contributions to our understanding of disease progression and generation of immune responses. Four *Plasmodium* species are commonly used in laboratories around the world, including, *P. berghei* ANKA (PbA), *P. chabaudi chabaudi* AS (PcAS), *P. yoelii* (YM-non-lethal and 17XNL-lethal) and *P. vinckei* [reviewed in[69, 70, 71, 72]]. While not all aspects of *P. falciparum* pathophysiology can be modelled using these experimental malaria models, they have provided valuable insights into host-parasite interactions and the generation of immune responses. An alternative approach is the development of humanised mouse models to study *P. falciparum* infection; however, the limitations associated with any experimental model still remain[73]. The challenge associated with the continued use of experimental malaria models is to ensure better translation of experimental findings into effective treatments for malaria.

1.2.6.1 Uncomplicated malaria

C57BL/6J mice injected intravenously (i.v.) with PcAS develop mild, uncomplicated malaria infections, where peripheral parasitemia reaches its peak 7-8 days post infection (p.i.) and is largely resolved by 12-13 days p.i.[72]. Symptoms of PcAS-infected mice include: ruffled fur, anaemia, weight loss and haemoglobinuria, which is generally observed 8-9 days p.i.. However, mice are generally able to resolve the infection and establish immunity to re-infection[72]. Interleukin (IL)-10 production by type I regulatory (Tr1) $CD4^+$ T cells has recently been shown to be important for the prevention of immune-mediated pathology in this model[74, 75]. Cell adoptive transfer experiments have demonstrated that the initial wave of parasitemia is controlled by $CD4^+$ T-helper 1 (Th1) cells[45], whereas resolution of infection occurs as a result of $CD4^+$ follicular T-helper (Tfh) cell-mediated help in the generation of antibody responses[76]. Tfh cells provide help to B cells by promoting proliferation, survival, class-switch recombination, plasma cell differentiation, somatic hyper-mutation, adhesion and attraction [reviewed in[77]]. It is through these processes that Tfh cells have been shown to be essential for antibody production and germinal centre formation which leads to the production of high-affinity antibodies and memory B cells[77]. A recent study found that inducible T-cell co-stimulator (ICOS) was not required for Tfh induction during PcAS infection or production of isotype-switched antibodies but is necessary for the maintenance of a sustained high-affinity, protective antibody response[78].

The involvement of ICOS in providing help to B cells, involves the induction of Bcl-6 expression[79] or protecting Bcl-6 from ubiquitin-mediated degradation[80], as well as promoting the mobilisation and entry of Tfh cells into B cell follicles[81]. Additionally, a role for IL-21 signalling was shown to be involved in orchestrating co-operation between T and B cell responses during PcAS infections[82].

1.2.6.2 Severe malaria

PbA-infected mice rapidly develop severe neurological symptoms including coma, paralysis and convulsions 6-7 days p.i., and has been commonly employed to model the pathogenesis of severe malaria symptoms observed in humans infected with *P. falciparum*. Five years ago, the relevance of this particular model was questioned[83], resulting in a debate amongst the malaria research community. Notably, the terms 'severe' and 'cerebral' have been used interchangeably when describing the pathogenesis of malaria, however, severe malaria, as defined by the WHO has a range of clinical syndromes which do not result in coma[21]. Therefore, a consistent definition of disease is required when interpreting findings from studies on severe malaria. Evidence of brain pathology in PbA-infected mice includes inflammation in the brain, breakdown of the blood-brain barrier (BBB)[84] and up-regulation of ICAM-1 and VCAM-1 on brain endothelium[85]. Numerous studies have identified a role for $CD8^+$ cytotoxic T cells in the immunopathology of the brain via perforin/granzyme B dependent mechanisms[86, 87, 88, 89]. Numerous features of the PbA model recapitulate the severe symptoms observed in humans with cerebral malaria, severe anaemia, metabolic acidosis, liver dysfunction and respiratory distress[84, 90, 91, 92, 93].

1.2.7 Leishmaniasis

Leishmaniasis has emerged as the one of the most prevalent parasitic diseases world-wide after malaria and filariasis[94]. The three main forms of leishmaniasis include cutaneous leishmaniasis (CL), the most common form classified by self-resolving, localised cutaneous lesions. In some instances, parasites disseminate throughout the skin resulting in multiple non-ulcerative nodules (also known as diffuse cutaneous leishmaniasis, DCL). A second form of disease occurs when parasites grow in macrophages in the lymphatics, contributing to nasobronchial and buccal mucosal tissue destruction, also known as mucocutaneous leishmaniasis (ML). This affects mucus secreting membranes of the nose, mouth and throat. The third, and most deadly form of disease is visceral leishmaniasis (VL), also known as kala-azar[5, 95]. The WHO estimates 310 million people to be at risk of infection[96] and 1 million cases of CL have been reported in the last 5 years[96].

VL is a parasitic disease that mainly affects the spleen, liver and bone marrow[97]. An estimated 1.3 million cases, results in up to 30,000 deaths occur annually according to the latest WHO estimates[96]. VL is endemic in 76 countries, but more than 90% of cases occur in India, Bangladesh, Ethiopia, Sudan and Brazil[96] (**Fig 3**). Clinical features of VL include persistent low-grade fever, pancytopenia, hypergammaglobulinemia, hepatosplenomegaly resulting in progressive distension of the abdomen and cachexia[97]. Immunocompromised individuals have a greater risk of developing VL and relapse is not uncommon after drug treatment[98, 99]. Recent efforts have focused on treatment regimens against VL in HIV/VL co-infected individuals, since people living with HIV have a much higher risk of developing active disease[100]. In addition, VL-mediated immune dysfunction accelerates progression to AIDS[101]. However, the toxicity associated with anti-leishmanial drugs, their long treatment course and cost, as well as lack of a vaccine to prevent VL, has renewed efforts to combat this neglected parasitic disease[100]. The main focus of recent studies include the development of novel, targeted therapies involving drug in conjunction with immunotherapy to enhance anti-parasitic responses to prevent disease[102].

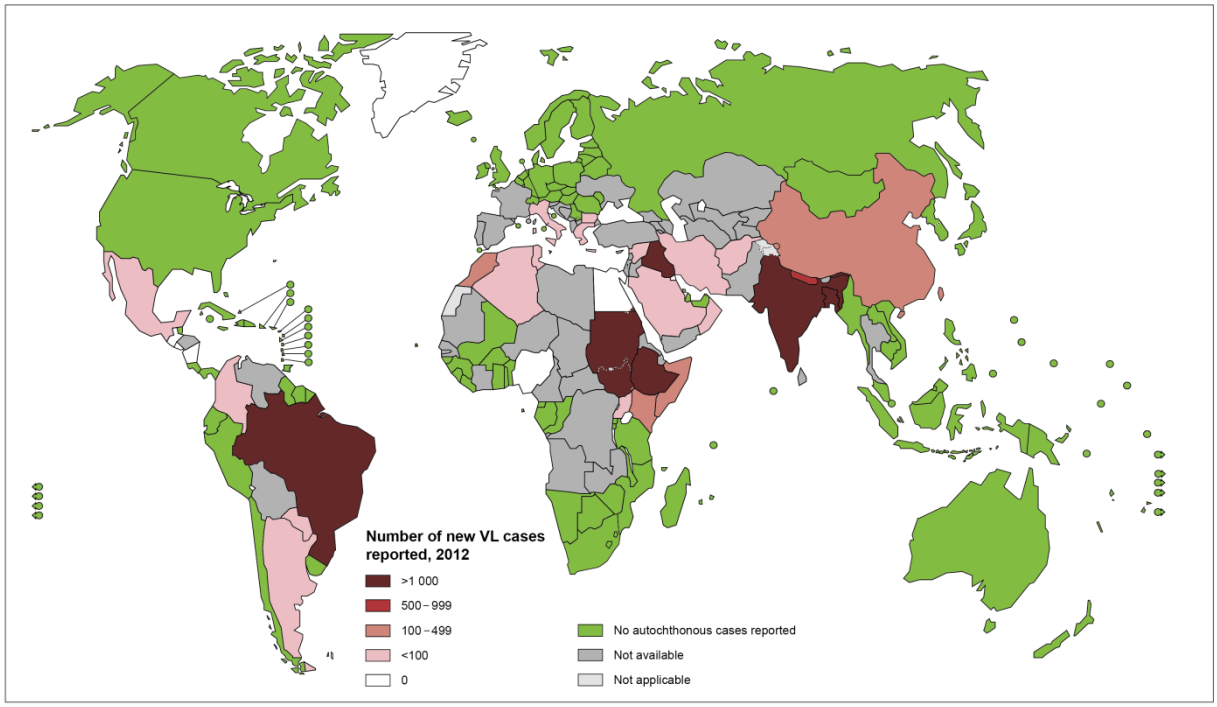


Figure 3: Global distribution of VL in 2013.

Figure source: Adapted from WHO Leishmaniasis control programme: Annual report 2013[103].

1.2.8 *Leishmania* species

Leishmania spp belongs to the group of flagellated Kinetoplastidae and 7 main species transmitted by *Phelbotomine* sandflies have been identified to cause disease in humans, including *L. mexicana, L. amazonensis, L. tropica* and *L. major* which cause CL, *L. braziliensis* which causes MCL, and *L. donovani* and *L. infantum* which cause VL[99, 104].

1.2.9 *Leishmania* life cycle

Leishmania parasites exist in two developmental stages: the flagellated, motile promastigote form and the non-motile amastigote form. The promastigote is found within the infected female *Phelobotomine* sandfly which progresses to the non-dividing, infectious 'metacyclic' promastigote form that is transmitted during a blood meal[104, 105]. Injected promastigotes replicate within host mononuclear phagocytes as amastigotes, which do not have an exterior flagellum (**Fig 4**)[104, 105]. Parasites can survive within the phagolysomes of these cells by resisting digestion by lysosomal enzymes. Within the host cell they multiply until the cell ruptures, allowing parasites that invade other macrophages[104, 105]. When a female sandfly takes up infected macrophages during a blood meal, amastigotes convert into the promastigote form within the mid-gut of the sandfly, thus completing the transmission cycle (**Fig 4**)[104, 105].

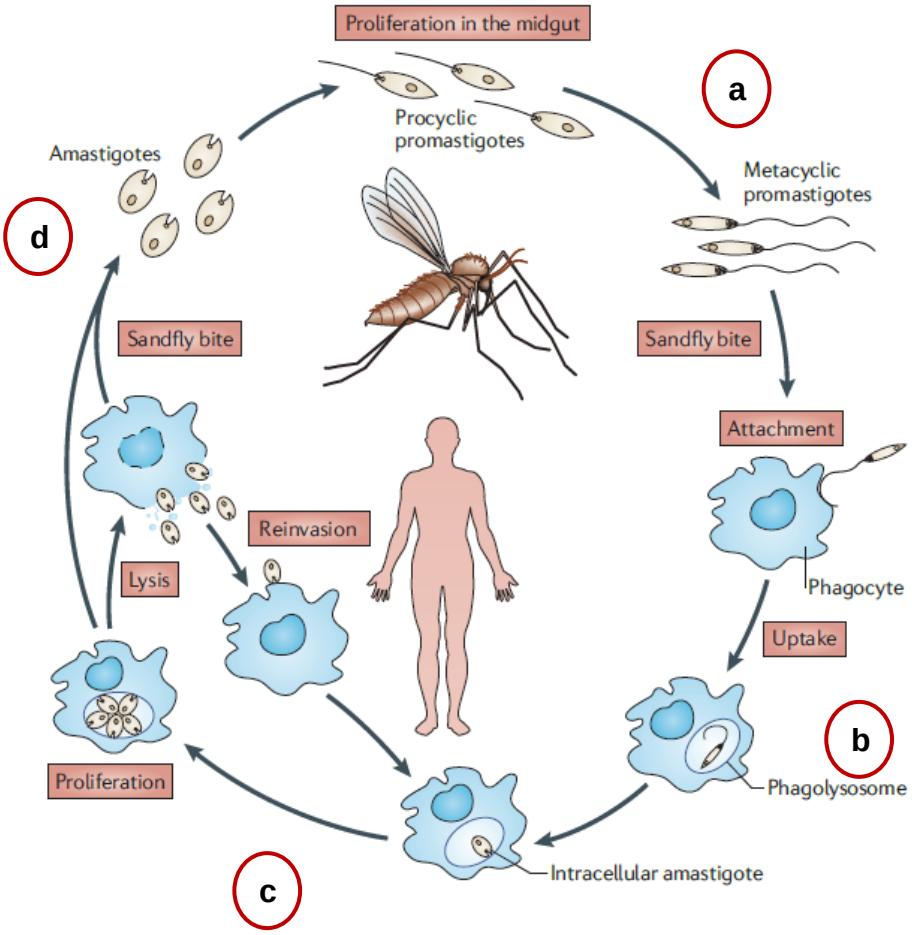


Figure 4: Lifecycle of the *Leishmania* parasite.

a) A female *Phelobotomine* sandfly injects the infective metacyclic promastigotes during a blood meal. **b)** The metacyclic promastigotes are taken up by phagocytes such as macropahges. **c)** Once inside the macrophage, the metacyclic promastigotes develop into their aflagellate amastigote form and undergo numerous cycles of replication causing the host cell to rupture and release amastigotes into the environment, so that they can be phagocytosed by other un-infected macrophages. **d)** Infected macrophages are taken up by another sandfly during a bloodmeal and amastigotes are converted into promastigotes within the mid-gut of the sandlfy, thus completing the transmission cycle. Adapted from Paul Kaye & Phillip Scott, 2011, *Nature Reviews Microbiology* 9, 604–615, permission number 3766460241587.

1.2.10 Immunopathology in VL

Experimental VL induced by infecting C57BL/6J mice with *L. donovani* results in dissemination of parasites throughout the viscera where the liver, spleen and bone marrow are the key sites of infection[106]. Injected *L. donovani* amastigotes are preferentially taken up by tissue-resident macrophages such as marginal zone macrophages (MZM's) and marginal zone metallophilic macrophages (MMM's) in the spleen, stromal macrophages in the bone marrow and Kupffer cells (KC's) in the liver[107]. Immune responses to *L. donovani* are tissue-specific, with hepatic infection being acute and transient whereas chronic infection becomes established in the spleen and bone marrow. The reasons for parasite persistence in the spleen and bone marrow remain unclear.

In experimental VL, liver parasite burdens peak at 14-21 days p.i., and begin to decline by 28 days p.i., and are largely resolved within 6-8 weeks p.i.[106]. Parasite clearance in the liver is driven by the formation of inflammatory granulomas around infected KC's, the activation of $CD4^+$ T cells to produce TNF and IFNγ which signal to macrophages to kill parasites via inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS)[108, 109, 110]. $CD4^+$ T cells control parasite growth in the liver[111, 112], whereas $CD8^+$ T cells are required for protection from reinfection[109]. $CD4^+$ T cell-derived IL-2, IL-12, IFNγ, TNF, lymphotoxin (LT) and granulocyte/macrophage colony-stimulating factor (GM-CSF) are required for effective granuloma formation around infected KC's[110, 113, 114, 115, 116, 117, 118].

The spleen is a unique, highly organised secondary lymphoid organ. The vascular networks of the spleen are arranged such that the branching arterial vessels are sheathed with lymphoid tissue and terminate in an avenous sinusoidal system, which is encapsulated by a fibrous capsule that supports the larger vasculature[119] (**Fig 5a**). This intricate system of interconnected vascular networks divide the spleen into two main compartments, known as the red pulp and white pulp regions, which are separated by a specialised collection of cells comprising the marginal zone (MZ)[119] (**Fig 5b**). The red pulp is important for the removal of pathogens and old RBC's from the circulation, as well as for recycling iron[119]. Within the white pulp, the branching arterial vessels are surrounded by B and T cell areas which are maintained by the chemokines. CXCL13 is produced by $CD35^+$ follicular DC's (FDC's) and stromal cells, and is important for maintenance of B cell zones[120]. In contrast, CCL19 and CCL21 (produced by stromal cells) are involved in recruiting T cells and DC's to T cell zones[121, 122]. CXCL13, CCL19 and CCL21 expression is regulated by LT-$\alpha_1\beta_2$ and TNF[123].

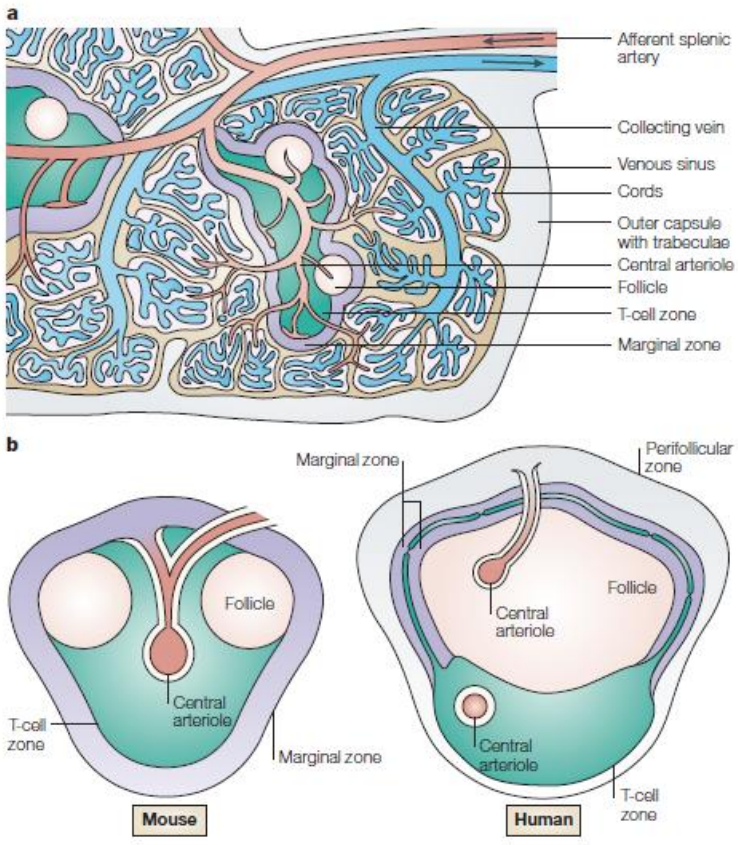


Figure 5: Structure of the spleen.

a) Structural layout of the spleen **b)** layout of white pulp regions in mice (left) and humans (right).
Figure source: Reina E. Mebius & Georg Kraal, 2005 *Nature Reviews Immunology* **5**, 606-616, permission number 3766781021951.

The close interaction between haematopoietic cells and the circulation makes for an efficient immune surveillance system[119]. The structure of the spleen facilitates this interaction through the MZ which acts as a transit area for cells entering the white pulp via the bloodstream[119, 124], and thus allows for T and B cell interactions to take place. The MZ is home to various specialised cell populations which assist in the maintenance of the splenic architecture and in particular two subsets of macrophages. MMM's line the inner border of the MZ, adjacent to the white pulp and the MZM's reside at the outer boundary of the MZ, adjacent to the red pulp (**Fig 6**)[119, 125, 126, 127].

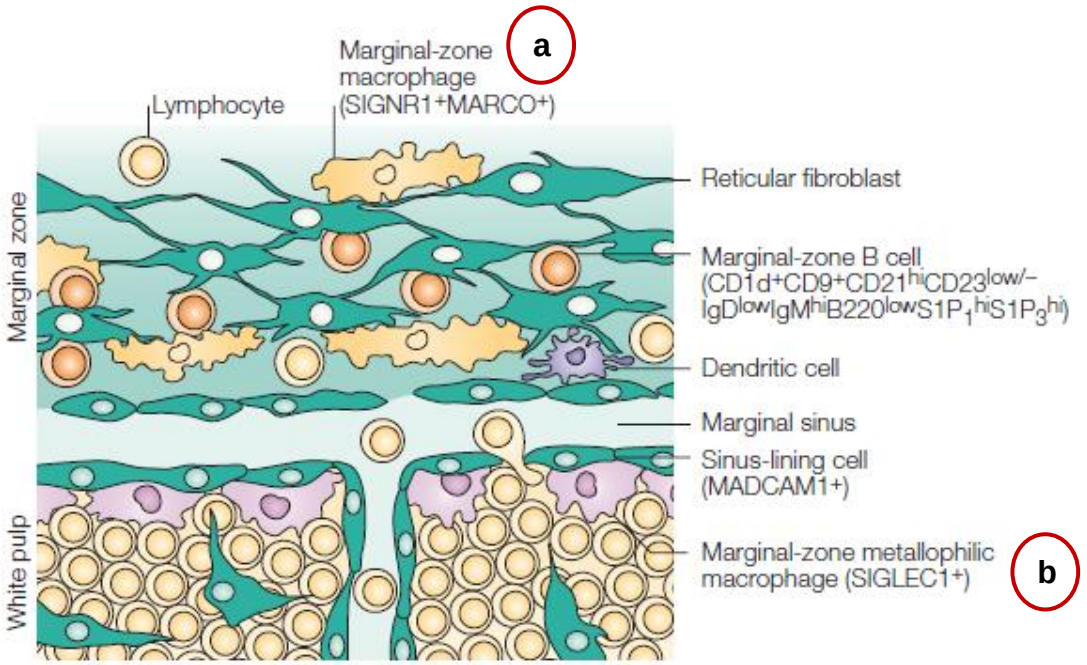


Figure 6: Layout of cells residing in the MZ of the spleen.

a) MZM's form the outer ring of macrophages **b)** MMM's form the inner ring around the white pulp.
Figure source adapted from: Reina E. Mebius & Georg Kraal, 2005 *Nature Reviews Immunology* **5**, 606-616, permission number 3766781021951.

In contrast to the response to infection in the liver during experimental VL, a chronic infection is established in the spleen and is thought to result in impaired immune responses[128]. Furthermore, TNF-mediated destruction of the splenic architecture consequently impedes lymphocyte trafficking through the spleen, and thus T cell priming is signficantly reduced[129]. Elevated levels of TNF in the spleen are associated with the induction of IL-10[130] which impedes anti-parasitic immunity and consequently allows parasites to persist. It is unclear why the anti-parasitic action of TNF is beneficial in the liver and aids control of parasite growth, yet exacerbates the inflammatory response in the spleen leading to architectural destruction and immunological dysfunction[128].

TNF and its related cytokine LTα are both required for initial parasite control in the liver during *L. donovani infection*[118], yet exhibit distinct roles despite their structural similarities as members of the TNF superfamily[131]. Specifically, LTα is required for leukocyte migration from the perivascular areas (surrounding the hepatic portal vein) of the liver to infected KC's[118]. In contrast, TNF was needed for leukocyte recruitment to the liver, but critically, was required for the survival of *L. donovani* infected mice[118].

Splenomegaly is also a feature of VL in humans and dogs. During *L. donovani* infection, TNF plays a critical role in vascular and architectural remodelling of the MZ of the spleen[129, 132], including expansion of the red pulp vascular system, neovascularisation of the white pulp and remodelling of stromal cell populations that define T and B cell areas[133].

1.2.11 Inflammation

1.2.11.1 TNF

TNF was first discovered in 1975, and is a key inflammatory cytokine for control of intracellular pathogen growth, as well as normal physiological functions in homeostasis and health[134]. Various cells are able to produce TNF during infection, including monocytes, macrophages, DC's, B cells and T cells[135, 136, 137, 138]. The inflammatory action of TNF occurs via two transmembrane receptors; tumour necrosis factor receptor (TNFR)-1 which is expressed ubiquitously on all cell types and TNFR2 which is preferentially expressed by cells of haematopoietic origin, as well as endothelial cells and can be induced upon cell activation[139, 140]. The pro-inflammatory and programmed cell death pathways activated by TNF via TNFR1 are associated with tissue injury[131, 141, 142]. Signalling via TNFR2 promotes tissue repair and angiogenesis[142, 143, 144]. Excessive TNF production has been linked to rheumatoid arthritis and other autoimmune diseases[145]. The soluble form of TNF (sTNF) is found in blood plasma and confers TNF with potent endocrine function and an ability to act at distant physiological sites away from the site of synthesis[146].

All major anti-TNF agents bind to soluble and membrane-bound forms of TNF with high affinity and specificity, thus preventing TNF from binding to TNFRs[137]. Anti-TNF agents like Enbrel™ are widely used to treat rheumatoid arthritis, ankylosing spondylitis and plaque psoriasis[137], however, immunosuppression is a major issue for patients receiving anti-TNF treatment[137]. Therefore, it is imperative to understand how TNF is regulated in these settings in order to provide better therapeutic options for these diseases.

1.2.11.2 IFNγ

IFNγ is predominantly known to aid Th1 cell differentiation, stimulate antigen presentation via MHCI and MHCII as well as induce inflammatory responses against intracellular pathogens such as *Plasmodium* and *Leishmania* [reviewed in[147, 148]]. One of the most important effects of IFNγ on macrophages is the activation of microbicidal effector functions[149, 150]. Earlier reports have shown that TNF is produced by *L. major* amastigote-infected macrophages and IFNγ dramatically enhances TNF secretion, suggesting that both TNF and IFNγ act synergistically to enhance the killing actions by macrophages[151]. IFNγ is also involved in orchestrating the trafficking of lymphocytes to sites of inflammation through up-regulating the expression of CXCL10, MCP-1, MIG, MIP-1α/β, CCL5, ICAM-1 and VCAM-1[147]. TNF and IL-1β synergistically regulate many of these molecules[148].

1.2.12 Immunoregulation

1.2.12.1 $CD4^+$ T cell differentiation

$CD4^+$ T cells play key roles in protection against *Plasmodium*, *Leishmania*, *Mycobacterium* and HIV infections[63, 152, 153, 154]. The diverse nature of these pathogens requires $CD4^+$ T cells to differentiate into specialised subsets and respond accordingly to the invading pathogen (**Fig 7**). While the field of $CD4^+$ T cell biology has progressed rapidly, increasing our understanding of the types of $CD4^+$ T cell responses required to provide protection against infectious diseases will aid the development of immune therapies and vaccines against diseases such as malaria and leishmaniasis.

The differentiation pathways of the various Th subsets of $CD4^+$ T cells continues to expand, since their initial discovery 29 years ago by Mosmann and Coffman[155]. Th cells were first characterised by their cytokine profiles which determined their function, however since then, the identification of master transcription factors for each subset re-defined the roles of Th1, Th2, Th17, Tfh and Treg (regulatory) cells (**Fig 7**). Upon encountering antigen, $CD4^+$ T cells acquire their effector function via the integration of signals from professional antigen presenting cells (APC's), such as DC's, as well as the local cytokine environment[156]. The integration of these signals activates or represses master transcription factors such as T cell-specific T-box (Tbet), "G-A-T-A" sequence binding protein 3 (GATA3), RAR-related orphan receptor gamma-T (RORγT), B-cell cll/lymphoma 6 (Bcl-6) and Forkhead box P3 (Foxp3) which drive and sustain the specialised functions exhibited by Th1, Th2, Th17, Tfh and Treg cells, respectively[156].

Th1 cell differentiation is driven by the master transcription factor Tbet (encoded by the *tbox21* gene) to produce Th1-signature cytokines, including IFNγ, TNF, LTα and IL-2 in order to promote cell-mediated immunity against intracellular pathogens such as *Plasmodium* and *Leishmania*[156, 157].

Th2 cells are programmed by the master transcription factor GATA3 and typically produce IL-4, IL-5, IL-13 and IL-10 to mediate humoral immune responses and resistance against extracellular pathogens, such as helminths[158, 159].

The Th17 cell differentiation pathway is driven by RORγT to induce IL-17 production by Th17 cells which are important for protection against bacterial and fungal pathogens such as *Klebsiella pneumoniae*, *Citrobacter rodentium* and *Candida albicans*[160]. Th17 cells typically produce IL-17A, IL-17F and IL-22 as signature cytokines, mediating neutrophil infiltration into peripheral tissues[156, 161]. Furthermore, Th17 responses are important in several autoimmune disease pathologies[162].

The discovery of Tfh cells brought about new insights into the development of antibody responses. Tfh cells assist B cell-mediated immune responses, including class switching, and germinal centre formation[163]. Tfh cells express PD1, SLAM, CXCR5, ICOS and their differentiation is driven by the master transcription factor Bcl-6[164, 165]. Tfh cells typically produce IL-21 in addition to other cytokines produced by Th2 cells. IL-21 production acts in an autocrine manner, that along with IL-6, transforming growth factor beta (TGFβ) and Bcl-6 promotes Tfh differentiation[164, 165]. Although all $CD4^+$ T cells migrate to follicular regions of the spleen, Tfh cells preferentially reside there due to the continuous expression of the CXCR5 chemokine receptor[164].

Recent findings using comparative analysis methods between the genome-wide landscapes of various transcription factors have identified a new layer of complexity that complements the plasticity and functional diversity of $CD4^+$ T cells in the face of disease. For example, the discovery of IL-10-producing Th1 cells, commonly referred to as Tr1 cells, has challenged the concept of a single transcription factor solely driving the differentiation of a single Th subset.

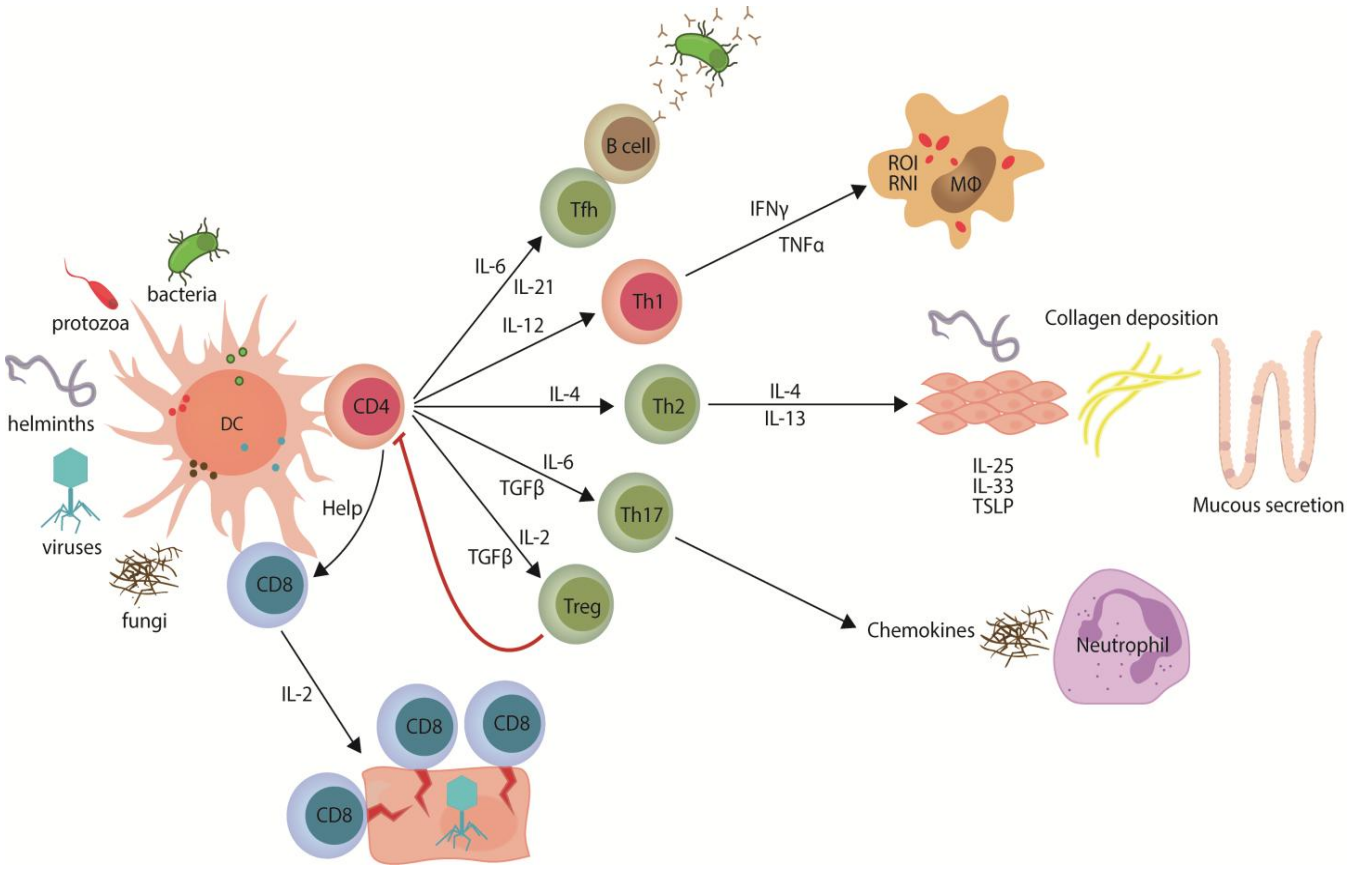


Figure 7: CD4$^+$ T cell differentiation pathways.

Depending on the nature of the invading pathogen, local tissue environment and expression of master transcription factors, CD4$^+$ T cells can differentiate into various Th subsets (Th1, Th2, Th17 and Tfh) that act to limit pathogen spread and confer protection, provide help to CD8$^+$ T cells to exert their cytotoxic effects or activate immunoregulatory mechanisms (Treg cells) that prevent immunopathology in the host. Figure drawn by: S. Ng, Immunology and Infection Laboratory, QIMR Berghofer.

1.2.12.2 Treg cells

The selection and deletion of self-reactive T cells occurs in the thymus and allows for the generation of potent immune responses with minimal damage to 'self'. However, in rare instances, self-reactive T cells enter the periphery, but are suppressed by a process known as peripheral tolerance[166]. In 1993, Powrie *et al*., demonstrated the regulatory ability of T cells (which would be later known as regulatory T cells or Tregs) in a series of adoptive transfer experiments[167]. $CD45RB^{lo}$ (regulatory) or $CD45RB^{hi}$ $CD4^{+}$ T cells were transferred into severe combined immunodeficient (SCID) mice, where they observed that the mice that received the $CD45RB^{lo}$ $CD4^{+}$ T cells did not develop colitis or wasting disease[167]. Later in 1995, Sakaguchi *et al*., went on to show that the suppressive ability of Treg cells was conferred by the expression of high levels of the interleukin 2-receptor alpha chain (IL-2Rα, CD25)[168]. Then in 2003, several groups identified the master transcription factor, Foxp3, as the driver of Treg cell differentiation and their suppressive abilities[169, 170, 171]. Treg cells can be classified into thymus-derived natural Tregs (nTregs) which control auto-reactivity, and peripherally-generated inducible Tregs (iTregs) which are thought to regulate immunity to tumours or invading pathogens [reviewed in[172, 173, 174]].

Mechanisms of Treg cell suppression include cell-cell contact via cytotoxic T-lymphocyte-associated protein (CTLA)-4, lymphocyte-activation gene (LAG)-3 or galectin-1, the production of immunoregulatory cytokines and suppressing effector T cell responses via IL-2 sequestration[175, 176, 177, 178, 179] (**Fig 8**). The phenotypic and functional diversity of Treg cells is broad and determined by the expression of molecules other than Foxp3. For example, in inflammatory settings, Tregs often employ the same transcriptional machinery as their target cells, thereby copying the migratory and tissue localising behaviour such that they are able to modulate specific Th cell subsets[180, 181, 182, 183, 184, 185].

Activation of effector T cells requires T cell receptor – major histocompatibility complex (TCR-MHC) engagement, as well as co-stimulatory signals from the APC, where CD28 on the T cell interacts with CD80 or CD86 on the APC[186, 187]. Cell-cell contact suppression involves the down-regulation of CD80 and CD86 on APC's during interaction between CTLA4 expressing T cells and APC's[188, 189]. Therefore, in the absence of co-stimulatory signals, T cells go into a state of anergy and are unable to respond to stimuli[190].

Treg cells produce immunoregulatory cytokines such as IL-10 and TGFβ as another means of suppressing effector T cell responses. IL-10 suppresses the ability of APC's to stimulate T cells via the inhibition of co-stimulatory signals[191]. Furthermore, Treg-derived IL-10 is critical for limiting inflammation-induced tissue pathology and in particular, suppressing Th17 inflammatory responses[192]. Similar functions have been reported for TGFβ, including the promotion of Treg cells in combination with IL-2[193].

Treg cells sequester IL-2 by up-regulating their expression of IL-2Rα and thus compete with activated T cells (which also express IL-2Rα) to consume IL-2 for their own proliferation, maintenance and induction of IL-10[194]. The down-regulation of IL-2Rα expression on activated T cells, decreases their IL-2 responsiveness and thus their survival in the surrounding environment is also decreased[194].

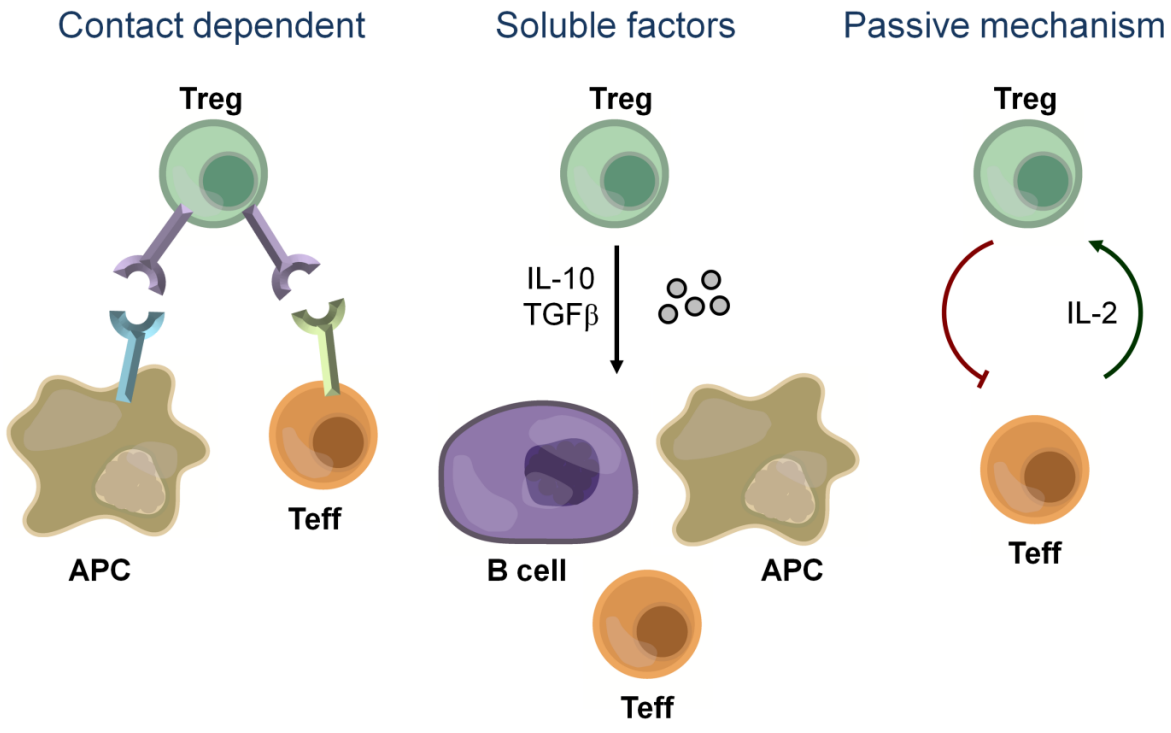


Figure 8: Mechanisms of Treg-mediated suppression.

Three main mechanisms that Treg cells use to exert their suppressive functions include, contact dependent, secretion of soluble factors (IL-10 and TGFβ) or sequestration of IL-2. Figure source adapted from: Lauren E Mays and Youhai H Chen, 2008 *Cell Research* **17:** 904–918, permission number 3767561095697.

1.2.12.3 Tr1 cells

Tr1 cells were first identified in 1988 in SCID patients and were associated with high levels of IL-10 production[195, 196]. In 1997, Groux *et al*., found that IL-10 producing $CD4^+$ T cells in both humans and mice were a distinct subset of T cells that were antigen-specific, exhibited immunosuppressive functions and were capable of mediating immune tolerance[197]. Since then, Tr1 cells have been characterised on the basis of their effector cytokine profile, whereby they secrete IL-10 in large amounts and IFNγ in variable amounts, simultaneously[198, 199]. Gene expression profiling experiments demonstrated that CD49b and LAG3 co-expression is specific to Tr1 cells in humans and mice[200]. Tr1 mediated suppression mechanisms include cell-cell contact suppression via CTLA4 or programmed cell death protein (PD)-1, secretion of IL-10, TGFβ, granzyme B and perforin, as well as disrupting the metabolic states of effector T cells via CD39 and CD73[201, 202].

The important immunoregulatory functions of Tr1 cells have been widely reported and they are being tested as an alternative immunotherapeutic approach in cases where Treg cell based therapies are not feasible[203, 204]. Tr1 cells are $Tbet^+$ $Foxp3^-$ $CD4^+$ T cells that co-produce the effector cytokines IL-10 and IFNγ simultaneously[205]. While no single transcription factor has been identified for Tr1 differentiation, it is thought that Tr1 cells derive from a common Th1 lineage, and under inflammatory conditions, acquire a self-regulating ability to produce IL-10 in addition to IFNγ. The induction of Tr1 cells begins with IL-27 production by APC's, such as DC's and macrophages which stimulates IL-21 production by $CD4^+$ T cells and acts in an autocrine manner for IL-10 producing Th1 cells[206, 207]. IL-27 induces the transcription factors c-Maf and aryl hydrocarbon receptor (Ahr) via STAT1 and STAT3-dependent mechanisms into order to activate *il10* and *il21* gene expression (**Fig 9**)[206, 208, 209, 210]. Importantly, the critical role for IL-27 in the induction of Tr1 cells in *Plasmodium* and *Leishmania*, *Toxoplasma* and *Mycobacterium* infections have been reported[74, 208, 211, 212, 213]. Furthermore, the predominant source of IL-10 in *Plasmodium* and *Leishmania* infections is derived from Th1 cells[205, 214].

While c-Maf and Ahr are required for inducing IL-10 production, the transcriptional regulator, B-lymphocyte induced maturation protein (Blimp)-1 also plays a role in the induction of IL-10. Treg cells require Blimp1 in order to produce IL-10[181] and more recently, IL-10 production by Th1 cells was reported to be Blimp1-dependent (**Fig 9**)[215, 216, 217].

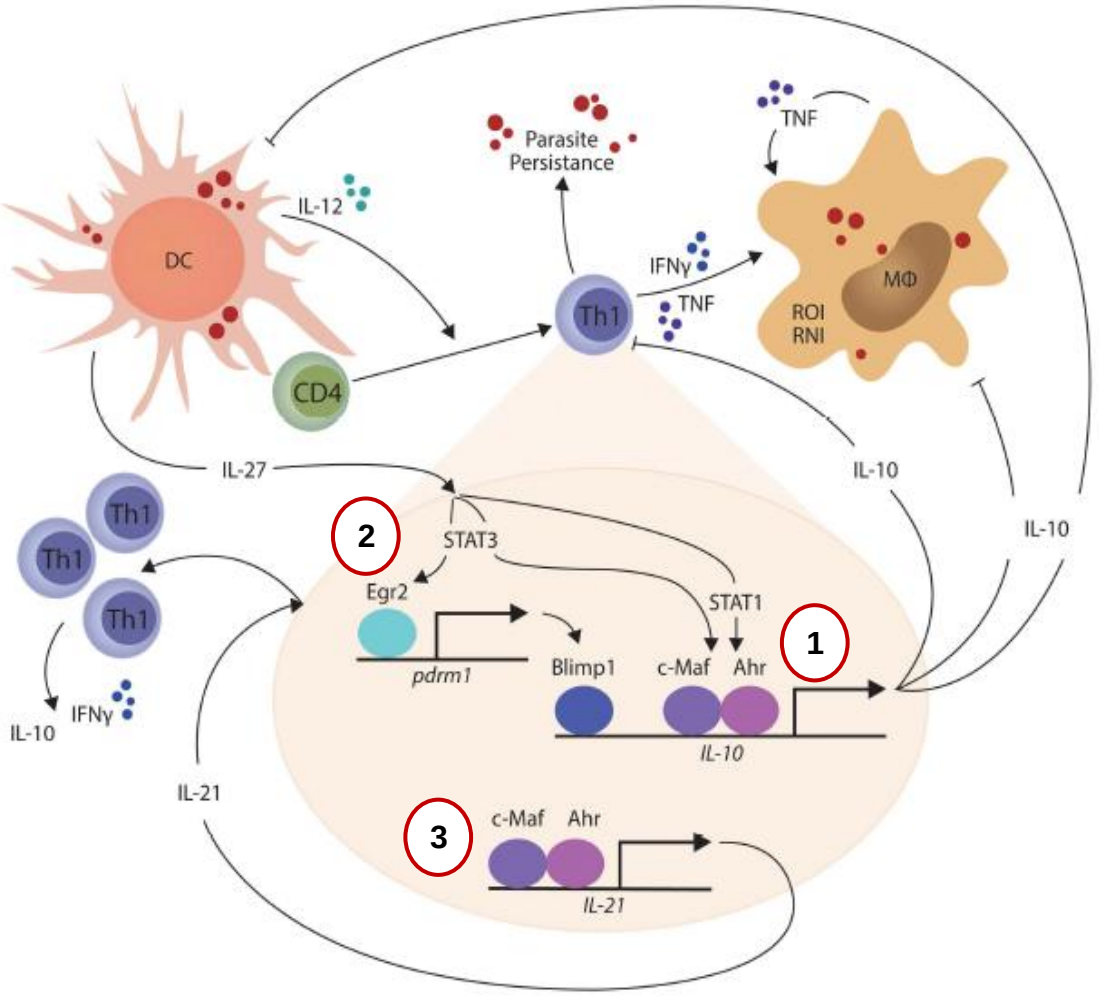


Figure 9: Induction of IL-10 producing Th1 cells.

Three mechanisms of IL-10 induction in Th1 cells, **1:** IL-27 produced by APC's (macrophages and DC's) activates c-Maf and Ahr transcription factors in Th1 cells via STAT1 and STAT3-dependent mechanisms to dive IL-10 transcription which then regulates Th1 responses and suppresses macrophages and DC's, **2:** IL-27 signalling activates the early growth response (Egr)-2 transcription factor to induce Blimp1 to bind to the *il10* locus. **3:** c-Maf and Ahr activates IL-21 to maintain IL-10 production by Th1 cells, in an autocrine manner. Figure source: Engwerda CR, Ng SS and Bunn PT, 2014, *Front. Immunol.***5**:498. doi: 10.3389/fimmu.2014.00498, open-access article under the Creative Commons Attribution License (CC BY).

1.2.12.4 IL-10

IL-10 was first identified 26 years ago and has shaped our current understanding of immune regulation in the context of inflammation, autoimmunity, infectious diseases and pathology[218]. IL-10 is produced by T cells, B cells, macrophages, DC's and epithelial cells[219] and is induced in response to excessive inflammation in order to prevent tissue damage[201, 220]. The immunoregulatory abilities of IL-10 involve the suppression of TNF and nitric oxide (NO) resulting in an overall reduced capacity of macrophages to activate their microbicidal mechanisms[219]. IL-10 can also inhibit the antigen-presentation abilities of APC's, inhibit DC maturation (via the down-regulation of co-stimulatory signals) and inhibit IL-12 production which is required for the activation of Th1 cells[219]. Thus, the regulation of IL-10 is critical during infection, since its expression is detrimental and promotes pathogen survival in *Leishmania*[214, 221], *Mycobacterium tuberculosis*[222] and *Candida*[223] infections, whereas lack of IL-10 correlates with fatal outcomes in *Plasmodium*[74, 224], *Toxoplasma gondii*[225] and *Listeria monocytogenes*[226] infections [reviewed in[205]].

In humans, a shift from an inflammatory response towards a persistent anti-inflammatory response may provide one explanation as to why individuals living in malaria endemic areas have reduced frequencies of severe malaria episodes[227, 228]. Field studies have shown that the proportion of IL-10 responders increases with age, and is inversely correlated with severe malaria cases[229, 230]. More recently, it was demonstrated that the ability of PBMC's (isolated from individuals from a low endemic area who had a documented clinical record of *P. falciparum* or *P. vivax* in the last 6 years) to respond to IL-10 was maintained with no significant decline over 6 years, whereas the response to IFNγ rapidly decreased over time[231]. However, the induction of IL-10 production, particularly by Tr1 cells, may offer an explanation into the limited efficacy rate of the current RTS,S/AS01 vaccine, where immunoregulatory mechanisms are induced instead of anti-parasitic responses. Therefore, while inflammatory responses are regulated, the induction of these immunoregulatory mechanisms may cause an impediment of parasite control and instead promote parasite persistence[47, 232].

While Treg cell-derived IL-10 plays a suppressive role, the frequency of Tregs were not elevated in the blood and did not accumulate in the spleen during active VL[233], suggesting a minimal role for Tregs in VL. Additionally, antigen-specific IFNγ responses were not restored when Treg cells were depleted[233]. Interestingly, Tr1 cells are the predominant source of IL-10 during *Leishmania*, as well as *Plasmodium* infections[47, 75, 205, 212, 214, 233, 234, 235].

Since IL-10 plays an immunosuppressive role in humans and mice with VL[236, 237], IL-10 blockade represents a logical therapeutic approach[238]. However, releasing the brake on potent anti-parasitic responses could result in inflammation-induced pathology, leading to immune dysfunction. Therefore, a better understanding of IL-10 regulation is required to maximise its therapeutic potential.

1.2.12.5 Blimp1

Blimp1 encoded by *prdm1*, functions as a transcriptional regulator by creating a more closed/repressive chromatin structure[239, 240, 241]. Initially reported to play a key role in plasma cell differentiation[242, 243], recent reports have identified a wider role for Blimp1 in various immune cell subsets including, DC's, NK cells, $CD8^+$ and $CD4^+$ T cells (**Fig 10**)[217, 244, 245, 246, 247]. Blimp1 is expressed by B cells, T cells, granulocytes, macrophages, epithelial cells and germ cells[248].

The five C_2H_2 zinc finger motifs in the C-terminus of Blimp1 contain DNA-binding sites. The consensus-binding site for Blimp1 is very similar to interferon regulatory factor (IRF)-1 and IRF2, and therefore, competes with these transcription factors to bind to the IFNβ promoter[249]. Blimp1 also contains a proline-rich region N-terminal to the zinc fingers and PR domain conserved between Blimp1 and the Rb-binding protein RIZI (encoded by *prdm2*)[250]. Both the proline-rich region and zinc fingers are required for transcriptional repression, where groucho transcription factors (hGroucho) and histone deacetylases (HDACs) 1 and 2 associate to form a co-repressor complex at this site[241, 250].

Blimp1 has been reported to solely function as a transcriptional repressor, but a study which showed Blimp1 could directly bind to a conserved region in intron 1 of the *il10* locus, has suggested that it may also play a positive role in gene expression[181]. One of the most important findings pertaining to Blimp1 has been its relation to IL-10[216, 251]. Recent studies have implicated an important role for Blimp1 in driving $CD4^+$ T cell differentiation, where Treg cells require Blimp1 and IRF4 in order to produce IL-10[181]. More recently, Blimp1 has been shown to drive the differentiation of Tr1 cells in various disease settings[215, 216, 251]. IL-12-induced Blimp1 expression in Tr1 cells via a STAT4 dependent manner[216], supports the emerging concept that Tr1 cells differentiate from a Th1-related lineage. Tr1 cells are induced in inflammatory conditions and while the transcriptional program/s they employ to produce IL-10 is not restricted to a single transcription factor, Tr1 cells respond to environmental signals and thus, their adaptive abilities are well-suited during infection, where the environment is constantly changing. Given the broad role of Blimp1 in various immune cells, it also highlights the potential for Blimp1 to regulate mechanisms of IL-10 induction by other cells, in addition to $CD4^+$ T cells.

While the transcriptional requirements for Tr1 cell induction have been reported and their immunoregulatory functions validated, the combined effect of these has not been studied in the context of infectious or inflammatory diseases. This is of paramount importance if immunoregulatory networks involving IL-10 and Tr1 cells are to be employed in therapeutic settings.

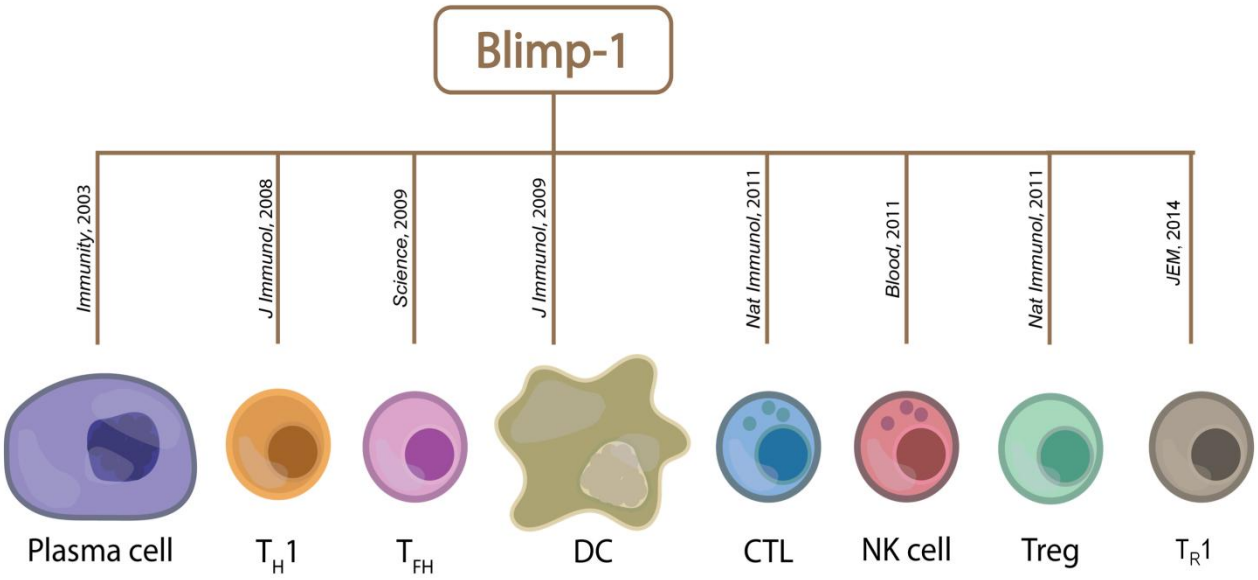


Figure 10: Diverse role of Blimp1 in immune cells.

Timeline of the expanding roles of Blimp1 from 2003 – 2014. Figure drawn by: S. Ng, Immunology and Infection Laboratory, QIMR Berghofer

1.2.12.6 Type I IFN's

In human malaria studies, host genetic factors play a central role in resistance mechanisms against malaria infection[252, 253]. Cytokines and their receptors, such as type I IFN alpha receptor (IFNαR)-1 and IFNαR2 have been shown to be involved in malaria pathogenesis, where an association between two polymorphisms in the IFNαR1 gene and increased risk of severe malaria was identified[252]. Furthermore, whole brain transcriptional responses during PbA infection in genetically resistant and susceptible mouse strains resulted in the identification of differentially expressed genes, implicated in interferon-regulated processes. Thus, IFN-dependent transcriptional programs may play a role in the pathogenesis of severe malaria syndromes[254]. In viral infections, type I IFN's induce the expression of a wide array of genes that prevent viral spread and replication. IFNα and IFNβ are early innate cytokines known to affect DC function in viral infections, whereby type I IFN's can act in an autocrine manner to induce DC maturation *in vivo*. Type I IFN's have been shown to suppress $CD4^+$ T cell-dependent parasite control during blood-stage *Plasmodium* infection[67]. Recent studies have shown that type I IFN's signalling directly, modulates the function of $CD8\alpha^-$ cDC's during severe blood-stage *Plasmodium* infection, and by doing so, limits their ability to drive IFNγ production by Th1 cells[68]. This provides a cellular mechanism to explain how $CD4^+$ T cell responses are impaired by type I IFN's during systemic disease.

1.3 DEVELOPMENT OF HYPOTHESIS AND AIMS

The human immune system in all its complexity has evolved to fight against some of the worst plagues and pandemics known to mankind. Given this, it is no wonder that $CD4^+$ T cells exhibit great functional diversity and plasticity in order to fight pathogens of varying virulence and complexity. *Plasmodium* and *Leishmania* parasites have continued to evade immune surveillance mechanisms and instead hijack the host's immune system for their own survival. The limited efficacy of the RTS,S/AS01 vaccine and increased toxicity of anti-leishmanial drugs highlights the need for novel strategies for modulating immune responses that ultimately provide long-term protection. Importantly, the translation of this research into therapies that extend beyond parasitic diseases has the potential to offer novel ways to treat a broad range of inflammatory diseases.

Based on the above background information, the following hypothesis has been formulated for testing in this thesis:

$CD4^+$ T cell responses are suppressed during *Plasmodium* and *Leishmania* infections via mechanisms involving the early development of immune regulatory mechanisms involving IL-10. Furthermore, anti-parasitic $CD4^+$ T cell responses can be improved to control infection without causing disease by targeting Tr1 cells that develop during infection.

This hypothesis was tested by addressing specific aims as follows:

Chapter 3: Investigates the role of type I IFN's and suppression of $CD4^+$ T cell responses during *P. falciparum* infections.

Chapter 4: Investigates the role of Blimp1 in $CD4^+$ T cell responses during *Plasmodium* infections.

Chapter 5: Describes the mechanism by which Blimp1-dependent IL-10 production by Tr1 cells limits TNF-mediated tissue pathology during *Leishmania* infections.

Chapter 2

Materials and Methods

This chapter describes the generic materials and methods used in Chapters 3, 4 and 5. Materials and methods specific to each chapter have been included separately within each chapter.

2.1 BUFFERS AND MEDIA

All buffers and media were prepared under sterile conditions and filtered before use

RPMI/PS: 990ml Roswell Park Institute Medium (QIMR Berghofer Medical Research Institute, QLD, Australia), 10ml Penicillin (100 U/ml) /Streptomycin (100µg/ml) (Life Technologies, Scoresby, VIC, Australia)

RPMI/PS+: 990ml Roswell Park Institute Medium (QIMR), 10ml Penicillin/Streptomycin Plus (Life Technologies), 2mM L-glutamine (QIMR), 40mM HEPES (QIMR)

FACS buffer: 5g AlbuMAX® II Lipid-Rich Bovine Serum Albumin (Life Technologies), 500µl Sodium Azide (QIMR), 500ml 1x PBS (QIMR)

FACS block buffer: 50ml 24G2 supernatant (QIMR), 50ml Fetal Bovine Serum (Life Technologies)

MACS buffer: 5g AlbuMAX® II Lipid-Rich Bovine Serum Albumin (Life Technologies), 5ml 0.5M EDTA (QIMR), 495ml Dulbecco's PBS (Life Technologies)

Freezing Medium: 10% DMSO (Sigma-Aldrich, Castle Hill, NSW, Australia), 90% Fetal Bovine Serum (Life Technologies)

Complete Media: 45 ml RPMI 1640 (Life Technologies), 5ml Fetal Bovine Serum (Life Technologies), 20µl Gentamicin (50mg/ml, Sigma-Aldrich)

LV9 Assay Medium: 395ml RPMI (QIMR), 100ml Fetal Bovine Serum (Life Technologies), 5ml Penicillin/Streptomycin Plus (Life Technologies)

2.2 MICE

The following strains of mice were used for experiments (details are provided in each respective chapter):

- C57BL/6J = Wild type (WT)
- C57BL/6J.IFNγR$^{-/-}$ = Interferon Gamma Receptor deficient

- C57BL/6J.*Prdm1*$^{gfp+}$ (GFP reporters)

- C57BL/6J.Lck-Cre^{+} x *Prdm1*$^{fl/fl}$ = ***Prdm1***$^{\mathbf{\Delta T}}$ (Cre positive) **T cell specific** Blimp1 deficient
- C57BL/6J.Lck-Cre^{-} x *Prdm1*$^{fl/fl}$ = *Prdm1*$^{fl/fl}$ (Cre negative) littermate controls

- C57BL/6J.Foxp3-Cre^{+} x *Prdm1*$^{fl/fl}$ = ***Prdm1***$^{\mathbf{\Delta F}}$ (Cre positive) **Treg cell specific** Blimp1 deficient
- C57BL/6J.Foxp3-Cre^{-} x *Prdm1*$^{fl/fl}$ = *Prdm1*$^{fl/fl}$ (Cre negative) littermate controls

- C57BL/6J.Lck-Cre^{+} x *Il10*$^{fl/fl}$ = ***Il10***$^{\mathbf{\Delta T}}$ (Cre positive) **T cell specific** IL-10 deficient
- C57BL/6J.Lck-Cre^{-} x *Il10*$^{fl/fl}$ = *Il10*$^{fl/fl}$ (Cre negative) littermate controls

- C57BL/6J.Lck-Cre^{+} x *Il10R*$^{fl/fl}$ = ***Il10R***$^{\mathbf{\Delta T}}$ (Cre positive) **T cell specific** IL-10R deficient
- C57BL/6J.Lck-Cre^{-} x *Il10R*$^{fl/fl}$ = *Il10R*$^{fl/fl}$ (Cre negative) littermate controls

- C57BL/6J.LysM-Cre^{+} x *Il10R*$^{fl/fl}$ = ***Il10R***$^{\mathbf{\Delta M}}$ (Cre positive) **Myeloid-derived specific** IL-10R deficient
- C57BL/6J.LysM-Cre^{-} x *Il10R*$^{fl/fl}$ = *Il10R*$^{fl/fl}$ (Cre negative) littermate controls

2.3 PARASITES AND INFECTIONS

2.3.1 Preparing PcAS parasites for infection

Cryopreserved stabilate of PcAS parasites was thawed on ice for 2-3 minutes. 12% (w/v) NaCl solution in MilliQ water (0.2x the initial frozen volume) was added drop-wise to the stabilate to wash any excess glycerolyte solution. Stabilate was placed back on ice for 5 minutes. 1.6% (w/v) NaCl solution in MilliQ water (10x the initial frozen volume) was added drop-wise and then centrifuged at 338xg (Eppendorf Centrifuge 5810R) for 10 minutes at 4°C. Supernatant was carefully removed using a Pasteur pipette and the red blood cell pellet was then gently resuspended in 500µl PBS. 200µL of this inoculum was then injected into a C57BL/6J mouse i.v. via the lateral tail vein. Parasites were detectable in this passage mouse after 3-4 days post injection, where parasitemia had reached 3-5%. At this time point, the passage mouse was subsequently euthanized via CO_2 asphyxiation and approximately 0.5-1ml of blood was collected via cardiac puncture into a 10ml conical tube containing 5ml of RPMI/PS + 5U/ml of heparin. An additional 5ml of RPMI/PS was added and then centrifuged at 338 x g for 7 minutes at room temperature. Supernatant was carefully removed and the RBC pellet was resuspended in 1ml of RPMI/PS. A 1:1000 dilution of the 1ml preparation was then prepared for RBC counts in a 0.1% Trypan Blue solution in PBS (0.4%, MP Biomedicals Pty Ltd, Seven Hills, NSW, Australia). RBC concentration was adjusted to $5x10^5$ pRBC's/ml in RPMI/PS, to inject 200µl per mouse (final concentration: $1x10^5$ pRBC's/mouse) i.v.

2.3.2 Preparation of PbA parasites for infection

Cryopreserved PbA stabilate was thawed at room temperature and 200µl was subsequently injected into a C57BL/6J mouse i.p. Parasites were detectable in this passage mouse after 4 days post injection, where parasitemia had reached 1-3%. At this time point, the passage mouse was subsequently euthanized via CO_2 asphyxiation and parasite inoculum was prepared as above (**2.3.1**).

2.3.3 Preparation of *Leishmania* parasites for infection

L. donovani (LV9 strain) amastigotes were harvested from the spleen of a chronically infected C57BL/6J.RAG1$^{-/-}$ mouse. A single cell suspension was prepared by homogenization of the spleen in RPMI/PS and then centrifuged at 128xg for 5 minutes at room temperature to remove any debris. Supernatant (containing parasites) was decanted into a separate tube and centrifuged at 1800xg for 15 minutes at room temperature. 1ml of RBC Lysis buffer (Sigma-Aldrich) was added to the pellet and incubated for 5 minutes at room temperature. 49ml of RPMI/PS was then added to wash the lysis buffer and centrifuged at 1800xg for 15 minutes at room temperature. The pellet containing the parasites was washed twice more by decanting the supernatant and adding 50ml RPMI/PS and centrifuging as above. Parasites were then resuspended in 10ml RPMI/PS and then passed through a blunt 26" gauge needle and 10ml syringe in order to minimise parasite clumping. Parasites were counted using the Helber counting chamber (Weber Scientific, Teddington, UK). Concentration was adjusted to 1x10^8 parasites/ml in RPMI/PS and 200µl (final concentration: 2x10^7 parasites/ml) was injected per mouse i.v. via the lateral tail vein.

2.4 COLLECTION OF MOUSE SAMPLES

At indicated time points, *Plasmodium* or *Leishmania* infected mice were euthanized by CO_2 asphyxiation and blood, spleens and livers were subsequently harvested and processed.

Blood was collected via cardiac puncture using 27" gauge insulin syringes (QIMR) into 1ml eppendorf tubes. Blood was then centrifuged at 775xg (Eppendorf Centrifuge 5418) for 10 minutes at 4°C. 200µl of serum was collected and transferred to a 96 well U-bottom plate, sealed with parafilm and stored at -20°C until required for analysis.

2.4.1 Collection of samples from *Plasmodium* infected mice

Spleens were collected into 10ml RPMI/PS.

2.4.2 Collection of samples from *L. donovani* infected mice

Spleens were harvested and a section of the spleen was collected into 4% (w/v) Paraformaldehyde (PFA, MP Biomedicals Pty Ltd, Seven Hills, NSW, Australia) for microscopy. A small section of the end of the spleen was cut laterally to make multiple impression smears on glass slides in order to determine parasite burden and a third piece was collected into 10ml RPMI/PS for flow cytometry.

Livers were perfused via the hepatic portal vein with 10ml of 1x PBS using a 26"gauge needle (QIMR). A portion of the liver from the left lateral lobe was cut laterally to make multiple impression smears on glass slides to determine parasite burdens. The remainder of the liver was collected into 5ml of 2% FBS solution in PBS for flow cytometry.

2.5 PROCESSING OF MOUSE SAMPLES FOR FLOW CYTOMETRIC ANALYSIS

Spleens were passed through a BD Falcon-100μm cell strainer (BD Biosciences, San Jose, CA, USA) to obtain a single cell suspension. Splenocyte suspensions were then centrifuged at 338xg for 7 minutes at room temperature. To obtain the splenic mononuclear cells, supernatants were decanted; pellets were resuspended in 1ml of RBC lysis buffer (Sigma-Aldrich) and incubated at room temperature for 8 minutes to ensure complete lysis of RBC's. 9ml of RPMI/PS was added to wash lysis buffer and then centrifuged at 338xg for 7 minutes at 4°C, to inhibit any further lysis. Supernatants were decanted and pellets were resuspended in a final volume of 10ml RPMI/PS for counting. Splenic mononuclear cells were then counted on a haemocytometer (BLAUBRAND®counting chamber Neubauer, Blackburn, VIC, Australia) after a 1:5 to 1:20 dilution in 0.1% Trypan Blue solution in PBS (0.4%, MP Biomedicals Pty Ltd).

Livers were passed through a SEFAR- 200µm metal mesh (SEFAR Pty Ltd, Meadowbrook, QLD, Australia) and strainer to obtain a single cell suspension. Hepatocyte suspensions were then washed with 2%FBS in PBS and centrifuged twice at 338xg for 7 minutes at room temperature. To obtain hepatic mononuclear cells, supernatants were decanted; pellets were resuspended in 25ml of a 33% (v/v) Percoll (VWR International, Tingalpa, QLD, Australia) density gradient solution and centrifuged for 578xg for 15 minutes at room temperature. After centrifugation, the hepatocyte layer was removed using pasteur pipettes, while the hepatic mononuclear cell pellet was resuspended in 1ml of RBC Lysis buffer (Sigma-Aldrich) and incubated for 5 minutes at room temperature. 9ml of RPMI/PS was added to wash lysis buffer and then centrifuged at 338xg for 7 minutes at 4°C, to inhibit any further lysis. Supernatants were decanted and pellets were resuspended in a final volume of 0.5 - 3ml RPMI/PS for counting. Hepatic mononuclear cells were then counted on a haemocytometer (BLAUBRAND® counting chamber Neubauer) after a 1:20 dilution in 0.1% Trypan Blue solution in PBS (0.4%, MP Biomedicals Pty Ltd).

2.6 FLOW CYTOMETRY

2.6.1 Surface staining

For surface staining 1-2x10^6 cells per well were plated into a 96 well U-bottom plate. Cells were centrifuged at 338xg for 3 minutes at 4°C, supernatant was decanted and 225µl of HBSS (Hank's Balanced Salt Solution, Life Technologies) was added per well and centrifuged at 338xg for 3 minutes at 4°C. 2.5µg/ml (50µl final volume) of LIVE/DEAD® Fixable Aqua dead cell stain (Life Technologies) diluted in HBSS was added per well and incubated for 15-20 minutes at room temperature, protected from light. 170µl of HBSS was added per well and centrifuged at 338xg for 3 minutes at 4°C. Supernatant was decanted and 100µl of FACS block (QIMR) was added per well and incubated on ice for 10 minutes on ice, protected from light in order to block non-specific Fc-mediated interactions. Cells were washed and 2.5-5µg/ml (50µl final volume) of primary surface antibodies (e.g. CD4, TCRβ) was added per well and incubated for 15-20 minutes on ice, protected from light. Primary antibodies were either directly conjugated to a fluorophore (e.g. CD49d PeCy7) or biotinylated (e.g. CD49d biotin, followed by Streptavidin PeCy7). Cells were washed twice by adding 150-200µl of FACS buffer (QIMR) and centrifuging at 338xg for 3 minutes at 4°C.
Supernatants were decanted and cells were resuspended in a final volume of 100µl of 1% PFA (MP Biomedicals Pty Ltd), stored at 4°C and protected from light until acquisition on a Fortessa 5 (BD Biosciences). Samples were generally acquired within 12-24 hours post staining.

2.6.2 Intracellular cytokine staining (ICCS)

For intracellular cytokine staining, 1-2x10^6 cells per well were plated into a 96 well U-bottom plate and incubated for 3 hours at 37°C in 10µg/ml Brefreldin A (10mg/mL, Sigma Aldrich) diluted in 5% FBS in RPMI/PS, to prevent the secretion of cytokines from the cell. For the detection of IL-10, 25ng/ml phorbol 12-myristate 13-acetate (PMA, 1mg, Sigma-Aldrich) and 500ng/ml Ionomycin calcium salt (1mg, Sigma-Aldrich) was added in addition to the Brefeldin A to re-stimulate cells to produce IL-10. After the 3 hour incubation, cells were centrifuged at 338xg for 3 minutes at room temperature. Supernatant was decanted and 225µl of HBSS was added per well for washing. 2.5µg/ml (50µl final volume) of LIVE/DEAD® Fixable Aqua dead cell stain (Life Technologies) diluted in HBSS was added per well and incubated for 15-20 minutes at room temperature, protected from light. Cells were then treated with FACS block for 10 minutes on ice and subsequently stained for surface markers with primary antibodies for 15-20 minutes on ice. Cells were then washed with FACS buffer prior to fixation with either 100µl of BD cytofix buffer (BD Biosciences) if only detecting cytokines or 100µl of 1x eBioscience fixation/permeabilisation buffer staining kit (4x, Jomar Bioscience, Kensington, SA, Australia) if detecting transcription factors in addition to intracellular cytokines. Cells were incubated in each respective cytofix buffer for 20 – 30 minutes on ice, protected from light. After fixation, 100µl of 1x BD permeabilisation buffer (10x, BD Biosciences) or 100µl of 1x eBioscience permeabilisation buffer (10x, Jomar Bioscience) was added per well and cells were then centrifuged at 338xg for 3 minutes at 4°C. Cells were washed a second time with 1x permeabilisation buffer and centrifuged at 338xg for 3 minutes at 4°C. 2.5-5µg/ml (50µl final volume) of intracellular surface antibodies (e.g. IFNγ, Tbet) were added per well and incubated for 45-60 minutes on ice, protected from light.

Cells were subsequently washed twice with 150-200µl of 1x permeabilisation buffer and centrifuged at 338xg for 3 minutes at 4°C. Cells were resuspended in a final volume of 100µl of 1% PFA (MP Biomedicals Pty Ltd), stored at 4°C and protected from light until acquisition on a Fortessa 5 (BD Biosciences). Samples were generally acquired within 12-24 hours post staining.

Acquisition of all samples was performed on a Becton Dickson Fortessa 5 (BD Biosciences) and the data acquired using FACS DIVA software (BD Biosciences) was analysed on FlowJo software v9 and v10.0.7 (Treestar, Ashland, OR, USA).

2.7 ASSESSMENT OF SUPERNATANT AND SERUM CYTOKINE LEVELS

Culture supernatants and serum from blood collected via cardiac bleeds were processed for cytokine analysis. A Flex set cytometric bead array (CBA) Kit (BD, Franklin Lakes, NJ, USA) was used to quantify cytokine levels (pg/mL) in culture supernatants or serum. Briefly, standards were prepared by serial dilutions (0-5000pg/mL) and subsequently the master mix containing antibody-coated beads along with the detection reagent (PE) was plated into a 96 V-bottom well-plate and standards and serum samples were added and incubated in the master mix for 2 hours at room temperature. Samples were then washed with the BD wash buffer and then acquired on a HTS system plate reader on the Fortessa 5 Flow cytometer (BD Biosciences) according to the manufacturer's instructions. Analysis was performed using FCAP Array v3.0 (Soft Flow, Minnesota, USA)

2.8 STATISTICAL ANALYSIS

All statistical methods have been reported separately in each chapter.

Chapter 3

Type I IFN’s promote the emergence of immunoregulatory networks during blood-stage *P. falciparum* infection

3.1 ACKNOWLEDGEMENTS

I would like to acknowledge and thank all 68 individuals who volunteered for the human malaria studies reported in this Chapter; your time and willingness to participate is most appreciated. I would like to especially thank Dr. James McCarthy and Q-Pharm Pty Ltd for their efforts in assisting us with these studies.

3.2 INTRODUCTION

Malaria represents a major global health burden, but the most recently tested RTS,S/AS01 vaccine has limited efficacy that wanes relatively quickly[12, 255]. Since its development began in 1984, the RTS,S/AS01 vaccine has progressed to show protection in young children in 2004 and infants in 2007 in malaria endemic regions[256, 257]. However, the maximum efficiency achieved with the RTS,S/AS01 vaccine is a modest 46% in recent Phase III clinical trials[12]. The limited vaccine efficacy highlights the urgent need for the development of novel therapeutic strategies, as well as developing a better understanding for this low efficacy rate. Although sterilising immunity is rarely achieved, if at all, a high degree of immune responsiveness resulting in low levels of parasitemia and an asymptomatic carrier status is a minimum requirement for malaria elimination programs[6, 258]. While potent immune responses limit parasite growth, immunoregulatory networks are essential for protection against clinical disease caused by excessive inflammation. Therefore, host survival is dependent on the fine balance achieved between these two processes.

An effective vaccine induces a combination of cellular and humoral immune responses to provide protection. Until very recently, registered vaccines with high efficacy records have been developed without a clear understanding of their mechanism of protection[259]. Early protection offered by a majority of existing vaccines is dependent on antibody responses, and therefore the efficacy of various vaccines under trial, including the RTS,S/AS01 vaccine, is determined by measuring antibodies in response to immunisation[259]. This is primarily due to past studies which demonstrated that the administration of concentrated human serum immunoglobulin could prevent and attenuate measles[260]. Similarly, passive serum transfer studies from immune adults to malaria-infected children resulted in parasite clearance and disease recovery[36]. However, the immunogenicity of antigens in vaccines poses a challenge that goes beyond the antibody response itself and therefore a greater understanding of immune responses against the pathogen in question is required[259]. Differentiating between immune responses that contribute to protection versus disease is imperative for better vaccine development and efficacy. In an effort to identify immune correlates of protection achieved by the current RTS,S vaccine, many groups have studied antibody levels as a measure of vaccine efficacy in addition to identifying immune correlates of protection. White *et al.*, used mathematical modelling to predict that RTS,S-induced protection from infection is dependent on both anti-CSP antibodies and CSP-specific $CD4^+$ T cells, with antibodies playing a dominant role in preventing infection[261].

It is important to note however, identifying immune correlates of protection in individuals living in malaria endemic areas is further confounded by whether the vaccine itself or naturally acquired responses account for protection from infection in older children and adults[262]. Furthermore, it is becoming increasingly apparent that the magnitude of antibody responses is insufficient to explain overall efficacy and as such, recent vaccine studies are now investigating the quality and specificity of antibody responses[263]. However, there is still debate regarding the rapid waning of protection in the absence of parasite exposure[32, 258, 264, 265, 266]. Nonetheless, various studies have indicated that once established, clinical immunity can in fact be long-lasting, particularly in trans-migrant populations and/or may vary depending on the parasite antigen; the immune response was generated against[267, 268, 269, 270, 271]. However, it still remains to be shown how and why if there is no or minimal defect in the generation and maintenance of humoral immune responses, then what is the underlying cause of the observed limited efficacy of the current RTS,S vaccine formulation? Thus, it will be important to identify the mechanisms that govern the induction and maintenance of humoral immunity, particularly in the vaccine setting.

The blood-stages of the malaria parasite *Plasmodium* induce a pro-inflammatory cascade that contributes to parasite clearance, but in the absence of immunoregulatory mechanisms, can lead to immunopathology[37, 74]. Early reports demonstrated a protective role for $CD4^+$ T cells during *Plasmodium* infections, but more recent studies have also reported on the pathogenic roles of $CD4^+$ T cells and their contribution to disease[37, 45, 67, 68]. The pro-inflammatory cytokine IFNγ has been shown to play protective roles in experimental malaria models, during uncomplicated and severe malaria. $CD4^+$ T cell derived IFNγ, in particular, is required for anti-parasitic immunity via the activation of macrophages, NK cells and differentiation of Th1 cells[45, 50, 54]. Recent studies have shown that type I IFN's suppress $CD4^+$ T cell-derived IFNγ production, via DC's in a model of severe malaria[67, 68].

In addition to suppressing $CD4^+$ T cell responses, type I IFN's have been shown to induce IL-10 production in monocytes/macrophages, DC's, B and T cells, via STAT3-dependent or independent pathways[272, 273]. The induction of type I IFN responses by *Plasmodium* species may be beneficial to the parasite, given the observed suppression of anti-parasitic $CD4^+$ T cell responses[67, 274]. The suppression of T cell responses during parasitic infections, allows for the preferential emergence or induction of immunoregulatory networks that can allow parasites to persist and cause disease.

$CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells are well known for their suppressive and immunoregulatory functions. While it is thought that these Treg cells are present and poised to limit damage to the host tissue caused by inflammation, this provides a simplistic picture which is in fact more complicated than previously thought. Given that cell-mediated immune responses play a dual role during malaria infections, whereby inflammation ultimately serves to limit parasite growth, but in doing so, contributes to pathogenesis and disease[37]. The potential involvement of Treg cells in promoting and orchestrating a balance between these two processes is still under intense investigation. Earlier studies have shown in experimental models[275] as well as humans[235] associations between Treg cell frequencies and reduced parasite-specific IFNγ memory responses. Recent studies however, have found very little evidence of associations between $CD4^+$ $Foxp3^+$ Treg cell frequencies and protection against severe malaria[276, 277]. Boyle *et al*., demonstrated a trend toward reduced risk of symptomatic malaria once infected with *P. falciparum*, amongst children with lower Treg cell frequencies[276]. This suggests that chronic malaria exposure may potentially alter Treg homeostasis, and thus impact the development of anti-malarial immunity in naturally exposed populations[276]. Furthermore, a recent study showed that neither Treg cell frequencies ($CD4^+$ $CD25^+$ $Foxp3^+$ $CD127^{lo}$) or absolute numbers were associated with malaria-related symptoms[277]. Unlike naturally occurring Treg cells which develop in the thymus, induced Treg cells are derived from conventional $CD4^+$ T cells which acquire their suppressive abilities upon activation [reviewed in[278, 279]]. Therefore, while the role of Treg cells remains to be further elucidated during *Plasmodium* infections, the involvement of other regulatory mechanisms, such as those involving IL-10 have been recently reported to play a role in *Plasmodium* infections.

Regulation of IL-10 induction, production and activity in $CD4^+$ T cells depends on local signals received by activated $CD4^+$ T cells in inflamed tissue [reviewed in[280]]. The emergence of $IFN\gamma^+$ $IL\text{-}10^+$ $CD4^+$ (Tr1) T cells in *Plasmodium* infections has been recently reported[47, 232, 235, 281]. Portugal *et al*., suggested that upon re-exposure, children acquire exposure-dependent immunoregulatory responses to regulate inflammation while enhancing anti-parasitic responses[232]. Jagannathan *et al*., proposed that while Tr1 cells displayed immunoregulatory characteristics and their emergence was largely dependent upon recent malaria exposure, they were not associated with protection from future disease, after controlling for prior malaria incidence[47]. Studies have shown that in naturally exposed populations, the effector phenotype of *P. falciparum*-specific $CD4^+$ T cells is largely influenced by age and transmission intensity[48]. In areas of high transmission, higher frequencies of Tr1 cells were observed in children, whereas adults displayed higher frequencies of $IFN\gamma^+$ TNF^+ $CD4^+$ T cells[48]. Interestingly, in areas of low transmission, both children and adults had higher frequencies of $IFN\gamma^+$ TNF^+ $CD4^+$ T cells[48].

This suggests that the emergence of immunoregulatory networks depends on the level of exposure to malaria parasites, with high exposure early in life possibly requiring the establishment of these networks to protect from inflammation caused by infection.

While the generation of cellular immunity against *Plasmodium* species contributes to the control of parasite growth, the induction of humoral immunity is required for long-term protection against malaria. Follicular T helper (Tfh) cells play critical roles in generating long lived antibody responses[165, 282, 283, 284]. Children in areas of high transmission rates of *P. falciparum* tend to have short-lived antibody responses to malaria[285, 286]. Obeng-Adjei *et al.*, recently showed that stimulation of PBMC's with *P. falciparum* antigen results in the production of Th1 cytokines and preferential activation of a 'Th1-like' $CXCR3^+$ Tfh subset, which was less efficient than the $CXCR5^+$ Tfh population in helping B cells[287]. It was suggested that this response to parasite antigen might contribute to the limited efficacy of the RTS,S/AS01 vaccine.

Moreover, in the human setting, the role of IFNγ, particularly in endemic regions is more complicated with regards to whether it plays protective, redundant or pathogenic roles. Evidence of a protective role for $CD4^+$ T cell derived IFNγ has largely been based on associations between IFNγ levels and protection from disease[35, 288, 289, 290]. This includes data from high versus low transmission areas, studies comparing immune responses in infants and young children versus adults, as well as investigations on the presence of other infections or complications such as HIV, tuberculosis and salmonella infections[48, 291, 292, 293, 294]. However, the magnitude of immune responses generated in response to parasite antigen in healthy volunteers versus individuals living in malaria endemic areas is unknown and may provide valuable insights into the limited efficacy of the current RTS,S/AS01 vaccine. Therefore, we studied $CD4^+$ T cell responses following first exposure to *P. falciparum* infection in a series of controlled human malaria infection (CHMI) studies.

CHMI's have been employed to test drugs and vaccines in an effort to gain a better understanding of protective immune responses against malaria[295]. CHMI's provide a unique situation where the generated immune responses are independent of previous or multiple exposures and thus represents a system where primary immune responses to malaria can be studied. Furthermore, this system may potentially be informative for gaining a better understanding of the immune responses observed during primary/early exposures (such as those observed in very young children living in malaria endemic areas). While the immune responses observed in adults may be considered to be more developed, these studies are nonetheless a stepping stone to understanding first/early exposures to *Plasmodium* infections. CHMI's can be divided into two systems including, sporozoite-induced malaria infection (SIM) and induced blood-stage malaria infection (IBSM)[296]. The CHMI studies described in this Chapter comprise of IBSM's, since it is the blood-stage of infection that is responsible for causing symptoms, disease and death, if left untreated. The IBSM system allows for the safe testing of anti-malarial drugs and compounds, as well as vaccine efficacy[296].

The limited efficacy of the RTS,S/AS01 vaccine has prompted investigation into interactions between the host and malaria parasites, and how this may impede the generation of immune responses that should provide long-term protection. In addition, there is an urgent need to identify reliable correlates of protective immune responses in malaria endemic regions. The RTS,S/AS01 vaccine was designed to target free sporozoites and intra-hepatic stages of the parasites, and by doing so, prevent the development of blood-stages responsible for all clinical symptoms of malaria. A blood-stage vaccine may extend the protection offered by pre-erythrocytic vaccines, but in order for these to be developed, a better understanding of host immune responses that control blood-stage malaria is needed.

This Chapter examines the development of host immune responses, including the emergence of immunoregulatory networks, following first exposure to *P. falciparum* in a series of CHMI studies.

3.3 MATERIALS AND METHODS

The material and methods presented here are specific to this chapter

3.3.1 CHMI

The studies described here comprise a series of sub-studies performed in conjunction with a main study (controlled, non-randomised interventional trials) aimed at characterising the effectiveness of various newly discovered anti-malarial compounds/drugs (**Table 1 and 2**) against early blood-stage *P. falciparum* infection in healthy individuals. The main study was undertaken by Professor James McCarthy (QIMR Berghofer Medical Research Institute) in association with Q-Pharm Pty Ltd (QIMR Berghofer) under approval of the QIMR Berghofer Human Research Ethics Committee (QIMR-HREC). HREC study numbers: P1444, P1470, P1530, P2012 and P2066. ANZCTR Trial IDs: ACTRN12612000323820, ACTRN12612000814875, ACTRN12613000565741, ACTRN12613001040752 and NCT02281344. All participants involved in the sub-study provided written consent to participate. The sub-study involved the collection of blood (20-40ml) at various time points including: day 0 (before challenge), day 7, day 14 and day 28-35 post challenge (**Fig 1**), in order to assess cellular immune responses (see **3.3.2** and **3.3.3**) and or collecting samples for gene array studies (see **3.3.6**). Participants were healthy males and females aged between 16 and 55 with no prior exposure to malaria or malaria endemic regions. All participants were infected i.v. with *P. falciparum* (clone 3D7, drug sensitive strain) which results in IBSM. Real-time quantitative PCR (qPCR), as described by Rockett *et al.*, was used to monitor peripheral parasitemia twice daily beginning at 4 days post challenge and subsequently monitored every 2 hours for the first 12 hours after drug treatment[297]. Drug was administered once parasitemia had exceed ≥1,000 parasites/ml and participants were monitored closely on-site (Q-Pharm). While this was a controlled human malaria infection, some participants did report symptoms of headaches, nausea, vomiting and increased body temperature which are all characteristic symptoms induced by the blood-stages of *P. falciparum* infection.

Figure 1

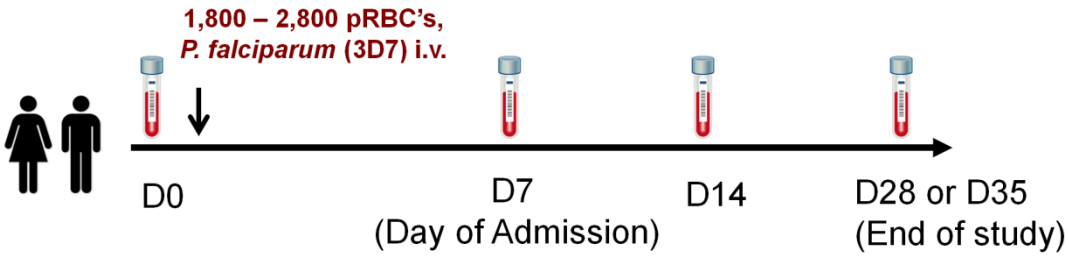


Figure 1: Blood collection at indicated time-points during CHMI studies

20-40ml of blood was collected from participants before infection (day 0). Participants were then infected with 1,800 – 2,800 pRBC's (*P. falciparum*, 3D7 clone) i.v. on day 0. 20-40ml of blood was collected at day 7 (day of admission – treated with experimental drug), 14 and 28 or 35 (end of study – treated with rescue drug, Riamet®) post challenge. At each time-point, PBMC's were isolated and cultured in the presence of nRBC's or pRBC's for 24-72 hours and Brefeldin A was added for the last 3 hours of culture.

Table 1: Cohorts used in Gene array studies n= 33

Year of study	Cohort	Subject	Age (years)	Gender	Drug, Dose
2012	QP12C02 C1	*S001*	31	M	Larium®, Mefloquine (5mg/kg)
		S006	23	M	
		S011	26	M	
2012	QP12C02 C2	*S015*	20	F	Larium®, Mefloquine (10mg/kg)
		S016	27	M	
		S017	22	F	
		S018	24	F	
		S019	20	F	
		S020	24	F	
		S022	23	M	
		S025	25	M	
2012	QP12C02 C3	*S028*	28	F	Larium® Mefloquine (15mg/kg)
		S032	27	F	
		S033	28	M	
		S035	25	M	
		S036	25	F	
		S038	37	M	
		S041	25	F	
		S042	34	M	
2012	QP12C10 C1	*S005*	22	F	OZ439, 100mg
		S009	23	M	
		S011	28	F	
		S012	25	M	
		S013	32	F	
		S017	16	M	
		S018	34	M	
2012	QP12C10 C2	*S024*	20	M	OZ439, 100mg
		S026	27	M	
		S027	27	F	
		S028	27	M	
		S029	35	M	
		S031	23	F	
		S032	23	M	

Table 2: Cohorts used in microarray validation n=18* and PBMC assay studies n= 35 §

Year of study	Cohort	Subject	Age (years)	Gender	Drug, Dose
2013	QP13C14 C1*,§	*S001*	25	M	Ferroquine, 800mg
		S002	22	M	
		S003	24	F	
		S007	22	F	
		S008	27	M	
		S009	41	F	
		S010	26	F	
2013	QP13C05 C1*	*S003*	19	M	Piperaquine, Piperaquine phosphate (960mg)
		S008	21	M	
		S012	20	M	
		S013	27	M	
2013	QP13C05 C2*	*S016*	26	F	Piperaquine, Piperaquine phosphate (640mg)
		S017	25	M	
		S018	23	M	
		S019	19	M	
		S022	24	M	
		S024	31	M	
		S025	25	M	
2014	QP13C05 3A§	*S030*	25	M	Piperaquine, Piperaquine phosphate (480mg)
		S033	21	F	
		S035	23	M	
		S036	26	F	
		S037	21	F	
2014	QP13C05 3B§	*S045*	18	M	Piperaquine, Piperaquine phosphate (480mg)
		S050	22	F	
		S052	32	F	
		S057	23	M	
2014	QP14C11§	*S001*	29	M	MMV390048, 20mg
		S002	28	M	
		S005	29	M	
		S006	24	M	
		S008	23	M	
		S011	19	M	
2015	QP14C12 C1§	*S018*	37	M	OZ439 (200mg) DSM265, (100mg)
		S019	55	M	
		S020	22	M	
		S017	25	M	
		S011	21	F	
		S021	19	F	
		S007	24	F	
2015	QP14C12 C2§	*S028*	27	F	OZ439 (200mg) DSM265, (50mg)
		S029	23	M	
		S022	24	F	

3.3.2 Human Peripheral Blood Mononuclear cell (PBMC) isolation

Human PBMC's were isolated by Ficoll-gradient separation from whole blood. Briefly, 20-40ml of blood was collected into Lithium Heparin tubes (BD Biosciences, San Jose, CA, USA) from participants of the following cohorts: QP13C14 Cohort 1 (2013, Ferroquine, n=5), QP13C05 Cohort 3A (2014, Piperaquine, n=5), QP13C05 Cohort 3B (2014, Piperaquine, n=4), QP14C11 Cohort 1 (2014, MMV390048, n=6), QP14C12 Cohort 1 (2015, OZ439 DSM265, n=7), QP14C12 Cohort 2 (2015, OZ439 DSM265, n=3). Whole blood was inverted gently a few times prior to centrifugation at 784 xg for 10 minutes at room temperature to remove 1-2ml of plasma that was stored at -20°C to -80°C until required. After plasma removal, 25ml of sterile 1x PBS (QIMR Berghofer) was added and tubes were inverted gently a few times to ensure a homogenous mixture. 13ml of Ficoll-Paque™ PLUS (GE Healthcare, Silverwater, NSW, Australia) was layered gently under the blood and centrifuged at 392xg for 30 minutes at room temperature without brake. After centrifugation the buffy coat was collected and PBMC's were washed with 50ml of 1x PBS, centrifuged at 392xg for 10 minutes at 4°C. The PBMC pellet was then re-suspended in 4ml complete media (**Chapter 2, 2.1**). Cells were diluted 1:10 in 0.1% Trypan Blue in PBS (0.4%, MP Biomedicals Pty Ltd, Seven Hills, NSW, Australia) and counted on a haemocytometer (BLAUBRAND®counting chamber Neubauer, Blackburn, VIC, Australia).

3.3.3 PBMC Assay

For fresh isolation, PBMC's were isolated as described in **3.3.2**, counted on a haemocytometer and then adjusted to a concentration of $2x10^6$ cells/ml, in complete media.

For cryopreserved samples (field samples collected from Timika, Indonesia), each vial underwent a "quick thaw" procedure (37°C for 5 minutes in a water bath). Once samples were thawed, complete media was added drop-wise (1ml), followed by the addition of 9ml of media. Cells were centrifuged for 600xg for 7 minutes at room temperature. Cells were then counted and viability of the cells was determined on a haemocytometer (BLAUBRAND® counting chamber Neubauer), after a 1:10 dilution in 0.1% Trypan Blue in PBS (0.4%, MP Biomedicals Pty Ltd). All samples were adjusted to a concentration of $2x10^6$ cells/ml, in complete media. Patient clinical data for samples from Timika is shown in **Table 3**.

Table 3: Timika patient clinical data

All Pf*	Age (years)	Gender	Weight (kg)	Ethnicity	Treatment
	43	F	55	Non-Papuan	Artekin® (Dihydroartemisinin and Piperaquine)
	43	M	51	Non-Papuan	Coart® (Artemether and Lumefantrine)
	30	M	45	Highland	Coart®
	25	M	46	Non-Papuan	Coart®
	27	M	49	Highland	Coart®
	35	F	71	Non-Papuan	Coart®
	37	M	55	Non-Papuan	Artekin®
	20	M	59	Highland	Artekin®
	36	M	59	Highland	Coart®
	20	M	49	Highland	Coart®
	33	M	47	Non-Papuan	Coart®
	40	F	55	Highland	Cq+SP (Chloroquine and sulfadoxine-pyrimethamine)

*Pf: All patients were *P. falciparum* positive

Normal red blood cells (nRBC's) and parasitised red blood cells (pRBC's) were prepared as follows for use as antigen in the PBMC assay which was set up in a 96 well U-bottom plate. Cryopreserved stocks of nRBC's and pRBC's were allowed to thaw on ice and then adjusted to a working concentration of $9x10^6$ nRBC's or pRBC's/ml in complete media. 25µl of this working stock was then added into each corresponding well (for a final concentration of $1x10^6$ nRBC's or pRBC's/ml). As a positive control, 25µl of a mitogen, phytohaemagglutinin (PHA, final concentration 10µg/ml, Sigma-Aldrich, Castle Hill, NSW, Australia) was added into corresponding wells. After all antigens and mitogens had been plated out, 200µl of PBMC's (final concentration $4x10^5$ cells/well) was added to each respective well. The following blocking antibody treatments were then added: Human anti-Interferon-α/ß Receptor Chain 2 antibody (clone MMHAR-2, final concentration: 5µg/ml, Merck Pty Ltd, Bayswater, VIC, Australia) or its mouse IgG2A isotype control (clone 20102, final concentration: 5µg/ml, Bio-scientific Pty Ltd, Kirrawee, NSW, Australia); Human anti-IL-10 antibody (clone 25209, final concentration: 20µg/ml, Sapphire Bioscience Pty Ltd, Redfern, NSW, Australia), or its mouse IgG2B isotype control (clone 20116, final concentration: 20µg/ml, Sapphire Bioscience Pty Ltd) and Ultra-LEAF™ Purified anti-human HLA-DR antibody (clone L243, final concentration 20µg/ml, Biolegend, San Diego, CA, USA) or its Ultra-LEAF™ purified mouse IgG2a, κ isotype control antibody (clone: MOPC-173, final concentration 20µg/ml, Biolegend). The plate was then placed in a humidified incubator at 37°C, 5% (v/v) CO_2 for 24-72 hours. Culture supernatants were harvested at 24 and 72 hours post- PBMC re-stimulation and stored at -20°C until required.

3.3.4 Flow cytometry

72 hours post PBMC re-stimulation, intracellular cytokine analysis for IFNγ and IL-10 was performed. Briefly, for detection of *ex vivo* IFNγ alone, 10µg/ml of Brefeldin A (BFA, Sigma-Aldrich) was prepared in complete media (with 10% FBS) and added to each well, for the remaining 3 hours (at 69 hours) of re-stimulation. To detect Tr1 cells ($IFN\gamma^{+}IL\text{-}10^{+}$), 10µg/ml of BFA, 25ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 500ng/ml of ionomycin calcium salt (Sigma-Aldrich) was prepared in complete media (with 10% FBS) and added to each well, for the remaining 3 hours of re-stimulation. 72 hours post re-stimulation; PBMC's were washed with HBSS (Life Technologies) and centrifuged at 338xg for 3 minutes at 4°C. 1µg/ml (in a final volume of 50µl) of Zombie NIR™ fixable viability kit (Biolegend) was diluted in HBSS, added to each well and incubated for 15-20 minutes at room temperature, protected from light. PBMC's were washed with HBSS and centrifuged at 338xg for 3 minutes at 4°C. 1-5µg/ml (in a final volume of 50µl) of surface antibodies (**Table 4**) were diluted in FACS buffer, added to each well and incubated for 15-20 minutes on ice, protected from light. PBMC's were then washed twice with 150 – 200µl of FACS buffer and centrifuged at 338xg for 3 minutes at 4°C. 100µl of BD cytofix buffer (BD Biosciences) was added to each well and incubated on ice for 20 minutes, protected from light. PBMC's were then washed twice with 100 - 200µl of 1x BD perm buffer (BD Biosciences) and then centrifuged at 338xg for 3 minutes at 4°C. 2µg/ml (final volume of 50µl) of intracellular antibody (IFNγ PeCy7 and IL-10 PE), diluted in BD perm buffer was then added per well and incubated on ice for 60 minutes, protected from light. PBMC's were then washed twice with 150 - 200µl of 1x BD perm buffer (BD Biosciences) and then centrifuged at 338xg for 3 minutes at 4°C. PBMC's were resuspended in a final volume of 100µl of 1% PFA (MP Biomedicals Pty Ltd), stored at 4°C and protected from light until acquisition on a Fortessa 5 (BD Biosciences). Samples were generally acquired within 12-24 hours post staining.

Cell populations of interest were defined as follows:

- $CD4^{+}$ T cells: $CD16^{-}CD56^{-}CD3^{+}CD4^{+}$
- $CD8^{+}$ T cells: $CD16^{-}CD56^{-}CD3^{+}CD8^{+}$
- NK cells: $CD16^{-}CD56^{+}CD3^{-}$

Table 4: Human monoclonal antibodies used for flow cytometry

Specificity	Clone	Fluorophore	Dilution	Supplier
BDCA-1	L161	PerCP/Cy5.5	1 in 50	Biolegend
CD3	SK7	Alexa Fluor 700	1 in 50	Biolegend
CD3	SK7	FITC	1 in 200	Biolegend
CD4	RPA-T4	APC Cy7	1 in 25	BD
CD4	RPA-T4	Horizon V500	1 in 30	BD
CD8	SK1	PerCP/Cy5.5	1 in 80	Biolegend
CD11c	B-ly6	Alexa Fluor 700	1 in 50	BD
CD14	TüK4	Qdot®605	1 in 100	Invitrogen
CD16	3G8	Alexa Fluor 700	1 in 50	Biolegend
CD19	H1B19	BV605	1 in 20	Biolegend
CD19	SJ25C1	FITC	1 in 20	BD
CD45RA	HI100	PeCy7	1 in 20	BD
CD56	NCAM16.2	Brilliant Violet 421	1 in 20	BD
CD123	6H6	PeCy7	1 in 50	Biolegend
CXCR3	1C6/CXCR3	Brilliant Violet 421	1 in 20	BD
CXCR5	RF8B2	Brilliant Violet 421	1 in 20	BD
HLA-DR	L243	APC Cy7	1 in 30	BD
IFNγ	4S.B3	PeCy7	1 in 100	BD
IL-6	MQ2-13A5	PE	1 in 20	BD
IL-10	JES3-19F1	PE	1 in 100	BD
Live/dead		APC/Cy7 (NIR)	1 in 100	Biolegend
PD1	EH12.2H7	APC	1 in 20	Biolegend

3.3.5 MACS purification of $CD4^+$ $CD8^+$ $CD56^+$ cells from whole blood

PBMC's were isolated as described in **3.3.2**. PBMC's were then washed in 10ml of sterile Dulbecco's PBS (Life Technologies) and centrifuged at 392xg for 10minutes at 4°C, to remove any FBS carryover from the complete media. Using the MACS Miltenyi bead kits (Miltenyi Biotec Australia Pty Ltd, Macquarie Park, NSW, Australia) for positive selection of $CD4^+$, $CD56^+$ and $CD8^+$ each sample was divided such that half was allocated for isolation of $CD4^+$ cells and the other half underwent 2 isolations, $CD56^+$ followed by $CD8^+$ cells, as per manufacturer's instructions (**Table 5**). For the positive selection of $CD4^+$ and $CD8^+$ cells, after the first elution, the sample was then loaded onto a fresh column and underwent a second round of elution in order to increase purity of selected population. Purity was ≥ 95-99%. Cells were then washed with 9ml of Dulbecco's PBS, centrifuged at 392xg for 10 minutes at 4°C and the pellet was gently re-suspended in 500µl of RNA-later® (Sigma-Aldrich). Samples were transferred to 4°C overnight and then stored at -80°C for long term storage until RNA was extracted.

Table 5: Magnetic labelling of $CD4^+$, $CD8^+$ and $CD56^+$ cells

Blood volume	# PBMC	Beads	MACS buffer	Total volume
10ml	2×10^7	40 µl	160 µl	200 µl
20ml	4×10^7	80 µl	320 µl	400 µl
30ml	6×10^7	120 µl	480 µl	600 µl
40ml	8×10^7	160 µl	640 µl	800 µl

3.3.6 Whole blood gene array

From May 2012 – October 2012 blood samples were collected from the following cohorts: QP12C02 Cohort 1 (2012, Larium, n=3), QP12C02 Cohort 2 (2012, Larium, n=8), QP12C02 Cohort 3 (2012, Larium, n=8), QP12C10 Cohort 1 (2012, OZ439, n= 7), QP12C10 Cohort 2 (2012, OZ439, n=7). A total of 33 paired samples were collected at day 0 and day 7 post challenge. 8ml of whole blood was collected from each subject into PAXgene® Blood RNA tubes (QIAGEN, Pty Ltd, Chadstone, VIC, Australia) and stored at -20°C within 2 hours after blood withdrawal. PAXgene tubes were thawed for 4 hours at room temperature before processing for RNA extractions. Automated RNA extractions were performed on the QIAcube (QIAGEN Pty Ltd, Valencia, CA, USA) using the PAXgene Blood miRNA kit (QIAGEN Pty Ltd, VIC, Australia) as per manufacturer's instructions. RNA integrity and concentration was determined using the Agilent RNA 6000 Nano LabChip (Agilent Technologies, Pacific Laboratory Products, Blackburn, VIC, Australia) on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA). RNA samples were stored at -80°C before processing for RNA amplification and biotinylation. The Illumina® TotalPrep RNA Amplification kit (Thermo Fisher, Scoresby, VIC, Australia) was used to amplify and label RNA for direct hybridization onto Illumina array platforms, as per manufacturer's instructions. Briefly, an input of 500ng of RNA was used in the first strand of cDNA synthesis (2 hours at 42°C) by reverse transcription, followed by the second strand (2 hours at 16°C). cDNA was purified using magnetic beads and eluted in water (heated to 55°C). *In vitro* transcription (IVT) was then used to synthesise the biotin-labelled cRNA by incubating cDNA with the IVT master mix for 14 hours at 37°C. cRNA was then purified using cRNA binding mix and cRNA binding beads, followed by 2 washes with cRNA wash solution. Finally, cRNA was eluted in 40µl of cRNA elution buffer (heated to 55°C). Labelled cRNA concentration was determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The Illumina® Whole-Genome Gene Expression Direct Hybridization Assay system (Direct Hybridization Assay) (Illumina Australia, Pty Ltd, Scoresby, VIC, Australia) was used to detect gene expression in our samples, as per manufacturer's instructions. Briefly, the labelled cRNA is hybridized to the bead on the BeadChip containing the complementary gene-specific sequence. A total of 6 Illumina® Human BeadChips (HumanHT-12_V4) were used for direct hybridization of all 33 paired samples. Samples were randomised in order to avoid sample bias, prior to loading onto the BeadChips and incubating the RNA-loaded BeadChips in an Illumina Hybridization Oven for 16 hours at 58°C. RNA-loaded BeadChips were then removed from the overnight hybridization and subsequently immersed and washed in 250ml of E1BC wash buffer, where the BeadChip cover-

seals were gently removed using tweezers. BeadChips were then washed in 1x High-temp was buffer (set to warm to 55°C, the day before) for 10 minutes before transferring back into fresh 250ml E1BC wash buffer for 5 minutes at room temperature. BeadChips were subsequently washed in 250ml of fresh 100% Ethanol for 10 minutes and then transferred into 250ml of E1BC wash buffer for 2 minutes at room temperature. Each BeadChip was then incubated in 4ml of Block E1 buffer for 10 minutes at room temperature on a rocker mixer to ensure even spread of block buffer across the surface of the chip. 1µg/ml (final volume of 2ml) of Cy3-Strepavidin (kindly supplied by Dr. Glen Boyle, QIMR Berghofer Medical Research Institute), diluted in Block E1 buffer was added per BeadChip and incubated at room temperature on the rocker mixer (set to medium) for 10 minutes, protected from light. All BeadChips were subsequently immersed and washed in fresh 250ml of E1BC wash buffer for 5 minutes. All BeadChips were then dried by centrifuging at 392xg for 4 minutes at room temperature (Eppendorf Centrifuge 5810R). Finally, BeadChips were scanned on the iScan microarray scanner (Illumina Inc, San Diego, CA, USA) which uses a laser to excite the Cy3 of the single base extension product on the beads of the BeadChip sections. Data was collected as TIFF files after appropriate calibration and quality control measures.

3.3.7 Gene array analysis

Agilent Genespring (Agilent Technologies Inc) was used to examine any statistical differences between day 0 and day 7 using a paired t-test with corrections for multiple testing (Benjamini-Hochberg method) and the fold change cut off set at 1.25[298, 299, 300, 301]. Ingenuity Pathway Analysis (IPA) (QIAGEN, Redwood City, CA, USA) was performed to determine the top 10 signalling pathways that were predominantly represented in our dataset with the fold change cut off set at 1.25.

3.3.8 Fluidigm® - micro fluidic RT qPCR (real time quantitative PCR) system

RNA was extracted from PAXgene tubes using the QIAcube as described above. RNA concentration and quality was determined on the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific). RNA was reverse transcribed into cDNA using Taqman Reverse Transcription Reagents (Life Technologies Pty Ptd, Mulgrave, VIC, Australia) and quantified on the Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific). Specific Target Amplification (STA) was carried out as per Fluidigm's recommendations (PN 68000133 Rev C**)** in order to increase target concentration. Fluidigm technology uses micro-volumes and in order to detect specific targets a minimum of 500 – 1,000 copies (of a specific target) is required in the original 5µl loading volume. Briefly, all 48 Taqman assays (Life Technologies Pty Ltd) of interest were pooled and diluted (final concentration 0.2x) in Tris-EDTA buffer (Sigma-Aldrich). 1.25µl of the pooled assay mix was mixed with 2.5µl of Taqman PreAmp master mix (Life Technologies Pty Ltd) and 1.25µl of cDNA. Reactions were cycled at the following conditions shown in **Table 6.**

Table 6: Thermal cycling conditions for Specific Target Amplification (STA)

	Temperature	Time	No. of cycles
Hold	95°C	10 minutes	1
Cycling Step 1	95°C	15 seconds	17
Cycling Step 2	60°C	4 minutes	

After cycling samples were diluted 1:4 by adding 15µl of Tris-EDTA buffer (final volume of 20µl post STA). Samples were then assayed on the same day as per manufacturer's instructions (PN 68000089 H1) using the 48.48 Dynamic Array^{TM} chips (Millennium Science Pty Ltd, Mulgrave, VIC, Australia). Briefly, 48.48 chips were primed by injecting 300µl of control line fluid into the accumulators on either side of the chip and was subsequently loaded into the Fluidigm MX Integrated Fluidic Circuit (IFC) controller (Millennium Science Pty Ltd), were the 'Prime 113x' script was selected and run for 10 minutes. Samples and Taqman assays were then pipetted into respective wells on the chip which was then loaded back into the MX IFC controller for 1 hour to allowing for sample and assay mixing, using the 'Load Mix (113x) script. Once the Load Mix script finished running, the chip was carefully removed and any dust particles or debris on the surface were removed prior to loading chip into the Biomark HD^{TM} (Millennium Science Pty Ltd). The 48.48 chips were cycled at the following conditions shown in **Table 7**. Gene expression was determined using the $2^{-\Delta\Delta CT}$ method and the GeoMean of the following three house-keeping genes: RPL13a, GAPDH and 18S, as reference.

Table 7: Thermal cycling conditions for Fluidigm® RT qPCR

	Temperature	Time	No. of cycles
Hold 1	50°C	2 minutes	1
Hold 2	95°C	10 minutes	1
Cycling Step 1	95°C	15 seconds	50
Cycling Step 2	60°C	1 minute	

3.3.9 Calculating Area Under the Curve (AUC)

To determine if there were any associations between cytokines and parasite growth, parasitemia was expressed as Area Under the Curve (AUC) to determine a more accurate indicator of parasite growth. Retrospective PCR parasitemia values were tested in duplicate and on the day of treatment, parasitemia was tested in triplicate. Importantly, AUC was only determined up until drug treatment. The limit of detection (LOD) was assumed to be 64parasites/ml[297]. After initial data checks for potential outliers, values below the LOD (between: 2 - 63) were substituted with LOD/2 (32) and values that were not-detected (ND) were set to 1. All parasitemia values were log-10 transformed, and the mean of the log-10 transformed parasitemia was used as a summary measure per time-point per participant. All regression analyses were performed using the mean log-10 parasitemia values and performed separately per participant. The parasitemia growth can be modelled as a sine-wave function, though it requires a minimum of 6 data points. As the majority of subjects (67%) had parasitemia data for less than 6 time-points, the area under the curve was estimated using the trapezoid rule, using GraphPad Prism version 6.

3.3.10 Statistical Analysis

Statistical differences between groups were determined using the Wilcoxon matched-pairs signed rank test (day 0 vs day 7 or day 0 vs day 14) and the linear regression function was used to analyse associations between IL-10 levels and Area Under the Curve (AUC) by GraphPad Prism version 6 for Windows (GraphPad, San Diego, CA, USA); $p<0.05$ was considered statistically significant. All data are presented as the mean ± SE, unless otherwise stated.

3.4 RESULTS

3.4.1 Whole blood gene array revealed a dominant type I IFN signature

Type I IFN's have been reported to suppress anti-parasitic $CD4^+$ T cell responses during blood-stage *Plasmodium* infections[67, 68]. Consistent with these findings in mice, here in malaria-naive individuals we observed a significant up-regulation of genes associated with the type I IFN signalling pathway, 7 days post challenge with *P. falciparum* (**Fig 2a, b**). Similarly, a recent study involving whole-blood transcriptional analysis in Rwandan adults with mild *P. falciparum* infection showed an up-regulation of the type I IFN pathway[302]. In these CHMI studies, the up-regulation of the type I IFN signalling pathway appeared to be associated with a down-regulation of genes in the T cell receptor signalling pathway (**Fig 2 b-d**). In order to validate results from the microarray, we employed use of the Fluidigm® 48.48 Chip platform to assess gene expression by RT qPCR, where we noted increases in *irf3, irf7* and *ifitm3* expression 7 days post challenge (**Fig 2 e**). Although these results show a similar pattern of gene expression as measured in gene array studies, it was critical that we functionally validated the link between the up-regulation of type I IFN genes and down-regulation of TCR signalling during blood-stage *P. falciparum* infection. To this end, we next performed a series of PBMC assays with or without type I IFN blockade, to assess the contribution of type I IFN to the suppression of immune responses during blood-stage *P. falciparum* infection.

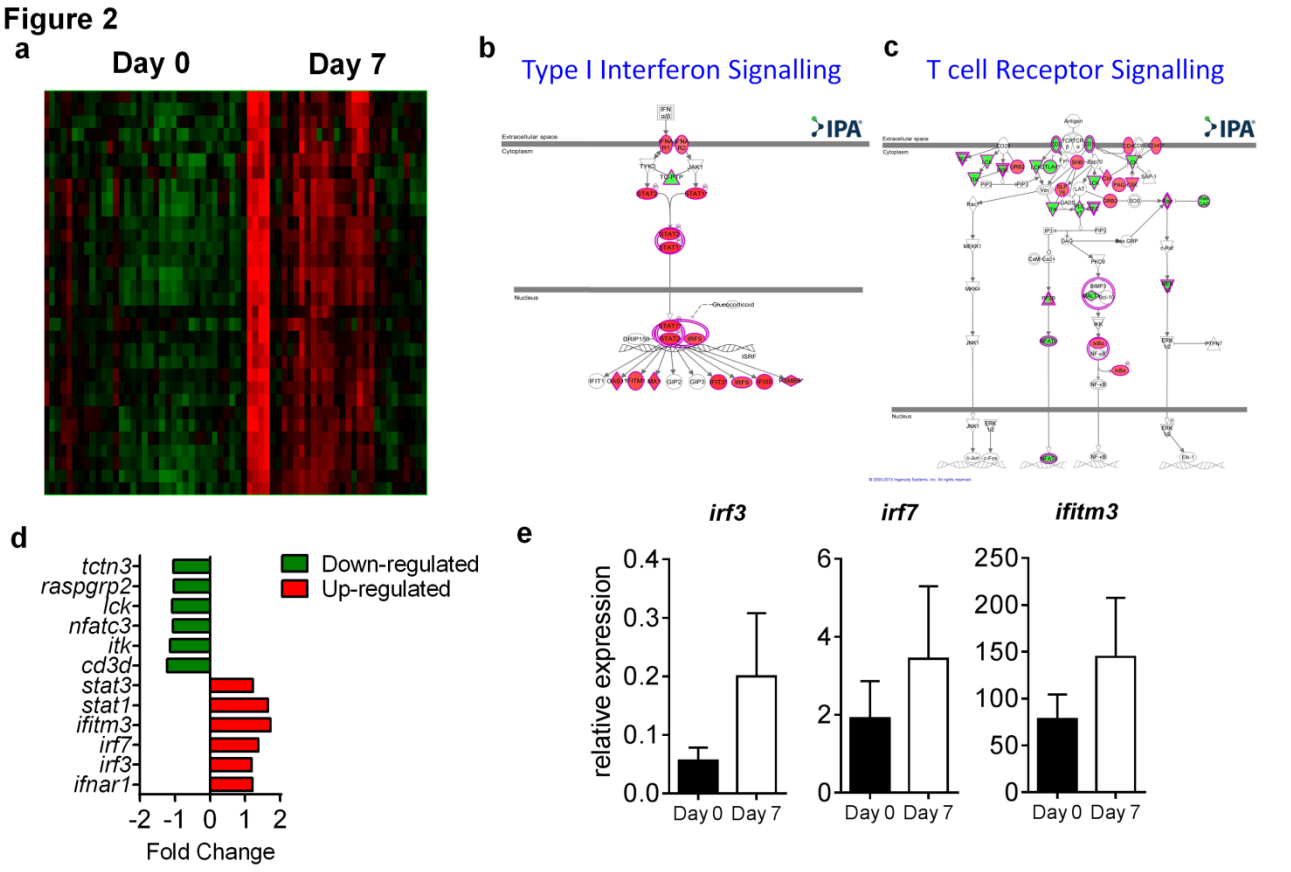


Figure 2: Whole blood gene array revealed a dominant type I IFN signature.

a) Whole blood from 33 participants (outlined in **Table 1**) was collected before and 7 days after infection with blood-stage *P. falcipaurm* (clone 3D7; drug sensitive strain) and processed for microarray analysis on GeneSpring®, heat map shows clustering analysis, fold change cut off set at ±1.25. Paired t-test matched for multiple test corrections using the Benjamini-Hochberg method. (*QP12C02 C1, C2, C3, QP12C10 C1 and C2*) **b), c)** Canonical pathway analysis tool in Ingenuity Pathway Analysis® used to analyse top 10 signalling pathways with a fold change cut off at ±1.25 (*QP12C02 C1, C2, C3, QP12C10 C1 and C2*). **d)** Genes selected for validation in GeneSpring (*QP12C02 C1, C2, C3, QP12C10 C1 and C2*). **e)** Preliminary validation of microarray data using whole blood RNA samples from 18 participants (outlined in **Table 2**, using the Fluidigm® platform for RT qPCR (*QP13C05 C1, C2 and QP13C14*). b) and c) enlarged in **Appendix 2**

3.4.2 Suppression of T cell responses during blood-stage *P. falciparum* infection

To gain an insight into the magnitude of $CD4^+$ T cell responses following first exposure to *P. falciparum*, PBMC's were isolated before and 7, 14 and 28-35 days after challenge. Blood parasitemia was monitored daily beginning at day 5 post challenge by PCR as described in Rockett *et al.*,[297]. Once parasite biomass had reached a physiological threshold of ≥1,000 pRBC's/ml (generally 7-8 days post challenge) (**Fig 3a**), individuals were treated with experimental drug (**see Table 2**) at day 7 or 8 and rescue drug (Riamet®) at 28-35 days post challenge. Overall frequencies of $CD4^+$ and $CD8^+$ T cells remained relatively unchanged between day 0 and day 7 post challenge (**Fig 3b**). This suggests that the decreased TCR signalling observed in the gene array could potentially result from intrinsic changes in the T cell populations, rather than changes in cell frequencies (**Fig 2c, Fig 3b**). Upon phenotypic analysis of $CD4^+$ T cell responses with particular focus on Th1 and Tfh cells, we noted a decrease in Th1 cells at day 7, whereas Tfh frequencies significantly increased at day 7 (**Fig 3c**). The early emergence of Tfh cells suggests the preferential selection of Tfh to Th1 given that the latter cell type promotes cellular immunity rather than humoral immunity which is required for long-term protection.

To determine if the reduced Th1 frequencies at day 7 resulted in a defect in production of the Th1 signature cytokine IFNγ, we first measured IFNγ levels in culture supernatants from PBMC's stimulated with *P. falciparum* antigen. Previous reports have shown cross reactive T cell responses in individuals who have never been exposed to malaria[303, 304] and consistent with these reports, we also detected significantly elevated IFNγ levels at day 0 (**Fig 3d**).72 hours post antigen re-stimulation we found that levels of IFNγ produced by PBMC's in response to parasite antigen declined from day 0 to day 7 post challenge (**Fig 3d**), suggesting T cell responses were suppressed upon first exposure to blood-stage *P. falciparum* infection. Parasite-specific IFNγ levels increased after drug treatment and were sustained up until at least day 28 post challenge (**Fig 3d**). $CD4^+$ T cells appeared to be the major source of IFNγ, since HLA-DR blockade resulted in significantly reduced IFNγ levels, 7 days post challenge (**Fig 3e**).

Figure 3

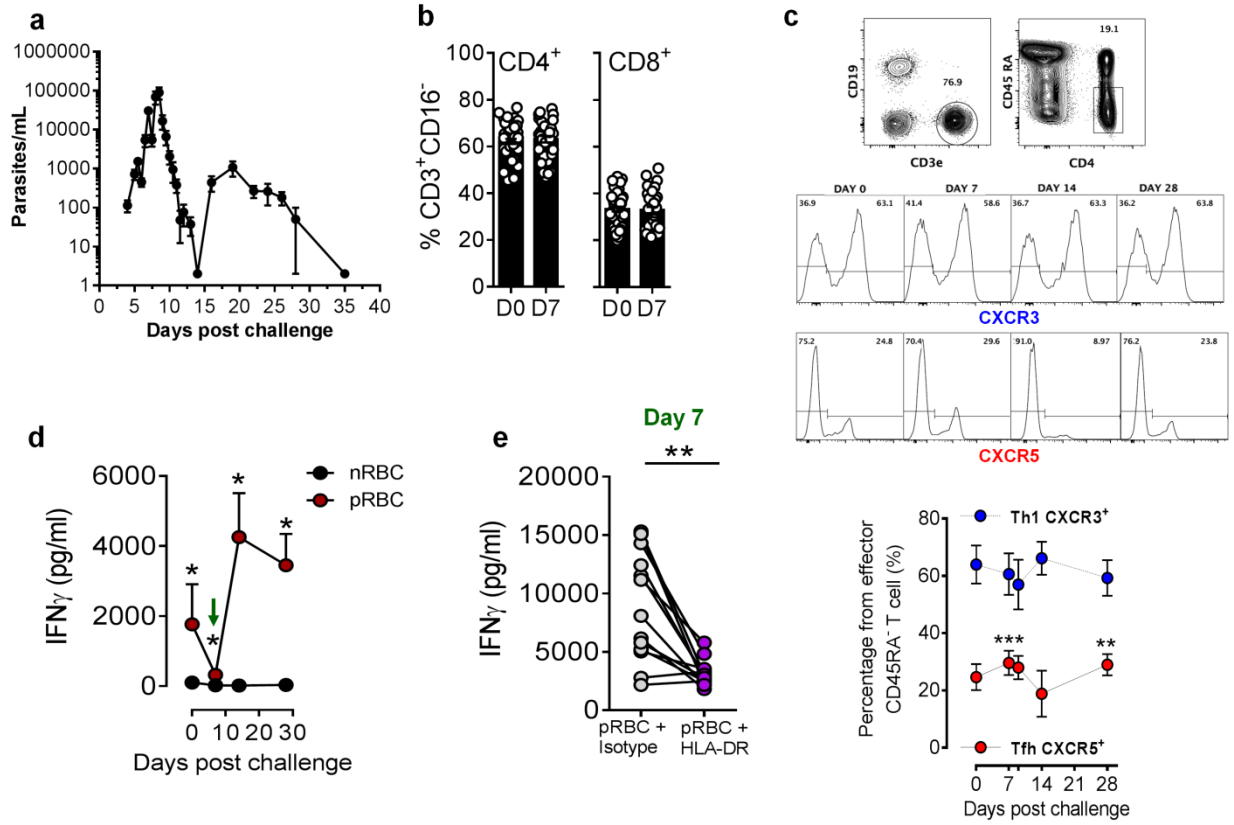


Figure 3: CD4$^+$ T cell responses are suppressed during blood-stage *P. falciparum* infection.

a) Participants were injected with 1,800 – 2,800 pRBC's/ml and blood parasitemia monitored by PCR beginning at 5 days post challenge (*QP13C14*) **b)** PBMC's isolated from participants before and 7 days post challenge and frequencies of CD4$^+$ and CD8$^+$ T cells were measured by flow cytometry (*QP12C02 C1, C2, C3, QP12C10 C1, C2*) **c)** PBMC's isolated from participants before and 7, 9, 14 and 28 days post challenge and frequencies of Tfh (CXCR5$^+$) and Th1 (CXCR3$^+$) cells measured at each time point by flow cytometry (*QP13C14*). **d)** PBMC's were isolated from participants throughout infection and cultured in the presence of nRBC's or pRBC's at 37°C, 5%CO_2 in an incubator for 72 hours, when culture supernatants were harvested and IFNγ levels were detected using the BD Human cytometric bead array (CBA) kit as per manufacturer's recommendations (*QP13C14*). **e)** PBMC's were isolated from participants 7 days post challenge and cultured in the presence of pRBC's and anti-HLADR antibody or its isotype control for 72 hours when culture supernatants were then harvested and IFNγ levels were detected using the BD Human CBA kit as per manufacturer's recommendations (*QP14C12 C1 and QP14C11*). Mean ±SEM, **=p<0.01, *= p<0.05, Wilcoxon matched-pairs signed rank test.

3.4.3 $CD4^+$ T cells are the predominant source of IFNγ during blood-stage *P. falciparum* infection

In order to ascertain the cell type responsible for the predominant source of parasite-specific IFNγ, we performed intracellular cytokine staining in PBMC cultures 72 hours post antigen re-stimulation (**Fig 4a**). Horowitz *et al*., showed that while NK cells were the main producers of IFNγ 12-18 hrs post antigen re-stimulation, T cells were the main producers of IFNγ 2-6 days post antigen re-stimulation[55]. Consistent with these results, we noted that $CD3^+$ $CD56^-$ (T cells) dominated the IFNγ response with minor contributions from $CD56^+$ $CD3^-$ (NK cells) and $CD56^+CD3^+$ (potentially NKT cells, γδ T cells and other activated T cells) 72 hours post antigen re-stimulation (**Fig 4b**). While γδ T cells have been shown to produce IFNγ in response to parasite antigen, they were greatly outnumbered by αβ T cells[55] and this was observed in our studies (**Fig 4 b**). Earlier studies have shown $CD4^+$ T cells to be a predominant source of IFNγ in response to parasite antigen[48, 55, 56]. While the contribution to IFNγ production by $CD4^+$ and $CD8^+$ T cells was relatively equal, $CD4^+$ T cells were the main producers of IFNγ in response to parasite antigen 14 days post challenge (**Fig 4c**).

Figure 4

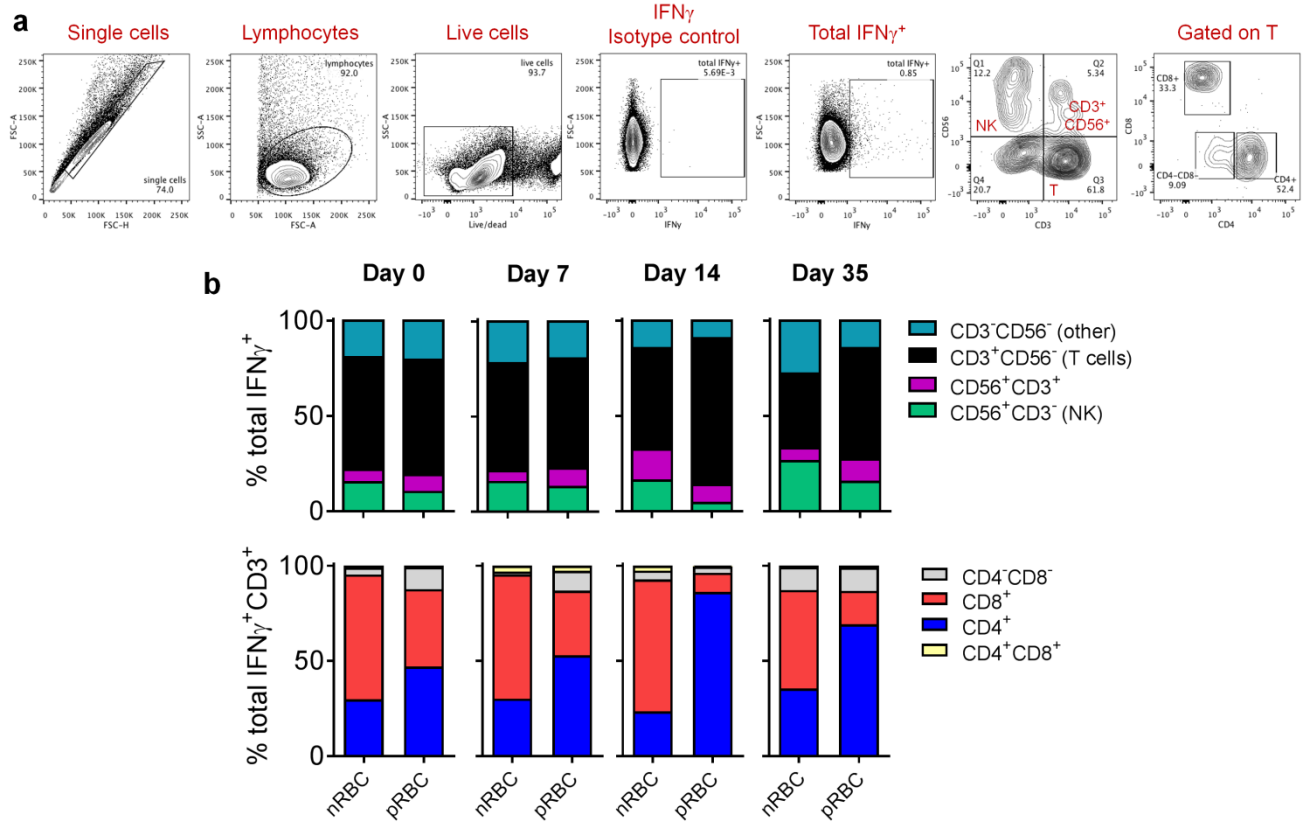


Figure 4: T cells are the predominant source of IFNγ during blood-stage *P. falciparum* infection.

a) PBMC's were isolated from participants before and 7, 14 and 35 days post challenge. PBMC's were culture in the presence of nRBC's or pRBC's for 72 hours and Brefeldin A was added for the last 3 hours of culture. Total frequencies of IFNγ producing cells were measured by flow cytometry, as per the gating strategy (left to right): single cells, lymphocytes, live/viable cells, total $IFN\gamma^+$, Q1:$CD3^-CD56^-$, Q2:$CD3^-CD56^+$ (NK cells), Q3: $CD3^+CD56^+$, Q4: $CD3^+CD56^-$ (T cells), $CD4^+$, $CD8^+$, $CD4^-CD8^-$ T cells. **b)** Frequencies of cells (coloured bars) as a percentage of total $IFN\gamma^+$ cells at 0, 7, 14 and 35 days post challenge from isolated PBMC's cultured in the presence of nRBC's or pRBC's for 72 hours. **c)** Frequencies of $IFN\gamma^+$ $CD3^+$ cells showing proportion of $CD4^+$ and $CD8^+$ T cells within the T cell compartment, throughout the course of infection. Representative of 2 independent cohorts (*QP13C05 3A and 3B*), data is shown from QP13C05 3A.

3.4.4 Type I IFN's were induced upon first exposure to *P. falciparum* and suppressed IFNγ production

Various studies have reported on the induction of type I IFN's during *Plasmodium* infection in various experimental malaria models[67, 68, 274, 305, 306, 307] as well as humans[308, 309]. Recently, type I IFN's have been reported to suppress $CD4^+$ T cell-derived IFNγ production, via DC's in a model of severe malaria[67, 68]. In accordance with these findings, we found significantly elevated levels of IFNα in response to parasite antigen, 7 days post challenge (**Fig 5a**). Importantly, blockade of type I IFN signalling showed a significant increase in IFNγ by PBMC's in response to parasite antigen, 7 days post challenge (**Fig 5b**). Collectively, these results suggest that type I IFN's suppressed anti-parasitic IFNγ responses.

Figure 5

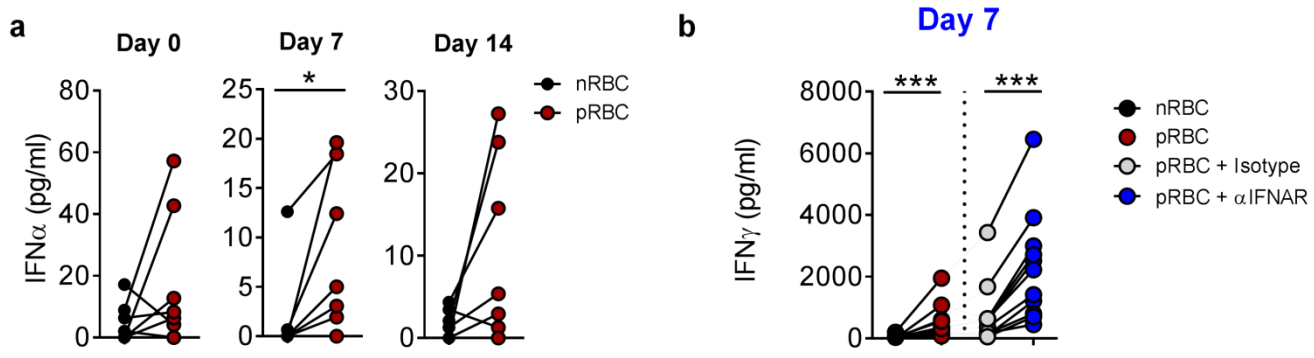


Figure 5: Blood-stage *P. falciparum* induces a type I IFN response that suppresses IFNγ production.

a) Isolated PBMC's cultured in the presence of nRBC's or pRBC's for 72 hours when culture supernatants were harvested and IFNα levels were measured by using the eBioscience Human IFNα detection kit (*QP14C12 C1*). **b)** PBMC's were isolated from participants 7 days post challenge and cultured in the presence of nRBC's, pRBC's or pRBC's + anti-IFNAR antibody or its isotype control for 72 hours and IFNγ levels were detected using the BD Human CBA kit (*QP13C14 and QP13C05 3A*). ***=p<0.001, *= p<0.05, Wilcoxon matched-pairs signed rank test.

3.4.5 Type I IFN's inhibit monocyte derived IL-6 production in response to *P. falciparum*

The dramatic increase in IFNγ levels, following type I IFN blockade, prompted us to investigate whether other cytokines might also be affected by IFN-I, during blood-stage *P. falciparum* infection. Notably, upon type I IFN signalling blockade IL-6 levels were significantly elevated throughout the course of infection (**Fig 6**). Interestingly, following type I IFN signalling blockade, IL-6 levels were highest prior to infection, during, suggesting that type I IFN's were potentially suppressing IL-6 production by innate cells[310] (**Fig 6**). Cellular sources of IL-6 were assessed by flow cytometry (**Fig 7a-d**), where IL-6 production by plasmacytoid DC's (pDC's) and myeloid DC's (mDC's) was found to be comparable between day 0 and day 7 (**Fig 7b, c**). However, IL-6 production by monocytes was significantly elevated upon type I IFN signalling blockade at day 0 (**Fig 7d**). Elevated IL-6 levels have been observed in patients with inflammatory diseases such as rheumatoid arthritis, Castleman's disease and systemic juvenile idiopathic arthritis[311]. Collectively, these results suggest that type I IFN's suppresses monocyte derived IL-6 production upon first exposure to *P. falciparum* infection.

Figure 6

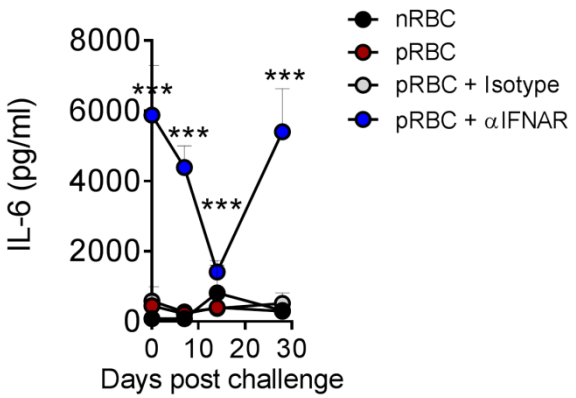


Figure 6: Type I IFN signalling blockade enhances IL-6 production in response to *P. falciparum.*

PBMC's were isolated from participants on 0, 7, 14 and 28 days post challenge and cultured in the presence of nRBC's, pRBC's or pRBC's + anti-IFNAR antibody or its isotype control for 72 hours and IL-6 levels were detected using the BD Human CBA kit, as per manufacturer's recommendations (*QP13C14 and QP13C05 3A*). Mean ±SEM, ***=p<0.001, Wilcoxon matched-pairs signed rank test.

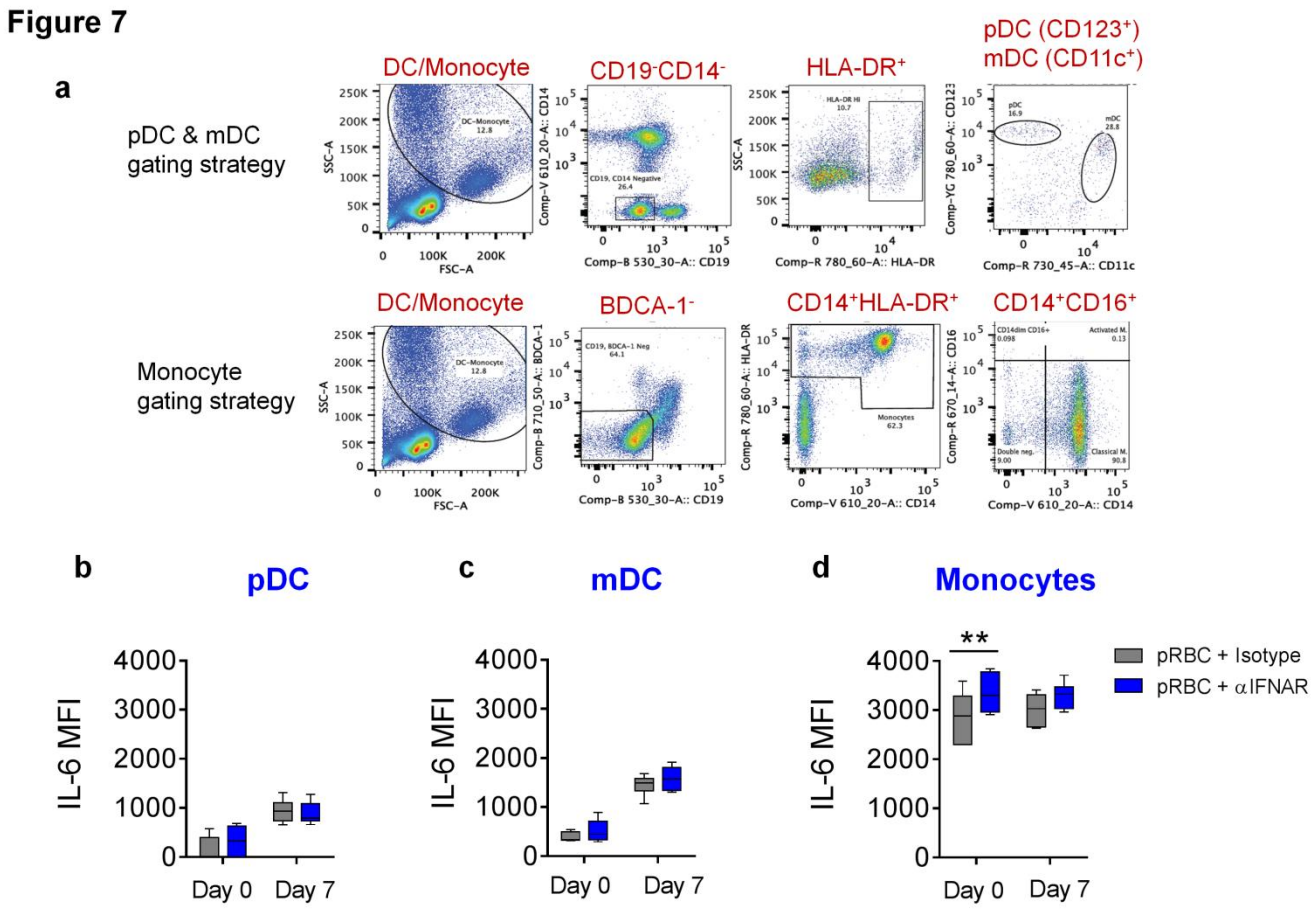


Figure 7: Type I IFN's suppress monocyte derived IL-6 production in response to *P. falciparum*.

a) Gating strategy shown for pDC's, mDC's (top row) and monocytes (bottom row) measured by flow cytometry (*QP13C14*). **b)** Isolated PBMC's were cultured in the presence of pRBC's + anti IFNAR or its isotype control for 24 hours and Brefeldin A added for the last 3 hours and IL-6 was measured by intracellular cytokine staining by flow cytometry. IL-6 Mean Fluorescence Intensity (MFI) shown on pDC's at day 0 and 7 post challenge (*QP13C14*). **c)** IL-6 MFI shown on mDC's at day 0 and 7 post challenge (*QP13C14*). **d)** IL-6 MFI shown on monocytes at day 0 and 7 post challenge (*QP13C14*). Median + Min and Max, **=p<0.01, Wilcoxon matched-pairs signed rank test.

3.4.6 Type I IFN's suppress early inflammatory cytokines in response to *P. falciparum*

Given that members of the type I IFN signalling pathway are involved in the regulation of inflammatory cytokine responses[312, 313], we next tested whether type I IFN blockade would affect other inflammatory cytokines. Strikingly, we observed significantly elevated levels of IL-1β and IL-17A production by PBMC's in response to parasite antigen, upon type I IFN signalling blockade, 7 days post challenge (**Fig 8 a, b**). It was interesting to note that the effects type I IFN signalling blockade was specific to IL-6, IL-1β and IL-17A, but not TNF (**Fig 6, Fig 8c**). Taken together, these data suggest that type I IFN's suppress major inflammatory networks early during *P. falciparum* infection.

Figure 8

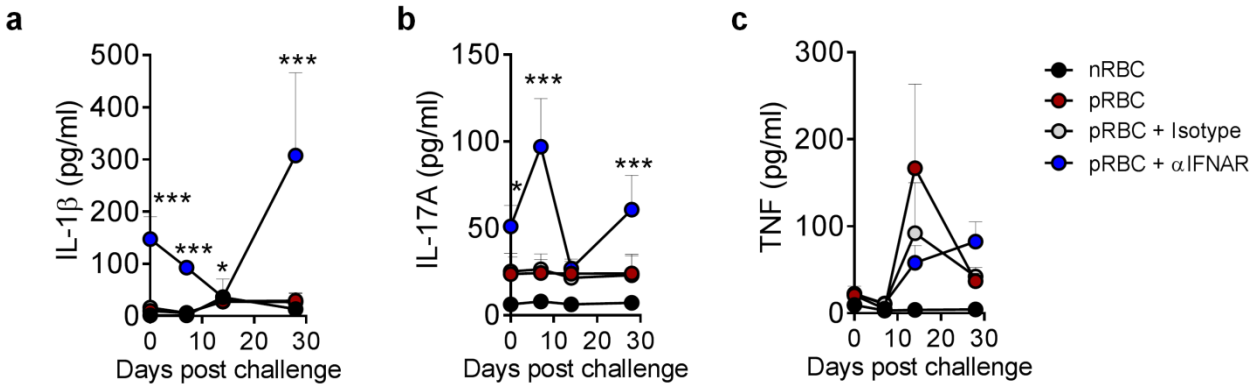


Figure 8: Type I IFN's suppress IL-1β, IL-17 but not TNF production in response to *P. falciparum.*

a) Isolated PBMC's were cultured in the presence of nRBC's, pRBC's, pRBC's + anti-IFNAR antibody or its isotype control for 72 hours when culture supernatants were harvested and levels of IL-1β, **b)** IL-17 or **c)** TNF were detected using the BD Human CBA kit, as per manufacturer's recommendations. Data shown from QP13C14 and QP13C05 3A, Mean ±SEM, ***=p<0.001, *=p<0.05, Wilcoxon matched-pairs signed rank test.

3.4.7 First exposure to *P. falciparum* induces parasite-specific Tr1 cells and IL-10 production 7 days post drug treatment

Earlier studies have shown that circulating levels of IFNγ and IL-10 were significantly higher in patients with severe malaria[314, 315]. More recently, it was shown that Tr1 cells emerge as the dominant $CD4^+$ T cell response in highly exposed children living in malaria endemic regions[47, 48, 232]. The gating strategy for Tr1 cells, in our CHMI studies is shown in **Fig 9**. Similar to field studies, in our CHMI studies, we found a significant increase in IL-10 production by PBMC's in response to parasite antigen throughout the course of infection (**Fig 10a**). IL-10 levels peaked 14 days post challenge (7 days post drug treatment) (**Fig 10a**), suggesting the generation of IL-10 occurred in response to increased antigen available as a result of parasite killing by drug. Recent studies have confirmed $CD4^+$ T cells as a major source of IL-10 production during *Plasmodium* infections[47, 74, 316]. Similarly, in our studies, this increase in IL-10 production by PBMC's in response to parasite antigen appeared to HLA-DR restricted (**Fig 10b**), suggesting that $CD4^+$ T cells were a major source of IL-10 during blood-stage *P. falciparum* infection. In addition to this increase in IL-10 production, we observed a significant increase in the frequency of Tr1 cells in response to parasite antigen 14 days post challenge (**Fig 10c, d**). Similar to total IL-10 production, the induction of Tr1 cells also appeared to be HLA-DR restricted (**Fig 10c, d**). Together these data indicate that a significant proportion of IL-10 production by PBMC's in response to parasite antigen was by $CD4^+$ T cells, 7 days post drug treatment (14 days post challenge), indicating the development of Tr1 cells during first exposure to blood-stage *P. falciparum* infection.

Figure 9

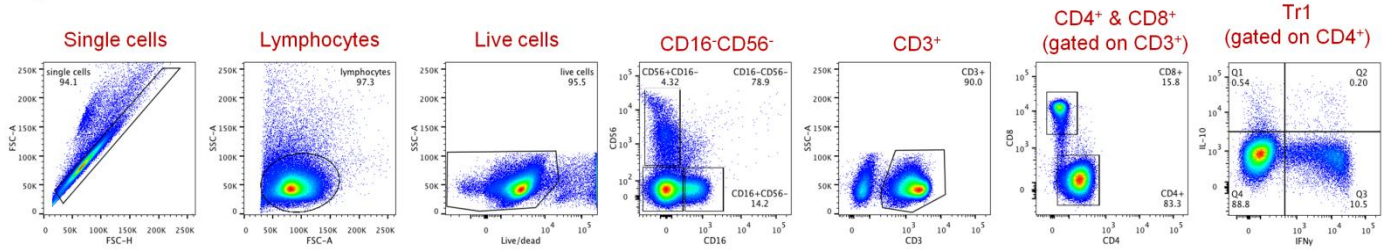


Figure 9: Gating strategy for Tr1 cells, 72 hours post *Pf* antigen re-stimulation.

a) Isolated PBMC's were cultured in the presence of nRBC's or pRBC's (*Pf*) for 72 hours and Brefeldin A, PMA/Ionomycin was added for the remaining 3 hours of culture before frequencies of Tr1 cells were analysed by flow cytometry. Gating strategy from left to right shows: single cells, lymphocytes, live/viable cells, $CD16^-CD56^-$ (exclusion of NK and γδ T cells), $CD3^+$, $CD4^+$, $IFN\gamma^+$ $IL\text{-}10^+$ (*QP13C05 3A*).

Figure 10

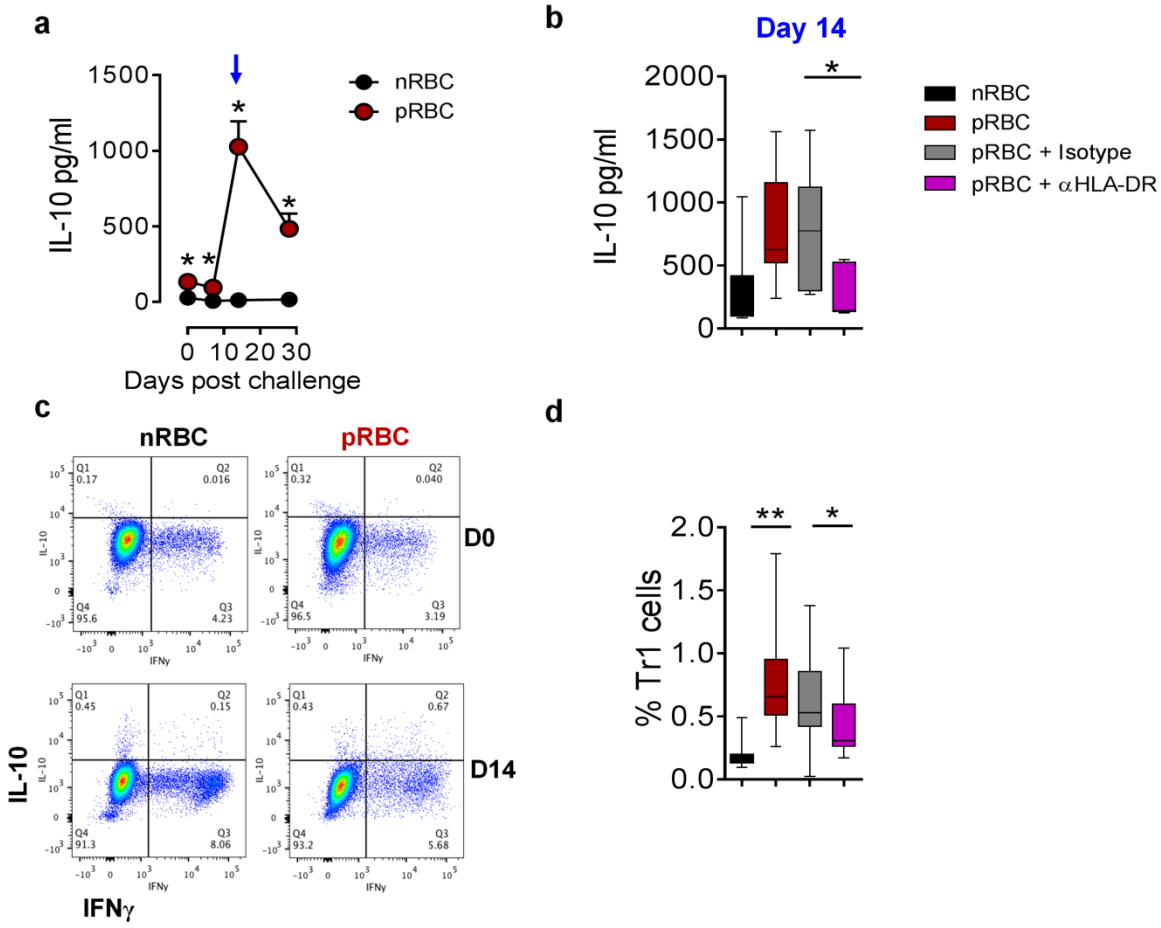


Figure 10: Blood-stage *P. falciparum* induces HLA-DR restricted Tr1 cells and IL-10 production.

a) PBMC's were isolated from participants at 0, 7, 14 and 28 days post challenge and cultured in the presence of nRBC's or pRBC's for 72 hours, when culture supernatants were harvested and levels of IL-10 were measured using the BD Human CBA kit (*QP13C14*). **b)** PBMC's were isolated from participants 14 days post challenge (7 days post drug treatment) and cultured in the presence of nRBC's, pRBC's or pRBC's + anti HLADR antibody or its isotype control for 72 hours, when culture supernatants were harvested and levels of IL-10 were measured using the BD Human CBA kit (*QP14C12 C1 and C2*). **c)** PBMC's were isolated from participants 14 days post challenge (7 days post drug treatment) and cultured in the presence of nRBC's, pRBC's or pRBC's + anti HLADR antibody or its isotype control for 72 hours and Brefeldin A, PMA/Ionomycin was added for the remaining 3 hours of culture before **d)** frequencies of Tr1 cells were measured by flow cytometry (*QP14C12 C1 and C2*). **a** Mean ±SEM, **b** and **d**, Median + Min and Max, **=p<0.01, *=p<0.05, Wilcoxon matched-pairs signed rank test.

3.4.8 Type I IFN dependent IL-10 production and Tr1 cell induction, during blood-stage *Plasmodium* infection

Interestingly, type I IFN's have been shown to induce IL-10 production in monocytes/macrophages, dendritic cells, B and T cells, via STAT3-dependent or independent pathways[272, 273]. We confirmed this, showing that upon blockade of type I IFN signalling, IL-10 production by PBMC's in response to parasite antigen was significantly reduced 7 days post drug treatment (**Fig 11a**). Interestingly, type I IFN's were also required for the induction of Tr1 cells (**Fig 11b**). Linear regression analysis showed a significant positive correlation between IL-10 levels and parasitemia, as determined by AUC at day 7 post challenge (**Fig 11c**). While IL-10 plays a key role in immunoregulation, this may contribute to suppression of anti-parasitic responses and thus impede parasite control. However, an alternative mechanism might be that higher parasite levels promote greater IL-10 production. To investigate this further, we neutralised IL-10 and observed significant increases in the production of pro-inflammatory cytokines IFNγ and TNF, 7 days post drug treatment (**Fig 11d, e**). Given the positive correlation between IL-10 and AUC and the significant increases in IFNγ and TNF levels upon neutralisation of IL-10, we investigated whether there was any association between IFNγ and AUC. Linear regression analysis showed a significant inverse correlation between IFNγ levels and parasitemia, as determined by AUC at day 7 post challenge (**Fig 11f**). Collectively, these data suggest that parasite-induced IL-10 can suppress the generation of potent anti-parasitic responses, contributing to increased parasitemia.

Figure 11

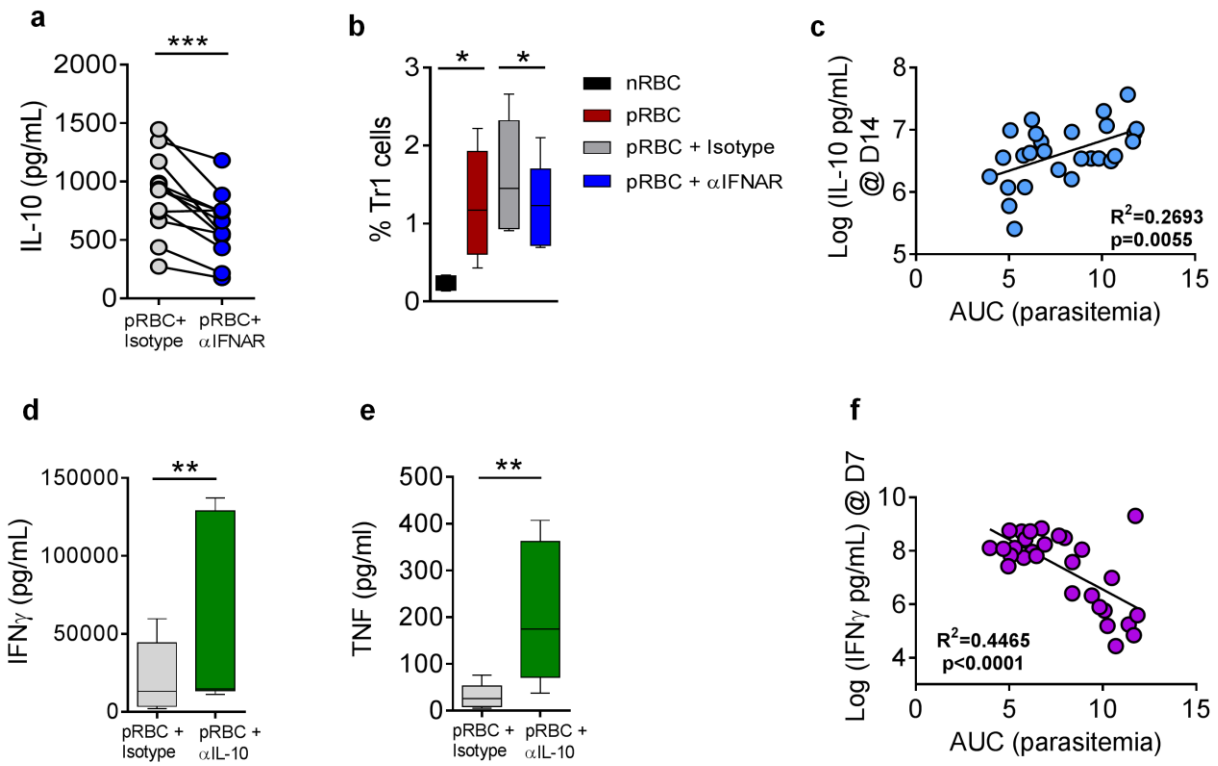


Figure 11: Emergence of immunoregulatory networks is type I IFN-dependent and may contribute to enhanced parasite growth.

a) PBMC's were isolated from participants 14 days post challenge (7 days post drug treatment) and cultured in the presence of nRBC's, pRBC's or pRBC's + anti-IFNAR antibody or its isotype control for 72 hours and IL-10 levels were detected using the BD Human CBA kit (*QP13C14 and QP13C05 3A*). **b)** PBMC's were isolated from participants 14 days post challenge (7 days post drug treatment) and cultured in the presence of nRBC's, pRBC's or pRBC's + anti IFNAR antibody or its isotype control for 72 hours and Brefeldin A, PMA/Ionomycin was added for the remaining 3 hours of culture before frequencies of Tr1 cells were analysed by flow cytometry (*QP13C05 3A*). **c)** Linear regression analysis performed to determine correlations between log-transformed IL-10 levels 14 days post challenge and parasitemia using area under the curve (AUC) (*QP13C14, QP13C05 3A, 3B, QP14C11 and QP14C12*). **d)** PBMC's were isolated from participants 14 days post challenge (7 days post drug treatment) and cultured in the presence of nRBC's, pRBC's or pRBC's + anti-IL-10 antibody or its isotype control for 72 hours when **d)** IFNγ levels (*QP13C05 3A and 3B*) and **e)** TNF levels were detected using the BD Human CBA kit (*QP13C05 3A and 3B*). **f)** Linear regression analysis performed to determine correlations between log-transformed IFNγ levels 7 days post challenge and parasitemia using area under the curve (AUC) (*QP13C14, QP13C05 3A, 3B, QP14C11 and QP14C12*). Median + Min and Max, ***=p<0.001, **=p<0.01, *=p<0.05, Wilcoxon matched-pairs signed rank test. **c, f** Linear regression function (goodness of fit).

3.4.9 Type I IFN-dependent IL-10 production in patients with natural *P. falciparum* exposure

To test whether type I IFN induced IL-10 production was limited to our CHMI studies we obtained PBMC samples from *P. falciparum* malaria patients from Timika, Papua, Indonesia (**Table 3, 3.3.3**) and measured IL-10 levels in response to parasite antigen. In these individuals, day 0 refers to the time of admission to the clinic, where parasites were detectable in the blood and day 7 refers to 7 days post-drug treatment. Upon type I IFN signalling blockade, IL-10 levels were significantly reduced 7 days post treatment (**Fig 12**). These data suggests that in individuals who have natural *P. falciparum* infection, type I IFN's were also required for inducing IL-10 production by PBMC's in response to parasite antigen.

Figure 12

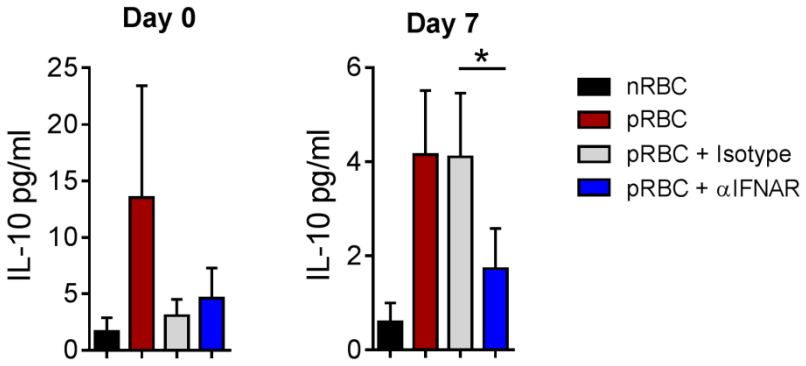


Figure 12: Emergence of immunoregulatory networks is also type I IFN-dependent in patients with natural *P. falciparum* exposure.

Cryopreserved PBMC's from Timika, Indonesia at day 0 (time of admission into the clinic) and day 7 (7 days post drug treatment) were thawed and cultured in the presence of nRBC's, pRBC's or pRBC's + anti-IFNAR antibody or its isotype control for 72 hours and IL-10 levels were detected using the BD Human CBA kit. Mean ±SEM, *=$p<0.05$, Wilcoxon matched-pairs signed rank test.

3.5 DISCUSSION

In this series of CHMI studies, whole blood gene array analysis revealed a dominant type I IFN signature associated with suppressed TCR signalling 7 days post challenge, relative to pre-challenge. Consistent with previous studies, parasite-specific IFNα levels were significantly elevated 7 days post challenge[308, 309]. Reduced T cell receptor signalling was not caused by reduced T cell frequencies, since $CD4^+$ and $CD8^+$ T cell frequencies were comparable between day 0 and day 7, thereby indicating intrinsic changes within these T cell sub-populations. Phenotypic analysis of Th1 and Tfh frequencies revealed a decrease in Th1 frequencies, but significant increase in Tfh frequencies at day 7 post challenge. While an increase in Tfh frequencies was observed at day 7 and 28 post challenge, despite a type I IFN-mediated reduction of IL-6, it is possible that other cytokines such as IL-21 play a compensatory role in the induction/maintenance of Tfh cells, since they both signal via STAT3[82, 317]. The induction of cellular immune responses induced by vaccination plays a key role in the activation of effector $CD4^+$ T cell subsets, including Th1 and Tfh cells[287]. Th1 cells mediate parasite killing, while Tfh cells provide B cell help in the generation of protective antibody responses[283, 284, 318]. Interestingly, Obeng-Adjei *et al*., demonstrated that in children with acute malaria, *Plasmodium* preferentially activates Th1-like $CXCR3^+$ Tfh cells that are unable to provide adequate B cell help[287]. Taken together with our results, this suggests that *Plasmodium* impedes the generation of protective immunity by manipulating the host's cellular and humoral immune responses early on in infection.

While there has been no direct evidence for a protective role for IFNγ during human *Plasmodium* infections, numerous studies have demonstrated associations between IFNγ levels and protection from disease. For example, previous studies have shown that $CD4^+$ T cell derived TNF and IFNγ induced by RTS,S vaccination has been associated with protection in naturally exposed children and experimentally challenged adults[319, 320, 321], suggesting that inflammatory $CD4^+$ T cell responses may contribute to protection from clinical disease[48]. We and others have shown $CD4^+$ T cells are the main source of IFNγ during blood-stage *Plasmodium* infection[48, 55, 56]. In line with previous studies[68], together these data suggest that upon first exposure to *P. falciparum,* type I IFN's suppress anti-parasitic $CD4^+$ T cell responses.

Field studies involving infants and young children, as well as these CHMI studies, provide substantial evidence that upon early or first exposures to *Plasmodium*, immunoregulatory networks emerge in response to the parasite. IL-10 production and Tr1 cell development seem to be induced in order to limit inflammation, but this has the additional effect of impeding the generation of anti-parasitic immune responses. The establishment of immunoregulatory networks in young children seems to increase their susceptibility to developing severe disease in the absence of effective treatment. Additionally, a recent study in western Kenya (holoendemic malaria region), showed that when IFNγ and IL-10 responses were both present in response to the merozoite surface protein 1 ($MSP1_{42}$), the protective effect of IFNγ was abrogated[35]. Taken together with our studies, this suggests that the induction of IL-10 limits the potentially protective effect of IFNγ and thus leads to parasite persistence and disease. On the other hand, a majority of adults living in malaria endemic regions, with repeated exposures to *Plasmodium* have high levels of IFNγ along with TNF, which ultimately serve to control parasite growth, such that these individuals are often asymptomatic. Indeed, while high levels of inflammatory cytokines do result in clinical symptoms, it has been shown in field studies, that over time and over a series of multiple exposures, adults have developed mechanisms to limit inflammation from causing pathology while maintaining low parasitemia levels [reviewed in[32, 258, 322]].

By using PBMC samples collected from Timika, Indonesia, where 125 million people live in malaria-endemic regions[323], we found that IL-10 production was dependent on type I IFN's, post drug treatment. The early emergence of immunoregulatory networks may provide an explanation for the limited efficacy of the RTS,S/AS01 vaccine and highlights the need for targeted therapeutic interventions to improve vaccine efficacy, possibly by manipulating immunoregulatory networks. In addition to this, a more comprehensive understanding of how Tr1 cells are induced in response to *Plasmodium* is required. Mapping the requirements and pathways for Tr1 cell induction during infection will provide mechanistic insights as to how these networks can be modulated when developing a vaccine. IL-27 has been shown to drive the generation of Tr1 cells in both humans and mice[209, 324, 325, 326]. Recently, Tyrosine kinase 2 (Tyk2) was reported to modulate IL-27 gene expression and release via a type I IFN dependent mechanism[327]. Tyk2-deficient mice exhibited significantly reduced IL-27 levels in the plasma following endotoxic shock or polymicrobial sepsis induced by cecal ligation puncture (CLP)[327]. While Tyk2 did not appear to be required to mediate the effects of IL-27 on target gene expression in $CD4^+$ T cells, the authors suggest that the association of Tyk2 with gp130 may not be required in $CD4^+$ T cells for IL-27 signalling[327]. Nonetheless, it would be interesting to investigate pathways involving type I IFN's and IL-27 and how they interact to induce Tr1 cells.

While vaccines currently in clinical trials have demonstrated excellent efficacy rates in healthy volunteers, it is unclear as to why this efficacy decreases dramatically, when tested in malaria endemic areas. Factors contributing to this reduced efficacy rate in malaria patients have been attributed to, transmission rates, degree of exposure to *Plasmodium* species, underlying medical conditions and age. A model of anti-malaria immunity proposed by Artavanis-Tsakonas *et al*., suggests that in infants from malaria endemic areas, low TNF and IFNγ levels are induced via an innate pathway and antigen-specific T cells are primed at the same time[322]. Upon re-infection, antigen-specific T cells expand and produce large amounts of IFNγ which synergises with TNF to control parasite growth, however, this inflammatory cascade increases their risk to developing severe malaria[322]. Further infections induce effective anti-parasitic immunity resulting in reduced parasite load and therefore the concomitant level of antigenic stimulation dampens the pro-inflammatory cascade[322]. Similar to recent field studies involving children in a high transmission setting, our findings show that in healthy adults from non-endemic areas with no previous history or exposure to malaria, anti-parasitic immune responses were suppressed and instead parasite-specific IL-10 production and Tr1 cells were induced[47, 48, 232, 235]. Boyle *et al*., suggest that in a low transmission setting, both children and adults predominantly produce IFNγ and TNF[48]. Contrastingly, in a high transmission setting, children have higher frequencies of parasite-specific $CD4^+$ T cells producing IL-10, whereas the $CD4^+$ T cells in adults produce IFNγ and TNF[48]. Furthermore, in highly exposed children, the dominant $CD4^+$ T cell response includes Tr1 cells[47, 232]. These data, along with the dramatic reduction of vaccine efficacy in field trials, strongly suggest that *Plasmodium* preferentially induces the immunoregulatory pathways to suppress the development of anti-parasitic immunity.

Collectively, these recent findings and the data described in this Chapter may provide insights into the limited efficacy of the current RTS, S/AS01 vaccine. We show that in healthy adult volunteers, upon first exposure to *P. falciparum*, type I IFN's promote the establishment of immunoregulatory networks. Given that these immunoregulatory networks also appear to be induced in young children living in malaria endemic areas who have not yet acquired an adequate level of immunity to malaria[47, 232, 235, 276], this suggests that first and/or early exposures to *Plasmodium* induces immunoregulatory responses rather than anti-parasitic responses . While these immunoregulatory networks may provide protection against pathology, they may also impede the generation of anti-parasitic immune responses. However, in order for this to be empirically demonstrated, participants from these CHMI studies would have to be re-infected with *P. falciparum* and their immune responses measured to determine if Tr1 cells still emerge after a second exposure to *P. falciparum*.

To understand how these immunoregulatory networks are established and regulated, the next Chapter investigates the requirements for the generation of Tr1 cells during parasitic infections including *Plasmodium,* but also examines whether Tr1 cells are induced in other parasitic diseases such as *Leishmania,* thereby probing the broader significance of these findings.

Chapter 4

Blimp1 modulates $CD4^+$ T cell responses during *Plasmodium* and *Leishmania* infections

4.1 INTRODUCTION

Polyclonal $CD4^+$ T cell responses against *Plasmodium* and *Leishmania* infections have provided valuable insights into host-parasite interactions, however, the study of antigen-specific $CD4^+$ T cell responses, particularly in infectious diseases, provide a more accurate insight into host-parasite interplay. From a vaccine development perspective, understanding the emergence and subsequent magnitude of antigen-specific $CD4^+$ T cell responses is imperative for the development of effective vaccines. The integrins CD11a and CD49d have been reported to aid the phenotypic identification of cells which have recently encountered antigen[328, 329]. CD11a is expressed on all leukocytes and is an important adhesion molecule involved in lymphocyte and granulocyte function[330, 331], while CD49d is directly involved in mononuclear leukocyte trafficking and is expressed by T cells, B cells, eosinophils and basophils[332, 333]. The utility of using CD11a and CD49d to track antigen-specific T cell responses has been confirmed in *Plasmodium*[328, 334], *Leishmania*[335], LCMV[329] and *Listeria* infections[336]. CD11a and CD49d expression requires the presence of cognate antigen and is not altered via TLR stimulation or an inflammatory environment[329], and therefore has been widely used to identify antigen-specific T cell responses.

Plasmodium and *Leishmania* infections elicit potent Th1 responses that promote parasite clearance via the production of IFNγ which activates macrophages[156, 337, 338]. An emerging paradigm is that in the presence of persisting pathogens, Th1 cells acquire the capacity to become immunoregulatory by transitioning into IL-10-producing Th1 cells, now commonly known as Tr1 cells. This transition involves the emergence of master transcription factors that determine cell fate. While various master transcription factors have been identified for the development of Th1, Th2, Th17, Tfh and Treg cells, as well as the environmental conditions and extrinsic factors that further promote their differentiation, other transcriptional regulators have been shown to play important roles in fine-tuning this process. For example, Th1 cell development requires Tbet, while Blimp1, c-maf, Ahr and Egr2 are all thought to play key roles in the development of Tr1 cells[206, 209, 216, 217]. Blimp1 has been shown to play key roles in T cell function, differentiation and homeostasis[339, 340]. In T cells, *prdm1* mRNA encoding the Blimp1 protein is induced upon antigen-dependent activation of naïve $CD4^+$ and $CD8^+$ T cells via IL-2-, activator protein (AP)-1, nuclear factor kappa (NFκ)B and IRF4-dependent signals[339, 340].

Several studies have demonstrated a strong association between Blimp1 expression and IL-10 production, where in conventional $CD4^+$ T cells Blimp1 induces IL-10 production[215, 216, 217, 251] whereas Blimp1 and IRF4 were both required for inducing IL-10 production by $CD4^+$ $Foxp3^+$ Treg cells[181]. This suggests that while Th cells have unique differentiation pathways, the mechanisms of IL-10 induction for each subset has been integrated within their differentiation program[201, 341]. The effects of IL-10 induction are of particular interest, given its immunoregulatory role during *Plasmodium* infections, suppressive role in *Leishmania* infections and how this influences disease outcome. In an effort to modulate infection-induced inflammatory responses, IL-10 is produced, but there is a delicate balance between the tissue protective role of IL-10 and the unwanted suppression of anti-parasitic immunity[37, 280]. In addition to IFNγ and IL-10 expression, the surface markers, CD49b and LAG3 have been reported to potentially aid in the phenotypic identification of Tr1 cells[200]. CD49b is an integrin which mediates adhesion and lymphocyte activation, and is expressed by NK cells, a subset of splenic $CD4^+$ T cells, NKT cells, intestinal intraepithelial and lamina propria lymphocytes, epithelial cells and platelets[200]. LAG3 is a CD4 homolog, binds with high affinity to MHCII and is up-regulated in T cells that have recently been activated, as well as exhausted T cells, in comparison to expression on effector or memory T cells[342]. LAG3 is not expressed by resting T cells[343]. Furthermore, a recent study reported an increase in the frequency of $CD49b^+$ $LAG3^+$ Tr1 cells in HIV patients; however, it was unclear as to whether elevated IL-10 levels had a beneficial or detrimental impact on disease progression[344]. The development of Tr1 cells during *Plasmodium*[47, 75, 235], *Leishmania*[214, 234, 345], LCMV[215] and *Toxoplasma*[216] infections provides an insight into how these pathogens modulate IL-10 to aid their survival by causing immunosuppression of the host. Further study into the effects of Blimp1 induced IL-10 production and Tr1 cells will provide a greater understanding of the suppression of anti-parasitic $CD4^+$ T cell responses, which consequently leads to the establishment of parasite persistence and pathology.

This chapter examines the roles for Blimp1 in the development of $CD4^+$ T cell responses during *Plasmodium* and *Leishmania* infections, with a particular focus on Th1 and Tr1 cells.

4.2 MATERIALS AND METHODS

The material and methods presented here are specific to this chapter

4.2.1 Mice

Female C57BL/6J mice 8-12 weeks old were purchased from either the Australian Resource Centre (Canning Vale, Perth, WA, Australia) or the Walter and Eliza Hall Institute (WEHI) (Parkville, Melbourne, VIC, Australia) and were maintained in the animal house facility at the QIMR Berghofer Medical Research Institute under conventional conditions. T cell specific Blimp1 deficient mice: C57BL/6J.Lck-Cre^{+} x *Prdm1*$^{fl/fl}$ = ***Prdm1***$^{\boldsymbol{\Delta T}}$ (Cre positive), Lck-Cre^{-} x *Prdm1*$^{fl/fl}$ = *Prdm1*$^{fl/fl}$ (Cre negative) and ***prdm1***$^{\boldsymbol{gfp}}$ (GFP reporter) mice were bred in-house under specific-pathogen free conditions. All animal procedures were approved and monitored by the QIMR Berghofer Animal Ethics Committee. This work was conducted under QIMR Berghofer animal ethics approval numbers: A02-633M and A02-634M, in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian National Health and Medical Research Council).

4.2.2 Parasites and infection

4.2.2.1 *Plasmodium* infections

PcAS and PbA strains were used in all experiments after one *in vivo* passage in a C57BL/6J mouse. Please see **Chapter 2, 2.3.1** and **2.3.2** for preparation of inoculums. All mice received a standard dose of $1x10^5$ pRBC's in RPMI/PS i.v. via the lateral tail vein. Thin blood smears from tail bleeds were stained with Clini Pure- stains (HD Scientific Supplies, Willawong, QLD, Australia). Parasitemia was used to monitor the course of infection and was determined by flow cytometry.

4.2.2.2 *Leishmania* infections

L. donovani parasites were maintained by passage in B6.RAG1$^{-/-}$ mice and amastigotes were isolated from the spleens of chronically infected mice. Mice were infected with $2x10^7$ *L. donovani* amastigotes in RPMI/PS i.v. via the lateral tail vein. Spleen and liver impression smears were used to determine parasite burdens and were expressed as Leishman Donovan Units (LDU; number of amastigotes per 1000 host nuclei multiplied by the organ weight (in grams))

4.2.3 Monitoring parasitemia (during *Plasmodium* infections) by flow cytometry

Briefly, 1-2 drops of blood, from a tail bleed was diluted and mixed in 250 µl RPMI/PS containing 5 U/ml heparin sulphate. Diluted blood was stained simultaneously with Syto84 (5 µM; Life Technologies) to detect RNA/DNA and Hoechst33342 (10 µg/ml; Sigma-Aldrich) to detect DNA for 30 minutes at room temperature, protected from light. 2 ml RPMI/PS was added to stop the reaction, and samples were immediately placed on ice until acquisition on a BD FACSCanto II Analyzer (BD Biosciences). Data was analysed using FlowJo software (Treestar), where pRBC were readily detected as being Hoechst33342$^+$ Syto84$^+$, with lymphocytes excluded on the basis of size, granularity, and higher levels of Hoechst33342/Syto84 staining compared with pRBC's (**Fig 1a,b**)

Figure 1

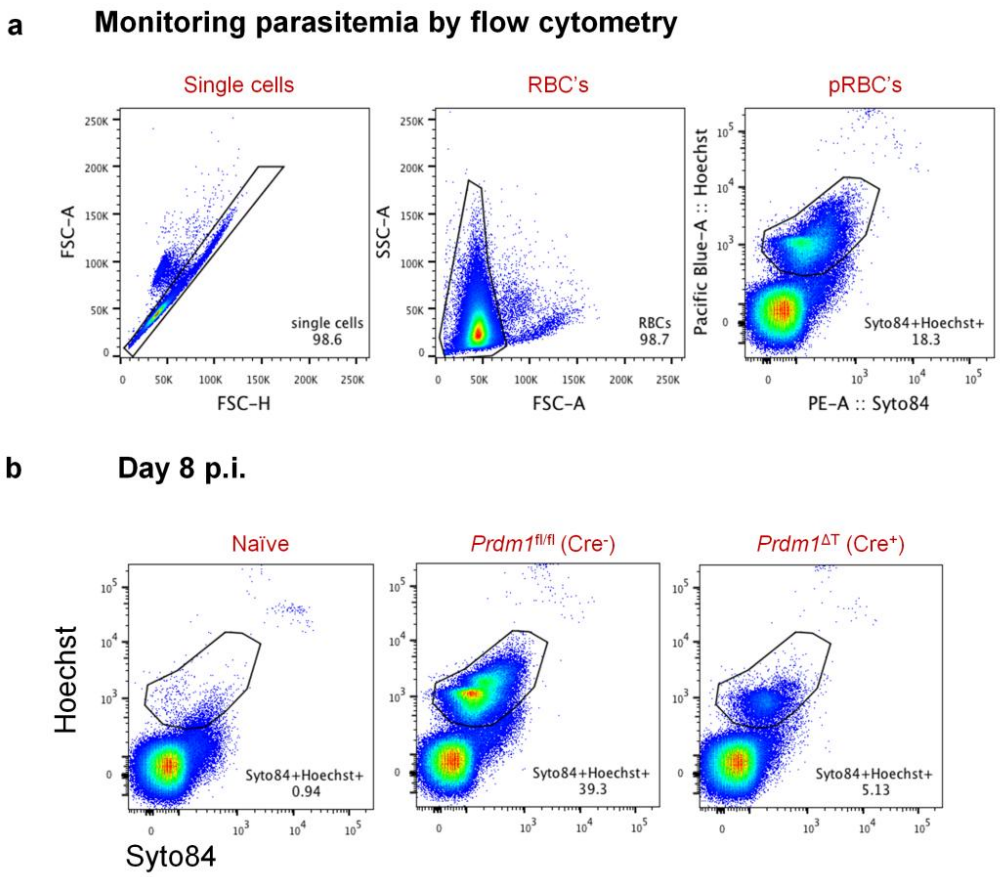


Figure 1: Monitoring peripheral parasitemia during PcAS infection.

a) C57BL/6J mice infected with $1x10^5$ PcAS pRBC's i.v. and parasitemia (1-2 drops of blood collected from the tail) was monitored by flow cytometry by gating on (top, left to right) **1:** single cells, **2:** RBC's and **3:** $Syto84^+$ $Hoechst^+$ pRBC's to identify parasite nucleic acid/DNA within RBC's. **b)** Representative Syto84/Hoechst staining profiles in naïve mice and *Prdm1*$^{fl/fl}$ (WT equivalent) and *Prdm1*$^{\Delta T}$ PcAS-infected mice 8 days p.i. when parasitemia peaks in the WT controls. Representative of 5 independent experiments, n = 5 mice per group.

4.2.4 Monitoring PbA infection and clinical scoring of ECM symptoms

A transgenic PbA line (clone 231c11) expressing luciferase (PbA-luc) and GFP under the control of the ef1-α promoter was used for all PbA experiments unless otherwise stated. From day 5 post-infection with PbA, mice are monitored twice daily to assess their level of infection/illness. When mice showed signs of experimental cerebral malaria (ECM), monitoring was increased to 2-3hr intervals. Monitoring mice involved assigning them a clinical score at each time point.

Mice were scored subjectively according to the following criteria:

1 Ruffling of fur

2 Hunched, still some mobility

3 Wobbly gait

4 Limb paralysis

5 Convulsions, coma, death

Each of the above criteria received a score of 1, and these symptoms usually presented in the order described above. A cumulative score of 4 and above required that mice were sacrificed. The next time point for sacrificed mice was a score of 5.

4.2.5 *in vivo* bioluminescence imaging

The *in vivo* imaging system 100 (Xenogen, Alameda, CA, USA) was used to detect the level of bioluminescence as a measure of whole body parasite burden in each mouse. At selected time-points, PbAluc-infected mice were anaesthetised with isofluorane and injected with 150mg/kg i.p. of D-luciferin (Xenogen) 5 minutes prior to imaging. Bioluminescence was measured in p/s/cm²/sr using Living Image Software (Xenogen).

4.2.6 Flow cytometry

List of antibodies used summarised in **Table 1**. Cell populations of interest were defined as follows:

- pRBC's: $Syto84^+$ $Hoechst^+$
- Activated $CD4^+$ T cells: $CD11a^+$ $CD49d^+$ $CD4^+$ $TCR\beta^+$
- Activated Th1 cells: $CD11a^+$ $CD49d^+$ $CD4^+$ $TCR\beta^+$ $Tbet^+$ $IFN\gamma^+$
- Activated Tr1 cells: $CD11a^+$ $CD49d^+$ $CD4^+$ $TCR\beta^+$ $IL\text{-}10^+$ $IFN\gamma^+$
- Treg cells: $CD4^+$ $TCR\beta^+$ $Foxp3^+$ $IL\text{-}10^+$
- Plasma cells: $B220^+$ $CD19^+$ $CD138^+$ Blimp1 $(GFP)^+$

Table 1: Mouse monoclonal antibodies used for flow cytometry

Specificity	Clone	Fluorophore	Dilution	Supplier
B220	RA3-6B2	APC	1 in 200	Biolegend
CD4	GK1.5	PerCPCy.5.5	1 in 200	Biolegend
CD4	GK1.5	FITC	1 in 200	Biolegend
CD8α	53-6.7	Alexa Fluor 700	1 in 200	Biolegend
CD19	6D5	Biotin	1 in 200	Biolegend
CD19	6D5	PerCP/Cy5.5	1 in 100	Biolegend
CD11a	M17/4	FITC	1 in 50	Biolegend
CD49b	DX5	APC	1 in 100	Biolegend
CD49d	R1-2	Biotin	1 in 50	Biolegend
CD138	281-2	PE	1 in 100	Biolegend
Foxp3	MF-14	APC or AF488	1 in 100	Biolegend
Hoechst 33342*		Pacific Blue	1 in 500	Sigma-Aldrich
IFNγ	XMG1.2	APC	1 in 200	BD
IFNγ	XMG1.2	BV421	1 in 200	Biolegend
IFNγ	XMG1.2	PE	1 in 200	Biolegend
IL-10	JES5-16E3	PE	1 in 200	Biolegend
LAG3	C9B7W	Biotin	1 in 200	Biolegend
Live/Dead		Aqua (Amcyan)	1 in 200	Invitrogen
Streptavidin		PE	1 in 100	Biolegend
Streptavidin		AF700	1 in 100	Biolegend
Streptavidin		PeCy7	1 in 200/ 1 in 400	Biolegend
Syto84*		PE	1 in 500	Life Technologies
Tbet	Ebio4B10	efluor 660 (APC)	1 in 10	Ebioscience
Tbet Isotype control (mouse IgG1κ)	P3.6.2.8.1	efluor 660 (APC)	1 in 10	Ebioscience
TCRβ	H57-597	APC	1 in 200	Biolegend
TCRβ	H57-597	BV421	1 in 200	Biolegend
TCRβ	H57-597	PerCP/Cy5.5	1 in 200	Biolegend
TNF	MP6-XT22	PE	1 in 400	Biolegend
TNF	MP6-XT22	PeCy7	1 in 400	Biolegend

*RNA/DNA dyes for detecting pRBC's in blood.

4.2.8 Antibodies for *in vivo* neutralisation of IL-12

For IL-12 neutralisation experiments, mice were administered 500µg of rat IgG (Sigma or Invitrogen) or anti-IL-12 (clone: C17.8, BioXcell, West Lebanon, NH, USA) i.p on the day of infection and every 3 days p.i. until day 14 p.i. (see **Fig 11a**)

4.2.9 Human VL patient samples

All patients in this study presented with symptoms of VL at the Kala-azar Medical Research Center (Muzaffarpur, Bihar, India). Diagnosis of VL was confirmed either by the microscopic detection of amastigotes in splenic aspirate smears or by the rk39 dipstick test. In total, 10 patients were enrolled in the study with their prior consent and ethical clearance from the Institutional Ethical Committee of Banaras Hindu University (Varanasi, Uttar Pradesh, India). Blood was collected from patients before and 28 days after commencement of drug treatment, and PBMC were isolated by Ficoll-Hypaque (GE Healthcare, NJ, USA) gradient centrifugation and used for the positive selection of $CD4^+$ T cells using magnetic beads and columns (Miltenyi Biotech, Bergisch Gladbach, Germany). After MACS purification, cells were transferred directly into RNAlater® (Sigma-Aldrich), and stored at -70°C until RNA was extracted. Total RNA was isolated using RNeasy mini kits and QiaShredder homogenizers (Qiagen, Valencia, CA), as per manufacturers protocol. The quality of RNA was assessed by denaturing agarose gel electrophoresis. cDNA synthesis was performed in 20 µL reactions on 0.5–1.0 µg RNA using High-Capacity cDNA Archive kit (Applied Biosystems, CA, USA). Real-time PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) using cDNA-specific FAM–MGB labelled primer/probe sets for human *PRDM1*. The relative quantification of products was determined by the number of cycles over 18S mRNA endogenous control required to detect *PRDM1* gene expression. Patient clinical data is shown in **Table 2**.

Table 2: Human VL patient clinical data

Patient Information	Recorded data
Number of individuals per group (n)	10
Gender (M/F)	5 males, 5 females
Age (years)	28.90± 18.19 (24)
Duration of illness (days)	29.75 ± 26.41 (22)
WBC counts (pre treatment)	4160 ± 1257 (3700)
WBC counts (post treatment)	9440 ± 2777 (10000)
Spleen size (cm), (pre treatment)	2.6 ± 1.5 (3)
Spleen size (cm), (post treatment)	0.1 ± 0.31 (0)
Values are given as mean + SD Median values are given within brackets. Post treatment values are 28 days post treatment.	

4.2.10 Statistical Analysis

Statistical differences between groups were determined using the Mann-Whitney U test or the Log -rank (Matel-Cox) test (survival) for mouse studies and the Wilcoxon matched-pairs signed rank test was used in the human studies by GraphPad Prism version 6 for Windows (GraphPad, San Diego, CA); $p<0.05$ was considered statistically significant. All data are presented as the mean ± SEM, unless otherwise stated.

4.3 RESULTS

4.3.1 Characterisation of CD4$^+$ T cell responses during *Plasmodium* infections

C57BL/6J mice infected with PcAS develop mild, uncomplicated malaria where peak parasitemia occurs 7-8 days p.i., and then resolves by day 12-13 p.i.. After this time, mice establish immunity to re-infection[346]. Common symptoms observed throughout the course of infection include ruffled fur and hunching, whereas anaemia and haemoglobinuria are observed at peak parasitemia or as parasitemia begins to resolve[347]. During blood-stage *Plasmodium* infections, CD4$^+$ T cells provide help for B cell responses, as well as producing IFNγ which is critical for parasite control[45, 54, 76, 348]. CD4$^+$ T cell help is not restricted to the blood-stage of infection, but is also required during the liver stage, where CD4$^+$ T cells help activate parasite-specific CD8$^+$ T cells, thus aiding control of infected hepatocytes[349, 350]. To better understand CD4$^+$ T cell responses during the course of *Plasmodium* infections, we employed the use of the integrins CD11a and CD49d, as reported by Butler *et al*., to monitor activated CD4$^+$ T cell responses[328], with a particular focus on Th1 and Tr1 cells, throughout the acute phase of PcAS infection (**Fig 2a-f**). We noted that the frequency of CD11a$^+$ CD49d$^+$ cells peaked 15 days p.i. in C57BL/6J mice and by day 34 p.i. had declined to almost similar frequencies in naive mice (day 0 p.i.) (**Fig 2a, b**). The frequency of Th1 cells (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ Tbet$^+$) reached a maximum at 7 days p.i. (**Fig 2c, d**), whereas a 2-fold increase was observed in Tr1 cell (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ IL-10$^+$) frequencies from day 7-15p.i., and then stabilised (**Fig 2e, f**). Importantly, we noted that at day 7 p.i., when parasitemia had reached its maximum, Th1 cells all resided within the CD11a$^+$CD49d$^+$ gate, indicating they were highly activated, recently antigen experienced cells (**Fig 2g**). The frequency of splenic CD4$^+$ Foxp3$^+$ Treg cells initially declined between day 5 to day 7 p.i., but then stabilised (**Fig 3a, b).** The relatively small frequency of CD4$^+$ Foxp3$^+$ IL-10$^+$ Tregs increased slightly between days 5-7 p.i., and then steadily declined from day 7 to day 34 p.i. (**Fig 3a, c),** suggesting a limited role for Tregs and Treg-derived IL-10 production during PcAS infection.

Figure 2

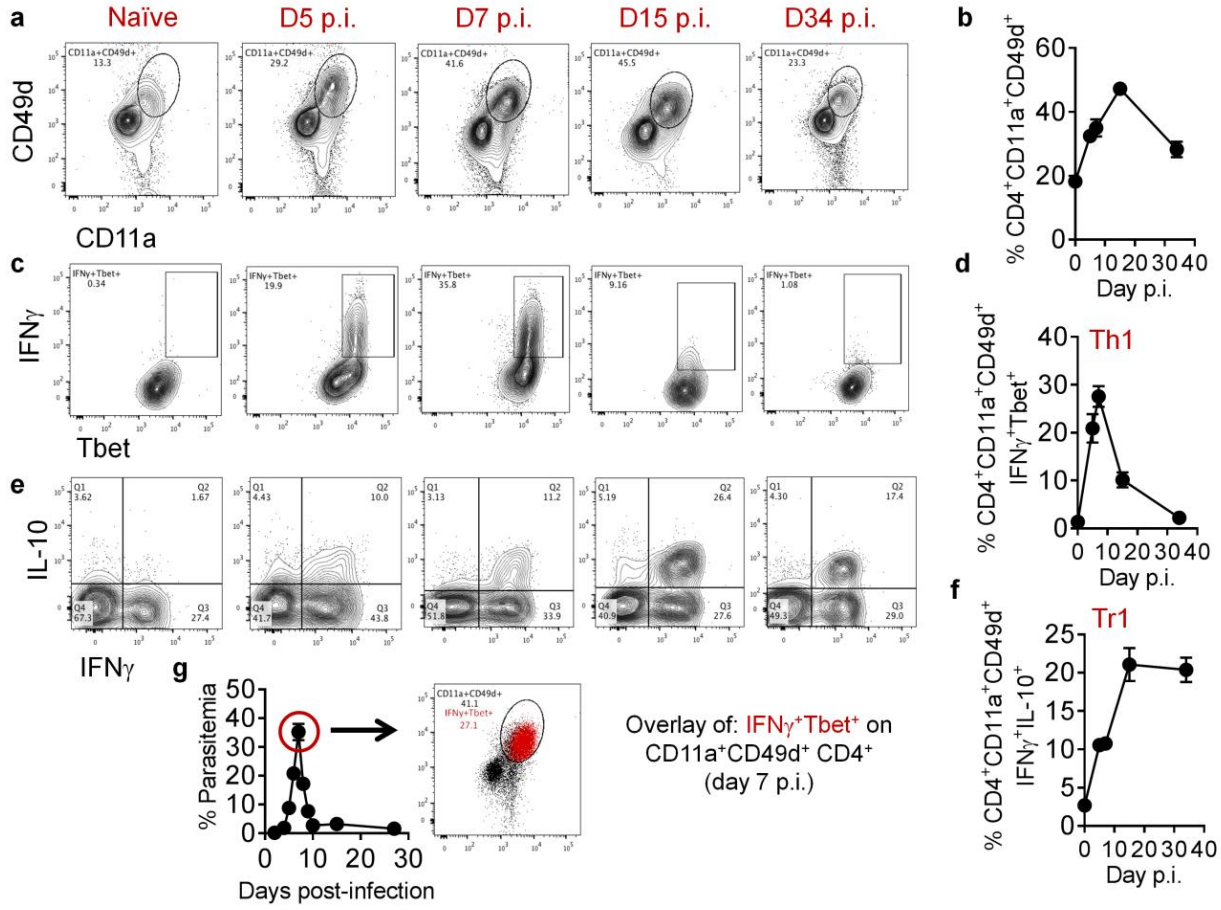


Figure 2: Characterisation of Th1 and Tr1 CD4$^+$ T cell responses during PcAS infection.

a) C57BL/6J mice infected with 1x10^5 PcAS pRBC's i.v. and splenic CD4$^+$ T cells assessed for CD11a and CD49d expression 5, 7, 15 and 34 days p.i. by flow cytometry. **b)** CD11a$^+$ CD49d$^+$ CD4$^+$ T cell frequencies in PcAS-infected mice. **c)** CD11a$^+$ CD49d$^+$ IFNγ$^+$ Tbet$^+$ expression on CD4$^+$ T cells and **d)** frequencies of Th1 cells throughout PcAS infection. **e)** CD11a$^+$ CD49d$^+$ IFNγ$^+$ IL-10$^+$ expression on CD4$^+$ T cells and **f)** frequencies of Tr1 cells throughout PcAS infection. **g)** Overlay of Th1 cells on CD11a$^+$ CD49d$^+$ gate 7 days p.i.. Representative of 2 independent experiments, mean ±SEM, n=5 mice per group.

Figure 3

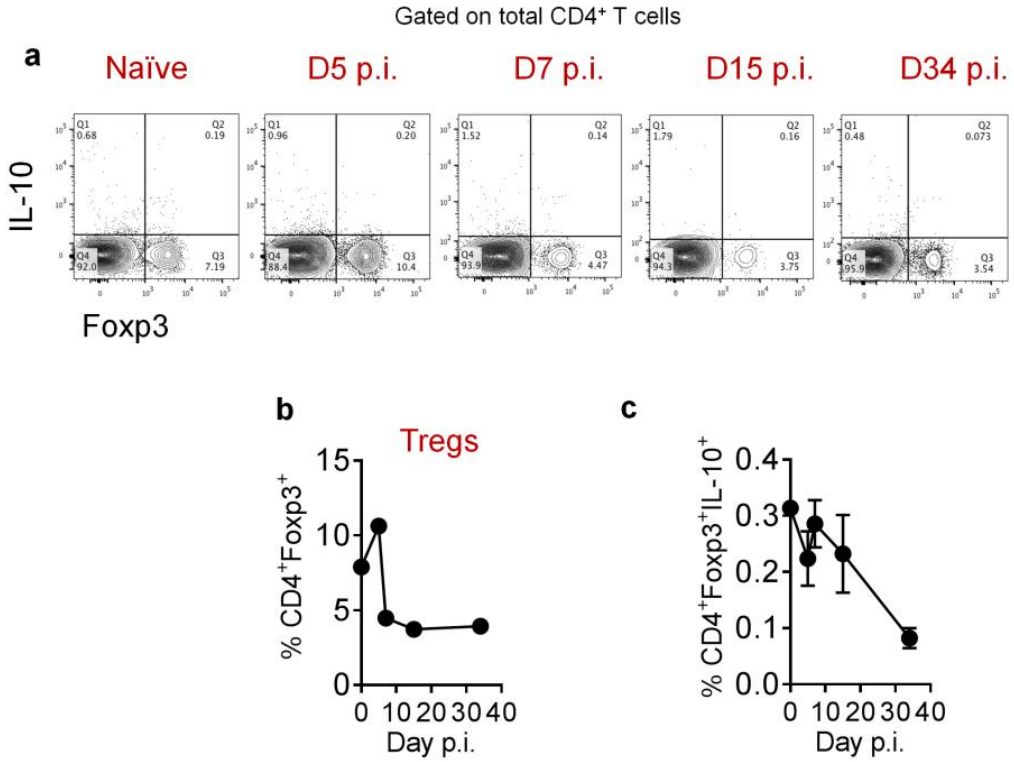


Figure 3: CD4$^+$ Foxp3$^+$ Treg cell kinetics during PcAS infection.

a) C57BL/6J mice infected with 1x10^5 PcAS pRBC's i.v. and splenic CD4$^+$ T cells assessed for IL-10 and Foxp3 expression 5, 7, 15 and 34 days p.i. by flow cytometry. **b)** Total CD4$^+$Foxp3$^+$ Treg cell frequencies in PcAS-infected mice. c) CD4$^+$ Foxp3$^+$ IL-10$^+$ Treg cell frequencies in PcAS-infected mice. Representative of 2 independent experiments, mean ±SEM, n=5 mice per group.

4.3.2 Blimp1 is required for IL-10 production by $CD4^+$ T cells during infection

We next examined IL-10 induction during infection. Recent studies indentified the transcriptional regulator Blimp1 as an inducer of IL-10 production by T cells. To study the kinetics of Blimp1 expression, we used Blimp1-GFP reporter mice, as described by Kallies *et al*., where they genetically targeted the Blimp1 locus to insert an IRES-GFP cassette 3' to exon 6 to produce the *prdm1*^{gfp} allele (**Fig 4a**)[242]. The targeted allele encodes a truncated Blimp1 protein lacking exons 7-8 and GFP from the same mRNA transcript. Thus, the truncated protein provides an effective way of measuring *prdm1* transcription, using GFP as a readout (**Fig 4a, b**)[242]. It is important to note that this truncated protein is transcriptionally inactive and unable to bind to any promoters or regulatory elements. Early studies in B cell differentiation showed a requirement for Blimp1 in plasma cell development, where increased Blimp1 expression was observed as plasmablasts matured into long-lived antibody-secreting cells (ASCs)[242]. Plasma cells ($CD19^+$ $B220^+$ $CD138^+$) constitutively express high levels of Blimp1 and therefore to validate Blimp1 GFP expression in our studies, we first measured Blimp1 GFP levels in these cells (**Fig 4b, c**). Plasma cells exhibited 3-5 fold higher levels of Blimp1 expression compared to T cells during PcAS infection (**Fig 4c**). Consistent with published reports[215, 216, 251], we were able to demonstrate that IL-10 producing $CD4^+$ T cells exhibited the highest level of Blimp1 expression (**Fig 4c**). Importantly, we showed that these IL-10^+ $CD4^+$ T cells express high Blimp1 levels throughout the course of PcAS infection (**Fig 4c**). IFNγ and to a far lesser extent, TNF producing $CD4^+$ T cells also expressed medium to low levels of Blimp1 (**Fig 4c**). This hierarchical expression of Blimp1 in $CD4^+$T cells highlighted the positive association between Blimp1 and IL-10. Consequently, IL-10^+ and IL-10^+ IFNγ^+ $CD4^+$ T cells exhibited the highest Blimp1 expression, relative to $CD4^+$ T cells producing IFNγ or TNF (**Fig 4c, d**).

Figure 4

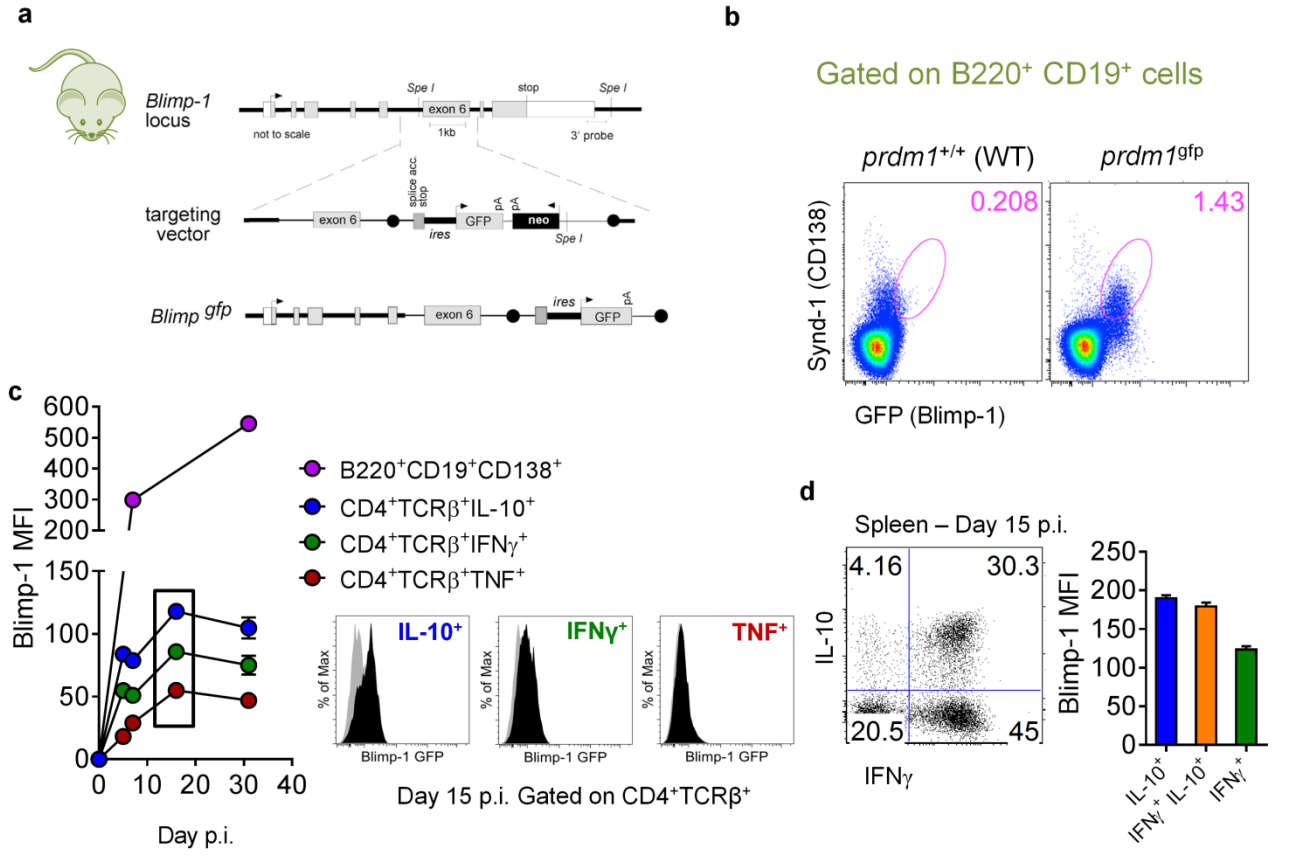


Figure 4: Kinetics of Blimp1 expression in plasma cells and cytokine producing CD4$^+$ T cells during PcAS infection.

a) Generation of *prdm1*^{gfp} reporter mice as reported by Kallies *et al*., 2004, JEM, 200 **8**: 967-977 permission number 3765180709708 **b)** Verification of Blimp1 GFP expression in plasma cells identified as B220$^+$ CD19$^+$ CD138$^+$ Blimp1(GFP)$^+$ by flow cytometry. **c)** *prdm1*^{gfp} and WT mice infected with 1x10^5 PcAS pRBC's i.v. Comparison of Blimp1 expression profiles in plasma cells (purple), IL-10$^+$ (blue), IFNγ$^+$ (green) and TNF$^+$ (red) CD4$^+$ T cells 5, 7, 15 and 34 days p.i.. Grey-filled histogram (WT) and black-filled histogram (*prdm1*^{gfp}) shows Blimp1 expression by cytokine producing CD4$^+$ T cells 15 days p.i.. **d)** Blimp1 Mean Fluorescence Intensity (MFI) in IL-10$^+$, IFNγ$^+$ and IL-10$^+$ IFNγ$^+$ CD4$^+$ T cells in the spleen 15 days p.i.. Representative of 3 independent experiments, mean ±SEM, n=3-5 mice per group.

4.3.3 Blimp1 regulates parasite growth and effector CD4$^+$ T cell responses during *Plasmodium* infections

The positive association between Blimp1 and IL-10 and the negative association between Blimp1 and TNF, suggested a potential role for Blimp1 in regulating inflammation, since TNF promotes inflammation while IL-10 limits this process. Therefore, since Blimp1 positively associated with IL-10, we hypothesised that Blimp1 dependent IL-10 played a key role in inflammation during infection. T cell specific, Blimp1-deficient (*Prdm1*$^{\Delta T}$) mice spontaneously develop inflammatory disease characterised by the infiltration of activated T cells into tissues as a result of significant alterations in T cell homeostasis[339, 340]. This highlights the contribution of Blimp1 to the regulation of tissue pathology, primarily driven by inflammation. Given that both *Plasmodium* and *Leishmania* elicit potent inflammatory responses we next assessed the contribution of Blimp1 mediated regulation of CD4$^+$ T cells on disease outcome. *Prdm1*$^{\Delta T}$ mice, infected with PcAS exhibited significantly reduced parasitemia levels throughout the course of infection, compared with *Prdm1*$^{fl/fl}$ litter mate control mice (**Fig 5a**), implicating a role for Blimp1 in parasite control. To assess splenic CD4$^+$ T cells with an activated phenotype, we used CD11a and CD49d expression to detect CD4$^+$ T cells that had recently encountered antigen[328]. The gating strategy for Th1 and Tr1 cells for this Chapter and Chapter 5 is shown in **Fig 6**. Blimp1 has been shown to play a fundamental role in T cell differentiation and homeostasis and consistent with these reports[339, 340], we observed a dramatic increase in the frequency of activated CD4$^+$ T cells (**Fig 5b, c**) and Th1 cells (**Fig 5 b, d**), but a severe impairment in the generation of Tr1 cells in the *Prdm1*$^{\Delta T}$ mice 15 days after PcAS infection (**Fig 5 b, e**). Despite the increased inflammatory responses observed in the *Prdm1*$^{\Delta T}$ mice, they showed very little if any signs of symptoms such as anaemia and haemoglobinuria, compared to the *Prdm1*$^{fl/fl}$ litter mate control mice which did display these symptoms. Since the deletion of Blimp1 using Cre under the control of the Lck promoter with the Cre-loxP system will also impact on CD8$^+$ T cells due to the CD4$^+$CD8$^+$ double positive stage in thymic development, we also measured IFNγ and IL-10 production by CD8$^+$ T cells during PcAS infection. CD8$^+$ T cells displayed significantly increased Tbet and IFNγ expression (**Fig 7a**) and an impaired ability to produce IFNγ and IL-10 simultaneously (**Fig 7b**). While these results trended in a similar manner to CD4$^+$ T cells, it should be noted that the frequencies of Tbet$^+$ IFNγ$^+$ and IL-10$^+$ IFNγ$^+$ CD8$^+$ T cells were 2-3 fold less, compared to CD4$^+$ T cells. These results confirmed the requirements for Blimp1 in IL-10 production by T cells[215, 216]. However, we found no such dependence on Blimp1-dependent IL-10 production by Treg cells during PcAS infection (**Fig 7c**).

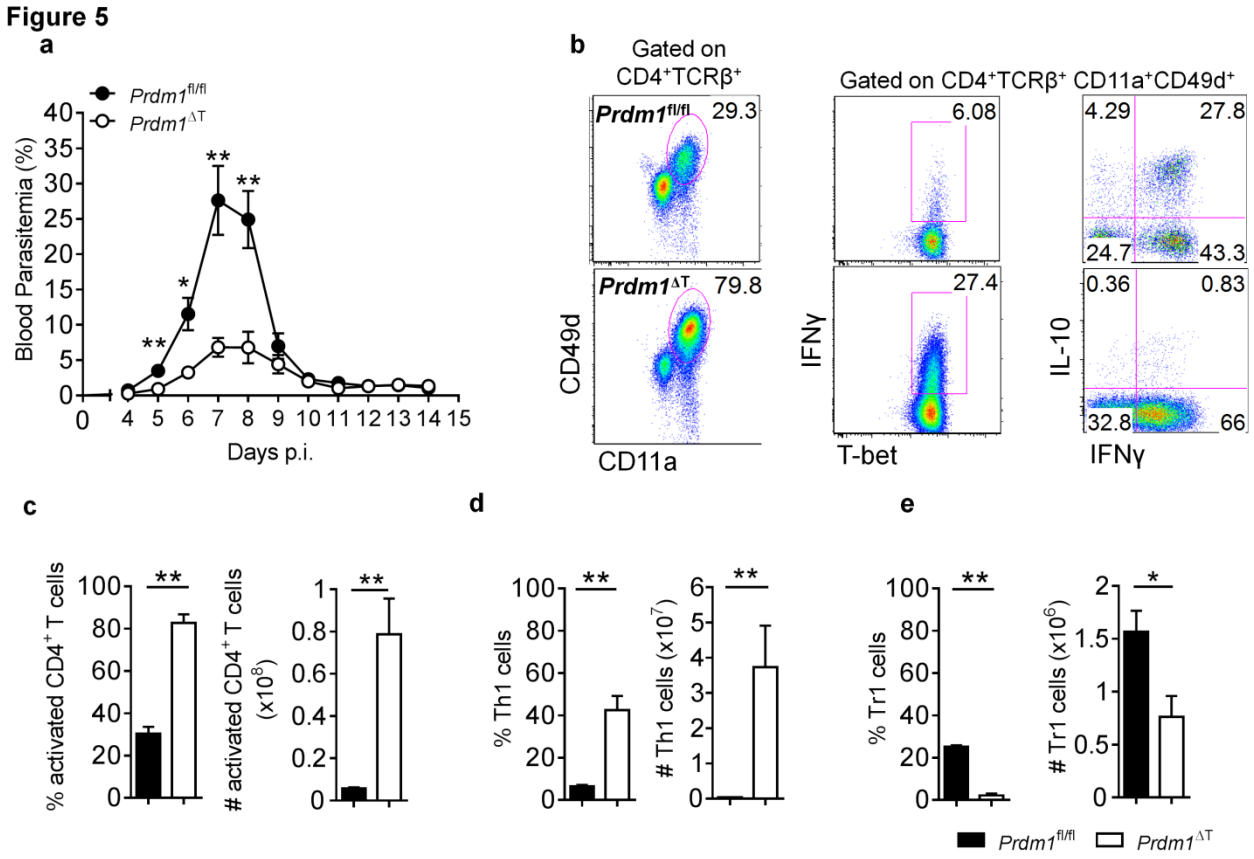


Figure 5: Blimp1 influences parasite control and is required for CD4$^+$ T cell-derived IL-10 production during PcAS infection.

a) *Prdm1*$^{\Delta T}$ (T cell specific Blimp1 deficient) and *Prdm1*$^{fl/fl}$ (littermate controls) mice were infected with 1x10^5 PcAS pRBC's i.v. and peripheral parasitemia was monitored by flow cytometry beginning at 4 days p.i.. **b)** Splenic CD4$^+$ T cell responses assessed at day 15 p.i.. **c)** Frequencies and numbers of activated (CD11a$^+$ CD49d$^+$) CD4$^+$ T cells were measured by flow cytometry 15 days p.i.. **d)** Frequencies and numbers of Th1 (IFNγ$^+$ Tbet$^+$) CD4$^+$ T cells were measured by flow cytometry 15 days p.i.. **e)** Frequencies and numbers of Tr1 (IFNγ$^+$ IL-10$^+$) CD4$^+$ T cells were measured by flow cytometry 15 days p.i.. Representative of 3 independent experiments, mean ±SEM, n=5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

Figure 6

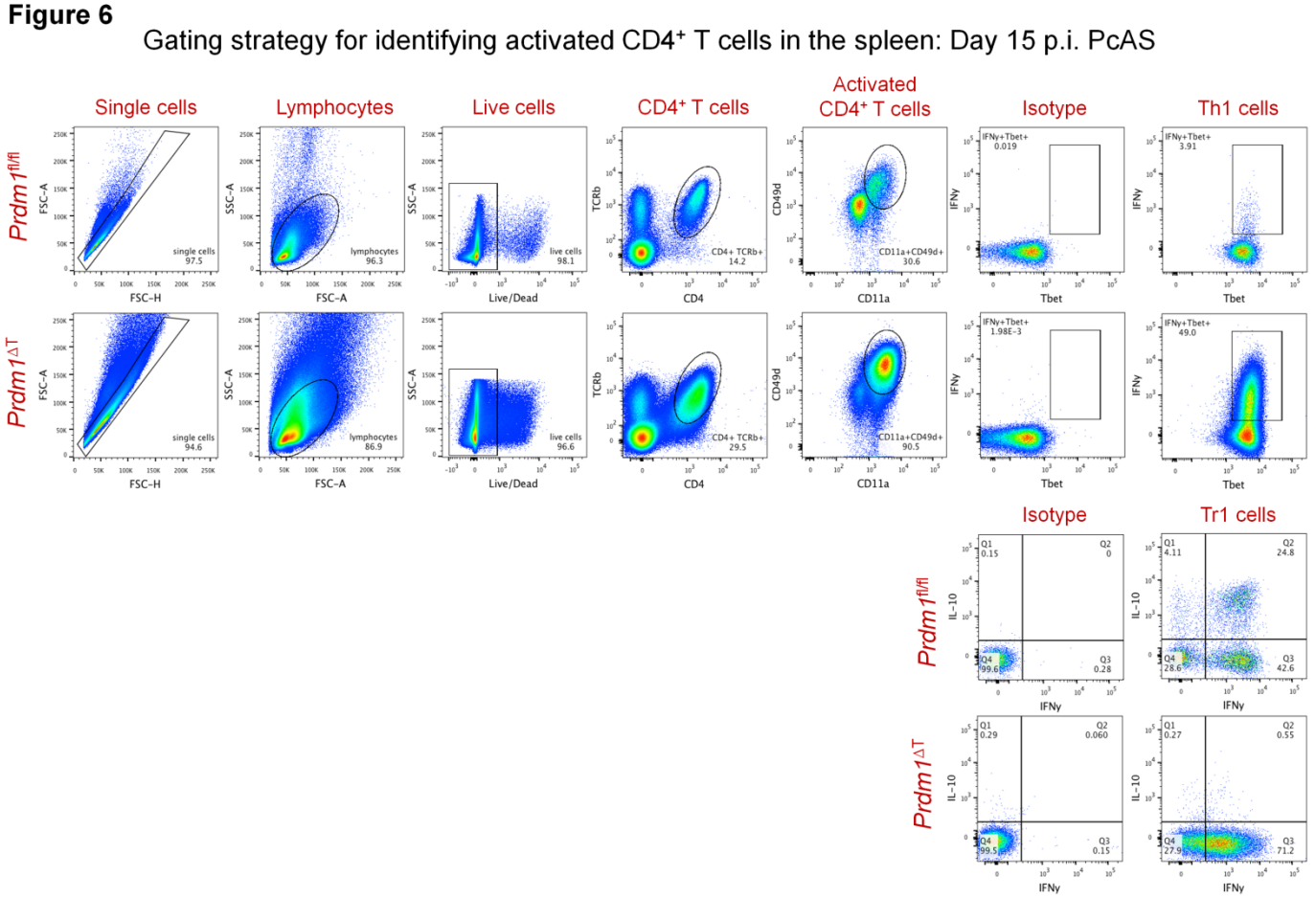


Figure 6: Gating strategy for identifying Th1 and Tr1 cells

Order of gating: left to right, beginning with single cells, lymphocytes, live/viable cells, $CD4^+$ T cells, $CD11a^+$ $CD49d^+$ $CD4^+$ T cells, isotypes for identifying Th1 ($IFN\gamma^+$ $Tbet^+$) and Tr1 ($IFN\gamma^+$ $IL\text{-}10^+$) $CD4^+$ T cells in the spleens of *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice. Representative of 5 independent experiments, n=5 mice per group.

Figure 7

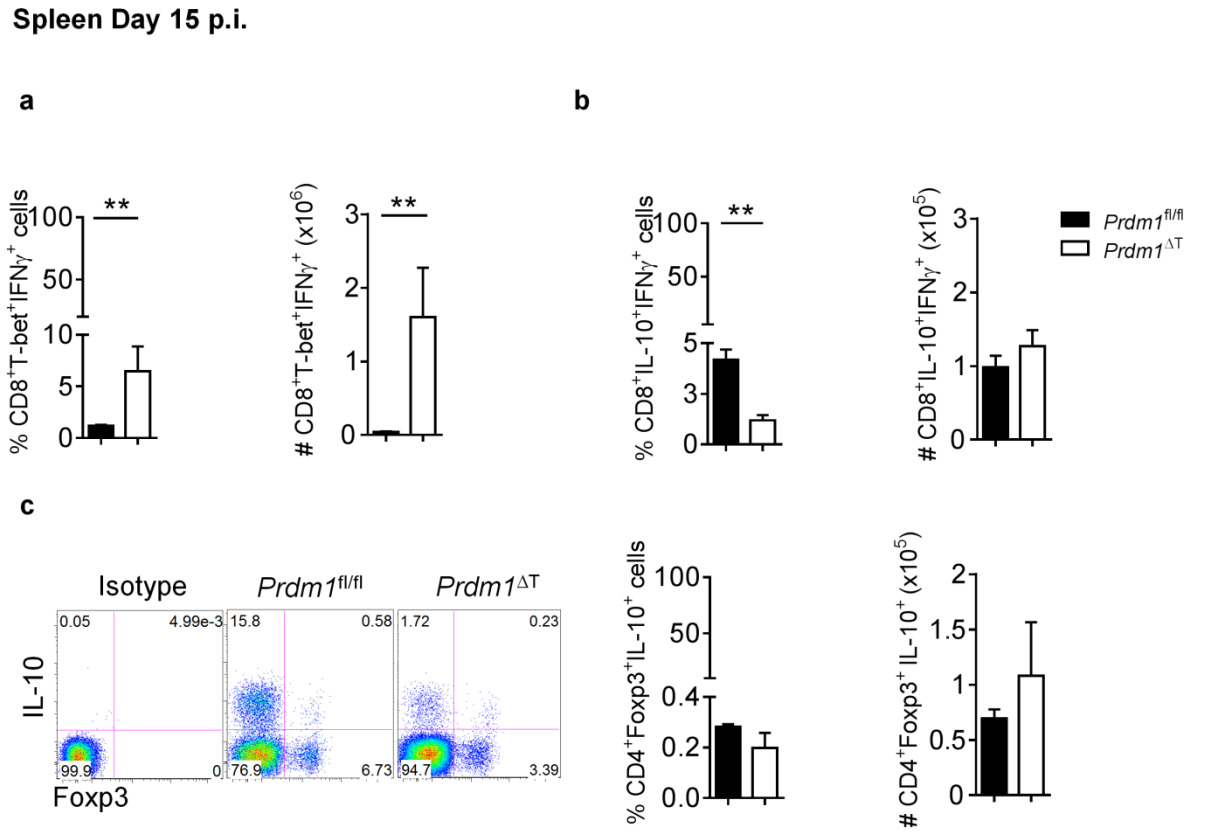


Figure 7: Blimp1 has a minor impact on CD8$^+$ T cells and Foxp3$^+$ IL-10$^+$ CD4$^+$ Treg cells during PcAS infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 1x10^5 PcAS pRBC's i.v. Frequencies and numbers of CD8$^+$ T cells expressing IFNγ and Tbet in the spleen at day 15 p.i.. **b)** Frequencies and numbers of CD8$^+$ T cells expressing IFNγ and IL-10 at day 15 p.i.. **c)** Frequencies and numbers of CD4$^+$ T cells expressing Foxp3 and IL-10 at day 15 p.i.. Representative of 3 independent experiments, mean ±SEM, n=5 mice per group, **=p<0.01, Mann-Whitney U test.

Since PcAS infections do not result in extensive tissue pathology, to assess the potential contribution of the Th1 response to pathology, we employed the use of a severe malaria model caused by infection with PbA. *Prdm1*$^{\Delta T}$ mice infected with PbA exhibited significantly reduced whole body parasite burdens at day 6 p.i. (onset of neurological symptoms in *Prdm1*$^{fl/fl}$ litter mate controls), and reduced blood parasitemia at day 4 p.i. (**Fig 8a**). *Prdm1*$^{\Delta T}$ mice had a small survival advantage compared to *Prdm1*$^{fl/fl}$ litter mate controls (**Fig 8b**), but when monitored for clinical scores, *Prdm1*$^{\Delta T}$ mice all eventually succumbed to the neurological complications associated with PbA infection (**Fig 8c**).

Interestingly, despite no beneficial impact on disease outcome, a significant increase in Th1 frequencies and numbers was observed in the absence of Blimp1 in T cells, and a severe impairment in the Tr1 response was observed (**Fig 8 d, e**).

Figure 8

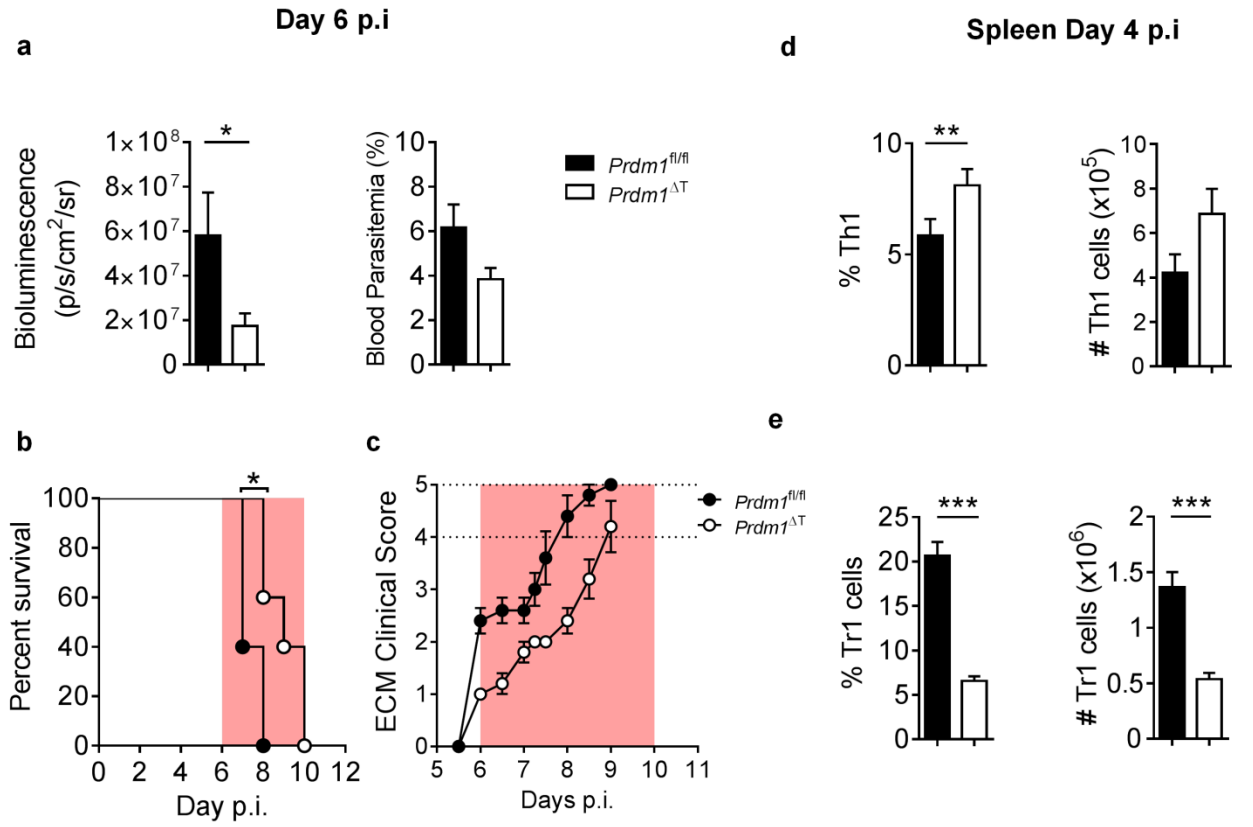


Figure 8: Blimp1 regulates CD4$^+$ T cell responses but does not result in protection against severe symptoms during PbA infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 1x10^5 PbA-luc pRBC's i.v. At day 6 p.i. when *Prdm1*$^{fl/fl}$ littermate mice controls began to exhibit symptoms such as ruffled fur and hunching, all mice were injected with 5mg/kg of D-luciferin to determine parasite biomass using an *in vivo* bioluminescence imaging system (Xenogen IVIS). Peripheral parasitemia was measured by flow cytometry **b)** Survival curves for *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ PbA-infected mice **c)** All mice were assigned a clinical score beginning at 5 days p.i. and a score of 4-5 indicated euthanasia or death, respectively. **d)** Splenic CD4$^+$ T cell responses were assessed 4 days p.i. prior to the onset of symptoms. Frequencies and numbers of Th1 cells were measured by flow cytometry 4 days p.i.. **e)** Frequencies and numbers of Tr1 cells measured by flow cytometry at day 4 p.i.. Representative of 3 independent experiments, mean ±SEM, n=5-9 mice per group, ***=p<0.001, **=p<0.01, *= p<0.05, Mann-Whitney U test.

4.3.4 Blimp1 expression is negatively associated with TNF producing CD4$^+$ T cells

Low Blimp1 expression in TNF producing CD4$^+$ T cells suggested an opposite and negative association between Blimp1 and TNF expression, relative to the relationship between Blimp1 and IL-10 (**Fig 4c**). B6.TNF$^{-/-}$ mice infected with *L. donovani* exhibited 3-fold higher parasite burdens with 100% mortality 11 weeks p.i.[351]. To date, TNF-deficient mice are one of the only genetically modified mouse strains to succumb to VL, suggesting that TNF plays a critical role in the survival of the host against the parasite. TNF has been shown to be required for controlling parasite growth during *Leishmania* infections, but is also a major contributor to splenic pathology[129]. To examine this intriguing association between Blimp1 and TNF more extensively, we infected *prdm1*^{gfp} mice with *L. donovani* and measured Blimp1 expression by CD4$^+$ T cells in the spleen and liver 14 days p.i. (**Fig 9a, b**). A similar Blimp1 expression profile to that found during PcAS infection was observed during *L. donovani* infection, whereby in both liver and spleen, IL-10 producing CD4$^+$ T cells displayed the highest levels of Blimp1 expression (**Fig 9a, b**). However, given that the liver is a site of acute infection and the spleen being a site of chronic infection[352], it was interesting to note that very similar Blimp1 expression profiles were observed in both tissues, which might suggest a requirement for Blimp1 expression in both acute and chronic experimental VL.

Figure 9

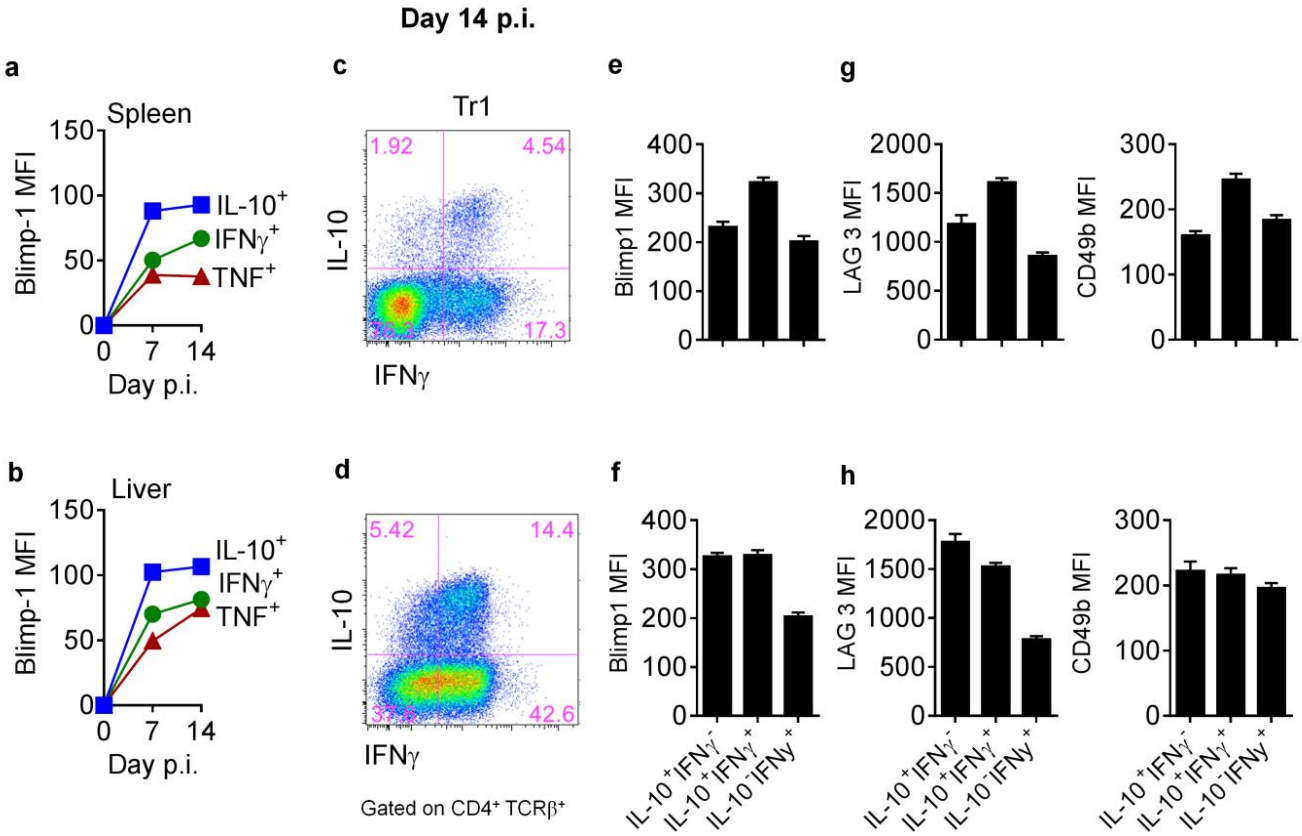


Figure 9: Positive associations between Blimp1, LAG3, CD49b and Tr1 CD4$^+$ T cells during *L. donovani* infection.

a) *prdm1*^{gfp} and WT mice infected with 2x10^7 *L. donovani* amastigotes i.v. Blimp1 GFP expression by IL-10$^+$, IFNγ$^+$ or TNF$^+$ CD4$^+$ T cells measured at day 7 and 14 p.i. in the spleen and **b)** liver by flow cytometry. **c)** Tr1 (IFNγ$^+$ IL-10$^+$) CD4$^+$ T cells were measured at day 14 p.i. in the spleen and **d)** liver. **e)** Blimp1 MFI on IFNγ$^-$ IL-10$^+$, IFNγ$^+$ IL-10$^+$ (Tr1) and IFNγ$^+$ IL-10$^-$ CD4$^+$ T cells was measured in both spleen and **f)** liver, 14 days p.i.. **g)** LAG3 and CD49b MFI on IFNγ$^-$ IL-10$^+$, IFNγ$^+$ IL-10$^+$ (Tr1) and IFNγ$^+$ IL-10$^-$ CD4$^+$ T cells were also measured by flow cytometry in both the spleen and **h)** liver at day 14 p.i.. Representative of 3 independent experiments, mean ±SEM, n=5 mice per group.

4.3.5 Tr1 preferentially cells express CD49b and LAG3 in the spleen during *L. donovani* infection

Recent reports have shown IL-10$^+$ IFNγ$^+$ Tr1 cells to express high amounts of Blimp1[251] and this was notably observed in the spleen, 14 days p.i. with *L. donovani* (**Fig 9c, e**). In the liver, however, comparable levels of Blimp1 expression was found between IL-10$^+$ single producing CD4$^+$ T cells and Tr1 cells (**Fig 9d, f**), suggesting that Blimp1 is not preferentially expressed solely by Tr1 cells and may potentially be expressed by other IL-10 producing T cell populations, such as Tregs. Tr1 cells have been predominantly characterised on the basis of their co-expression of IL-10 and IFNγ[47, 199, 201]. Recent reports, however, have identified surface markers that may aid the phenotypic characterisation of these cells. For example, CD49b and LAG3 co-expression has been reported in mouse and human Tr1 cells[200]. In an effort to identify specific surface markers to detect Tr1 cells, CD49b$^+$ and LAG3$^+$ T cells were tested for IL-10 expression, as previously reported[200]. In the spleen 14 days p.i., Tr1 cells exhibited the highest levels of LAG3 and CD49b, compared to IL-10 and IFNγ single producing CD4$^+$ T cells (**Fig 9g**), which is consistent with published literature[200]. However, in the liver, LAG3 and CD49b expression was comparable between IL-10 single producing CD4$^+$ T cells and Tr1 cells (**Fig 9h**), highlighting potential different tissue requirements for Blimp1, LAG3 and CD49b during *L. donovani* infection. These results support the notion that Blimp1 expression during *Plasmodium* and *Leishmania* infections is required for the generation of Tr1 cells to potentially suppress parasite-induced inflammatory responses.

4.3.6 VL patients have increased *PRDM1* expression in PBMC's and CD4$^+$ T cells

After establishing a hierarchical expression pattern for Blimp1 in two models of parasitic infections, we next determined if there was any clinical relevance for Blimp1 expression in humans with active VL disease. Importantly, we found that *PRDM1* mRNA expression was higher in total PBMC's (**Fig 10a**), as well as CD4$^+$ T cells (**Fig 10b**) in active VL patients, compared to levels found 28 days after drug treatment. It is interesting to note that human VL patients that report to this clinic are all at different stages of disease and despite this, *PRDM1* mRNA levels were elevated in most patients (**Table 1**). While it would have been interesting to investigate the expression levels of other transcription factors, due to limitations associated with human samples, no other transcription factors were analysed in these studies.

Figure 10

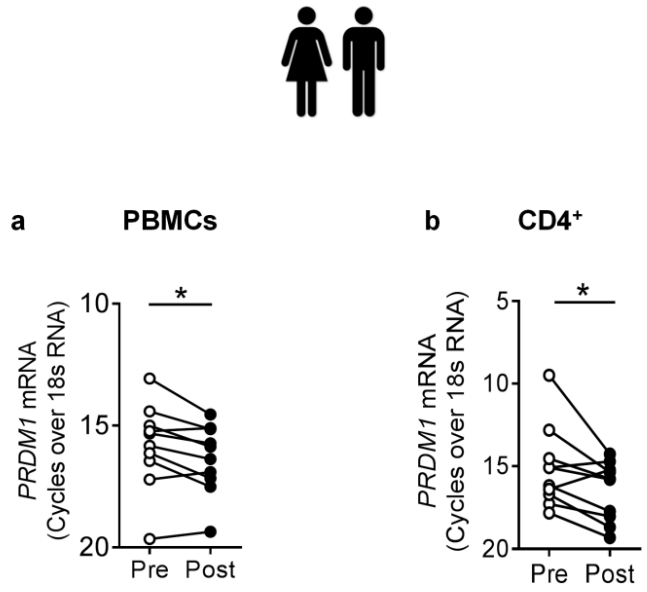


Figure 10: *PRDM1* mRNA is up-regulated in PBMC's and CD4$^+$ T cells isolated from VL patients.

a) Human *PRDM1* mRNA measured by qPCR from PBMC's and **b)** MACS purified CD4$^+$ T cells isolated of patients before and 28 days after drug treatment. n= 10 patients per group as shown by the individual black lines, *= $p<0.05$, Wilcoxon matched-pairs signed rank test.

4.3.7 IL-12 induces Blimp1 expression during *L. donovani* infection

The IL-12 cytokine family plays central roles in the regulation of $CD4^+$ T cell responses, whereby Th1 differentiation is largely driven by Tbet expression, resulting in IFNγ production[156, 157]. The $CD4^+$ T cell differentiation pathway is dependent on IL-12 produced by DC's[156, 353]. *L. donovani* infection induces a potent Th1 response which aids the activation of microbicidal killing mechanisms in macrophages[108, 354, 355]. In support of the notion that Tr1 cells emerge from a terminally differentiated Th1 cell subset, we investigated the relationship between IL-12, Blimp1 and Tr1 induction during *L. donovani* infection. We administered *prdm1*^{gfp} mice with control rat IgG or anti-IL-12 on the day of infection and every 3 days after, until 14 days p.i. (**Fig 11a**). IL-12 neutralisation significantly reduced hepatosplenomegaly (**Fig 11b, c**), and this was associated with a significant reduction in IFNγ producing $CD4^+$ T cells and increases in parasite burdens (**Fig 11d-g**). *Prdm1*^{gfp} mice treated with anti-IL-12 also had significantly reduced Tr1 frequencies, and Blimp1 expression on these Tr1 cells was also impaired (**Fig 11h, i**). Together, these results demonstrate a requirement for IL-12 in the induction of Blimp1 expression by Tr1 cells, as well as IL-10 production by these cells.

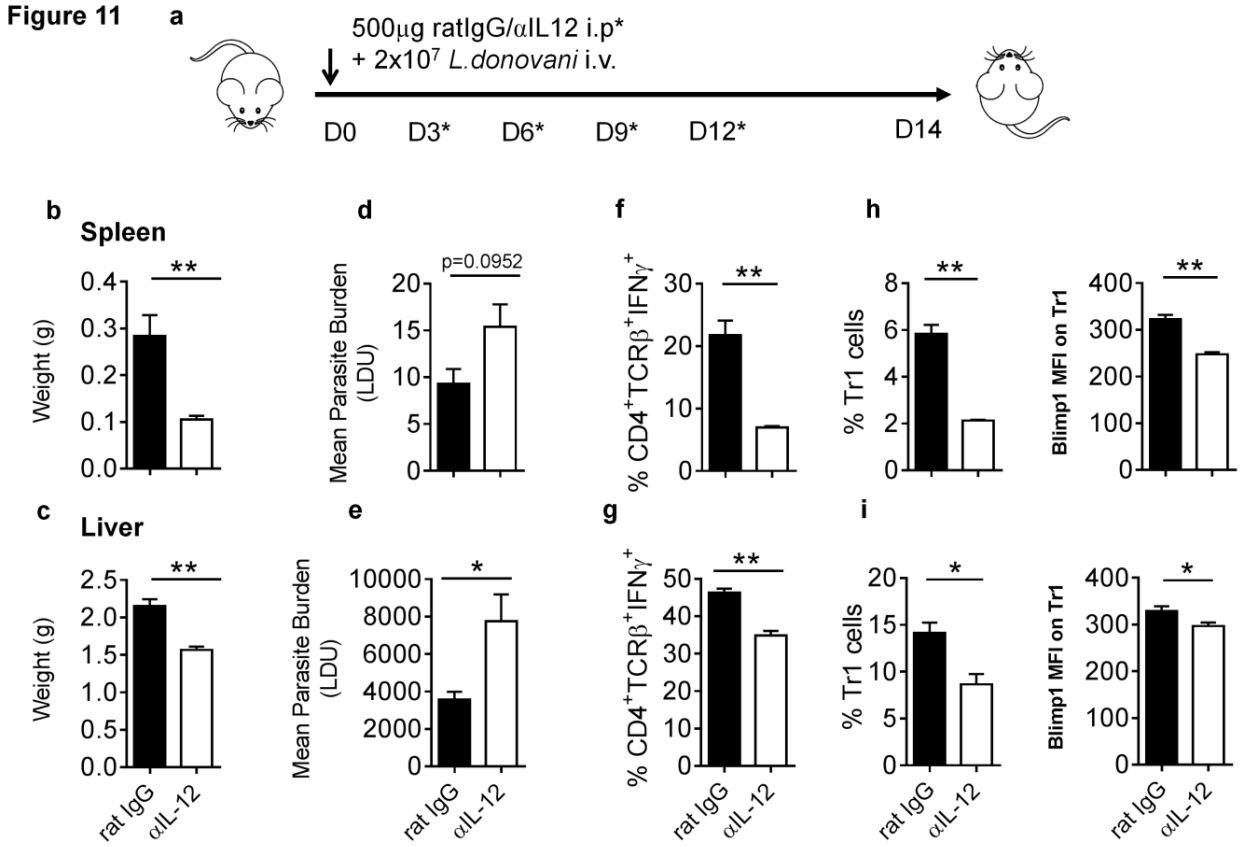


Figure 11: IL-12 is required for Blimp1 expression and Tr1 induction during *L. donovani* infection.

a) *prdm1*^{gfp} and WT mice treated with 1 dose of 500µg rat IgG or anti-IL-12 (C17.8) prior to infection with 2x10^7 *L. donovani* amastigotes i.v. and every 3 days following infection (* denotes 1 dose). **b)** Spleen and **c)** liver weights measured at day 14 p.i.. **d)** Mean parasite burdens expressed as LDU were measured in the spleen and **e)** liver at day 14 p.i.. **f)** Frequencies of IFNγ$^+$ CD4$^+$ T cells were measured by flow cytometry in both spleen and **g)** liver 14 days p.i.. **h)** Frequencies of Tr1 cells as well as Blimp1 MFI (GFP) on Tr1 cells were measured in the spleen and **i)** liver by flow cytometry 14 days p.i.. Representative of 1 independent experiment, mean ±SEM, n=5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

4.4 DISCUSSION

Our data presented here demonstrates that the transcriptional regulator Blimp1 modulates $CD4^+$ T cell responses during *Plasmodium* and *Leishmania* infections. Specifically, *PRDM1* mRNA was up-regulated in $CD4^+$ T cells from VL patients. Employing experimental models of malaria we were able to show that Blimp1 limited the generation of Th1 cells, but promoted Tr1 cell development. Furthermore, the observation that Blimp1 expression was strongly associated with IL-10 production, suggests that Blimp1 promotes immunoregulatory functions, as opposed to inflammatory responses. Notably, IFNγ and TNF producing $CD4^+$ T cells exhibited lower Blimp1 expression compared to IL-10 single producing $CD4^+$ T cells and Tr1 cells, suggesting that Blimp1 expression promotes immunoregulatory activity during infection-induced inflammation as a means of limiting host-tissue damage.

PbA infected control mice develop severe malaria symptoms as a result of $CD4^+$ and $CD8^+$ T cells promoting parasite sequestration in vital organs, including the lungs, liver, brain and spleen[37]. Interestingly, T cell specific Blimp1-deficient mice succumbed to PbA infection, at a time when neurological complications began during infection, potentially suggesting that the intrinsic inflammatory response in these mice is either only protective for a short term, before parasite biomass exceeds the immune system's capacity to exert control or this inflammatory response aids parasite sequestration via an alternate mechanism. Collectively, these results show a cell intrinsic requirement for Blimp1 in modulating T cell responses during *Plasmodium* infections. While it was tempting to speculate that the increased Th1 frequencies and numbers were the cause of reduced parasitemia levels, and therefore played a protective role, we cannot exclude the possibility that this potent Th1 response may also promote pathology. Given that PbA infected mice rapidly develop severe neurological symptoms, it may be difficult to detect differences in malarial pathogenesis, in this model. As such, in a model of uncomplicated malaria (PcAS), the significant reduction in parasitemia in the absence of T cell Blimp1 expression was associated with a strong Th1 response which could potentially contribute to protection. However, this same response in the more severe malaria model (PbA) had minimal impact on the final outcome of disease. This may reflect different requirements of $CD4^+$ T cells in both models. $CD4^+$ T cells have been shown to play protective roles in PcAS infections[45], whereas in the PbA model, have been shown to contribute to pathology[37]. The vastly different requirements for $CD4^+$ T cells in lethal and non-lethal experimental malaria emphasises the delicate balance between protective responses that result in control of parasite growth versus inflammatory responses that contribute to disease.

Upon parasite invasion, inflammation forms the first line of defence against the spread of infection. However, inflammation can also damage host-tissues[37, 129]. Infection-induced inflammation is a major driver of parasitic disease, and we employed an experimental VL model caused by *L. donovani* infection, where pathology and parasite control, particularly in the spleen, is largely driven by TNF[129]. Understanding the factors that modulate these inflammatory processes during infection is critical to limit the subsequent immunopathology associated with excessive inflammation. Importantly, we found that Tr1 cells expressed the highest levels of Blimp1, compared to IL-10$^+$ IFNγ$^-$ and IFNγ$^+$ IL-10$^-$ CD4$^+$ T cells in the spleen. The hierarchical distribution in Blimp1 expression in CD4$^+$ T cells during *Plasmodium* and *Leishmania* infections suggested that Blimp1 expression is more strongly associated with immunoregulatory responses. Cimmino *et al.*, reported that Blimp1 repressed Th1 differentiation[247], which was associated with pro-inflammatory functions, including IFNγ and TNF production. The strong association between Blimp1 expression and IL-10 production in our studies and others[215, 216, 251], supports the notion that this transcriptional regulator is important in promoting the emergence of immunoregulatory pathways during infection-induced inflammation to counteract pro-inflammatory environments, and thus aid in the prevention of host tissue damage.

For many years, IL-10 signalling has been known to play important roles in limiting immunopathology in the host[74, 214, 225, 226, 356]. In the absence of this regulatory mechanism, tissue damage occurs[74, 357]. IL-10 can be produced by multiple cell types, including conventional CD4$^+$ T cells and Foxp3$^+$ Tregs[181, 214, 356, 358]. Depletion studies of Treg cells during PbA infections have identified a protective requirement for IL-10 against severe malaria[46]. However, adoptive transfer studies of CD4$^+$T cells from Foxp3Tg mice (Foxp3 is over-expressed in these mice) or CD4$^+$ CD25$^+$ T cells from wild-type (WT) mice into naive control recipients compromised parasite control during uncomplicated experimental malaria caused by PcAS[359]. While Foxp3$^+$ CD4$^+$ Tregs have been shown to be a cellular source of IL-10 during *Plasmodium* infections[360], recent studies have identified Tr1 cells as a more critical source of IL-10 required to protect against severe immunopathology during PcAS infection[74]. B cells and macrophages/monocytes are also a predominant source of IL-10, but only T cell specific, IL-10 deficient mice exhibited significantly reduced survival and exacerbated pathology, relative to controls[74]. Similarly, during *Leishmania* infections, Tr1 cells, and not CD4$^+$ Foxp3$^+$ Treg cells, were also the predominant source of IL-10[214, 234, 345].

Additionally, other studies have shown that Tr1 cells, and not Foxp3$^+$ Treg cells, were the main suppressors of inflammation[361], further supporting a role for Blimp1-induced IL-10 in limiting immunopathology via Tr1 cells. While Blimp1 expression was not measured in Treg cells, data from our group suggests that depletion of Treg cells during infection results in a complementary increase Tr1 cell numbers. Studies have shown that IRF4 is induced in Treg cells, which acts downstream of Foxp3 to target Blimp1 to then induce IL-10 production[181]. While the role of Treg cells has been extensively studied in many disease settings, Tr1 cells and their role in immunoregulation, and how this differs from Treg cell-mediated regulation, is less well understood. Our data here and studies by others in different inflammatory and disease settings, demonstrate that Tr1 cells exhibit high levels of Blimp1 expression during *Plasmodium* and *Leishmania* infections. In malaria endemic regions, recent field studies have suggested that while Tr1 cells display immunoregulatory characteristics, they were not independently associated with protection against future malaria infections and may in fact be associated with increased risk[47], implying a suppressive role for Tr1 cells during *Plasmodium* infections. Data presented in this Chapter supports this idea because mice lacking Blimp1 expression by T cells generated few Tr1 cells, and this was associated with improved control of non-lethal PcAS, lethal PbA and *L. donovani* growth. Thus, Tr1 cells are induced in response to human and mouse *Plasmodium* and *Leishmania* infections and their ability to produce IL-10 is an important mechanism for limiting inflammation, but may also suppress anti-parasitic CD4$^+$ T cell responses.

The extrinsic and intrinsic requirements for the generation of Tr1 cells have been widely reported[196, 200, 209, 251]. Consistent with these findings, our data shows that IL-12 and Blimp1 were required for the generation of Tr1 cells. In addition, IL-27 is required for driving IL-10 production by Tr1 cells, and also belongs to the IL-12/IL-23 family of cytokines[208]. Iwasaki *et al.*, showed that IL-12 and IL-23 only modestly induced Blimp1 expression, compared to IL-27, suggesting that a more severe impairment in Tr1 generation would be expected with IL-27 blockade[217]. Given that IL-12 and IL-27 contribute to Th1 differentiation, a recent study using a model of *M. tuberculosis* showed that IL-27Rα$^{-/-}$ mice do not display major defects in IFNγ-mediated responses, suggesting that where IL-12 is not limiting, IL-27 is most likely redundant for this function[362]. These studies imply that IL-12 and Blimp1 are sufficient to induce Th1 cells that have the potential to become self-regulatory and give rise to Tr1 cells. Collectively, the data reported in this Chapter and by other groups suggest that Tr1 cells emerge from Blimp1-dependent, terminally differentiated effector Th1 cells, rather than an independent CD4$^+$ T cell lineage[216, 251].

The plasticity of CD4$^+$ T cell responses during *Plasmodium* and *Leishmania* infections is essential for survival of the host, and numerous feedback mechanisms in response to infection-induced inflammation promote the emergence of Tr1 cells preferentially over Th1 cells when parasites persist. Current studies aim to gain a better understanding about what factors controls the emergence of Tr1 from Th1 cells at the transcriptional level and under what conditions. Our studies here have shown that Blimp1 plays a key role in modulating the emergence of both Th1 and Tr1 responses, and the next Chapter provides a mechanistic insight into the subsequent effects on splenic pathology as a result of Tr1 induction.

Chapter 5

Blimp1-dependent IL-10 production by $CD4^+$ T cells protects against TNF-mediated pathology during *Leishmania* infections

"It is the pervading law of all things organic and inorganic, of all things physical and metaphysical, of all things human and all things superhuman, of all true manifestations of the head, of the heart, of the soul, that the life is recognizable in its expression, that ***form ever follows function****...*
This is the law"

– Louis Sullivan 1896 –

5.1 INTRODUCTION

A key function of Tr1 cells, involves protecting host tissue against inflammation-induced pathology[200, 203, 363]. Harnessing the therapeutic potential of Tr1 cells has quickly gained interest and thus a better understanding of Tr1 induction during disease will aid the development of novel therapeutic strategies against chronic inflammatory diseases. Infection-induced inflammation has been reported to contribute to host tissue remodelling[129, 364]. It is thought that Tr1 cells may help preserve tissue integrity by limiting inflammatory-mediated pathology, but Tr1 cells may also suppress anti-parasitic $CD4^+$ T cell responses, thus allowing pathogen persistence. These different roles for Tr1 cells emphasises the need to better understand their functions, and in particular, the transcriptional regulators that determine their development and function.

Leishmania infections cause potent $CD4^+$ T cell responses, including the secretion of pro-inflammatory cytokines aimed at killing intracellular parasites and limiting parasite spread. TNF is a potent pro-inflammatory cytokine that alters epithelial cell barrier permeability, and also initiates tissue remodelling, macrophage activation and cellular recruitment during inflammation, particularly in response to pathogen invasion[365]. While TNF serves to limit the spread of infection and rid the host from the invading pathogen, excess production can be detrimental to host tissue. Infliximab, and adalimumab (anti-TNF monoclonal antibodies) and Enbrel (soluble TNF receptor Fc fusion protein) were amongst the first TNF inhibitors licensed to treat chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, ankylosing spondylitis, inflammatory bowel disease and ulcerative colitis[366, 367, 368, 369, 370, 371, 372, 373]. While TNF blockade can reduce inflammation, it can also increase susceptibility to established and opportunistic pathogens. Thus, in order to develop TNF blocking therapies with increased specificity, a greater understanding of how TNF is regulated during inflammation is required.

The architecture of the spleen reflects its function and role in orchestrating immune responses against invading pathogens. The spleen is a site for the removal of old RBC's from the bloodstream, as well as the removal of pathogens and cellular or foreign debris[119, 126]. Importantly, the highly organised lymphoid compartment allows for the effective interaction between T cells and B cells. In addition, the spleen comprises specialised macrophage populations which help maintain splenic architecture by allowing efficient trafficking of lymphocytes into and out of the organ[119, 129]. The two main areas in the spleen are the red pulp, which is involved in filtering the blood, recycling iron and removing pathogens and other foreign materials, and the white pulp, where T and B cell responses are initiated and maintained. The organisation of the white pulp is controlled by the chemokines CCL19, CCL21 and CXCL13, which guide T cells and B cells to their respective zones[120, 121, 122]. Within the T cell zone, T cells interact with DC's , while in B cell zones, activated B cells undergo clonal expansion and maturation through isotype switching and somatic hypermutation[119]. The MZ, also known as a transit area for cells entering the white pulp from the bloodstream, is home to various cells that work together to maintain the integrity of the spleen[119]. In particular, specialised subsets of macrophages can be found in the MZ, including MZM's which form the outer ring around the white pulp and the MMM's which comprise the inner ring of the white pulp[119, 127]. In between these macrophage populations reside the MZ B cells and DC's[374, 375].

During *L. donovani* infection, TNF is required for parasite control, but can also contribute to the disruption of the splenic architecture, resulting in the characteristic splenomegaly observed in VL patients. TNF causes the selective destruction of MZM's and $gp38^{+}$ stromal cells lining the splenic white pulp areas, and consequently, this contributes to splenomegaly observed 28 days p.i. in C57BL/6J mice infected with *L. donovani*[128, 129]. The functional consequence of this breakdown in splenic architecture involves impaired lymphocyte trafficking[129]. These changes in splenic organisation are associated with impaired immune function, and as such, are thought to contribute to parasite persistence. In contrast to parasite control in the liver, which is a site of acute, resolving infection, parasites persist in the spleen contributing to chronic infection. Disruptions in tissue organisation are also evident in other chronic inflammatory diseases, including cancer, autoimmune diseases, a range of other infectious diseases and other disorders where vascular remodelling occurs during the chronicity of inflammation[132, 364]. In fact, vascular remodelling is thought to contribute to splenomegaly associated with leishmaniasis[376]. Therefore, a better understanding about how TNF is regulated during inflammatory settings may allow for more selective approaches to modulate TNF activity, where host tissue is preserved without compromising protection against intracellular pathogens.

This Chapter investigates the requirements for the development of Blimp1-dependent Tr1 cells during *Leishmania* infections, which appears to impair the control of parasite growth, but limit TNF-mediated pathology, thus helping to preserve splenic architecture during *L. donovani* infection.

5.2 MATERIALS AND METHODS

The material and methods presented here are specific to this chapter

5.2.1 Mice

Female C57BL/6J mice 8-12 weeks old were purchased from either the Australian Resource Centre (Canning Vale, Perth, WA, Australia) or the WEHI (Parkville, Melbourne, VIC, Australia) and were maintained in the animal house facility at the QIMR Berghofer Medical Research Institute under conventional conditions. C57BL/6J.IFNγR$^{-/-}$ mice were maintained in the animal house facility at the QIMR Berghofer Medical Research Institute under conventional conditions. T cell specific Blimp1 deficient mice: C57BL/6J.Lck-Cre^{+} x *Prdm1*$^{fl/fl}$ = ***Prdm1***$^{\mathbf{\Delta T}}$(Cre positive), Lck-Cre^{-} x *Prdm1*$^{fl/fl}$ = *Prdm1*$^{fl/fl}$ (Cre negative), T cell specific IL-10R deficient mice: C57BL/6J.Lck-Cre^{+} x *Il10R*$^{fl/fl}$ = ***Il10R***$^{\mathbf{\Delta T}}$ (Cre positive) , Lck-Cre^{-} x *il10R*$^{fl/fl}$ = *Il10R*$^{fl/fl}$ (Cre negative) , myeloid-derived specific IL-10R deficient mice: C57BL/6J.LysM-Cre^{+} x *Il10R*$^{fl/fl}$ = ***Il10R***$^{\mathbf{\Delta M}}$ (Cre positive) , LysM-Cre^{-} x *Il10R*$^{fl/fl}$ = *Il10R*$^{fl/fl}$ (Cre negative) , T cell specific IL-10 deficient mice: C57BL/6J.Lck-Cre^{+} x *Il10R*$^{fl/fl}$ = ***Il10***$^{\mathbf{\Delta T}}$ (Cre positive) , Lck-Cre^{-} x *Il10*$^{fl/fl}$ = *Il10*$^{fl/fl}$ (Cre negative) mice were bred in-house under specific-pathogen free conditions. Treg cell specific Blimp1 deficient mice: C57BL/6J.Foxp3-Cre^{+} x *Prdm1*$^{fl/fl}$ = ***Prdm1***$^{\mathbf{\Delta F}}$ (Cre positive), Foxp3-Cre^{-} x *Prdm1*$^{fl/fl}$ = *Prdm1*$^{fl/fl}$ (Cre negative) mice were sourced from the WEHI and maintained at QIMR Berghofer Medical Research Institute under conventional conditions. All animal procedures were approved and monitored by the QIMR Berghofer Animal Ethics Committee. This work was conducted under QIMR Berghofer animal ethics approval number A02-634M, in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian National Health and Medical Research Council).

5.2.2 Parasites and infection

L. donovani parasites were maintained by passage in B6.RAG1$^{-/-}$ mice and amastigotes were isolated from the spleens of chronically infected mice. Mice were infected with 2x10^{7} *L. donovani* amastigotes in RPMI/PS i.v. via the lateral tail vein. Spleen and liver impression smears were used to determine parasite burdens and were expressed as LDU; number of amastigotes per 1000 host nuclei multiplied by the organ weight (in grams).

5.2.3 Preparation of *L. donovani* amastigotes for use as antigen

L. donovani infected spleens were prepared as per **Chapter 2, 2.3.3.** Parasites were resuspended in 5ml of 4% PFA (MP Biomedicals Pty Ltd) and placed on ice for 30 minutes. Parasites were then washed once with 50ml RPMI/PS and centrifuged at 1800xg for 15 minutes at room temperature. Parasites were resuspended in 1ml of RPMI/PS and passed through a blunt 26" gauge needle and 10ml syringe in order to minimise parasite clumping. Parasites were then counted using the Helber counting chamber (Weber Scientific, Teddington, UK). Concentration was adjusted to 1x10^{8} parasites/ml in appropriate volumes of freezing medium (**Chapter 2, 2.1**) and 1ml aliquots were frozen and stored at -80°C, until required.

5.2.4 *L. donovani* antigen re-stimulation assay

Spleens were collected aseptically into 10ml RPMI/PS and subsequently passed through a BD Falcon-100µm cell strainer (BD Biosciences, San Jose, CA, USA) to obtain a single cell suspension. Splenocyte suspensions were then centrifuged at 338xg for 7 minutes at room temperature. To obtain splenic mononuclear cells, supernatants were decanted; pellets were resuspended in 1ml of RBC lysis buffer (Sigma-Aldrich) and incubated at room temperature for 8 minutes to ensure complete lysis of RBC's. 9ml of RPMI/PS was added to wash lysis buffer and then centrifuged at 338xg for 7 minutes at 4°C, to inhibit any further lysis. Supernatants were decanted and pellets were resuspended in a final volume of 10ml RPMI/PS for counting. Splenic mononuclear cells were then counted on a haemocytometer (BLAUBRAND® counting chamber Neubauer, Blackburn, VIC, Australia) after a 1:5 to 1:20 dilution in 0.1% Trypan Blue solution in PBS (0.4%, MP Biomedicals Pty Ltd). Splenic mononuclear cells were then adjusted to a concentration of 2 x 10^6 cells/ml. *L. donovani* fixed amastigotes (4% PFA) were thawed and washed in RPMI/PS and then counted and adjusted to a final concentration of 4 x 10^7/ml. Cells and parasites were plated into a 96 well U-bottom plate at a 1:20 ratio, where each well contained 1 x 10^5 cells and 2 x 10^6 parasites. Cells were cultured in the presence of antigen for a period of 24 and 72 hours. Culture supernatants were harvested at 24 to 72 hours and intracellular cytokine staining was performed at both time points (**Table 1**).

5.2.5 Flow cytometry

Splenic and hepatic mononuclear cells were stained using antibodies in **Table 1**.

Cell populations of interest were defined as follows:

- Activated $CD4^+$ T cells: $CD11a^+$ $CD49d^+$ $CD4^+$ $TCR\beta^+$
- Activated Th1 cells: $CD11a^+$ $CD49d^+$ $CD4^+TCR\beta^+$ $Tbet^+$ $IFN\gamma^+$
- Activated Tr1 cells: $CD11a^+$ $CD49d^+$ $CD4^+$ $TCR\beta^+$ $IL\text{-}10^+$ $IFN\gamma^+$
- Treg cells: $CD4^+$ $TCR\beta^+$ $Foxp3^+$
- Inflammatory monocyte-like populations: $B220^-$ $CD11b^+Ly6C^{hi}$
- Conventional dendritic cells (cDC's): $B220^-$ $CD11c^{hi}$ $MHCII^{hi}$
- B cells: $CD19^+B220^+$

Table 1: Mouse monoclonal antibodies used for flow cytometry or immunofluorescence*

Specificity	Clone	Fluorophore	Dilution	Supplier
B220	RA3-6B2	APC	1 in 200	Biolegend
B220	RA3-6B2	APC/Cy7	1 in 200	Biolegend
B220*	RA3-6B2	PE	1 in 40	Biolegend
CD3*	17A2	biotin	1 in 100	Biolegend
CD4	GK1.5	PerCPCy.5.5	1 in 200	Biolegend
CD4	GK1.5	FITC	1 in 200	Biolegend
CD8α	53-6.7	Alexa Fluor 700	1 in 200	Biolegend
CD19	6D5	Biotin	1 in 200	Biolegend
CD19	6D5	PerCP/Cy5.5	1 in 100	Biolegend
CD11a	M17/4	FITC	1 in 100	Biolegend
CD11b	M1/70	PerCP/Cy5.5	1 in 200	Biolegend
CD11c	N418	APC	1 in 200	Biolegend
CD49b	DX5	APC	1 in 100	Biolegend
CD49d	R1-2	Biotin	1 in 100	Biolegend
Foxp3	MF-14	APC or AF488	1 in 100	Biolegend
IFNγ	XMG1.2	APC	1 in 200	BD
IFNγ	XMG1.2	BV421	1 in 200	Biolegend
IFNγ	XMG1.2	PE	1 in 200	Biolegend
IL-10	JES5-16E3	PE	1 in 200	Biolegend
LAG3	C9B7W	Biotin	1 in 200	Biolegend
Live/Dead		Aqua (Amcyan)	1 in 200	Invitrogen
Ly6C	HK1.4	FITC	1 in 200	Biolegend
MHCII (I-A/I-E)	M5/114-15.3	Pacific Blue	1 in 200	Biolegend
Streptavidin		PE	1 in 100	Biolegend
Streptavidin*		AF594	1 in 100	Biolegend
Streptavidin		AF700	1 in 100	Biolegend
Streptavidin		PeCy7	1 in 200/ 1 in 400	Biolegend
Tbet	Ebio4B10	efluor 660 (APC)	1 in 50	Ebioscience
Tbet Isotype control (mouse IgG1κ)	P3.6.2.8.1	efluor 660 (APC)	1 in 50	Ebioscience
TCRβ	H57-597	APC	1 in 200	Biolegend

Specificity	Clone	Fluorophore	Dilution	Supplier
TCRβ	H57-597	BV421	1 in 200	Biolegend
TCRβ	H57-597	PerCP/Cy5.5	1 in 200	Biolegend
TNF	MP6-XT22	PE	1 in 400	Biolegend
TNF	MP6-XT22	PeCy7	1 in 400	Biolegend

5.2.6 Antibodies for *in vivo* TNF blockade

For TNF blockade experiments, mice were administered 200µg of Human Normal Immunoglobulin (INTRAGAM® P; CSL, Melbourne, VIC, Australia) or anti-TNF (Enbrel^{TM}; Amgen, Thousand Oaks, CA, USA) i.p., on the day of infection and every 2 days p.i. until day 14 p.i..

5.2.7 Fluorescence Microscopy

Mice were injected with 100µg i.v. of FITC dextran (Life Technologies, Melbourne, Australia) one day prior to collection of organs. Spleen tissue was collected into 4% PFA, incubated at room temperature for 1-2 hours and then transferred to a 30% sucrose solution (in MilliQ water) (Sigma-Aldrich, Sydney, Australia) overnight at 4°C. Fixed spleen tissue was then preserved in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA, USA). Splenic architecture and distribution of MZM's were analysed in 20 µm sections counter-stained with DAPI and imaged on the Aperio FL slide scanner (Leica Biosystems, North Ryde, NSW, Australia). Image analysis was performed using Image Scope v11.2.0.780 (Leica Biosystems) to determine area of the sections and Metamorph® v7.8 (Integrated Morphometry analysis tool; Molecular Devices, Sunnyvale, CA, USA) to count the MZM's. For lymphocyte trafficking experiments, mice were injected i.v. with 2 x 10^7 naïve splenocytes labelled with Cell Trace Far Red DDAO-SE (Life Technologies, Mulgrave, Australia), 2 hours prior to sacrifice. 20 µm sections were used to assess cell trafficking, where sections were stained with CD3 biotin (5 µg/ml) + SA AF594 (5 µg/ml), B220 PE (5 µg/ml) (Biolegend, San Diego, CA, USA), counter-stained with DAPI (1:25000, Sigma-Aldrich, Castle Hill, Australia) and mounted with Pro-Long Gold anti-fade (Life Technologies).

5.2.8 Cell trafficking experiments

Spleen tissue was processed as above in **5.2.7**. Sections were cut (20µm) and subsequently stored at -80°C until processing. Briefly, slides were equilibrated to room temperature for 30 minutes and an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories, Burlingame, CA, USA) was used to encircle the sections. Slides were allowed to dry for 10-15 minutes before washing them in 1x PBS for 3 minutes. 100µl of 5% goat serum (in PBS) was added and slides incubated at room temperature for 30 minutes in the dark, to block any non-specific binding. Slides were then washed once in 1x PBS and 100µl of Avidin (Vector Laboratories) solution was added and slides were incubated for 15 minutes at room temperature. Slides were subsequently washed and 100µl of biotin solution was added and incubated for 15 minutes at room temperature. 5µg/ml of mouse anti-CD3 biotin (17A2) was added per slide and incubated for 1 hour at room temperature, protected from light. Slides were washed 3 times in 1x PBS (3 minutes per wash) and then incubated in 5µg/ml SA-AF594 and 5µg/ml of B220 PE for 1 hour at room temperature. After 3 washes, DAPI solution (1:25000) was added to each slide and incubated for 10 minutes at room temperature. Slides were washed (as before) and then mounted with 100µl of ProLong Gold (Invitrogen) and 22 x 50mm cover-slips (Menzel Gläser, Braunschweig, Germany).

5.2.9 Microscopy Analysis

5.2.9.1 Quantifying MZM's

Splenic architecture and distribution of MZM's were analysed in 20µm sections counter-stained with DAPI and imaged on the Aperio FL slide scanner under 20x magnification (Leica Biosystems). Image analysis was performed using Image Scope (Leica Biosystems) to determine section area in mm^2 and the 'Integrated Morphometry analysis tool' using MetaMorph® v7.8 software (Molecular Devices, Sunnyvale, CA, USA) to count the MZM's. Briefly, the AF488 (FITC) image panel showing only the MZM's was extracted from Aperio FL software (Spectrum, Leica Biosystems). Using the Integrated Morphometry analysis tool in MetaMorph®, the 'Measure' function was used to count all MZM's in each section. For consistency, cell area limits were set from 5-500 (based on fluorescence threshold). Section areas were determined by drawing around the perimeter of the spleen transverse section and then using the 'Positive pixel count v9 algorithm' in Image Scope, to measure the area in mm^2. Finally to normalise MZM counts to splenic area, MZM counts were divided by the area to give 'Number of MZM's per mm^2, (as shown in graphs in **5.3**)

5.2.9.2 Quantifying MZM's in white pulp areas – cell trafficking

For cell trafficking experiments, slides were imaged on a Carl Zeiss 780 NLO laser scanning confocal microscope (Zeiss, North Ryde, NSW, Australia) under 10x magnification. Image analysis was performed using the 'Counting App and Region Measurement tool' in MetaMorph® v7.8 software (Molecular Devices). Briefly, two image panels were extracted from ZEN Black (Imaging acquisition software), the first panel comprised of only labelled splenocytes. Once the fluorescence threshold was set, the 'count cells' app was used to count cells in each field of view (4 FOVs per mouse, 5 mice per group = 20 images in total per group) and since each image was identical in size (1416.30μm x 1416.30μm), the total number of cells was counted in each image. The second panel comprised of T and B cell zones (CD3 and B220 staining, respectively) in the white pulp (WP). The drawing tool was used to define T and B cell zones in each image. Defined regions from the second panel (WP) were transferred onto the first panel (cells) and the total number of cells in each region was also counted. Total cells in T and B cell zones were then normalised to total WP area. To do this, pixel calibration was performed by setting the X and Y calibration to 0.692μm, where 1 pixel would now be equal to $1mm^2$. The 'Morphometry' tool was used to determine the WP area (in mm^2) within each image. Finally, the number of cells in WP per mm^2 (**Fig 13g**) equals the total number of cells in WP (**Fig 13f**), divided by the WP area (**Fig 13h**).

5.2.10 Statistical Analysis

Statistical differences between two groups were determined using the non-parametric Mann-Whitney U test. Statistical differences between multiple groups were made using a Kruskal-Wallis test (Dunn's multiple comparisons test) by GraphPad Prism version 6 for Windows (GraphPad, San Diego, CA, USA); $p<0.05$ was considered statistically significant. All data are presented as the mean ± SEM, unless otherwise stated.

5.3 RESULTS

5.3.1 *L. donovani* infection results in tissue-specific responses

C57BL/6J mice infected with *L. donovani* develop experimental VL where the liver, spleen and bone marrow are the main sites of infection[352] as shown in **Fig 1a** with the aid of a transgenic bioluminescent parasite (tdTomato – expressing *L. donovani*). The liver is a site of acute infection, where peak parasite burden occurs around day 14 - 21 p.i., and is largely resolved by day 56 p.i. (**Fig 1b**). Early infection triggers rapid IL-12 production by $CD8\alpha^+$ DC's, $CD4^+$ and $CD8^+$ T cell activation and production of cytokines such as IL-2, IFNγ and TNF[377]. IL-12 signals to $CD4^+$ Th1 cells to produce IFNγ and TNF in order to activate macrophages to produce iNOS and ROS[108, 354, 377]. Hepatic infection is generally resolved within 6-8 weeks post-infection, a process that is largely driven by granuloma formation around infected KC's[109]. The bone marrow and spleen are sites of chronic infection characterised by parasite persistence and associated with changes in the splenic architecture leading to impaired lymphocyte trafficking[97, 129]. Parasite burdens in the spleen steadily increase throughout infection and plateau around day 56 p.i. (**Fig 1c**). One of the advantages of this model of experimental VL is that the kinetics of both acute and chronic infection can be studied within the same animal. A hallmark feature observed in VL patients is the progressive distension of the abdomen as a result of extensive hepatosplenomegaly. Similarly, the spleen and liver of *L. donovani* infected mice progressively enlarge throughout the course of infection (**Fig 1d**). As infection progresses, the acute infection resolves in the liver (**Fig 1b**), but a chronic infection becomes established in the spleen (**Fig 1c**). It is unclear why potent inflammatory responses promote parasite clearance in the liver but not in the spleen.

Figure 1

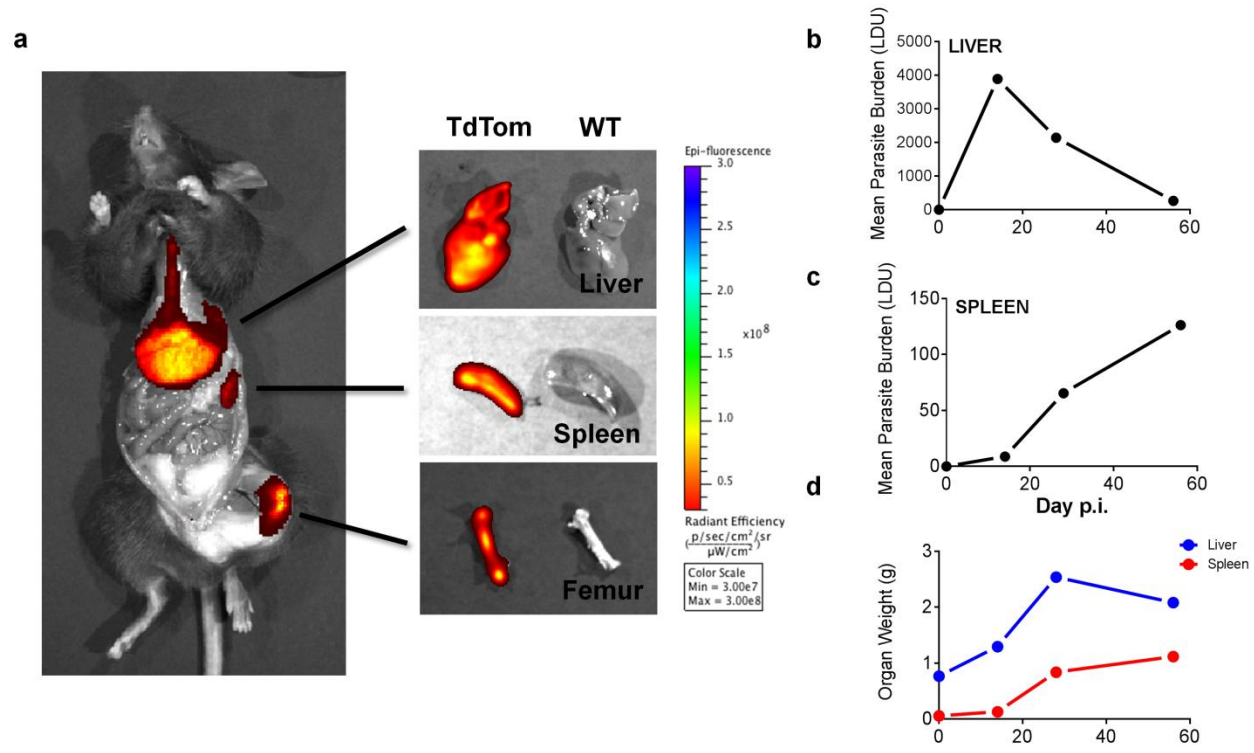


Figure 1: Immune responses during *L. donovani* infection are tissue-specific.

a) C57BL/6J mice infected with either $2x10^7$ tdTomato- expressing *L. donovani* bioluminescent amastigotes or WT – *L. donovani* amastigotes i.v.. Main sites of infection observed in the liver, spleen and bone marrow. *In vivo* bioluminescent imaging of infected mice performed on the Xenogen IVIS 100 imaging system. **b)** Liver impression smears taken from infected mice at indicated time-points and mean parasite burden expressed in LDU. **c)** Spleen impression smears taken from infected mice at indicated time-points and mean parasite burden expressed in LDU. **d)** Liver and spleen weights recorded in grams from infected mice at indicated time-points, throughout the course of infection. Representative of 5 independent experiments, n= 5 mice per time-point. Figure source: Immunology and Infection Lab, QIMR Berghofer

5.3.2 Blimp1 impairs parasite control and regulates CD4$^+$ T cell responses during *L. donovani* infection

Data presented in Chapter 4 demonstrated a requirement for Blimp1 in the development of Th1 and Tr1 responses during *Plasmodium* infections. These results were consistent with previous reports for Blimp1 in regulating CD4$^+$ T cell responses[215, 216, 247], and we next sought to examine whether Blimp1 also played a key role in modulating Th1 and Tr1 responses in another important parasitic disease to establish the broad relevance of these findings to infection. T cell specific Blimp1-deficient (*Prdm1*$^{\Delta T}$) mice infected with *L. donovani* had significantly reduced parasite burdens in the spleen 28 days p.i. (**Fig 2a**), which was associated with significant increases in the frequencies of activated Th1 cells (**Fig 2b**), and a severe impairment in the generation of Tr1 cells (**Fig 2c**). Parasite persistence in the spleen leads to a breakdown in the splenic architecture, and as a functional consequence, impedes the generation, priming and maintenance of effective immune responses[106, 129]. Surprisingly, *Prdm1*$^{\Delta T}$ mice had significantly larger spleens, indicative of extensive splenomegaly throughout the course of infection (**Fig 2d**), and was associated with a significant increase in the frequency of CD4$^+$ T cells producing TNF (**Fig 2e**). Similar responses were observed in the livers of *Prdm1*$^{\Delta T}$ mice infected with *L. donovani* (**Fig 3**). The significant reduction in parasite burdens (**Fig 3a**), was associated with increases in Th1 cell frequencies (**Fig 3b**) and impaired Tr1 cell responses (**Fig 3c**), in the *Prdm1*$^{\Delta T}$ mice. Hepatomegaly (**Fig 3d**) in the *Prdm1*$^{\Delta T}$ mice was also associated with a significant increase in TNF production by CD4$^+$ T cells, particularly 14 days p.i. (**Fig 3e**). Collectively, these results implicate a broader role for Blimp1 in parasitic diseases, where in a second parasitic model Blimp1 impaired parasite control, modulated effector CD4$^+$ T cell responses, but was required to limit pathology during *L. donovani* infection. Therefore, these results suggested that Blimp1-dependent IL-10 production by CD4$^+$ T cells was required to limit tissue pathology, but may also contribute to impaired parasite clearance.

Figure 2

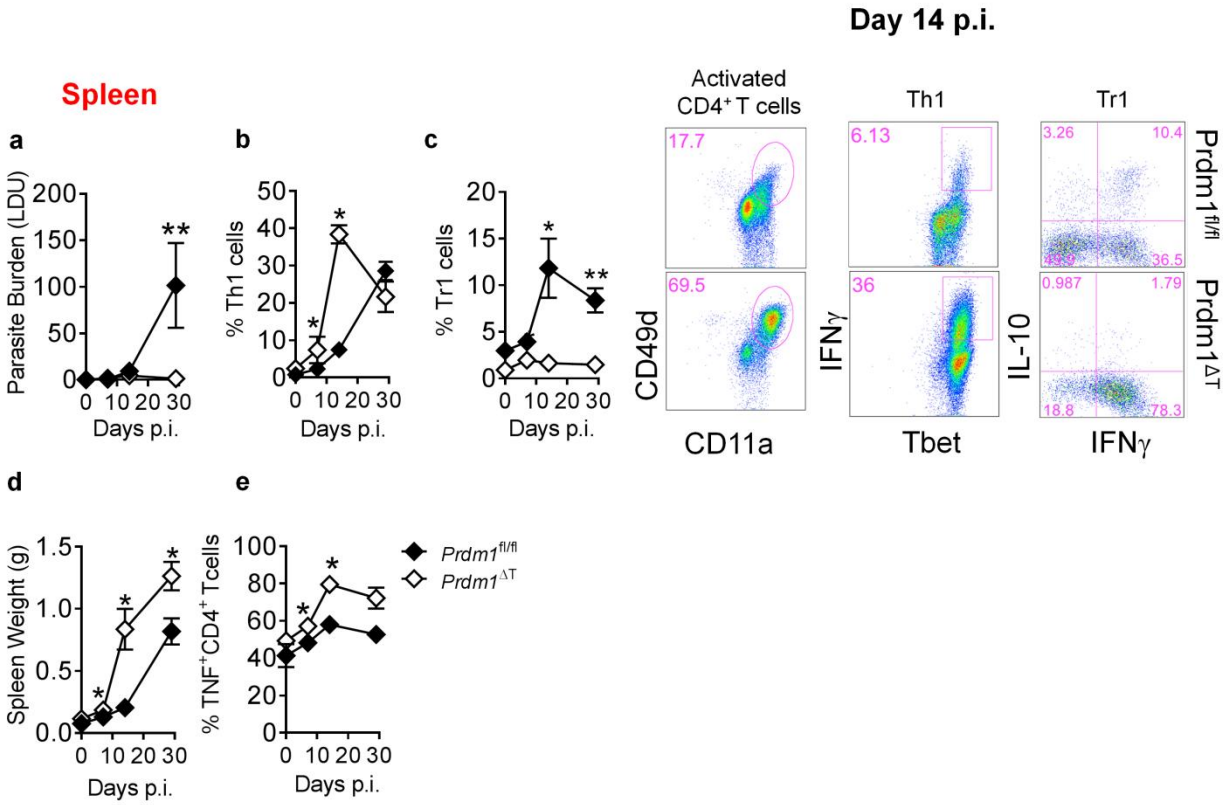


Figure 2: Blimp1 impairs parasite control in the spleen and regulates CD4$^+$ T cell responses during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and spleen parasite burdens expressed in LDU. **b)** Frequency of Th1 cells (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ Tbet$^+$) measured by flow cytometry at indicated time-points. **c)** Frequency of Tr1 cells (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ IL-10$^+$) measured by flow cytometry at indicated time-points. **d)** Spleen weights recorded in grams at indicated time-points. **e)** Frequency of TNF producing CD4$^+$ T cells in the spleen were assessed by flow cytometry at indicated time-points. Representative of 6 independent experiments, mean ± SEM, n= 4-6 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

Figure 3

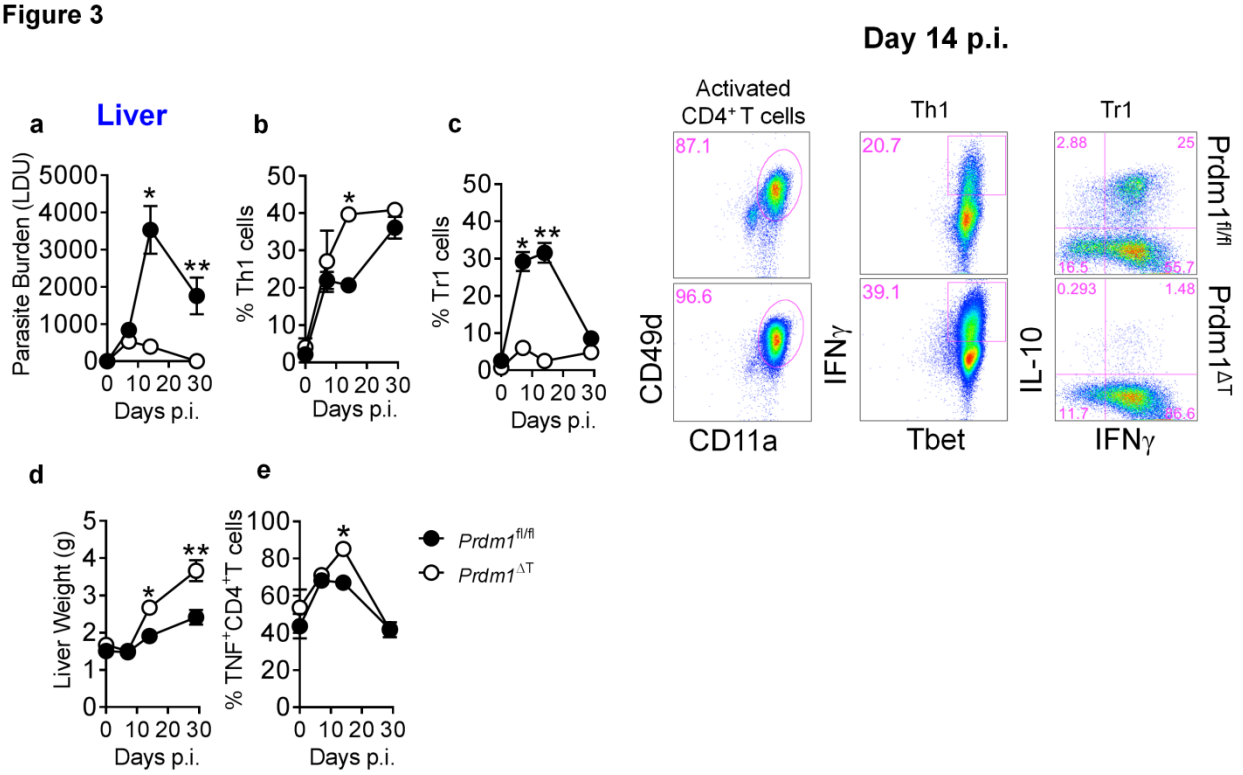


Figure 3: Blimp1 impairs parasite control in the liver and regulates CD4$^+$ T cell responses during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and liver parasite burdens expressed in LDU. **b)** Frequency of Th1 cells (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ Tbet$^+$) measured by flow cytometry at indicated time-points. **c)** Frequency of Tr1 cells (CD4$^+$ TCRβ$^+$ CD11a$^+$ CD49d$^+$ IFNγ$^+$ IL-10$^+$) measured by flow cytometry at indicated time-points. **d)** Liver weights recorded in grams at indicated time-points. **e)** Frequency of TNF producing CD4$^+$ T cells in the liver were assessed by flow cytometry at indicated time-points. Representative of 6 independent experiments, mean ± SEM, n= 4-6 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

5.3.3 Blimp1 modulates parasite-specific inflammatory responses during *L. donovani* infection

The pro-inflammatory cytokines, TNF and IFNγ play critical roles in parasite clearance by activating macrophages to kill resident parasites[354, 378, 379]. While TNF is a potent mediator of parasite killing, it has also been shown to contribute to pathology, particularly in the spleen[129]. *L. donovani* infected *Prdm1*$^{\Delta T}$ mice exhibited significantly elevated levels of TNF and IFNγ in the serum at day 14 p.i. (**Fig 4a**). We next tested whether the increase in the frequencies of Th1 cells was parasite-specific by culturing splenocytes in the presence of fixed *L. donovani* amastigotes for 24 hours and subsequently assessing the frequencies of Th1 cells by flow cytometry (**Fig 4b**). Similar to the responses observed in **Figs 2 and 3**; we observed a significant increase in Th1 cells in response to parasite antigen 14 days p.i. (**Fig 4b**). Additionally, cytokine analysis of culture supernatants revealed that Blimp1 deficiency resulted in significant increases in parasite-specific TNF and IFNγ levels (**Fig 4c**), and significantly reduced IL-10 levels 14 days p.i. (**Fig 4d**). Taken together, these data indicate that Blimp1 is required for regulating parasite-induced inflammation and promoting antigen-specific Th1 cell responses during *L. donovani* infection.

Figure 4

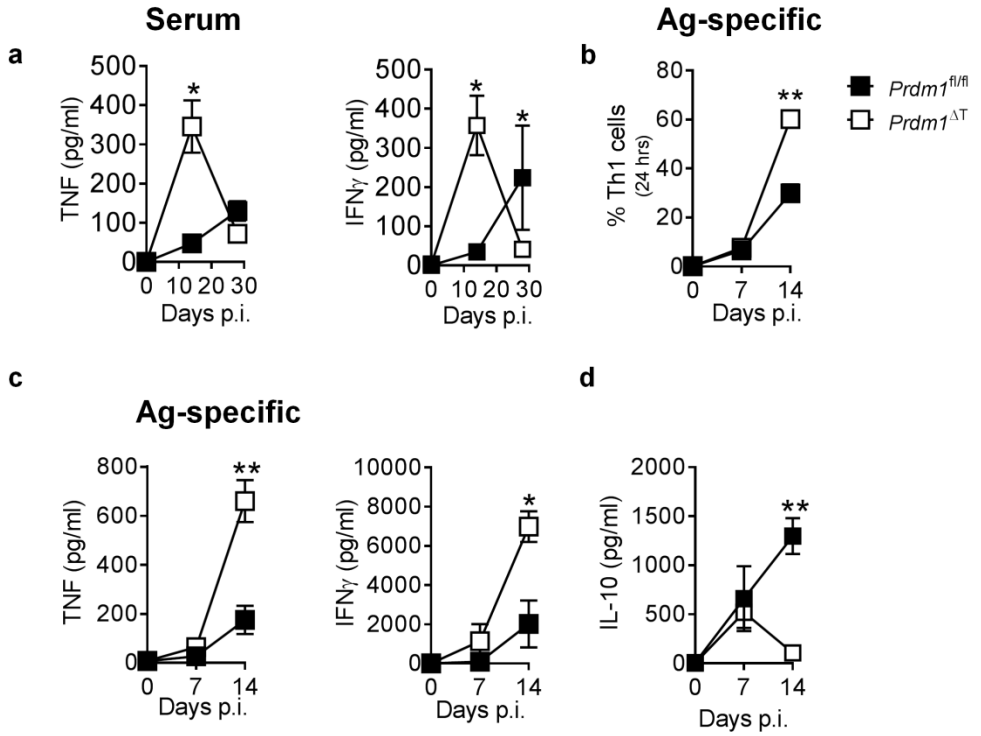


Figure 4: Blimp1 modulates parasite-specific inflammatory responses during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^7 *L. donovani* amastigotes i.v.. Serum TNF and IFNγ levels measured by using a BD Flex set cytometric bead array (CBA) kit, as per manufacturer's instructions at day 14 and 28 p.i.. **b)** Splenocytes from *L. donovani*-infected *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were cultured in the presence of fixed *L. donovani* (4% PFA) amastigotes for 24 hours before ICCS was performed to measure frequencies of Th1 cells by flow cytometry at day 7 and 14 p.i.. **c)** Splenocytes from *L. donovani*-infected *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were cultured in the presence of fixed *L. donovani* (4% PFA) amastigotes for 24 hours (TNF and IFNγ) or **d)** 72 hours (IL-10) when culture supernatants were collected and cytokine levels measured by using a BD Flex set CBA kit. Representative of 2 independent experiments, mean ± SEM, n= 4-5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

5.3.4 Blimp1 deficiency in Treg cells does not influence parasite control or CD4$^+$ T cell responses during *L. donovani* infection

Previous reports demonstrated a requirement for Blimp1 in conjunction with interferon regulatory factor (IRF) 4 to induce IL-10 production by Treg cells[181]. T cell derived IL-10 plays a suppressive role in *Leishmania* infections[205, 214]. Given that Treg cell derived IL-10 can act as a potent immunoregulator[380, 381], we investigated whether Blimp1 was required by Treg cells for inducing IL-10 production during infection. *L. donovani* infected *Prdm1*$^{\Delta T}$ mice exhibited significantly reduced frequencies of IL-10$^+$ Foxp3$^-$ CD4$^+$ T cells (**Fig 5a**), 28 days p.i. in the spleen (**Fig 5b**) and liver (**Fig 5c**). Consistent with previous reports, we found a significant reduction in IL-10$^+$ Foxp3$^+$ CD4$^+$ Treg cell frequencies in the spleens of *Prdm1*$^{\Delta T}$ mice (**Fig 5b**); suggesting Blimp1 is required for IL-10 production by Treg cells[181]. Interestingly, *Prdm1*$^{\Delta T}$ mice had significant increases in the frequencies of IL-10$^-$ Foxp3$^+$ CD4$^+$ T cells in the liver (**Fig 5c**). Since Treg cells required Blimp1 to produce IL-10, we next investigated whether Treg cell specific Blimp1 deficiency influenced control of parasite growth and/or development of tissue pathology. Interestingly, organ weights (**Fig 6a**), spleen and liver parasite burdens (**Fig 6b**), were comparable between the Treg cell specific Blimp1 deficient (*Prdm1*$^{\Delta F}$) mice and their littermate controls 28 days p.i.. Furthermore, the frequency of Th1 cells in both the spleen and liver were similar in *Prdm1*$^{\Delta F}$ mice and controls (**Fig 6c**), suggesting Blimp1 deficiency in Treg cells does not influence the generation of Th1 cells during *L. donovani* infection. While no difference in the frequency of Tr1 cells was observed in the spleen, a significant increase in Tr1 cells was observed in the liver in the absence of Blimp1 in Treg cells at day 28 p.i. (**Fig 6d**). This latter observation suggests that in the absence of Blimp1 in Treg cells, a compensatory immunoregulatory mechanism emerges, whereby Tr1 cells develop from a conventional CD4$^+$ T cell population to counteract the reduced Treg cell function due to the absence of IL-10. This data is supported by the significant increase in IL-10$^-$ Foxp3$^+$ CD4$^+$ Treg cells in the liver, 28 days p.i. in the *Prdm1*$^{\Delta T}$ mice (**Fig 5c**). Importantly, Treg cell-specific Blimp1 deficiency did not alter IFNγ or TNF serum levels at day 28 p.i. (**Fig 6e**). Collectively, these results suggest Blimp1-dependent IL-10 production by Treg cells does not influence control of parasite growth or regulate anti-parasitic immune responses during *L. donovani* infection.

Figure 5

Day 28 p.i.

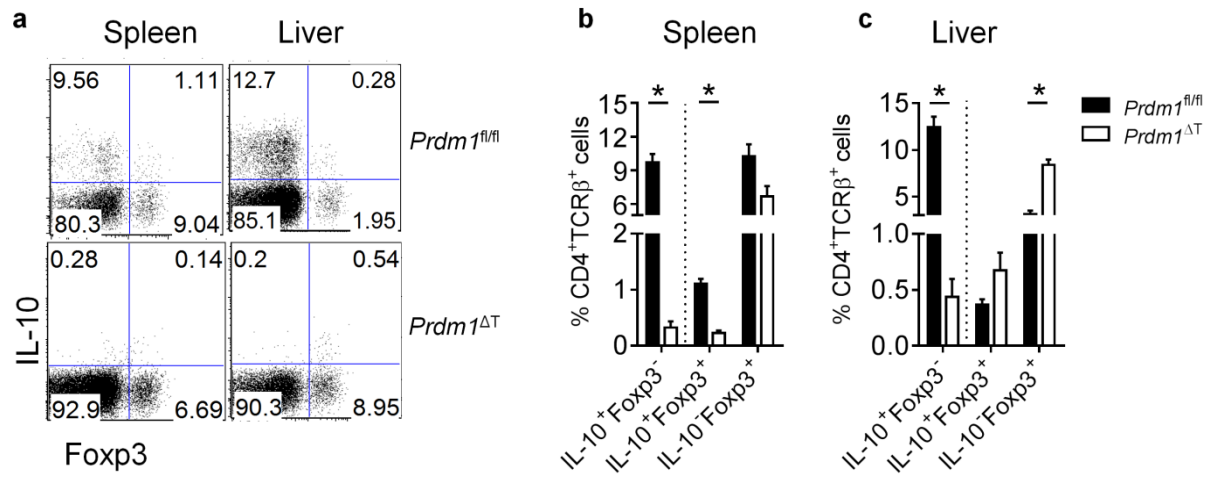


Figure 5: Blimp1 is required for IL-10 production by Treg cells during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^{7} *L. donovani* amastigotes i.v. and frequencies of IL-10 producing Foxp3^{+} CD4^{+} T cells were measured by flow cytometry in the spleen and liver, 28 days p.i.. **b)** Frequencies of IL-10^{+} (single positive), IL-10^{+} Foxp3^{+} (double positive) and IL-10^{-} Foxp3^{+} (single positive) CD4^{+} T cells were measured in the spleen by flow cytometry 28 days p.i.. **c)** Frequencies of IL-10^{+} (single positive), IL-10^{+} Foxp3^{+} (double positive) and IL-10^{-} Foxp3^{+} (single positive) CD4^{+} T cells were measured in the spleen by flow cytometry 28 days p.i.. Representative of 2 independent experiments, mean ± SEM, n= 4-5 mice per group, *= $p<0.05$, Mann-Whitney U test.

Figure 6

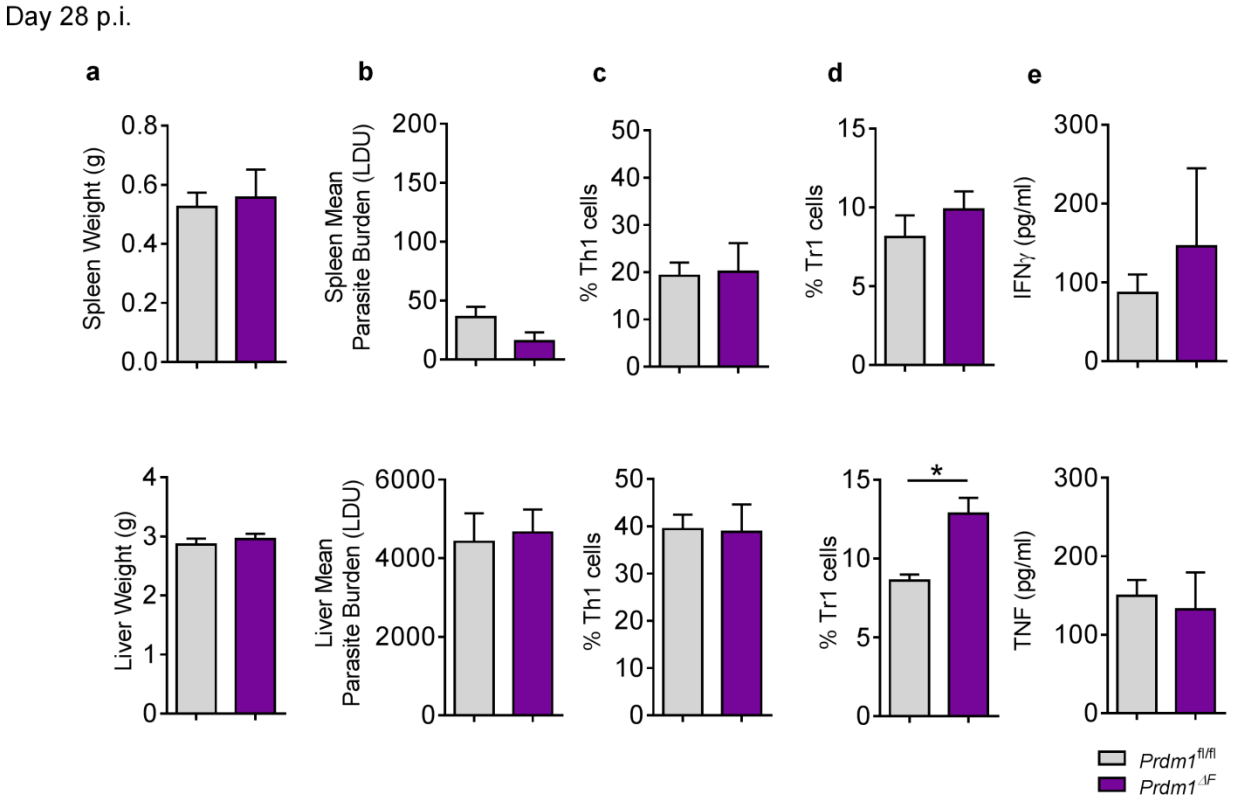


Figure 6: Blimp1 deficiency in Treg cells does not influence parasite control or CD4$^+$ T cell responses during *L. donovani* infection.

a) *Prdm1*$^{\Delta F}$ (Treg cell specific Blimp1 deficient) and *Prdm1*$^{fl/fl}$ (littermate controls) mice were infected with 2x10^7 *L. donovani* amastigotes i.v. Spleen and liver weights recorded in grams, 28 days p.i.. **b)** Spleen and liver parasite burdens expressed in LDU **c)** Frequency of Th1 cells in the spleen and liver measured by flow cytometry 28 days p.i.. **d)** Frequency of Tr1 cells in the spleen and liver, measured by flow cytometry 28 days p.i.. **e)** Serum IFNγ and TNF levels measured using a BD Flex set CBA kit at day 28 p.i.. Representative of 1 independent experiment, mean ± SEM, n= 4-5 mice per group, *= $p<0.05$, Mann-Whitney U test.

5.3.5 Blimp1-dependent IL-10 production by T cells is required to limit pathological changes in the spleen during *L. donovani* infection

TNF-producing $CD4^+$ T cells displayed a much lower level of Blimp1 expression compared to IL-10 producing $CD4^+$ T cells (**Chapter 4 Fig 9a, b**), and in addition to this, mice lacking Blimp1 specifically in T cells exhibited significantly higher levels of serum TNF, antigen-specific TNF production, as well as higher frequencies of TNF-producing $CD4^+$ T cells in both the spleen and liver (**Fig 2e, 3e, 4a, 4c**). A recent study demonstrated that TNF blockade induced IL-10 expression in human $CD4^+$ T cells[382], supporting earlier reports of IL-10 mediated regulation of TNF[383, 384, 385]. Together, these data suggest that Blimp1 in T cells regulates TNF production during *L. donovani* infection via IL-10. The architecture of the spleen is highly organised and by taking a transverse cross-section of the spleen (**Fig 7a**), it is possible to delineate the white pulp areas by fluorescence microscopy using FITC dextran and DAPI molecular probes. In a normal WT, un-infected mouse, white pulp areas are clearly visible (**Fig 7b**), whereas 28 days p.i., the spleen enlarges and destruction of the white pulp areas is clearly evident (**Fig 7c**). The white pulp is surrounded by the MMM's, and outside of this layer the MZM's, form the outermost perimeter of the white pulp (**Fig 7d**). To investigate the effects of TNF-mediated tissue pathology and how IL-10 may regulate this process, we employed the use of the T cell specific Blimp1-deficient and IL-10-deficient ($Il10^{\Delta T}$) mice. $Prdm1^{\Delta T}$ and $Il10^{\Delta T}$ mice infected with *L. donovani* exhibited significantly reduced parasite burdens (**Fig 8a**), and significant increases in spleen weights (**Fig 8b**), indicative of pathology 14 days p.i.. Furthermore, associated with improved parasite control, but increased pathology, we also observed significant increases in the frequencies of Th1 cells in both the $Prdm1^{\Delta T}$ and $Il10^{\Delta T}$ mice (**Fig 8c**), suggesting that Blimp1 induced IL-10 is required to regulate inflammatory responses in order to limit pathology. To examine the extent of architectural disruption, a process that is largely mediated by TNF[129], we injected mice with FITC dextran i.v. to determine whether the MZM's lining the white pulp areas of the spleen would be disrupted in the $Prdm1^{\Delta T}$ and $Il10^{\Delta T}$ mice. Given that in a WT mouse, loss of these MZM's occurs at day 28 p.i., it was interesting to observe a significant reduction in number of MZM's per mm^2 of tissue in both $Prdm1^{\Delta T}$ and $Il10^{\Delta T}$ mice as early as day 14 p.i. (**Fig 8d**), suggesting that both Blimp1 and IL-10 in T cells were required to protect against TNF-mediated pathology in the spleen. These observations are consistent with previous reports for a protective role for IL-10 against tissue damage mediated by parasite-induced inflammation[74, 356, 386], but also identifies loss of MZM's as a specific manifestation of this pathology.

Figure 7

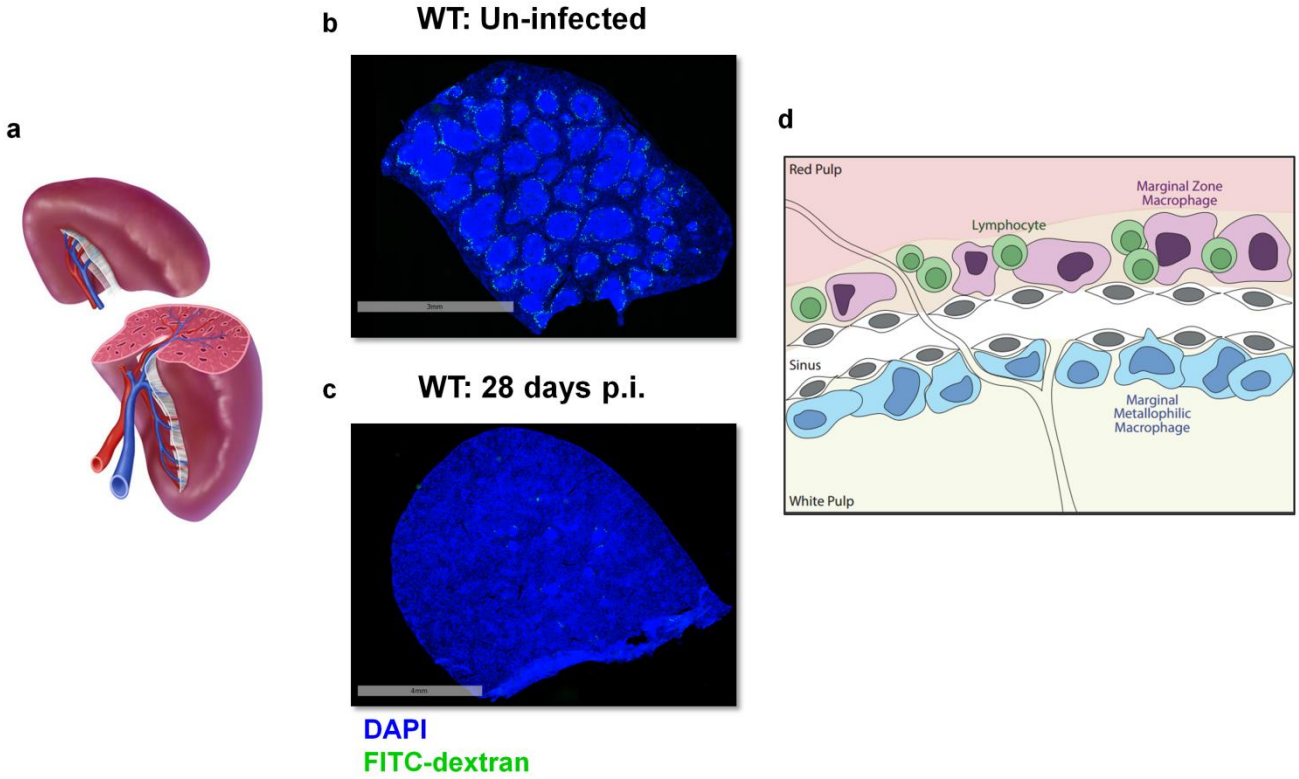


Figure 7: *L. donovani* infection results in disruption of the splenic architecture 28 days p.i..

a) Transverse cross-section of the spleen. Image source: Body Scientific International LLC website: http://www.semayjohnston.com/ **b)** C57BL/6J mice (un-infected) were injected with 100µg of FITC-dextran (in saline) i.v. spleens harvested and fixed in 4% PFA (1-2 hours) followed by overnight incubation at 4°C in 30% sucrose solution (in MilliQ water) before freezing in Tissue Tek OCT. 20µm sections were cut and stained with DAPI, mounted with Pro-Long Gold and imaged on the Aperio FL slide scanner under 20x magnification, scale bar 3mm. **c)** C57BL/6J mice were infected with $2x10^7$ *L. donovani* amastigotes i.v. and 27 days p.i. were subsequently injected with 100µg of FITC-dextran (in saline) i.v., spleen tissue was processed and imaged on the Aperio FL slide scanner under 20x magnification, scale bar 4mm. **d)** Schematic representation of the splenic MZ, adapted from Georg Kraal, 1992, *Int Rev Cytol*. **132**: 31-74, permission number 3763951413543

Figure 8

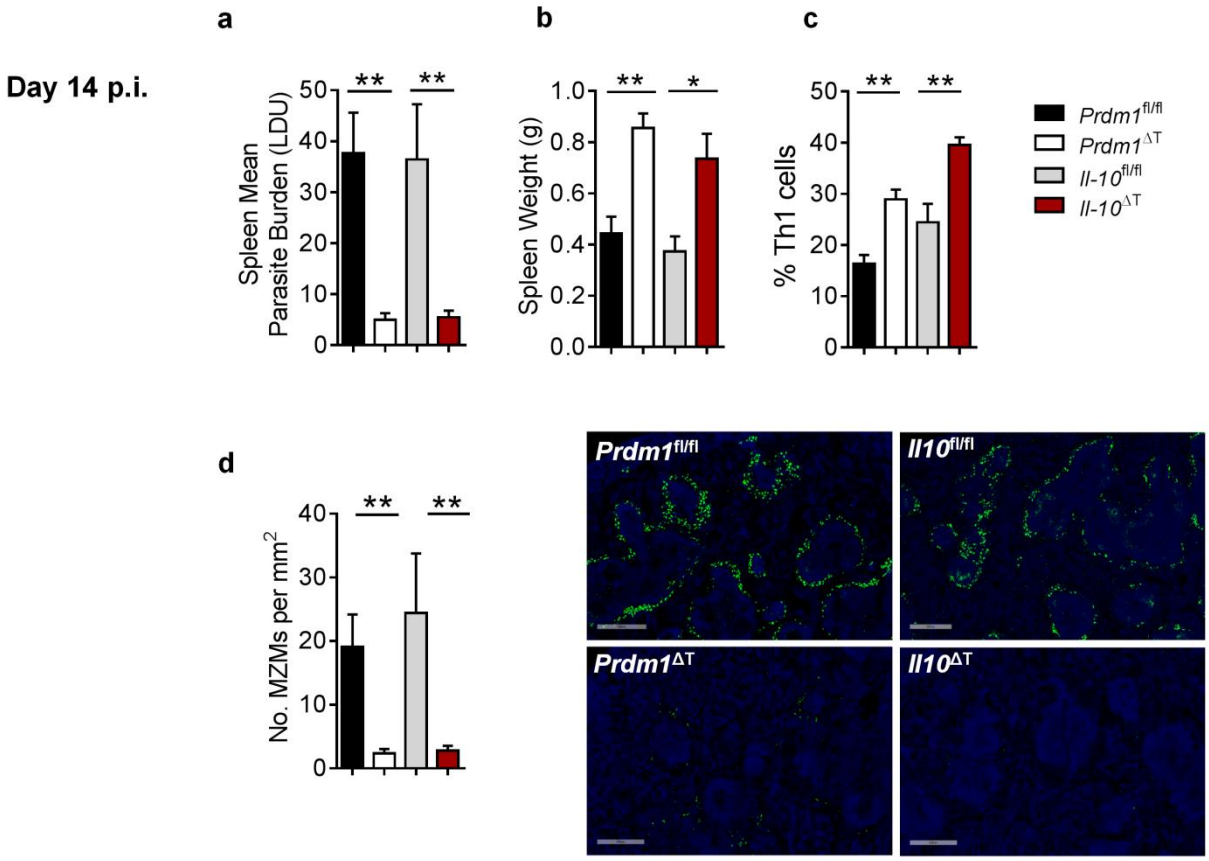


Figure 8: Blimp1-dependent IL-10 production by T cells limits pathological changes in the spleen during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$, *Prdm1*$^{fl/fl}$ mice and *Il10*$^{\Delta T}$ (T cell specific IL-10 deficient), *Il10*$^{fl/fl}$ (littermate controls) mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and spleen parasite burdens were expressed in LDU, 14 days p.i.. **b)** Spleen weights were recorded in grams **c)** frequency of Th1 cells were measured by flow cytometry in the spleen 14 days p.i.. **d)** number of MZM's per mm^2 was determined by injecting mice with 100µg of FITC-dextran 13 days p.i. and spleen tissue was processed for imaging on the Aperio FL slide scanner under 20x magnification, scale bars 500µm, 14 days p.i.. Representative of 2 independent experiments, mean ± SEM, n= 5-6 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

5.3.6 Blimp1is required to regulate TNF production by CD11b^{+}Ly6C^{hi} cells in the spleen during *L. donovani* infection

During remodelling of the MZ, the distribution of TNF production by various cells is widespread, where monocytes, macrophages, DC's, B cells and T cells can all produce varying amounts of TNF[135, 136, 137, 138]. It remains unclear whether a particular cellular source of TNF plays protective and or pathogenic roles. Previously, we had shown that Blimp1 was required for regulating TNF production by CD4^{+} T cells (**Fig 2, 3**). We next investigated whether Blimp1 was also involved in modulating TNF production by non-T cell populations, including inflammatory monocyte-like populations (CD11b^{+} Ly6C^{hi}), B cells (B220^{+} CD19^{+}) and cDC's (CD11c^{hi} MHCII^{hi}) (**Fig 9**). For the purposes of this basic analysis, the CD11b^{+} Ly6C^{lo} population was not analysed due to the absence of the Ly6G surface marker, to discriminate between neutrophils and patrolling monocytes. Interestingly, Blimp1 impaired TNF production by CD11b^{+} Ly6C^{hi} cells in the spleen 14 days p.i. (**Fig 10a**), similar to CD4^{+} T cells (**Fig 2e**), but did not suppress TNF production by B cells (**Fig 10b**) or cDC's (**Fig 10c**) 14 days p.i.. These findings suggest that there are cell-specific requirements for Blimp1-mediated regulation of TNF production, at a time when there is accelerated disruption to the splenic architecture. We next assessed levels of chemokines and cytokines involved in monocyte recruitment and function[387, 388] in supernatants of splenocytes from *Prdm1*$^{\Delta T}$ mice and their littermate controls cultured in the presence of fixed *L. donovani* amastigotes for 72 hours. *Prdm1*$^{\Delta T}$ mice exhibited significantly elevated levels of parasite-specific MCP-1 (**Fig 10d**), modest increases in IL-1β (**Fig 10e**) and significantly elevated levels of IL-6 (**Fig 10f**), further supporting the idea that Blimp1 regulates inflammatory responses during *L. donovani* infection. Taken together, these data suggest that at 14 day p.i., when the early loss of MZM's is observed in the *Prdm1*$^{\Delta T}$ mice, CD11b^{+} Ly6C^{hi} cells from these mice produce significantly more TNF, compared with controls, which along with CD4^{+} T cell-derived TNF may contribute to MZM loss. Furthermore, significant increases in parasite-specific MCP-1 and IL-6 production by splenocytes from *Prdm1*$^{\Delta T}$ mice, compared to controls, also points to the involvement of monocytes in the early MZM loss in the spleen.

Figure 9

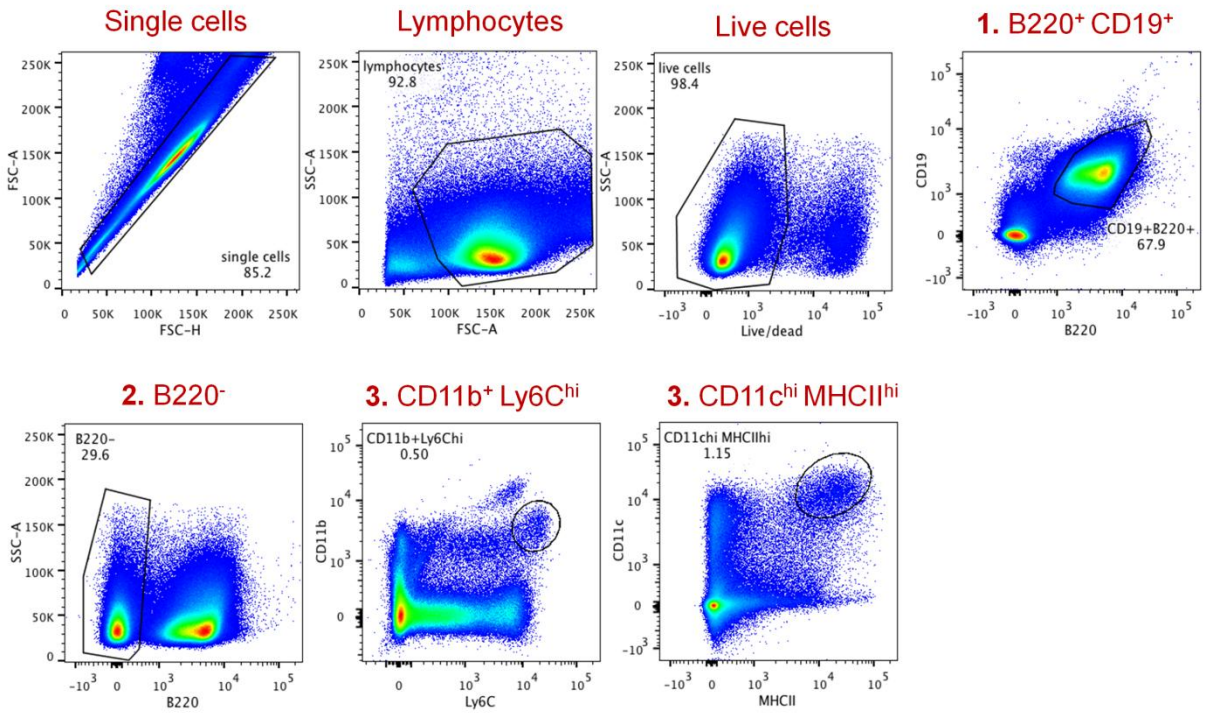


Figure 9: Gating strategy for B cells, monocytes and cDC's in the spleen during *L. donovani* infection.

Left to right: Single cells, lymphocytes, viable (live) cells, **1.** (From live cells gate): B cells ($B220^+$ $CD19^+$), **2.** (From live cells gate): $B220^-$ (exclusion of B cells and pDC's), **3.** (From $B220^-$ gate): inflammatory monocyte-like cells ($CD11b^+$ $Ly6C^{hi}$) and cDC's ($CD11c^{hi}$ $MHCII^{hi}$).

Figure 10

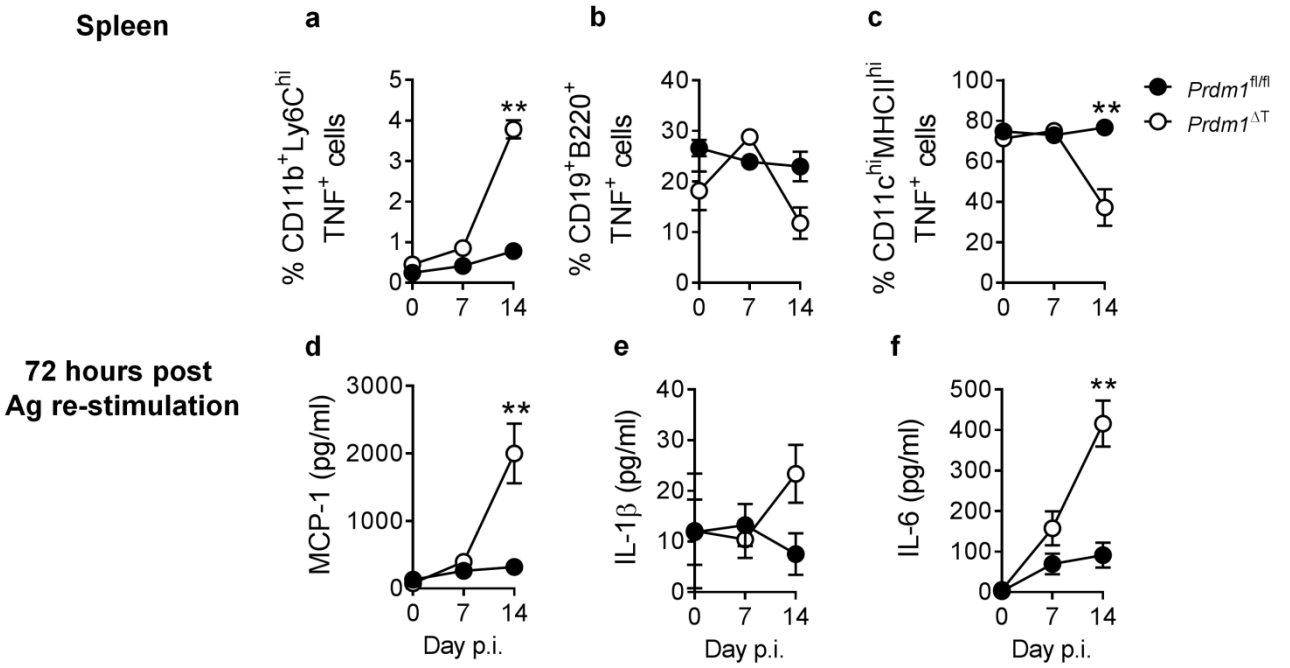


Figure 10: Blimp1 regulates TNF production by CD11b^{+}Ly6C^{hi} cells in the spleen during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^{7} *L. donovani* amastigotes i.v. and frequency of CD11b^{+} Ly6C^{hi} (inflammatory monocyte-like cells), **b)** B cells (B220^{+} CD19^{+}) and **c)** conventional DC's (CD11c^{hi} MHCII^{hi}) were measured by flow cytometry on days 7 and 14 p.i.. Splenocytes from *L. donovani*-infected *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were cultured in the presence of fixed *L. donovani* (4% PFA) amastigotes for 72 hours when culture supernatants were collected and **d)** MCP-1, **e)** IL-1β and **f)** IL-6 levels measured by using a BD Flex set CBA kit at 7 and 14 days p.i.. Representative of 2 independent experiments, mean ± SEM, n= 4-5 mice per group, **=p<0.01, Mann-Whitney U test.

5.3.7 IL-10 signalling to T cells and myeloid-derived cells protects MZM's against TNF mediated killing during *L. donovani* infection

The accelerated loss of MZM's in the absence of IL-10 (**Fig 8d),** prompted us to investigate whether IL-10 signalling to macrophages or other myeloid derived populations provided protection against exacerbated tissue pathology. Myeloid-specific IL-10R deficient (*Il10R*$^{\Delta M}$) mice infected with *L. donovani* exhibited significantly reduced parasite burdens (**Fig 11a**), and pronounced splenomegaly 14 days p.i. (**Fig 11b**), compared to litter mate controls. Improved parasite control and pathology was associated with impaired generation of Tr1 cells (**Fig 11c**), significantly increased frequencies of Th1 cells (**Fig 11d**), as well as significantly elevated levels of serum TNF and IFNγ (**Fig 11e**). Additionally, accelerated loss of MZM's was evident in the *Il10R*$^{\Delta M}$ mice (**Fig 11f**) and was associated with a significant increase in the frequency of TNF producing CD11b^{+} Ly6C^{hi} cells, 14 days p.i. (**Fig 11g**). We then asked whether IL-10 was signalling to the T cells themselves to protect host tissue from exacerbated pathology by infecting T cell specific IL-10R deficient (*Il10R*$^{\Delta T}$) mice with *L. donovani*. *Il10R*$^{\Delta T}$ mice also exhibited improved parasite control (**Fig 12a**), pronounced splenomegaly (**Fig 12b**), impaired Tr1 responses (**Fig 12c**) and significantly increased Th1 cell frequencies (**Fig 12d**). Interestingly, *Il10R*$^{\Delta T}$ mice did not have elevated serum TNF and IFNγ production (**Fig 12e**), yet displayed accelerated MZM loss 14 days p.i.(**Fig 12f)**, which was associated with significant increases in TNF production by CD11b^{+} Ly6C^{hi} (**Fig 12g**), suggesting that IL-10 signalling in T cells and myeloid-derived cells was required to limit TNF production by myeloid populations. Collectively, these results suggest that Blimp1-dependent IL-10 produced by Tr1 cells acts on the T cells themselves, as well as myeloid cells, possibly including MZM's, in order to protect the host tissue against TNF-mediated pathology.

Figure 11

Day 14 p.i.

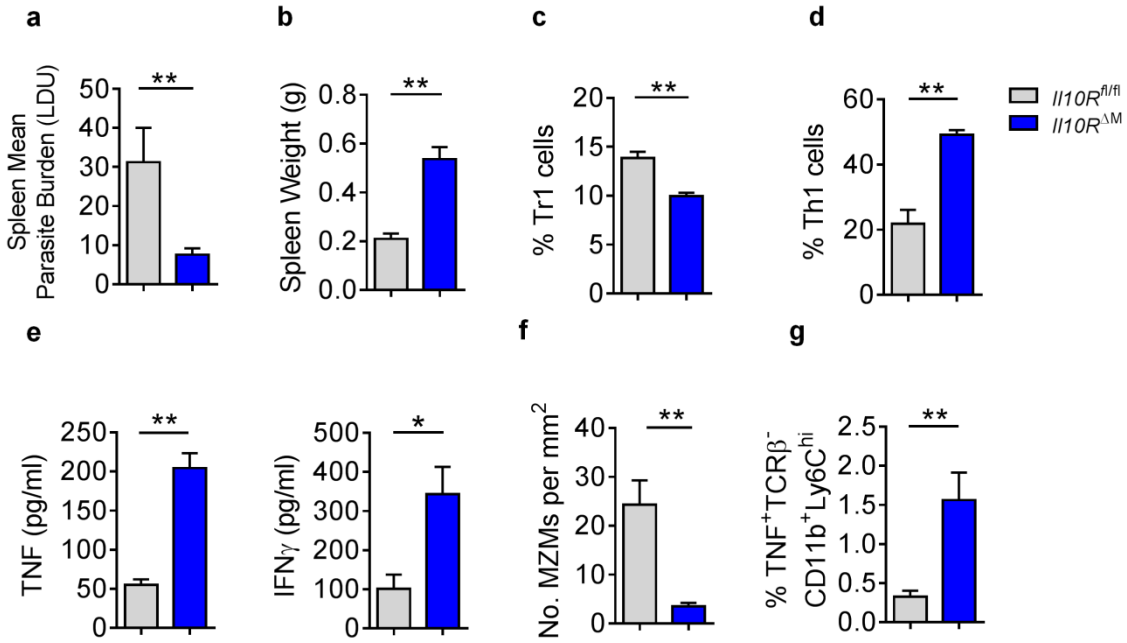


Figure 11: IL-10 signalling to myeloid-derived cells protects MZM's against TNF mediated killing during *L. donovani* infection.

a) *Il10R*$^{\Delta M}$ (Myeloid-specific IL-10R deficient) and *Il10R*$^{fl/fl}$ (littermate controls) mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and spleen parasite burdens were expressed in LDU, 14 days p.i.. **b)** Spleen weights recorded in grams **c)** Frequency of Tr1 cells measured by flow cytometry in the spleen 14 days p.i.. **d)** Frequency of Th1 cells measured by flow cytometry in the spleen 14 days p.i.. **e)** Serum TNF and IFNγ levels measured at day 14 p.i. using a BD Flex CBA set kit **f)** number of MZM's per mm^2 determined as above and imaged on the Aperio FL slide scanner under 20x magnification 14 days p.i.. **g)** Frequency of TNF producing TCRβ$^-$ CD11b$^+$ Ly6C^{hi} cells measured by flow cytometry in the spleen 14 days p.i.. Representative of 2 independent experiments, mean ± SEM, n= 5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

Figure 12

Day 14 p.i.

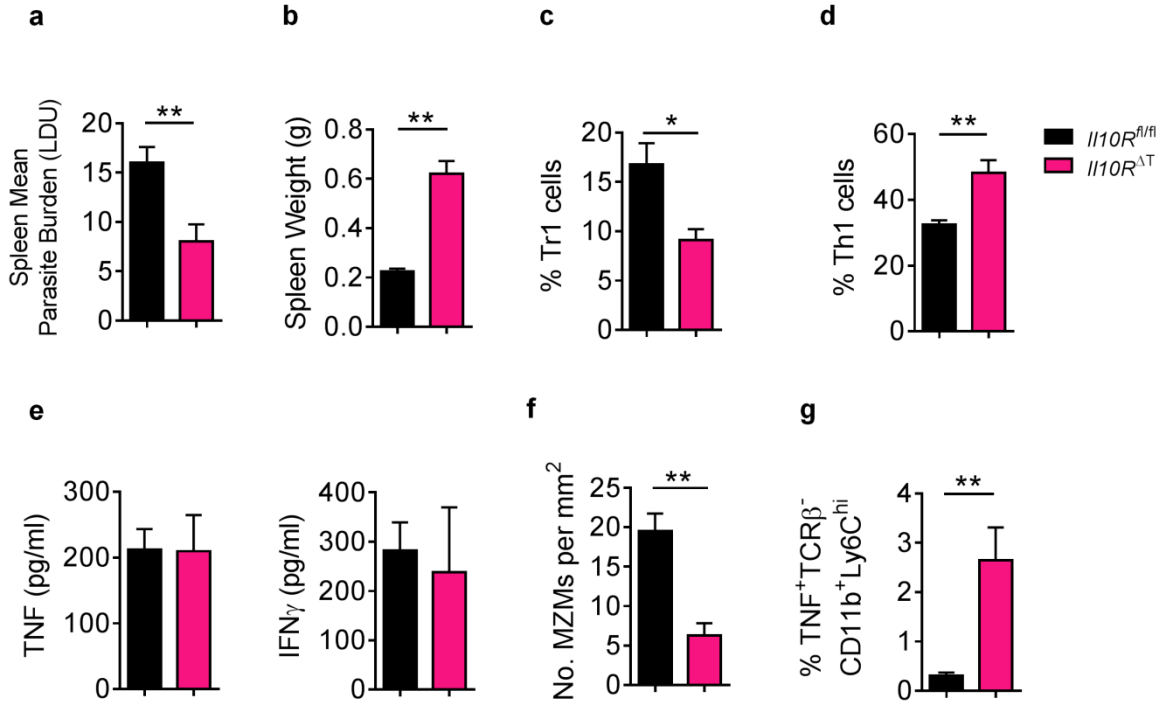


Figure 12: IL-10 signalling to T cells protects MZM's against TNF mediated killing during *L. donovani* infection.

a) *Il10R*$^{\Delta T}$ (T cell specific IL-10R deficient), *Il10R*$^{fl/fl}$ (littermate controls) mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and spleen parasite burdens were expressed in LDU. **b)** Spleen weights recorded in grams **c)** Frequency of Tr1 cells measured by flow cytometry in the spleen 14 days p.i.. **d)** Frequency of Th1 cells measured by flow cytometry in the spleen 14 days p.i.. **e)** Serum TNF and IFNγ levels measured at day 14 p.i. using a BD Flex set CBA kit **f)** number of MZM's per mm^2 determined as above and imaged on the Aperio FL slide scanner under 20x magnification 14 days p.i.. **g)** Frequency of TNF producing TCRβ$^-$ CD11b$^+$ Ly6C^{hi} cells measured by flow cytometry in the spleen 14 days p.i.. Representative of 1 independent experiment, mean ± SEM, n= 5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

5.3.8 Early MZM loss in the absence of IL-10 is TNF-dependent during *L. donovani* infection

Earlier studies demonstrated TNF was a major factor involved in the killing of splenic MZM's leading to a breakdown of the highly organised structure of the spleen and thereby impairing the trafficking of lymphocytes[129]. In order to test whether this early loss of MZM's in the *Prdm1*$^{\Delta T}$ mice was TNF-dependent, we infected *Prdm1*$^{\Delta T}$ mice with *L. donovani* and administered 200µg of Enbrel™ (anti-TNF) or control Intragam® (HuIgG) on the day of infection and every 2 days until day 14 p.i.. *Prdm1*$^{\Delta T}$ mice that underwent TNF blockade displayed significantly higher parasite burdens compared to the control HuIgG-treated mice (**Fig 13a**). In addition, TNF blockade resulted in *Prdm1*$^{\Delta T}$ mice exhibiting significantly reduced splenomegaly, relative to controls (**Fig 13b**), and this was associated with a significant increase in the number of MZM's per mm^2 at 14 days p.i. (**Fig 13c, d**). Thus, these data confirm that the early loss of MZM's in the absence of Blimp1 in T cells was TNF-dependent.

To address whether the breakdown in splenic architecture impaired the trafficking of lymphocytes in the *Prdm1*$^{\Delta T}$ mice, we transferred fluorescently-labelled splenocytes (grey) and employed the use of fluorescence microscopy to image T and B cell areas using CD3(blue) and B220 (yellow) antibodies to mark T and B cell zones, respectively (**Fig 13e**). *Prdm1*$^{\Delta T}$ mice treated with control HuIgG had significantly lower numbers of total lymphocytes, while TNF blockade partially restored the ability to retain lymphocytes in the spleen (**Fig 13e, panel 1, 13f**). Additionally, we found that TNF blockade also improved the trafficking of lymphocytes to the T and B cell areas (white pulp) in these mice (**Fig 13e panel 2, 13g**). Measurement of total white pulp areas showed that all areas were comparable between different treatment groups (**Fig 13h**). Therefore, the increase in the number of labelled-cells trafficking to these white pulp areas following TNF blockade did not result from changes in white pulp areas. Taken together, these results show that Blimp1-dependent IL-10 protects against TNF-mediated MZM loss, thereby preserving trafficking of lymphocytes through the T and B cell zones of the spleen. Furthermore, increased parasite burdens following TNF blockade, suggests that while Blimp1-dependent IL-10 protected against pathology, it also impaired parasite control.

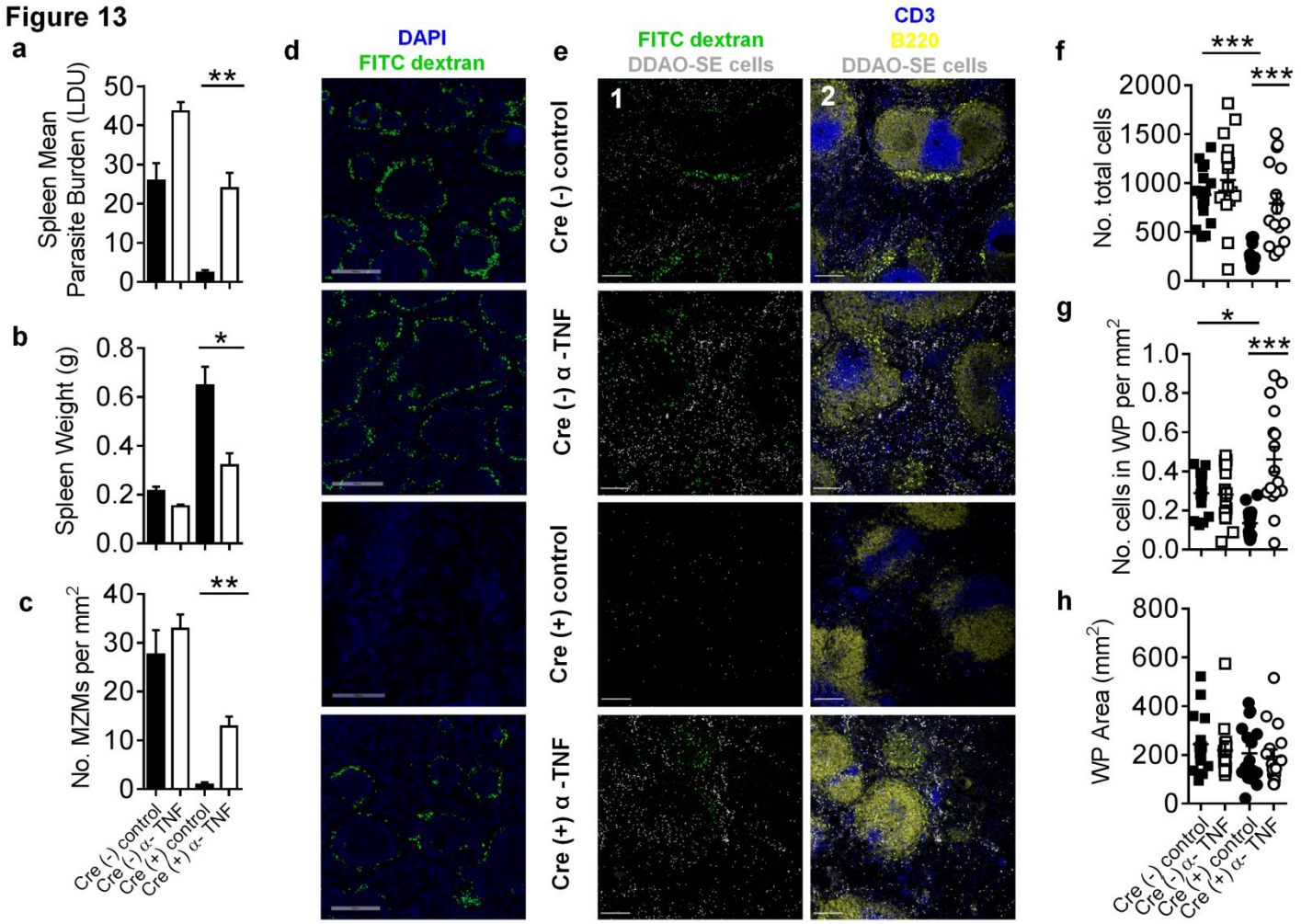


Figure 13: Early MZM loss in the absence of IL-10 is TNF-dependent during *L. donovani* infection.

a) *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and administered with 200µg of control HuIgG or anti-TNF (Enbrel^{TM}) i.p. before infection and every 2 days until day 14 p.i. Spleen parasite burdens expressed in LDU. **b)** Spleen weights recorded in grams 14 days p.i.. **c)** number of number of MZM's per mm^2 determined as above and **d)** imaged on the Aperio FL slide scanner under 20x magnification 14 days p.i. scale bars 500µm **e) Panel 1:** mice injected with FITC-dextran 13 days p.i. (one day prior to tissue collection). 2x10^7 DDAO-SE-labelled naive splenocytes (white-grey) were injected i.v. into mice 2 hours prior to collection of spleens on day 14 p.i.. **Panel 2:** All spleens were processed and 20µm sections were cut, stained with anti-CD3 (blue) and anti-B220 (yellow) antibodies before being mounted and imaged on the Carl Zeiss 780 NLO laser scanning confocal microscope under 10x magnification, scale bars 200µm. **f)** Total number of DDAO-SE$^+$ cells (4 fields of view per mouse). **g)** Number of total cells in white pulp (WP) divided by WP area in mm^2. **h)** Area of WP in mm^2 in all sections. Representative of 2 independent experiments, mean ± SEM, n= 5 mice per group (f = 4 images per mouse, therefore: 20 data points to obtain an average representation of each section), ***=p<0.001, **=p<0.01, *= p<0.05, Kruskal-Wallis test.

5.3.9 TNF blockade combined with anti-parasitic drug partially rescues pathology while maintaining anti-parasitic immunity

Rheumatoid arthritis patients or patients with inflammatory diseases are treated with drugs that inhibit TNF activity[389, 390, 391]. One important side-effect in these patients is their susceptibility to opportunistic infections[392, 393]. Given that TNF blockade resulted in increased parasite burdens, yet preserved splenic architecture, we next asked whether TNF blockade in combination with an anti-parasitic drug could serve as a potential therapeutic intervention. We hypothesised that the anti-parasitic drug, sodium stibogluconate (SSG, pentavalent antimonial) would combat parasite growth and the TNF blockade would preserve splenic architecture. C57BL/6J mice were infected with *L. donovani* and treated with TNF blockade or SSG beginning at day 14 p.i. in order to represent a situation where VL patients present to clinics in endemic areas with existing disease and or pathology. High dose of drug (500mg/kg of SSG) was administered on day 14 and day 21 p.i. and TNF blockade was administered every 2 days from day 14 p.i. until day 28 p.i. (**Fig 14**). Drug treatment alone and TNF blockade combined with drug resulted in significantly reduced parasite burdens in the liver and spleen 28 days p.i. (**Fig 15a, b**), but did not reduce splenomegaly (**Fig 15c**). Interestingly, while spleen weights remained unaffected, the number of MZM's per mm^2 was significantly improved upon TNF blockade combined with drug treatment, compared to drug alone (**Fig 15d**). Additionally, parasite-specific Th1 responses, measured by flow cytometry, revealed that TNF blockade combined with drug did not diminish the anti-parasitic benefit of drug treatment alone (**Fig 15e**). Furthermore, IFNγ levels produced in response to parasite were comparable between drug treatment alone and TNF blockade combined with drug (**Fig 15f**), suggesting that while TNF blockade alone would contribute to impaired immune responses, when administered in combination with drug, anti-parasitic immune responses could be sustained. Taken together, these data show the potential of employing TNF blockade in combination with drug to treat pathology in VL patients without compromising parasite control.

Figure 14

Figure 14: Combination treatment regimen of anti-TNF (Enbrel™) and drug (SSG).

C57BL/6J mice infected with $2x10^7$ *L. donovani* amastigotes i.v. and then administered with either 1: 200µg of control HuIgG (Intragam), 2: anti- TNF (Enbrel)* beginning at day 14 p.i. and every 2 days until day 28 p.i.. 3: 50mg/kg of sodium stibogluconate (SSG)# on day 14 p.i. and a second dose administered on day 21 p.i. or 4: anti-TNF + SSG (at indicated time-points)

Figure 15

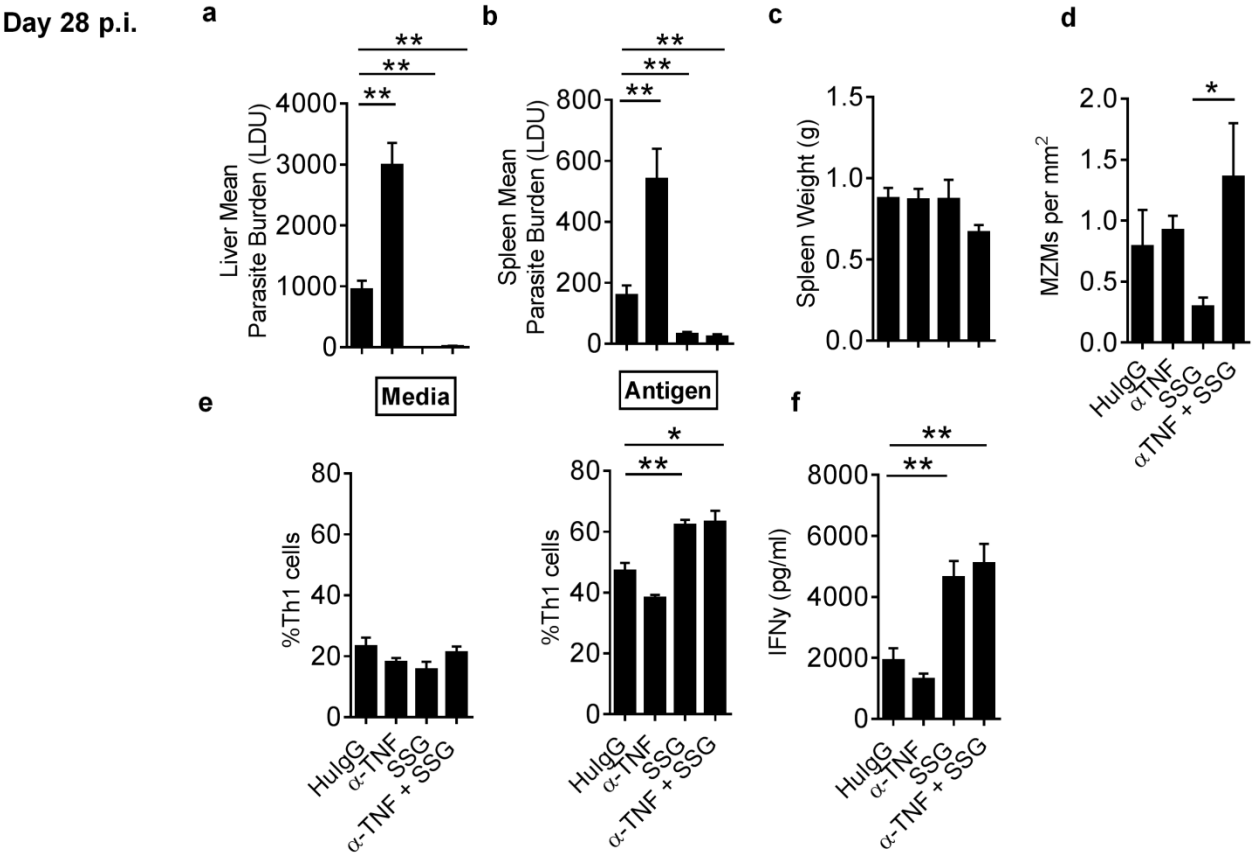


Figure 15: TNF blockade combined with anti-parasitic drug partially rescues pathology while maintaining anti-parasitic immunity.

a) Following the treatments outlined in Fig 14, liver parasite burdens were expressed in LDU, 28 days p.i.. **b)** Spleen parasite burdens also expressed in LDU 28 days p.i.. **c)** Spleen weights recorded in grams. **d)** Number of MZM's per mm^2 measured (as above) 28 days p.i.. **e)** Splenocytes from *L. donovani* infected C57BL/6J mice were cultured in the presence (antigen) or absence (media) of fixed *L. donovani* (4% PFA) amastigotes for 24 hours and subsequently, the frequency of Th1 cells was measured by flow cytometry. **f)** Culture supernatants were collected at 24 hours post antigen re-stimulation from (e) and IFNγ levels measured using a BD Flex set CBA kit on day 28 p.i.. Representative of 2 independent experiments, mean ± SEM, n= 4-5 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

5.3.10 IFNγ signalling is required for TNF production and consequently drives TNF-mediated tissue damage

Prdm1$^{\Delta T}$ mice had significantly higher serum TNF and IFNγ levels following *L. donovani* infection (**Fig 4a, c**), and following TNF blockade, splenic pathology was greatly reduced (**Fig 13**). Therefore, since improved parasite control and exacerbated pathology in mice lacking Blimp1 or IL-10 in T cells was associated with enhanced Th1 responses and elevated IFNγ and TNF levels, we examined the contribution of IFNγ signalling to the control of parasite growth and splenic pathology during *L. donovani* infection. Previous reports have shown that IFNγ is critical for controlling parasite growth in the liver[394]. Consistent with these reports, we also observed a significant increase in parasite burdens in the liver in the absence of IFNγ signalling (**Fig 16a**). Interestingly, while there was a modest decrease in splenic parasite burdens (**Fig 16b**), splenomegaly was significantly reduced in the IFNγR-deficient mice throughout the course of infection (**Fig 16c**). Associated with the reduction in splenomegaly, we also noted that MZM's were clearly retained and preserved in the IFNγR-deficient mice, 28 days p.i. compared to WT controls (**Fig 16d, e**). Furthermore, serum TNF levels were significantly reduced in the IFNγR-deficient mice, throughout the course of infection (**Fig 16f**), whereas IFNγ levels were significantly elevated 28 days p.i. (**Fig 16g**). Collectively, these results suggest that IFNγ signalling was required for inducing TNF production, and therefore, contributed to splenic pathology during *L. donovani* infection. Importantly, these results show that following *L. donovani* infection, IFNγ promoted TNF production, and this pathway was regulated by Blimp-1-mediated IL-10 production by T cells. Importantly, this regulatory pathway determined the balance between control of parasite growth and TNF-mediated pathology.

Figure 16

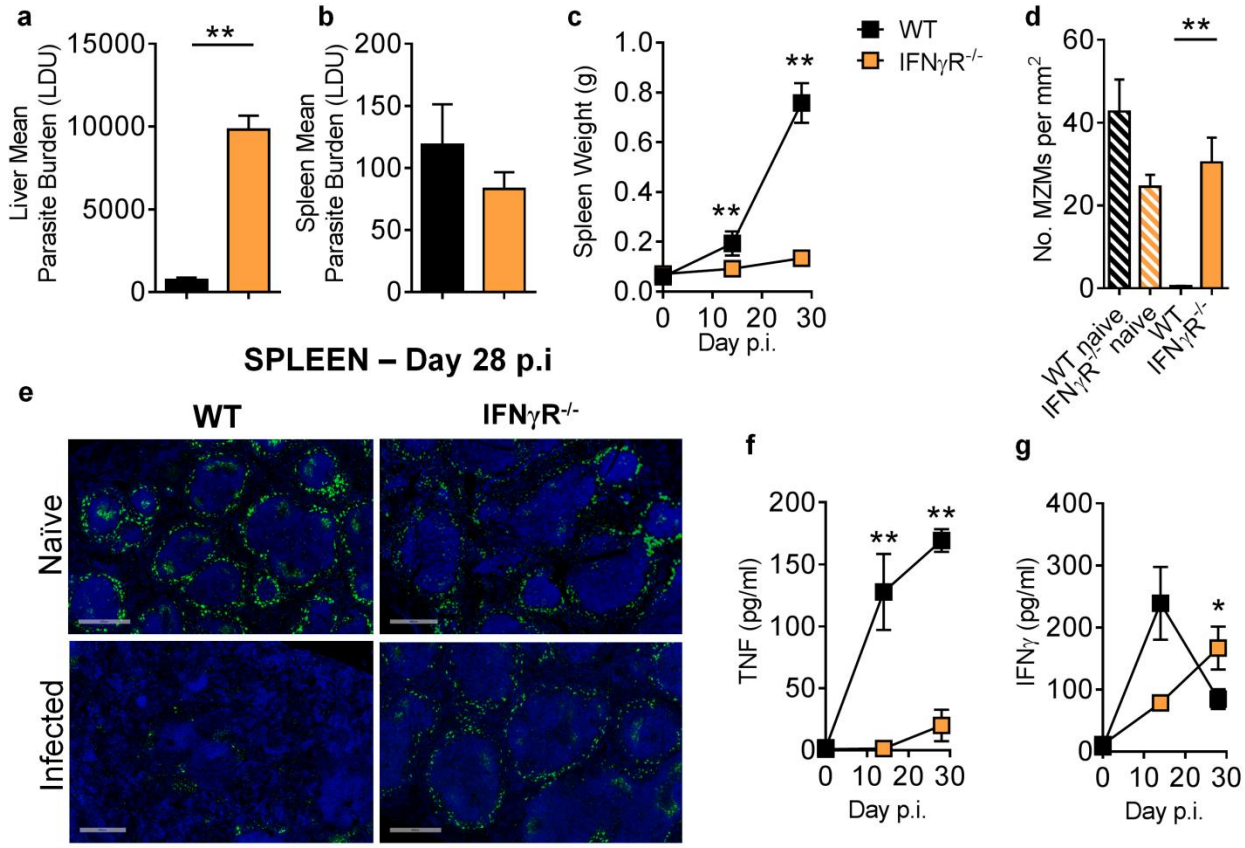


Figure 16: IFNγ signalling is required for TNF production and consequently drives TNF-mediated tissue damage.

a) WT and IFNγR deficient (IFNγR$^{-/-}$) mice were infected with 2x10^7 *L. donovani* amastigotes i.v. and liver parasite burdens expressed in LDU, 28 days p.i.. **b)** Spleen parasite burdens expressed in LDU, 28 days p.i.. **c)** Spleen weights recorded in grams at day 14 and 28 p.i. **d)** Number of MZM's per mm^2 were measured as described previously and **e)** imaged on the Aperio FL slide scanner under 20x magnification, scale bars 400µm 28 days p.i.. **f)** Serum TNF and **g)** IFNγ levels measured using a BD Flex set CBA kit 14 and 28 days p.i.. Representative of 2 independent experiments, mean ± SEM, n= 5-6 mice per group, **=p<0.01, *= p<0.05, Mann-Whitney U test.

Figure 17

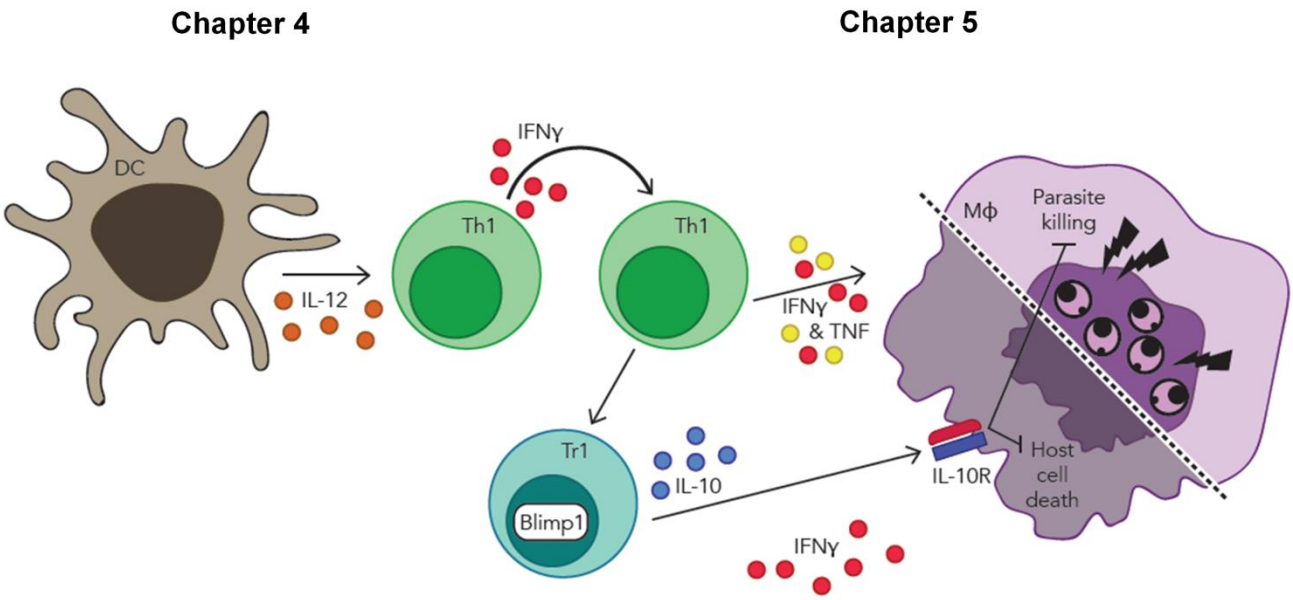


Figure 17: Blimp1-dependent IL-10 production by CD4$^+$ T cells protects against TNF-mediated pathology in the spleen.

IL-12 produced by DC's signals and is required to drive Th1 differentiation (Chapter 4) which subsequently induces Blimp1 expression in the Th1 cell population and therefore drives the induction of Tr1 cells during *Plasmodium* and *Leishmania* infections. Tr1 cell derived IL-10 limits the inflammatory cascade by signalling to macrophages to protect against TNF- mediated killing during *L. donovani* infection. Figure drawn by: S. Ng, Immunology and Infection Laboratory, QIMR Berghofer.

5.4 DISCUSSION

Blimp1 is central to various aspects of immune cell differentiation[395]. First identified in plasma cell function, the expanding roles for Blimp1 now includes involvement in T cell homeostasis, NK cell and DC maturation[242, 244, 245, 339]. Moreover, Blimp1 is required for the induction of T cell and Treg cell derived IL-10 production[181, 215, 216]. Here, we show that Blimp1-dependent IL-10 production by Tr1 cells is necessary to limit IFNγ-dependent, TNF-mediated tissue damage in the spleen during *L. donovani* infection (summarised in **Fig 17**). In this model of experimental visceral leishmaniasis, where splenomegaly is a TNF-mediated process, our results suggest that the highly inflammatory environment mediates control of parasite growth while at the expense of contributing host-tissue pathology. Collectively, these results suggest that while parasitic infections such as malaria and leishmaniasis induce potent inflammatory responses which are required for parasite control, in the absence of immunoregulatory molecules such as IL-10, excess inflammation exacerbates tissue-pathology and thus disrupts the splenic architecture.

IL-10 plays suppressive roles in multiple parasitic diseases, including *Leishmania* infections[218, 280, 385, 396]. T cell-specific Blimp1-deficient and IL-10-deficient mice exhibited significantly reduced parasite burdens, suggesting that Blimp1-dependent IL-10 impaired parasite control. However, these experiments also showed that the induction of IL-10 was important for preserving splenic architecture against inflammatory-mediated destruction. Furthermore, we found a significant increase in IL-10$^-$ Foxp3$^+$ CD4$^+$ Treg cells in the liver, 28 days p.i. in the *Prdm1*$^{\Delta T}$ mice. Additionally, we observed a significant increase in Tr1 frequencies in the liver of *Prdm1*$^{\Delta F}$ mice 28 days p.i.. Taken together, these data provide evidence for a compensatory mechanism where in the absence of Blimp1 in Treg cells, Tr1 cells emerge to counteract reduced Treg cell function due to the lack of IL-10. Mechanistically, this may occur via IL-2 consumption, since *Prdm1*$^{\Delta T}$ mice exhibit higher levels of IL-2[397] and thus contributing to the increase in Foxp3$^+$ CD4$^+$ Treg cell frequencies in the liver, 28 days p.i.. Interestingly, while Treg cell-derived IL-10 is known to play immunoregulatory roles in inflammatory settings, our results using the Treg cell-specific Blimp1-deficient mice showed that parasite burdens, organ weights and TNF and IFNγ levels were comparable with littermate controls, and therefore, the striking differences between the *Prdm1*$^{\Delta T}$ and littermate control mice were not caused by Blimp1 deficiency in Treg cells. Therefore, given that Treg cells appeared to play a minor role during infection, we focused on Tr1 cells and IL-10 signalling during *L. donovani* infection. IL-10 signals to T cells and myeloid cells, including MZM's, to provide protection against parasite induced inflammation.

In addition, the significant increase in Th1 cell frequencies in the *Il10R*$^{\Delta T}$ and *Il10R*$^{\Delta M}$ mice suggests that T cell derived-IFNγ signals to monocytes/macrophages to produce more TNF, and consequently contributes to the destruction of MZM's. TNF has been shown to be a mediator of splenic white pulp remodelling during *L. donovani* infection[129], and previously Ly6C^{+} inflammatory monocytes have been shown to be involved in the vascular remodelling of the spleen during *L. donovani* infection[133].

We also found that TNF production by inflammatory monocyte-like populations (CD11b^{+} Ly6C^{hi}) was significantly elevated in the absence of IL-10 signalling to T cells and myeloid populations, suggesting that IL-10-mediated control of TNF production by CD11b^{+} Ly6C^{hi} cells is important in minimising tissue damage during infection. However, in order to establish this definitively, studies need to be conducted with mice deficient for TNF specifically in myeloid and T cell populations. The early loss of MZM's in the T cell-specific Blimp1-deficient mice resulted in impaired trafficking of lymphocytes into the T and B cell areas of the white pulp compartment which was rescued by TNF blockade. Consistent with these results, earlier studies have shown MZM's to be key players in directing the trafficking of lymphocytes into white pulp areas[126, 398, 399]. Furthermore, in the absence of Blimp1 and IL-10 expression in T cells and IL-10R in T cells and myeloid cells, splenomegaly and loss of MZM was accelerated. During experimental VL, splenomegaly is accompanied by extensive vascular remodelling[132], mediated by neurotrophic receptor (Ntrk2) expression on splenic endothelial cells and interactions with ligands expressed by F4/80^{hi} CD11b^{lo} CD11c^{+} macrophages[364]. Moreover, macrophage-derived TNF has been previously reported to be involved in angiogenesis and vascular remodelling[400, 401], and therefore, taken together with our data, suggests that vascular remodelling in the spleen caused by TNF is regulated by Blimp1-dependent IL-10 production by Tr1 cells during *L. donovani* infection. Given that vascular remodelling is induced by inflammation[402, 403, 404], it is possible that the implications of these findings extend beyond infection-induced inflammation, and may apply to other chronic inflammatory diseases.

The immunoregulatory roles of IL-10 are well known, however, the cellular requirements for the induction of IL-10 in $CD4^+$ T cells has only recently been investigated in detail. Blimp1 was reported to play key roles in inducing IL-10 production by Tr1 cells (in LCMV and *T. gondii* models), which are thought to arise from a Th1 cell precursor that acquires self-regulatory capacity[215, 216]. However, these findings mainly focused on the upstream requirements for the generation of Tr1 cells, and while the authors discussed the self-regulating capacity of Tr1 cells, the biological and physiological consequence of this self-regulation was not studied. Parasitic infections such as those caused by *Plasmodium* and *Leishmania* species induce robust immune responses to kill parasites; however, parasite-induced inflammation also contributes to host tissue damage. Our results here show that Tr1 cells are induced in both *Plasmodium* (**Chapter 4**) and *Leishmania* infections as a means of limiting inflammation-induced immunopathology. Consistent with these findings, other studies have also reported on the induction of Tr1 cells in *Plasmodium*[47, 48, 232, 235] and *Leishmania*[214, 233, 234, 345] infections. A previous study had demonstrated that in the absence of IL-10, IL-17 was responsible for mediating immunopathology during *L. major* infection[405]. However, upon assessing serum IL-17 levels, as well as parasite-specific IL-17 production in the T cell specific Blimp1 deficient mice, no Blimp1-mediated effects on IL-17 production were observed (**Appendix 3**). Therefore, at least in our VL model, in the absence of Blimp1-dependent IL-10, IL-17 does not appear to contribute to the exacerbated pathology observed in the T cell-specific Blimp1-deficient mice. Furthermore, our data shows that Blimp1 induces IL-10 production by Tr1 cells which signals to macrophages to protect against TNF-mediated destruction of the splenic architecture. While limiting tissue pathology may seem like an obvious function of Tr1 cells, it will be important to fully understand the mechanism by which these immunoregulatory pathways develop and are maintained. A greater understanding of these pathways and networks will provide insight into how Tr1 cells can be modulated to protect against the inflammatory activities of TNF in inflammatory disorders.

The majority of VL patients that present to clinics exhibit pronounced signs of disease, and in particular, splenomegaly is a key pathological feature. We show that TNF is a key promoter this pathology, but also show that TNF blockade can exacerbate control of parasite growth. Therefore, development of targeted therapies that do not compromise parasite control is required. Given that TNF is a main driver of pathology and disease in VL patients[106], we explored the possibility of blocking TNF in conjunction with anti-parasitic drug in order to limit parasite growth, but protect host tissue against parasite-induced inflammation. Our results show that while TNF blockade did not compromise anti-parasitic immunity when combined with drug, it did not diminish parasite-specific IFNγ levels compared to drug alone. Thus, we have shown a potentially promising therapeutic application involving anti-TNF treatment combined with anti-parasitic drug. While, we showed a significant increase in the number of MZM's in mice that received drug and TNF blockade, it remains to be determined whether this partial preservation of MZM's is enough to restore lymphocyte trafficking to white pulp areas. Various cells are able to produce TNF during infection, including monocytes, macrophages, DC's, B cells and T cells[135, 136, 137, 138]. We found that disruption to the splenic architecture was associated with significant increases in TNF production by $CD4^+$ T cells and $CD11b^+$ $Ly6C^{hi}$ cells in the absence of Blimp1 and IL-10. Whether these cellular sources of TNF play protective and or pathogenic roles remains to be determined, and should be taken into careful consideration when developing therapeutic combination treatments against TNF.

Another promising immunotherapy approach being tested to treat inflammatory diseases is the use of Treg cells due to their immunoregulatory capacities[363, 406, 407]. However, some challenges of this approach include the fact that intranuclear expression of Foxp3 makes Treg cells difficult to purify. In addition, their frequencies range between 1-2% of human $CD4^+$ T cells and thus must be expanded *ex vivo* to gain sufficient numbers for therapeutic use and even then, repeated *in vitro* expansion may alter the phenotype and function of these cells[406, 408, 409]. The expansion of Treg cells from patients with rheumatoid arthritis or multiple sclerosis indicates there are phenotypic changes in the Treg cell populations isolated from these patients, leading to Treg instability[406]. Therefore, in a situation where Treg cell-based therapy is not feasible, the use of Tr1 cells may offer an alternative solution to treat disease. Data presented in this Chapter identifies Tr1 cells as critical regulators of inflammation-induced pathology and provides mechanistic insights into how Blimp1-induced IL-10 in Tr1 cells protects against TNF-mediated tissue damage.

Chapter 6

Final Discussion

The proposal of the Th1/Th2 paradigm in 1986[410] offered a simplistic view of $CD4^+$ T cell differentiation and regulation, based on their opposing actions. However, the discovery of Th1 and Th2 cells was only a small insight into the unique plasticity of $CD4^+$ T cells. While the Th1/Th2 paradigm provided a conceptual framework, the discovery of Th17, Tfh and Treg cells has propelled the field of $CD4^+$ T cell biology into an era where personalised medicine is becoming an attractive approach for the treatment of autoimmune, inflammatory and infectious diseases, as well as cancers. Additionally, the identification of IL-10 producing Th1 cells[199, 215, 216, 411, 412], as well as the ability of Treg cells to limit Th cell activity via mechanisms of migratory mimicry of the Th cell in question[180, 182, 184, 185], are examples of the ability of $CD4^+$ T cells to respond accordingly to their immediate environment.

Plasmodium and *Leishmania* infections activate potent $CD4^+$ T cell subsets that drive a myriad of inflammatory responses that mediate parasite control. However, in the absence of immunoregulatory mechanisms (IL-10 and Treg cells), the resulting immunopathology can influence disease outcome. Today, vaccine development and therapeutic medicine are at the forefront of medical research and it is therefore important to differentiate immune responses that are beneficial from those that pose detrimental outcomes. Therefore, the use of Treg cells has changed the field of immunotherapy by providing options for immunoregulation in a therapeutic setting.

Evidence of $CD4^+$ T cell suppression during malaria and VL has been extensively reported, thus highlighting the importance of identifying mechanisms to enhance anti-parasitic responses for vaccine development[47, 67, 275, 413, 414, 415]. Here, we have shown that type I IFN's suppress anti-parasitic $CD4^+$ T cell responses during first exposure to *P. falciparum* infection and instead promotes Tr1 cell development. In association with previous studies, results in this thesis show that first and/or early exposures induces immunoregulatory responses rather than anti-parasitic responses, since these same immunoregulatory networks appear to be established in young children living in malaria-endemic regions who have not yet acquired an adequate level of immunity to malaria[47, 48, 232, 235]. The implications of these findings extend to the limited efficacy (46%) of the RTS,S/A01 vaccine[12], where the early establishment of these immunoregulatory networks may impede anti-parasitic responses that serve to control parasite growth and thus limit disease progression .

Collectively, these findings provide valuable insights into vaccine development for other infectious diseases, such as leishmaniasis, but also immunotherapy where the therapeutic potential of Tr1 cells can be harnessed to treat inflammatory disease.

Recent studies have focused on the requirements (i.e. IL-27, IL-21, Blimp1, IL-10) for the generation of Tr1 cells during *T. gondii*[216] and LCMV infections[215]. Importantly, the combined effects of Tr1 cell induction and their regulatory actions on host tissue have not been studied in great detail. It is tempting to speculate on the immunoregulatory functions of Tr1 cells in limiting tissue pathology; however, as is the case with various chronic infectious diseases, where host tissue is destroyed, it is not clear whether Tr1 cells are able to restore tissue architecture as well as function. Tr1 cells limit pathology, however, whether this is enough to rescue protective immune responses at effector tissue sites remains to be empirically demonstrated.

In this thesis, it was demonstrated that the generation of Tr1 cells is dependent on IL-12 and Blimp1 which supports the concept that they represent a terminally differentiated Th1 $CD4^+$ T cell subset. T cell-specific Blimp1-deficient mice were employed to show the consequences of a failure to generate Tr1 cells in both *Plasmodium* and *Leishmania* infections. Impaired Tr1 responses were associated with extensive hepatosplenomegaly during *L. donovani* infection. In addition to this, TNF has been shown to be a driving force of pathology observed in the spleen[106, 129], suggesting that Tr1 cells play a protective role against TNF-mediated pathology. Taken together, these results suggest that during infection, DC's produce IL-12 to drive Th1 $CD4^+$ T cell differentiation. This results in an up-regulation of Blimp1 in Th1 cells to induce IL-10 production by these cells such that they acquire the capacity to secrete IL-10 in addition to IFNγ. Importantly, we show IL-10 derived from Tr1 cells is an important regulator of TNF-associated pathology. Furthermore, the preservation of splenic architecture during infection by TNF blockade rescued lymphocyte trafficking, suggesting Tr1 cells are involved in limiting pathology as well as restoring functional immune responses to homeostatic balance. Collectively, these findings show for the first time that the therapeutic potential of Tr1 cells extends beyond limiting inflammation and may be used to restore immune responses during infection. Moreover, these findings are of critical importance, where the use of Tr1 cells may be extended to other chronic inflammatory diseases.

TNF blockade as therapy is widely used to treat inflammatory disorders such as rheumatoid arthritis ankylosing spondylitis and plaque psoriasis[137]. However, the issue of immunosuppression is strikingly evident in patients treated with anti-TNF agents. Given that TNF is a main driver of pathology and disease in VL patients[106], we explored the possibility of blocking TNF in conjunction with anti-parasitic drug in order to limit parasite growth, but protect host tissue against parasite-induced inflammation. Mice that received drug plus anti-TNF exhibited significantly reduced parasite burdens, preserved splenic architecture and the frequency of IFNγ-producing Th1 cells was also increased relative to the control group. These findings demonstrate that TNF blockade in conjunction with drug treatment can potentially serve as a therapeutic strategy, where drug controls parasite growth and TNF blockade limits tissue pathology. These findings have broader implications, where an alternative strategy may be to distinguish cellular sources of TNF that promote pathology from those that control pathogen growth and then specifically target them with small molecules such as TNF inhibitors, as being tested by others[137].

According to the WHO, 80% of chronic disease deaths occur in low-middle income countries[416]. The National Institutes of Health (NIH) have identified 80-100 different autoimmune diseases and annual health care costs of up to $100 billion (USD) to treat these diseases[417, 418]. Furthermore, current first line therapies are not effective in all patients with autoimmune diseases and their different mechanisms of action make it difficult to choose the best treatment option for each patient[419]. Taken together, this presents an urgent need for the development of better treatment options for individuals with chronic infectious diseases such as malaria and leishmaniasis, as well as individuals suffering from autoimmune diseases. Importantly, the findings presented in this thesis have identified key immunoregulatory pathways in two important parasitic diseases that have the potential to be manipulated not only to improve infectious disease outcomes, but also have impacts on a broad range of chronic inflammatory diseases.

References

1. Kirchner W. The Black Death: New Insights into 18th Century Attitudes Toward Bubonic Plague. *Clinical Pediatrics* 1968, **7**(7)**:** 432-436.

2. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bulletin of the history of medicine* 2002, **76**(1)**:** 105-115.

3. Ebbesen P. The global epidemic of AIDS. *AIDS research* 1986, **2 Suppl 1:** S23-28.

4. Murray CJ, Ortblad KF, Guinovart C, Lim SS, Wolock TM, Roberts DA, *et al.* Global, regional, and national incidence and mortality for HIV, tuberculosis, and malaria during 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet (London, England)* 2014, **384**(9947)**:** 1005-1070.

5. Pigott DM, Bhatt S, Golding N, Duda KA, Battle KE, Brady OJ, *et al.* Global distribution maps of the leishmaniases. *eLife* 2014, **3:** e02851.

6. Carter R, Mendis KN. Evolutionary and historical aspects of the burden of malaria. *Clin Microbiol Rev* 2002, **15**(4)**:** 564-594.

7. World-Health-Organisation. World Malaria Report 2014. Geneva, Switzerland, World Health Organisation; 2014.

8. Centers-for-Disease-Control-and-Prevention. Impact of Malaria. 2015 [cited 1st December 2015]Available from: http://www.cdc.gov/malaria/malaria_worldwide/impact.html

9. Chanda E, Mzilahowa T, Chipwanya J, Mulenga S, Ali D, Troell P, *et al.* Preventing malaria transmission by indoor residual spraying in Malawi: grappling with the challenge of uncertain sustainability. *Malar J* 2015, **14:** 254.

10. Pluess B, Tanser FC, Lengeler C, Sharp BL. Indoor residual spraying for preventing malaria. *The Cochrane database of systematic reviews* 2010(4)**:** Cd006657.

11. Aponte JJ, Schellenberg D, Egan A, Breckenridge A, Carneiro I, Critchley J, *et al.* Efficacy and safety of intermittent preventive treatment with sulfadoxine-pyrimethamine for malaria in African infants: a pooled analysis of six randomised, placebo-controlled trials. *Lancet (London, England)* 2009, **374**(9700)**:** 1533-1542.

12. RTS SCTP. 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 (London, England)* 2015, **386**(9988)**:** 31-45.

13. Celli A. The history of malaria on the Roman Campagna from ancient times. *London, John Bale, Sons Danielsson* 1933.

14. Shortt HE, Fairley NH, Covell G, G. SP, Garnham PCC. The Pre-Erythrocytic Stage of *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1951, **44**(4)**:** 405-419.

15. Meis JF, Verhave JP, Jap PH, Sinden RE, H. MJ. Malaria Parasites-Discovery of the Early Liver Form *Nature* 1983, **302**(5907)**:** 424-426.

16. Perez-Mazliah D, Langhorne J. CD4 T-cell subsets in Malaria: TH1/TH2 revisited. *Frontiers in immunology* 2015, **5**.

17. Dvorak J, Miller L, Whitehouse W, Shiroishi T. Invasion of erythrocytes by malaria merozoites. *Science* 1975, **187**(4178)**:** 748-750.

18. Feldman RM, Singer C. Noncardiogenic Pulmonary Edema and Pulmonary Fibrosis in Falciparum Malaria. *Reviews of Infectious Diseases* 1987, **9**(1)**:** 134-139.

19. Joshi Y, Tandon B, Acharya S, Babu S, Tandon M. Acute hepatic failure due to Plasmodium falciparum liver injury. *Liver* 1986, **6**(6)**:** 357-360.

20. Oo M, Aikawa M, Than T, Aye T, Myint P, Igarashi I, *et al.* Human Cerebral Malaria: A Pathological Study *Journal of Neuropathology and Experimental Neurology* 1987, **46**(2)**:** 223-231.

21. World-Health-Organisation. Severe falciparum malaria. World Health Organisation, Communicable Diseases Cluster. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2000, **94:** S1-90.

22. Krishnan A, Karnad DR. Severe falciparum malaria: An important cause of multiple organ failure in Indian intensive care unit patients. *Critical Care Medicine* 2003, **31**(9)**:** 2278-2284 2210.1097/2201.CCM.0000079603.0000082822.0000079669.

23. Krishna S, Waller DW, Kuile Ft, Kwiatkowski D, Crawley J, Craddock CFC, *et al.* Lactic acidosis and hypoglycaemia in children with severe malaria: pathophysiological and prognostic significance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1994, **88**(1)**:** 67-73.

24. White NJ, Warrell DA, Chanthavanich P, Looareesuwan S, Warrell MJ, Krishna S, *et al.* Severe Hypoglycemia and Hyperinsulinemia in Falciparum Malaria. *New England Journal of Medicine* 1983, **309**(2)**:** 61-66.

25. Kraemer SM, Smith JD. A family affair: var genes, PfEMP1 binding, and malaria disease. *Current opinion in microbiology* 2006, **9**(4)**:** 374-380.

26. Hunt NH, Ball HJ, Hansen AM, Khaw LT, Guo J, Bakmiwewa S, *et al.* Cerebral malaria: gamma-interferon redux. *Frontiers in cellular and infection microbiology* 2014, **4:** 113.

27. Flick K, Chen Q. var genes, PfEMP1 and the human host. *Molecular and biochemical parasitology* 2004, **134**(1)**:** 3-9.

28. Turner GD, Morrison H, Jones M, Davis TM, Looareesuwan S, Buley ID, *et al.* An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. *Am J Pathol* 1994, **145**(5)**:** 1057-1069.

29. Berendt AR, Simmons DL, Tansey J, Newbold CI, Marsh K. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature* 1989, **341**(6237)**:** 57-59.

30. Smith T, Felger I, Tanner M, Beck HP. 11. Premunition in Plasmodium falciparum infection: insights from the epidemiology of multiple infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1999, **93, Supplement 1**(0)**:** 59-64.

31. Soe S, Khin Saw A, Htay A, Nay W, Tin A, Than S, *et al.* Premunition against Plasmodium falciparum in a malaria hyperendemic village in Myanmar. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2001, **95**(1)**:** 81-84.

32. Langhorne J, Ndungu FM, Sponaas A-M, Marsh K. Immunity to malaria: more questions than answers. *Nat Immunol* 2008, **9**(7)**:** 725-732.

33. Hansen DS, Schofield L. Natural Regulatory T Cells in Malaria: Host or Parasite Allies? *PLoS Pathog* 2010, **6**(4)**:** e1000771.

34. Garnham PCC. Malarial immunity in Africans; effects in infancy and early childhood. *Annals of tropical medicine and parasitology* 1949, **43**(1)**:** 47-61.

35. Moormann AM, Sumba PO, Chelimo K, Fang H, Tisch DJ, Dent AE, *et al.* Humoral and cellular immunity to Plasmodium falciparum merozoite surface protein 1 and protection from infection with blood-stage parasites. *The Journal of infectious diseases* 2013, **208**(1)**:** 149-158.

36. Cohen S. Gamma-globulin and acquired immunity to human malaria. *Nature (London)* 1961, **192:** 733-737.

37. Amante FH, Haque A, Stanley AC, Rivera FD, Randall LM, Wilson YA, *et al.* Immune-Mediated Mechanisms of Parasite Tissue Sequestration during Experimental Cerebral Malaria. *Journal of Immunology* 2010, **185**(6)**:** 3632-3642.

38. Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *Journal of immunology (Baltimore, Md : 1950)* 1996, **157**(4)**:** 1620-1624.

39. Hermsen C, van de Wiel T, Mommers E, Sauerwein R, Eling W. Depletion of CD4+ or CD8+ T-cells prevents Plasmodium berghei induced cerebral malaria in end-stage disease. *Parasitology* 1997, **114 (Pt 1):** 7-12.

40. Voller A. Immunopathology of malaria. *Bulletin of the World Health Organization* 1974, **50**(3-4)**:** 177-186.

41. Winkel KD, Good MF. Inability of Plasmodium vinckei-immune spleen cells to transfer protection to recipient mice exposed to vaccine 'vectors' or heterologous species of plasmodium. *Parasite Immunol* 1991, **13**(5)**:** 517-530.

42. Kumar S, Good MF, Dontfraid F, Vinetz JM, Miller LH. Interdependence of CD4+ T cells and malarial spleen in immunity to Plasmodium vinckei vinckei. Relevance to vaccine development. *Journal of immunology (Baltimore, Md : 1950)* 1989, **143**(6)**:** 2017-2023.

43. Weiss L. Mechanisms of splenic control of murine malaria: cellular reactions of the spleen in lethal (strain 17XL) Plasmodium yoelii malaria in BALB/c mice, and the consequences of pre-infective splenectomy. *Am J Trop Med Hyg* 1989, **41**(2)**:** 144-160.

44. Engwerda CR, Beattie L, Amante FH. The importance of the spleen in malaria. *Trends in Parasitology* 2005, **21**(2)**:** 75-80.

45. Langhorne J, Meding SJ, Eichmann K, Gillard SS. THE RESPONSE OF CD4+ T-CELLS TO PLASMODIUM-CHABAUDI-CHABAUDI. *Immunological Reviews* 1989, **112:** 71-94.

46. Amante FH, Stanley AC, Randall LM, Zhou Y, Haque A, McSweeney K, *et al.* A Role for Natural Regulatory T Cells in the Pathogenesis of Experimental Cerebral Malaria. *The American Journal of Pathology* 2007, **171**(2)**:** 548-559.

47. Jagannathan P, Eccles-James I, Bowen K, Nankya F, Auma A, Wamala S, *et al.* IFNγ/IL-10 Co-producing Cells Dominate the CD4 Response to Malaria in Highly Exposed Children. *PLoS Pathog* 2014, **10**(1)**:** e1003864.

48. Boyle MJ, Jagannathan P, Bowen K, McIntyre TI, Vance HM, Farrington LA, *et al.* Effector Phenotype of Plasmodium falciparum-Specific CD4+ T Cells Is Influenced by Both Age and Transmission Intensity in Naturally Exposed Populations. *The Journal of infectious diseases* 2015, **212**(3)**:** 416-425.

49. Cabantous S, Poudiougou B, Traore A, Keita M, Cisse MB, Doumbo O, *et al.* Evidence that interferon-gamma plays a protective role during cerebral malaria. *The Journal of infectious diseases* 2005, **192**(5)**:** 854-860.

50. Charoenvit Y, Majam VF, Corradin G, Sacci JB, Wang R, Doolan DL, *et al.* CD4(+) T-Cell- and Gamma Interferon-Dependent Protection against Murine Malaria by Immunization with Linear Synthetic Peptides from a Plasmodium yoelii 17-Kilodalton Hepatocyte Erythrocyte Protein. *Infection and Immunity* 1999, **67**(11)**:** 5604-5614.

51. Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage Plasmodium chabaudi AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. *Journal of immunology (Baltimore, Md : 1950)* 1995, **155**(5)**:** 2545-2556.

52. Artavanis-Tsakonas K, Riley EM. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes. *Journal of immunology (Baltimore, Md : 1950)* 2002, **169**(6)**:** 2956-2963.

53. Robinson LJ, D'Ombrain MC, Stanisic DI, Taraika J, Bernard N, Richards JS, *et al.* Cellular Tumor Necrosis Factor, Gamma Interferon, and Interleukin-6 Responses as Correlates of Immunity and Risk of Clinical Plasmodium falciparum Malaria in Children from Papua New Guinea. *Infection and Immunity* 2009, **77**(7)**:** 3033-3043.

54. Meding SJ, Cheng SC, Simon-Haarhaus B, Langhorne J. Role of gamma interferon during infection with Plasmodium chabaudi chabaudi. *Infect Immun* 1990, **58**(11)**:** 3671-3678.

55. Horowitz A, Newman KC, Evans JH, Korbel DS, Davis DM, Riley EM. Cross-talk between T cells and NK cells generates rapid effector responses to Plasmodium falciparum-infected erythrocytes. *Journal of immunology (Baltimore, Md : 1950)* 2010, **184**(11)**:** 6043-6052.

56. Chizzolini C, Grau GE, Geinoz A, Schrijvers D. T lymphocyte interferon-gamma production induced by Plasmodium falciparum antigen is high in recently infected non-immune and low in immune subjects. *Clinical and experimental immunology* 1990, **79**(1)**:** 95-99.

57. Ing R, Stevenson MM. Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage Plasmodium chabaudi AS infection in mice. *Infect Immun* 2009, **77**(2)**:** 770-782.

58. Roestenberg M, McCall M, Hopman J, Wiersma J, Luty AJ, van Gemert GJ, *et al.* Protection against a malaria challenge by sporozoite inoculation. *The New England journal of medicine* 2009, **361**(5)**:** 468-477.

59. Teirlinck AC, McCall MB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, *et al.* Longevity and composition of cellular immune responses following experimental Plasmodium falciparum malaria infection in humans. *PLoS Pathog* 2011, **7**(12)**:** e1002389.

60. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BJ, *et al.* Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 2007, **13**(7)**:** 843-850.

61. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-Cytokine-Producing Antiviral CD4 T Cells Are Functionally Superior to Single-Cytokine-Producing Cells. *Journal of Virology* 2007, **81**(16)**:** 8468-8476.

62. Beveridge NER, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, *et al.* Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional Mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations. *European Journal of Immunology* 2007, **37**(11)**:** 3089-3100.

63. Boaz MJ. Presence of HIV-1 Gag-specific IFN-gamma IL-2 and CD28 IL-2 CD4 T cell responses is associated with nonprogression in HIV-1 infection. *The Journal of immunology (1950)* 2002, **169**(11)**:** 6376-6385.

64. Younes S-A, Yassine-Diab B, Dumont AR, Boulassel M-R, Grossman Z, Routy J-P, *et al.* HIV-1 Viremia Prevents the Establishment of Interleukin 2–producing HIV-specific Memory CD4+ T Cells

Endowed with Proliferative Capacity. *The Journal of Experimental Medicine* 2003, **198**(12)**:** 1909-1922.

65. Bogdan C, Moll H, Solbach W, Röllinghoff M. Tumor necrosis factor-α in combination with interferon-γ, but not with interleukin 4 activates murine macrophages for elimination of Leishmania major amastigotes. *European Journal of Immunology* 1990, **20**(5)**:** 1131-1135.

66. Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *The Journal of Experimental Medicine* 1992, **175**(4)**:** 1111-1122.

67. Haque A, Best SE, Ammerdorffer A, Desbarrieres L, de Oca MM, Amante FH, *et al.* Type I interferons suppress CD4+ T-cell-dependent parasite control during blood-stage *Plasmodium* infection. *European Journal of Immunology* 2011, **41**(9)**:** 2688-2698.

68. Haque A, Best SE, Montes de Oca M, James KR, Ammerdorffer A, Edwards CL, *et al.* Type I IFN signaling in CD8- DCs impairs Th1-dependent malaria immunity. *The Journal of clinical investigation* 2014, **124**(6)**:** 2483-2496.

69. Stevenson MM, Riley EM. Innate immunity to malaria. *Nature Reviews Immunology* 2004, **4**(3)**:** 169-180.

70. Engwerda CE, Belnoue E, C. GA, Renia L. Experimental Models of Cerebral Malaria. *Current Topics in Microbiology and Immunology* 2005, **297:** 103-143.

71. Taylor-Robinson AW. Regulation of immunity to Plasmodium: Implications from mouse models for blood stage malaria vaccine design. *Experimental Parasitology* 2010, **126**(3)**:** 406-414.

72. Stephens R, Culleton RL, Lamb TJ. The contribution of Plasmodium chabaudi to our understanding of malaria. *Trends in Parasitology* 2012, **28**(2)**:** 73-82.

73. Vaughan AM, Kappe SH, Ploss A, Mikolajczak SA. Development of humanized mouse models to study human malaria parasite infection. *Future microbiology* 2012, **7**(5)**:** 657-665.

74. Freitas do Rosário AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, *et al.* IL-27 Promotes IL-10 Production by Effector Th1 CD4+ T Cells: A Critical Mechanism for Protection from Severe Immunopathology during Malaria Infection. *The Journal of Immunology* 2012, **188**(3)**:** 1178-1190.

75. Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, Kamanaka M, *et al.* IL-10 from CD4+CD25-Foxp3-CD127- adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog* 2008, **4**(2)**:** e1000004.

76. Meding SJ, Langhorne J. CD4+ T-CELLS B-CELLS ARE NECESSARY FOR THE TRANSFER OF PROTECTIVE IMMUNITY TO PLASMODIUM-CHABAUDI-CHABAUDI. *European Journal of Immunology* 1991, **21**(6)**:** 1433-1438.

77. Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol* 2015, **15**(3)**:** 185-189.

78. Wikenheiser DJ, Ghosh D, Kennedy B, Stumhofer JS. The Costimulatory Molecule ICOS Regulates Host Th1 and Follicular Th Cell Differentiation in Response to Plasmodium chabaudi chabaudi AS Infection. *Journal of immunology (Baltimore, Md : 1950)* 2016, **196**(2)**:** 778-791.

79. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 2011, **34**(6)**:** 932-946.

80. Leavenworth JW, Verbinnen B, Yin J, Huang H, Cantor H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* 2015, **16**(1)**:** 96-106.

81. Xu H, Li X, Liu D, Li J, Zhang X, Chen X, *et al.* Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. *Nature* 2013, **496**(7446)**:** 523-527.

82. Perez-Mazliah D, Ng DH, Freitas do Rosario AP, McLaughlin S, Mastelic-Gavillet B, Sodenkamp J, *et al.* Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria. *PLoS Pathog* 2015, **11**(3)**:** e1004715.

83. White NJ, Turner GDH, Medana IM, Dondorp AM, Day NPJ. The murine cerebral malaria phenomenon. *Trends in Parasitology* 2010, **26**(1)**:** 11-15.

84. Thumwood CM, Hunt NH, Clark IA, Cowden WB. Breakdown of the blood-brain barrier in murine cerebral malaria. *Parasitology* 1988, **96**(03)**:** 579-589.

85. Etienne-Manneville S, Manneville J-B, Adamson P, Wilbourn B, Greenwood J, Couraud P-O. ICAM-1-Coupled Cytoskeletal Rearrangements and Transendothelial Lymphocyte Migration Involve Intracellular Calcium Signaling in Brain Endothelial Cell Lines. *The Journal of Immunology* 2000, **165**(6)**:** 3375-3383.

86. Haque A, Best SE, Unosson K, Amante FH, de Labastida F, Anstey NM, *et al.* Granzyme B Expression by CD8+ T Cells Is Required for the Development of Experimental Cerebral Malaria. *The Journal of Immunology* 2011, **186**(11)**:** 6148-6156.

87. Nitcheu J, Bonduelle O, Combadiere C, Tefit M, Seilhean D, Mazier D, *et al.* Perforin-Dependent Brain-Infiltrating Cytotoxic CD8+ T Lymphocytes Mediate Experimental Cerebral Malaria Pathogenesis. *The Journal of Immunology* 2003, **170**(4)**:** 2221-2228.

88. Potter S, Chan-Ling T, Ball HJ, Mansour H, Mitchell A, Maluish L, *et al.* Perforin mediated apoptosis of cerebral microvascular endothelial cells during experimental cerebral malaria. *International Journal for Parasitology* 2006, **36**(4)**:** 485-496.

89. Belnoue E, Kayibanda M, Vigario AM, Deschemin J-C, Rooijen Nv, Viguier M, *et al.* On the Pathogenic Role of Brain-Sequestered αβ CD8+ T Cells in Experimental Cerebral Malaria. *The Journal of Immunology* 2002, **169**(11)**:** 6369-6375.

90. Lovegrove FE, Gharib SA, Peña-Castillo L, Patel SN, Ruzinski JT, Hughes TR, *et al.* Parasite Burden and CD36-Mediated Sequestration Are Determinants of Acute Lung Injury in an Experimental Malaria Model. *PLoS Pathog* 2008, **4**(5)**:** e1000068.

91. Epiphanio S, Campos MG, Pamplona A, Carapau D, Pena AC, Ataíde R, *et al.* VEGF Promotes Malaria-Associated Acute Lung Injury in Mice. *PLoS Pathog* 2010, **6**(5)**:** e1000916.

92. Jacobs T, Plate T, Gaworski I, Fleischer B. CTLA-4-dependent mechanisms prevent T cell induced-liver pathology during the erythrocyte stage of Plasmodium berghei malaria. *European Journal of Immunology* 2004, **34**(4)**:** 972-980.

93. Holloway PAH, Krishna S, White NJ. Plasmodium berghei: Lactic acidosis and hypoglycaemia in a rodent model of severe malaria; effects of glucose, quinine, and dichloroacetate. *Experimental Parasitology* 1991, **72**(2)**:** 123-133.

94. Pourahmad M, Hooshmand F, Rahiminejad M. Cutaneous leishmaniasis associated with visceral leishmaniasis in a case of acquired immunodeficiency syndrome (AIDS). *International journal of dermatology* 2009, **48**(1)**:** 59-61.

95. Bhattacharya P, Ali N. Involvement and interactions of different immune cells and their cytokines in human visceral leishmaniasis. *Revista da Sociedade Brasileira de Medicina Tropical* 2013, **46**(2)**:** 128-134.

96. World-Health-Organisation. Leishmaniasis, Factsheet No. 375. In: WHO, editor. Geneva, Switzerland: WHO; 2015.

97. Zijlstra EE. 3. Visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2001, **95:** S27-S58.

98. Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet J-P, *et al.* The Relationship between Leishmaniasis and AIDS: the Second 10 Years. *Clinical Microbiology Reviews* 2008, **21**(2)**:** 334-359.

99. World-Health-Organisation. Control of the leishmaniases. *World Health Organisation technical report series* 2010, **949**(XII-XIII)**:** 1-186.

100. Roatt BM, Aguiar-Soares RD, Coura-Vital W, Ker HG, Moreira N, Vitoriano-Souza J, *et al.* Immunotherapy and Immunochemotherapy in Visceral Leishmaniasis: Promising Treatments for this Neglected Disease. *Frontiers in immunology* 2014, **5:** 272.

101. Okwor I, Uzonna JE. The immunology of Leishmania/HIV co-infection. *Immunologic research* 2013, **56**(1)**:** 163-171.

102. Faleiro RJ, Kumar R, Hafner LM, Engwerda CR. Immune regulation during chronic visceral leishmaniasis. *PLoS Negl Trop Dis* 2014, **8**(7)**:** e2914.

103. World-Health-Organisation. WHO Leishmaniasis control programme, annual country reports (2013). Geneva, Switzerland; 2013.

104. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nature reviews Microbiology* 2011, **9**(8)**:** 604-615.

105. Veress B. Morphological observations on visceral leishmaniasis in the Sudan. *Tropical and geographical medicine* 1974, **26**(2)**:** 198-203.

106. Engwerda ACSaCR. Balancing immunity and pathology in visceral leishmaniasis. *Immunology and Cell biology* 2007, **85**(2)**:** 138-147.

107. Engwerda CR, Ato M, Kaye PM. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. *Trends Parasitol* 2004, **20**(11)**:** 524-530.

108. Murray HW, Nathan CF. Macrophage Microbicidal Mechanisms In Vivo: Reactive Nitrogen versus Oxygen Intermediates in the Killing of Intracellular Visceral Leishmania donovani. *The Journal of Experimental Medicine* 1999, **189**(4)**:** 741-746.

109. McElrath MJ, Murray HW, Cohn ZA. The dynamics of granuloma formation in experimental visceral leishmaniasis. *The Journal of Experimental Medicine* 1988, **167**(6)**:** 1927-1937.

110. Squires KE, Schreiber RD, McElrath MJ, Rubin BY, Anderson SL, Murray HW. Experimental visceral leishmaniasis: role of endogenous IFN-gamma in host defense and tissue granulomatous response. *Journal of immunology (Baltimore, Md : 1950)* 1989, **143**(12)**:** 4244-4249.

111. Murray HW, Hariprashad J, Aguero B, Arakawa T, Yeganegi H. Antimicrobial response of a T cell-deficient host to cytokine therapy: effect of interferon-gamma in experimental visceral leishmaniasis in nude mice. *The Journal of infectious diseases* 1995, **171**(5)**:** 1309-1316.

112. Alexander CE, Kaye PM, Engwerda CR. CD95 is required for the early control of parasite burden in the liver of Leishmania donovani-infected mice. *Eur J Immunol* 2001, **31**(4)**:** 1199-1210.

113. Murray HW. Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis. *The Journal of infectious diseases* 1997, **175**(6)**:** 1477-1479.

114. Tumang MC, Keogh C, Moldawer LL, Helfgott DC, Teitelbaum R, Hariprashad J, *et al.* Role and effect of TNF-alpha in experimental visceral leishmaniasis. *Journal of immunology (Baltimore, Md : 1950)* 1994, **153**(2)**:** 768-775.

115. Ghalib HW, Whittle JA, Kubin M, Hashim FA, el-Hassan AM, Grabstein KH, *et al.* IL-12 enhances Th1-type responses in human Leishmania donovani infections. *Journal of immunology (Baltimore, Md : 1950)* 1995, **154**(9)**:** 4623-4629.

116. Murray HW, Cervia JS, Hariprashad J, Taylor AP, Stoeckle MY, Hockman H. Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. *The Journal of clinical investigation* 1995, **95**(3)**:** 1183-1192.

117. Murray HW, Miralles GD, Stoeckle MY, McDermott DF. Role and effect of IL-2 in experimental visceral leishmaniasis. *Journal of immunology (Baltimore, Md : 1950)* 1993, **151**(2)**:** 929-938.

118. Engwerda CR, Ato M, Stager S, Alexander CE, Stanley AC, Kaye PM. Distinct roles for lymphotoxin-alpha and tumor necrosis factor in the control of Leishmania donovani infection. *Am J Pathol* 2004, **165**(6)**:** 2123-2133.

119. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol* 2005, **5**(8)**:** 606-616.

120. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, *et al.* A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000, **406**(6793)**:** 309-314.

121. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, *et al.* Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* 1999, **189**(3)**:** 451-460.

122. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, *et al.* CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999, **99**(1)**:** 23-33.

123. Ngo VN, Korner H, Gunn MD, Schmidt KN, Riminton DS, Cooper MD, *et al.* Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med* 1999, **189**(2)**:** 403-412.

124. Aichele P, Zinke J, Grode L, Schwendener RA, Kaufmann SH, Seiler P. Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. *Journal of immunology (Baltimore, Md : 1950)* 2003, **171**(3)**:** 1148-1155.

125. Kang YS, Yamazaki S, Iyoda T, Pack M, Bruening SA, Kim JY, *et al.* SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *International immunology* 2003, **15**(2)**:** 177-186.

126. Kraal G. Cells in the marginal zone of the spleen. *International review of cytology* 1992, **132:** 31-74.

127. Geijtenbeek TB, Groot PC, Nolte MA, van Vliet SJ, Gangaram-Panday ST, van Duijnhoven GC, *et al.* Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens in vivo. *Blood* 2002, **100**(8)**:** 2908-2916.

128. Stanley AC, Engwerda CR. Balancing immunity and pathology in visceral leishmaniasis. *Immunol Cell Biol* 2007, **85**(2)**:** 138-147.

129. Engwerda CR, Ato M, Cotterell SEJ, Mynott TL, Tschannerl A, Gorak-Stolinska PMA, *et al.* A Role for Tumor Necrosis Factor-α in Remodeling the Splenic Marginal Zone during Leishmania donovani Infection. *The American Journal of Pathology* 2002, **161**(2)**:** 429-437.

130. Ato M, Stager S, Engwerda CR, Kaye PM. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat Immunol* 2002, **3**(12)**:** 1185-1191.

131. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001, **104**(4)**:** 487-501.

132. Dalton JE, Maroof A, Owens BM, Narang P, Johnson K, Brown N, *et al.* Inhibition of receptor tyrosine kinases restores immunocompetence and improves immune-dependent chemotherapy against experimental leishmaniasis in mice. *The Journal of clinical investigation* 2010, **120**(4)**:** 1204-1216.

133. Yurdakul P, Dalton J, Beattie L, Brown N, Erguven S, Maroof A, *et al.* Compartment-Specific Remodeling of Splenic Micro-Architecture during Experimental Visceral Leishmaniasis. *The American Journal of Pathology* 2011, **179**(1)**:** 23-29.

134. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences* 1975, **72**(9)**:** 3666-3670.

135. Falvo JV, Tsytsykova AV, Goldfeld AE. Transcriptional control of the TNF gene. *Current directions in autoimmunity* 2010, **11:** 27-60.

136. Tsai EY, Yie J, Thanos D, Goldfeld AE. Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol Cell Biol* 1996, **16**(10)**:** 5232-5244.

137. Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine & growth factor reviews* 2014, **25**(4)**:** 453-472.

138. Kelker HC, Oppenheim JD, Stone-Wolff D, Henriksen-DeStefano D, Aggarwal BB, Stevenson HC, *et al.* Characterization of human tumor necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. *International Journal of Cancer* 1985, **36**(1)**:** 69-73.

139. Santee SM, Owen-Schaub LB. Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. *The Journal of biological chemistry* 1996, **271**(35)**:** 21151-21159.

140. Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. *The New England journal of medicine* 1996, **334**(26)**:** 1717-1725.

141. Boutaffala L, Bertrand MJM, Remouchamps C, Seleznik G, Reisinger F, Janas M, *et al.* NIK promotes tissue destruction independently of the alternative NF-[kappa]B pathway through TNFR1/RIP1-induced apoptosis. *Cell Death Differ* 2015, **22**(12)**:** 2020-2033.

142. Bradley JR. TNF-mediated inflammatory disease. *The Journal of pathology* 2008, **214**(2)**:** 149-160.

143. Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* 2005, **115**(1)**:** 1-20.

144. Luo Y, Xu Z, Wan T, He Y, Jones D, Zhang H, *et al.* Endothelial-specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis. *Arteriosclerosis, thrombosis, and vascular biology* 2010, **30**(7)**:** 1307-1314.

145. Shanahan JC, St Clair W. Tumor necrosis factor-alpha blockade: a novel therapy for rheumatic disease. *Clinical immunology (Orlando, Fla)* 2002, **103**(3 Pt 1)**:** 231-242.

146. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA, Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proceedings of the National Academy of Sciences* 1991, **88**(20)**:** 9292-9296.

147. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004, **75**(2)**:** 163-189.

148. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol* 1997, **15:** 749-795.

149. Karupiah G, Xie QW, Buller RM, Nathan C, Duarte C, MacMicking JD. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science* 1993, **261**(5127)**:** 1445-1448.

150. Green SJ, Nacy CA, Meltzer MS. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. *J Leukoc Biol* 1991, **50**(1)**:** 93-103.

151. Green SJ, Crawford RM, Hockmeyer JT, Meltzer MS, Nacy CA. Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *Journal of immunology (Baltimore, Md : 1950)* 1990, **145**(12)**:** 4290-4297.

152. Orme IM. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. *Journal of immunology (Baltimore, Md : 1950)* 1987, **138**(1)**:** 293-298.

153. White MT, Bejon P, Olotu A, Griffin JT, Riley EM, Kester KE, *et al.* The Relationship between RTS,S Vaccine-Induced Antibodies, CD4⁺ T Cell Responses and Protection against <italic>Plasmodium falciparum</italic> Infection. *PloS one* 2013, **8**(4)**:** e61395.

154. Kar S, Metz C, McMahon-Pratt D. CD4(+) T Cells Play a Dominant Role in Protection against New World Leishmaniasis Induced by Vaccination with the P-4 Amastigote Antigen. *Infection and Immunity* 2005, **73**(6)**:** 3823-3827.

155. Mosmann TR. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of immunology (1950)* 1986, **136**(7)**:** 2348-2357.

156. Zhu J, Yamane H, Paul WE. Differentiation of Effector CD4 T Cell Populations*. *Annual Review of Immunology* 2010, **28**(1)**:** 445-489.

157. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, *et al.* Role of T-bet in Commitment of TH1 Cells Before IL-12-Dependent Selection. *Science* 2001, **292**(5523)**:** 1907-1910.

158. Pai S-Y, Truitt ML, Ho I-C. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(7)**:** 1993-1998.

159. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, *et al.* Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. *Nat Immunol* 2004, **5**(11)**:** 1157-1165.

160. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology* 2009, **126**(2)**:** 177-185.

161. Wan YSY. Multi-tasking of helper T cells. *Immunology* 2010, **130**(2)**:** 166-171.

162. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005, **6**(11)**:** 1123-1132.

163. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular Helper T Cells: Lineage and Location. *Immunity* 2009, **30**(3)**:** 324-335.

164. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, *et al.* The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. *Immunity* 2009, **31**(3)**:** 457-468.

165. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, *et al.* Bcl6 and Blimp-1 Are Reciprocal and Antagonistic Regulators of T Follicular Helper Cell Differentiation. *Science* 2009, **325**(5943)**:** 1006-1010.

166. Walker LSK, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol* 2002, **2**(1)**:** 11-19.

167. Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *International immunology* 1993, **5**(11)**:** 1461-1471.

168. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of immunology (Baltimore, Md : 1950)* 1995, **155**(3)**:** 1151-1164.

169. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003, **4**(4)**:** 330-336.

170. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003, **299**(5609)**:** 1057-1061.

171. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003, **4**(4)**:** 337-342.

172. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003, **3**(3)**:** 253-257.

173. Lin X, Chen M, Liu Y, Guo Z, He X, Brand D, *et al.* Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. *Int J Clin Exp Pathol* 2013, **6**(2)**:** 116-123.

174. Shevach EM. Mechanisms of foxp3 T regulatory cell-mediated suppression. *Immunity (Cambridge, Mass)* 2009, **30**(5)**:** 636-645.

175. Kling JC, Korner H. Different regulatory mechanisms in protozoan parasitic infections. *Int J Parasitol* 2013, **43**(6)**:** 417-425.

176. Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. *European Journal of Immunology* 2004, **34**(2)**:** 366-376.

177. Garín MI, Chu C-C, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 2007, **109**(5)**:** 2058-2065.

178. Read S. Blockade of CTLA-4 on CD4 CD25 regulatory T cells abrogates their function in vivo. *The Journal of immunology (1950)* 2006, **177**(7)**:** 4376-4383.

179. Thornton AM, Shevach EM. CD4+CD25+ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2 Production. *The Journal of Experimental Medicine* 1998, **188**(2)**:** 287-296.

180. Wang Y, Su Maureen A, Wan Yisong Y. An Essential Role of the Transcription Factor GATA-3 for the Function of Regulatory T Cells. *Immunity* 2011, **35**(3)**:** 337-348.

181. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, *et al.* The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 2011, **12**(4)**:** 304-311.

182. Koch MA, Tucker-Heard Gs, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 2009, **10**(6)**:** 595-602.

183. Fragale A. IFN regulatory factor-1 negatively regulates CD4 CD25 regulatory T cell differentiation by repressing Foxp3 expression. *The Journal of immunology (1950)* 2008, **181**(3)**:** 1673-1682.

184. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, *et al.* CD4+ Regulatory T Cells Control TH17 Responses in a Stat3-Dependent Manner. *Science* 2009, **326**(5955)**:** 986-991.

185. Zheng Y. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature (London)* 2009, **458**(7236)**:** 351-356.

186. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1994, **1**(9)**:** 793-801.

187. Harris NL, Ronchese F. The role of B7 costimulation in T-cell immunity. *Immunol Cell Biol* 1999, **77**(4)**:** 304-311.

188. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends in molecular medicine* 2007, **13**(3)**:** 108-116.

189. Schmidt EM, Wang CJ, Ryan GA, Clough LE, Qureshi OS, Goodall M, *et al.* Ctla-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. *Journal of immunology (Baltimore, Md : 1950)* 2009, **182**(1)**:** 274-282.

190. Frauwirth KA, Alegre ML, Thompson CB. Induction of T cell anergy in the absence of CTLA-4/B7 interaction. *Journal of immunology (Baltimore, Md : 1950)* 2000, **164**(6)**:** 2987-2993.

191. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, *et al.* IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *Journal of immunology (Baltimore, Md : 1950)* 1991, **146**(10)**:** 3444-3451.

192. Chaudhry A, Samstein Robert M, Treuting P, Liang Y, Pils Marina C, Heinrich J-M, *et al.* Interleukin-10 Signaling in Regulatory T Cells Is Required for Suppression of Th17 Cell-Mediated Inflammation. *Immunity* 2011, **34**(4)**:** 566-578.

193. Takeuchi M, Alard P, Streilein JW. TGF-beta promotes immune deviation by altering accessory signals of antigen-presenting cells. *Journal of immunology (Baltimore, Md : 1950)* 1998, **160**(4)**:** 1589-1597.

194. Barthlott T, Moncrieffe H, Veldhoen M, Atkins CJ, Christensen J, O'Garra A, *et al.* CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *International immunology* 2005, **17**(3)**:** 279-288.

195. Roncarolo MG, Yssel H, Touraine JL, Betuel H, De Vries JE, Spits H. Autoreactive T cell clones specific for class I and class II HLA antigens isolated from a human chimera. *J Exp Med* 1988, **167**(5)**:** 1523-1534.

196. Bacchetta R, Bigler M, Touraine JL, Parkman R, Tovo PA, Abrams J, *et al.* High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *The Journal of Experimental Medicine* 1994, **179**(2)**:** 493-502.

197. Groux H. A CD4 T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature (London)* 1997, **389**(6652)**:** 737-742.

198. Vieira PL. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4 CD25 regulatory T cells. *The Journal of immunology (1950)* 2004, **172**(10)**:** 5986-5993.

199. Häringer B, Lozza L, Steckel B, Geginat J. Identification and characterization of IL-10/IFN-γ–producing effector-like T cells with regulatory function in human blood. *The Journal of Experimental Medicine* 2009, **206**(5)**:** 1009-1017.

200. Gagliani N. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nature medicine* 2013, **19**(6)**:** 739-746.

201. Simon Fillatreau AOG. *Interleukin-10 in Health and Disease*, 1 edn, vol. 380. Springer-Verlag Berlin Heidelberg, 2014.

202. Magnani CF, Alberigo G, Bacchetta R, Serafini G, Andreani M, Roncarolo MG, *et al.* Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur J Immunol* 2011, **41**(6)**:** 1652-1662.

203. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, *et al.* Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. *Frontiers in immunology* 2014, **5:** 16.

204. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Curr Top Microbiol Immunol* 2014, **380:** 39-68.

205. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, *et al.* Conventional T-bet+Foxp3− Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *The Journal of Experimental Medicine* 2007, **204**(2)**:** 273-283.

206. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, *et al.* Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *Journal of immunology (Baltimore, Md : 1950)* 2009, **183**(2)**:** 797-801.

207. Spolski R, Kim HP, Zhu W, Levy DE, Leonard WJ. IL-21 mediates suppressive effects via its induction of IL-10. *Journal of immunology (Baltimore, Md : 1950)* 2009, **182**(5)**:** 2859-2867.

208. Stumhofer JS, Silver JS, Laurence A, Porrett PM, Harris TH, Turka LA, *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 2007, **8**(12)**:** 1363-1371.

209. Apetoh L, Quintana FJ, Pot C, Joller N, Xiao S, Kumar D, *et al.* The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* 2010, **11**(9)**:** 854-861.

210. Xu J, Yang Y, Qiu G, Lal G, Wu Z, Levy DE, *et al.* c-Maf regulates IL-10 expression during Th17 polarization. *Journal of immunology (Baltimore, Md : 1950)* 2009, **182**(10)**:** 6226-6236.

211. Findlay EG, Greig R, Stumhofer JS, Hafalla JC, de Souza JB, Saris CJ, *et al.* Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection. *Journal of immunology (Baltimore, Md : 1950)* 2010, **185**(4)**:** 2482-2492.

212. Anderson CF, Stumhofer JS, Hunter CA, Sacks D. IL-27 Regulates IL-10 and IL-17 from CD4+ Cells in Nonhealing Leishmania major Infection. *The Journal of Immunology* 2009, **183**(7)**:** 4619-4627.

213. Kumar NP, Moideen K, Banurekha VV, Nair D, Sridhar R, Nutman TB, *et al.* IL-27 and TGFβ mediated expansion of Th1 and adaptive regulatory T cells expressing IL-10 correlates with bacterial burden and disease severity in pulmonary tuberculosis. *Immunity, Inflammation and Disease* 2015, **3**(3)**:** 289-299.

214. Anderson CF, Oukka M, Kuchroo VJ, Sacks D. CD4+CD25−Foxp3− Th1 cells are the source of IL-10–mediated immune suppression in chronic cutaneous leishmaniasis. *The Journal of Experimental Medicine* 2007, **204**(2)**:** 285-297.

215. Parish IA, Marshall HD, Staron MM, Lang PA, Brustle A, Chen JH, *et al.* Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. *The Journal of clinical investigation* 2014, **124**(8)**:** 3455-3468.

216. Neumann C, Heinrich F, Neumann K, Junghans V, Mashreghi MF, Ahlers J, *et al.* Role of Blimp-1 in programing Th effector cells into IL-10 producers. *J Exp Med* 2014, **211**(9)**:** 1807-1819.

217. Iwasaki Y, Fujio K, Okamura T, Yanai A, Sumitomo S, Shoda H, *et al.* Egr-2 transcription factor is required for Blimp-1 mediated IL-10 production in IL-27 stimulated CD4+ T cells. *European Journal of Immunology* 2013**:** n/a-n/a.

218. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *Journal of immunology (Baltimore, Md : 1950)* 2008, **180**(9)**:** 5771-5777.

219. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. *Trends Immunol* 2007, **28**(9)**:** 378-384.

220. Mege JL, Meghari S, Honstettre A, Capo C, Raoult D. The two faces of interleukin 10 in human infectious diseases. *The Lancet Infectious diseases* 2006, **6**(9)**:** 557-569.

221. Anderson CF. IL-10 and TGF-beta control the establishment of persistent and transmissible infections produced by Leishmania tropica in C57BL/6 mice. *The Journal of immunology (1950)* 2008, **180**(6)**:** 4090-4097.

222. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, *et al.* Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *European Journal of Immunology* 2010, **40**(8)**:** 2200-2210.

223. Roilides E, Anastasiou-Katsiardani A, Dimitriadou-Georgiadou A, Kadiltsoglou I, Tsaparidou S, Panteliadis C, *et al.* Suppressive Effects of Interleukin-10 on Human Mononuclear Phagocyte Function against Candida albicans and Staphylococcus aureus. *Journal of Infectious Diseases* 1998, **178**(6)**:** 1734-1742.

224. Li C, Corraliza I, Langhorne J. A Defect in Interleukin-10 Leads to Enhanced Malarial Disease in Plasmodium chabaudi chabaudi Infection in Mice. *Infection and Immunity* 1999, **67**(9)**:** 4435-4442.

225. Gazzinelli RT. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4 T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *The Journal of immunology (1950)* 1996, **157**(2)**:** 798-805.

226. Deckert M, Soltek S, Geginat G, Lütjen S, Montesinos-Rongen M, Hof H, *et al.* Endogenous Interleukin-10 Is Required for Prevention of a Hyperinflammatory Intracerebral Immune Response in Listeria monocytogenes Meningoencephalitis. *Infection and Immunity* 2001, **69**(7)**:** 4561-4571.

227. Rhee MSM, Akanmori BD, Waterfall M, Riley EM. Changes in cytokine production associated with acquired immunity to Plasmodium falciparum malaria. *Clinical & Experimental Immunology* 2001, **126**(3)**:** 503-510.

228. Struik SS, Riley EM. Does malaria suffer from lack of memory? *Immunological Reviews* 2004, **201**(1)**:** 268-290.

229. Moormann AM, Sumba PO, Tisch DJ, Embury P, King CH, Kazura JW, *et al.* Stability of Interferon-Gamma and Interleukin-10 Responses to Plasmodium falciparum Liver Stage Antigen 1 and Thrombospondin-Related Adhesive Protein Immunodominant Epitopes in a Highland Population from Western Kenya. *The American Journal of Tropical Medicine and Hygiene* 2009, **81**(3)**:** 489-495.

230. MOORMANN AM, JOHN CC, SUMBA PO, TISCH D, EMBURY P, KAZURA JW. Stability of Interferon-gamma and interleukin-10 responses to *Plasmodium falciparum* liver stage antigen-1 and thrombospondin-related adhesive protein in residents of a malaria holoendemic area. *The American Journal of Tropical Medicine and Hygiene* 2006, **74**(4)**:** 585-590.

231. Wipasa J, Okell L, Sakkhachornphop S, Suphavilai C, Chawansuntati K, Liewsaree W, *et al.* Short-Lived IFN-γ Effector Responses, but Long-Lived IL-10 Memory Responses, to Malaria in an Area of Low Malaria Endemicity. *PLoS Pathog* 2011, **7**(2)**:** e1001281.

232. Portugal S, Moebius J, Skinner J, Doumbo S, Doumtabe D, Kone Y, *et al.* Exposure-dependent control of malaria-induced inflammation in children. *PLoS Pathog* 2014, **10**(4)**:** e1004079.

233. Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med* 2007, **204**(4)**:** 805-817.

234. Costa DL, Cardoso TM, Queiroz A, Milanezi CM, Bacellar O, Carvalho EM, *et al.* Tr-1-like CD4+CD25-CD127-/lowFOXP3- cells are the main source of interleukin 10 in patients with cutaneous leishmaniasis due to Leishmania braziliensis. *The Journal of infectious diseases* 2015, **211**(5)**:** 708-718.

235. Walther M, Jeffries D, Finney OC, Njie M, Ebonyi A, Deininger S, *et al.* Distinct Roles for FOXP3+and FOXP3- CD4+ T Cells in Regulating Cellular Immunity to Uncomplicated and Severe *Plasmodium falciparum* Malaria. *PLoS Pathog* 2009, **5**(4)**:** e1000364.

236. Gasim S, Elhassan AM, Khalil EA, Ismail A, Kadaru AM, Kharazmi A, *et al.* High levels of plasma IL-10 and expression of IL-10 by keratinocytes during visceral leishmaniasis predict subsequent development of post-kala-azar dermal leishmaniasis. *Clinical and experimental immunology* 1998, **111**(1)**:** 64-69.

237. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP. IL-10 mediates susceptibility to Leishmania donovani infection. *Eur J Immunol* 2001, **31**(10)**:** 2848-2856.

238. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, *et al.* Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. *Infect Immun* 2002, **70**(11)**:** 6284-6293.

239. Turner Jr CA, Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 1994, **77**(2)**:** 297-306.

240. Mock BA, Liu L, Paslier DL, Huang S. The B-Lymphocyte Maturation Promoting Transcription Factor BLIMP1/PRDI-BF1 Maps to D6S447 on Human Chromosome 6q21–q22.1 and the Syntenic Region of Mouse Chromosome 10. *Genomics* 1996, **37**(1)**:** 24-28.

241. Ren B, Chee KJ, Kim TH, Maniatis T. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes & Development* 1999, **13**(1)**:** 125-137.

242. Kallies A, Hasbold J, Tarlinton DM, Dietrich W, Corcoran LM, Hodgkin PD, *et al.* Plasma Cell Ontogeny Defined by Quantitative Changes in Blimp-1 Expression. *The Journal of Experimental Medicine* 2004, **200**(8)**:** 967-977.

243. Shapiro-Shelef M, Lin K-I, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells. *Immunity* 2003, **19**(4)**:** 607-620.

244. Chan Y-H, Chiang M-F, Tsai Y-C, Su S-T, Chen M-H, Hou M-S, *et al.* Absence of the Transcriptional Repressor Blimp-1 in Hematopoietic Lineages Reveals Its Role in Dendritic Cell Homeostatic Development and Function. *The Journal of Immunology* 2009, **183**(11)**:** 7039-7046.

245. Kallies A. A role for Blimp1 in the transcriptional network controlling natural killer cell maturation. *Blood* 2011, **117**(6)**:** 1869-1879.

246. Sun J, Dodd H, Moser EK, Sharma R, Braciale TJ. CD4+ T cell help and innate-derived IL-27 induce Blimp-1-dependent IL-10 production by antiviral CTLs. *Nat Immunol* 2011, **12**(4)**:** 327-334.

247. Cimmino L, Martins GA, Liao J, Magnusdottir E, Grunig G, Perez RK, *et al.* Blimp-1 Attenuates Th1 Differentiation by Repression of ifng, tbx21, and bcl6 Gene Expression. *The Journal of Immunology* 2008, **181**(4)**:** 2338-2347.

248. Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, *et al.* Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 2005, **436**(7048)**:** 207-213.

249. Kuo TC, Calame KL. B lymphocyte-induced maturation protein (Blimp)-1, IFN regulatory factor (IRF)-1, and IRF-2 can bind to the same regulatory sites. *Journal of immunology (Baltimore, Md : 1950)* 2004, **173**(9)**:** 5556-5563.

250. Yu J, Angelin-Duclos C, Greenwood J, Liao J, Calame K. Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol Cell Biol* 2000, **20**(7)**:** 2592-2603.

251. Heinemann C, Heink S, Petermann F, Vasanthakumar A, Rothhammer V, Doorduijn E, *et al.* IL-27 and IL-12 oppose pro-inflammatory IL-23 in CD4+ T cells by inducing Blimp1. *Nature communications* 2014, **5:** 3770.

252. Aucan C, Walley AJ, Hennig BJW, Fitness J, Frodsham A, Zhang L, *et al.* Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in The Gambia. *Genes Immun* 2003, **4**(4)**:** 275-282.

253. Khor CC, Vannberg FO, Chapman SJ, Walley A, Aucan C, Loke H, *et al.* Positive replication and linkage disequilibrium mapping of the chromosome 21q22.1 malaria susceptibility locus. *Genes Immun* 2007, **8**(7)**:** 570-576.

254. Lovegrove FE, Gharib SA, Patel SN, Hawkes CA, Kain KC, Liles WC. Expression Microarray Analysis Implicates Apoptosis and Interferon-Responsive Mechanisms in Susceptibility to Experimental Cerebral Malaria. *The American Journal of Pathology* 2007, **171**(6)**:** 1894-1903.

255. RTS S-C-T-P. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. *PLoS Med* 2014, **11**(7)**:** e1001685.

256. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, *et al.* Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. *Lancet (London, England)* 2004, **364**(9443)**:** 1411-1420.

257. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, *et al.* Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *Lancet (London, England)* 2007, **370**(9598)**:** 1543-1551.

258. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev* 2009, **22**(1)**:** 13-36, Table of Contents.

259. Lambert PH, Liu M, Siegrist CA. Can successful vaccines teach us how to induce efficient protective immune responses? *Nat Med* 2005, **11**(4 Suppl)**:** S54-62.

260. Janeway CA. Use of Concentrated Human Serum gamma-Globulin in the Prevention and Attenuation of Measles. *Bulletin of the New York Academy of Medicine* 1945, **21**(4)**:** 202-222.

261. White MT, Bejon P, Olotu A, Griffin JT, Riley EM, Kester KE, *et al.* The relationship between RTS, S vaccine-induced antibodies, CD4(+) T cell responses and protection against Plasmodium falciparum infection. *PloS one* 2013, **8**.

262. Moorthy VS, Ballou WR. Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. *Malar J* 2009, **8:** 312.

263. Chaudhury S, Ockenhouse CF, Regules JA, Dutta S, Wallqvist A, Jongert E, *et al.* The biological function of antibodies induced by the RTS,S/AS01 malaria vaccine candidate is determined by their fine specificity. *Malar J* 2016, **15:** 301.

264. Yazdanbakhsh M, Sacks DL. Why does immunity to parasites take so long to develop? *Nat Rev Immunol* 2010, **10**(2)**:** 80-81.

265. Checkley AM, Smith A, Smith V, Blaze M, Bradley D, Chiodini PL, *et al.* Risk factors for mortality from imported falciparum malaria in the United Kingdom over 20 years: an observational study. *BMJ (Clinical research ed)* 2012, **344:** e2116.

266. Jennings RM, JB DES, Todd JE, Armstrong M, Flanagan KL, Riley EM, *et al.* Imported Plasmodium falciparum malaria: are patients originating from disease-endemic areas less likely to develop severe disease? A prospective, observational study. *Am J Trop Med Hyg* 2006, **75**(6)**:** 1195-1199.

267. Pistone T, Diallo A, Mechain M, Receveur MC, Malvy D. Epidemiology of imported malaria give support to the hypothesis of 'long-term' semi-immunity to malaria in sub-Saharan African migrants living in France. *Travel medicine and infectious disease* 2014, **12**(1)**:** 48-53.

268. Migot F, Chougnet C, Raharimalala L, Astagneau P, Lepers JP, Deloron P. Human immune responses to the Plasmodium falciparum ring-infected erythrocyte surface antigen (Pf155/RESA) after a decrease in malaria transmission in Madagascar. *Am J Trop Med Hyg* 1993, **48**(3)**:** 432-439.

269. Migot F, Chougnet C, Henzel D, Dubois B, Jambou R, Fievet N, *et al.* Anti-malaria antibody-producing B cell frequencies in adults after a Plasmodium falciparum outbreak in Madagascar. *Clinical and experimental immunology* 1995, **102**(3)**:** 529-534.

270. Moncunill G, Mayor A, Jiménez A, Nhabomba A, Casas-Vila N, Puyol L, *et al.* High Antibody Responses against <italic>Plasmodium falciparum</italic> in Immigrants after Extended Periods of Interrupted Exposure to Malaria. *PloS one* 2013, **8**(8)**:** e73624.

271. Ndungu FM, Lundblom K, Rono J, Illingworth J, Eriksson S, Farnert A. Long-lived Plasmodium falciparum specific memory B cells in naturally exposed Swedish travelers. *Eur J Immunol* 2013, **43**(11)**:** 2919-2929.

272. Gonzalez-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type I interferons. *Nat Rev Immunol* 2012, **12**(2)**:** 125-135.

273. Piconese S, Pacella I, Timperi E, Barnaba V. Divergent effects of type-I interferons on regulatory T cells. *Cytokine & growth factor reviews* 2015, **26**(2)**:** 133-141.

274. Sharma S, DeOliveira Rosane B, Kalantari P, Parroche P, Goutagny N, Jiang Z, *et al.* Innate Immune Recognition of an AT-Rich Stem-Loop DNA Motif in the *Plasmodium falciparum* Genome. *Immunity* 2011, **35**(2)**:** 194-207.

275. Nie CQ, Bernard NJ, Schofield L, Hansen DS. CD4+ CD25+ Regulatory T Cells Suppress CD4+ T-Cell Function and Inhibit the Development of Plasmodium berghei-Specific TH1 Responses Involved in Cerebral Malaria Pathogenesis. *Infect Immun* 2007, **75**(5)**:** 2275-2282.

276. Boyle MJ, Jagannathan P, Farrington LA, Eccles-James I, Wamala S, McIntyre TI, *et al.* Decline of FoxP3+ Regulatory CD4 T Cells in Peripheral Blood of Children Heavily Exposed to Malaria. *PLoS Pathog* 2015, **11**(7)**:** e1005041.

277. Torres KJ, Villasis E, Bendezu J, Chauca J, Vinetz JM, Gamboa D. Relationship of regulatory T cells to Plasmodium falciparum malaria symptomatology in a hypoendemic region. *Malar J* 2014, **13:** 108.

278. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008, **133**(5)**:** 775-787.

279. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* 2013, **38**(3)**:** 414-423.

280. Engwerda CR, Ng SS, Bunn PT. The Regulation of CD4(+) T Cell Responses during Protozoan Infections. *Frontiers in immunology* 2014, **5:** 498.

281. Gitau EN, Tuju J, Karanja H, Stevenson L, Requena P, Kimani E, *et al.* CD4+ T cell responses to the Plasmodium falciparum erythrocyte membrane protein 1 in children with mild malaria. *Journal of immunology (Baltimore, Md : 1950)* 2014, **192**(4)**:** 1753-1761.

282. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, *et al.* Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. *Immunity* 2008, **29**(1)**:** 138-149.

283. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, *et al.* Follicular B Helper T Cells Express Cxc Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production. *The Journal of Experimental Medicine* 2000, **192**(11)**:** 1545-1552.

284. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. Cxc Chemokine Receptor 5 Expression Defines Follicular Homing T Cells with B Cell Helper Function. *The Journal of Experimental Medicine* 2000, **192**(11)**:** 1553-1562.

285. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, *et al.* Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *The Lancet*, **366**(9502)**:** 2012-2018.

286. Riley EM, Stewart VA. Immune mechanisms in malaria: new insights in vaccine development. *Nat Med* 2013, **19**(2)**:** 168-178.

287. Obeng-Adjei N, Portugal S, Tran TM, Yazew TB, Skinner J, Li S, *et al.* Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children. *Cell Rep* 2015, **13**(2)**:** 425-439.

288. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, *et al.* Interferon-gamma responses are associated with resistance to reinfection with Plasmodium falciparum in young African children. *The Journal of infectious diseases* 1999, **179**(4)**:** 980-988.

289. John CC, Moormann AM, Sumba PO, Ofulla AV, Pregibon DC, Kazura JW. Gamma interferon responses to Plasmodium falciparum liver-stage antigen 1 and thrombospondin-related adhesive protein and their relationship to age, transmission intensity, and protection against malaria. *Infect Immun* 2004, **72**(9)**:** 5135-5142.

290. Reece WH, Pinder M, Gothard PK, Milligan P, Bojang K, Doherty T, *et al.* A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease. *Nat Med* 2004, **10**(4)**:** 406-410.

291. Boraschi D, Abebe Alemayehu M, Aseffa A, Chiodi F, Chisi J, Del Prete G, *et al.* Immunity against HIV/AIDS, Malaria, and Tuberculosis during Co-Infections with Neglected Infectious Diseases:

Recommendations for the European Union Research Priorities. *PLoS Neglected Tropical Diseases* 2008, **2**(6)**:** e255.

292. Saracino A, Nacarapa EA, da Costa Massinga EA, Martinelli D, Scacchetti M, de Oliveira C, *et al.* Prevalence and clinical features of HIV and malaria co-infection in hospitalized adults in Beira, Mozambique. *Malar J* 2012, **11:** 241.

293. Valadas E, Gomes A, Sutre A, Brilha S, Wete A, Hanscheid T, *et al.* Tuberculosis with malaria or HIV co-infection in a large hospital in Luanda, Angola. *Journal of infection in developing countries* 2013, **7**(3)**:** 269-272.

294. Cunnington AJ, de Souza JB, Walther M, Riley EM. Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. *Nat Med* 2012, **18**(1)**:** 120-127.

295. Laurens MB, Duncan CJ, Epstein JE, Hill AV, Komisar JL, Lyke KE, *et al.* A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines. *Vaccine* 2012, **30**(36)**:** 5302-5304.

296. Engwerda CR, Minigo G, Amante FH, McCarthy JS. Experimentally induced blood stage malaria infection as a tool for clinical research. *Trends in Parasitology* 2012, **28**(11)**:** 515-521.

297. Rockett R, Tozer S, Peatey C, Bialasiewicz S, Whiley D, Nissen M, *et al.* A real-time, quantitative PCR method using hydrolysis probes for the monitoring of Plasmodium falciparum load in experimentally infected human volunteers. *Malaria Journal* 2011, **10**(1)**:** 48.

298. Anacker C, Cattaneo A, Luoni A, Musaelyan K, Zunszain PA, Milanesi E, *et al.* Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 2013, **38**(5)**:** 872-883.

299. Rieger KE, Chu G. Portrait of transcriptional responses to ultraviolet and ionizing radiation in human cells. *Nucleic Acids Research* 2004, **32**(16)**:** 4786-4803.

300. Tangen SE, Tsinajinnie D, Nuñez M, Shaibi GQ, Mandarino LJ, Coletta DK. Whole Blood Gene Expression Profiles in Insulin Resistant Latinos with the Metabolic Syndrome. *PloS one* 2013, **8**(12)**:** e84002.

301. De Boever P, Wens B, Forcheh AC, Reynders H, Nelen V, Kleinjans J, *et al.* Characterization of the peripheral blood transcriptome in a repeated measures design using a panel of healthy individuals. *Genomics* 2014, **103**(1)**:** 31-39.

302. Subramaniam KS, Spaulding E, Ivan E, Mutimura E, Kim RS, Liu X, *et al.* The T-Cell Inhibitory Molecule Butyrophilin-Like 2 Is Up-regulated in Mild Plasmodium falciparum Infection and Is Protective During Experimental Cerebral Malaria. *The Journal of infectious diseases* 2015.

303. Good MF. Immunological responses from non-exposed donors to malaria antigens: implications for immunity and pathology. *Immunology letters* 1994, **41**(2-3)**:** 123-125.

304. Ndungu FM, Sanni L, Urban B, Stephens R, Newbold CI, Marsh K, *et al.* CD4 T Cells from Malaria-Nonexposed Individuals Respond to the CD36-Binding Domain of Plasmodium falciparum Erythrocyte Membrane Protein-1 via an MHC Class II-TCR-Independent Pathway. *The Journal of Immunology* 2006, **176**(9)**:** 5504-5512.

305. Wu J, Cai B, Sun W, Huang R, Liu X, Lin M, *et al.* Genome-wide Analysis of Host-Plasmodium yoelii Interactions Reveals Regulators of the Type I Interferon Response. *Cell Rep* 2015, **12**(4)**:** 661-672.

306. Liehl P, Zuzarte-Luis V, Chan J, Zillinger T, Baptista F, Carapau D, *et al.* Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection. *Nat Med* 2014, **20**(1)**:** 47-53.

307. Edwards CL, Zhang V, Werder RB, Best SE, Sebina I, James KR, *et al.* Coinfection with Blood-Stage Plasmodium Promotes Systemic Type I Interferon Production during Pneumovirus Infection but Impairs Inflammation and Viral Control in the Lung. *Clinical and vaccine immunology : CVI* 2015, **22**(5)**:** 477-483.

308. Krupka M, Seydel K, Feintuch CM, Yee K, Kim R, Lin CY, *et al.* Mild Plasmodium falciparum malaria following an episode of severe malaria is associated with induction of the interferon pathway in Malawian children. *Infect Immun* 2012, **80**(3)**:** 1150-1155.

309. Grangeiro de Carvalho E, Bonin M, Kremsner PG, Kun JF. Plasmodium falciparum-infected erythrocytes and IL-12/IL-18 induce diverse transcriptomes in human NK cells: IFN-alpha/beta pathway versus TREM signaling. *PloS one* 2011, **6**(9)**:** e24963.

310. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL. Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. *The Journal of cell biology* 2007, **178**(1)**:** 57-69.

311. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. *Clinical science (London, England : 1979)* 2012, **122**(4)**:** 143-159.

312. Schindler C, Levy DE, Decker T. JAK-STAT Signaling: From Interferons to Cytokines. *Journal of Biological Chemistry* 2007, **282**(28)**:** 20059-20063.

313. Rauch I, Müller M, Decker T. The regulation of inflammation by interferons and their STATs. *JAK-STAT* 2013, **2**(1)**:** e23820.

314. Ho M, Sexton MM, Tongtawe P, Looareesuwan S, Suntharasamai P, Webster HK. Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific lymphoproliferation in acute Plasmodium falciparum malaria. *The Journal of infectious diseases* 1995, **172**(3)**:** 838-844.

315. Ochola LA, Ng'wena GM, Noland GS, Ondigo BN, Ayodo G, John CC. The Plasmodium falciparum Antigen MB2 Induces Interferon-gamma and Interleukin-10 Responses in Adults in Malaria Endemic Areas of Western Kenya. *Journal of global infectious diseases* 2013, **5**(4)**:** 131-137.

316. Villegas-Mendez A, Shaw TN, Inkson CA, Strangward P, de Souza JB, Couper KN. Parasite-specific CD4+IFN-gamma+IL-10+ T cells distribute within both lymphoid and non-lymphoid compartments and are controlled systemically by IL-27 and ICOS during blood-stage malaria infection. *Infect Immun* 2015.

317. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, *et al.* IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PloS one* 2011, **6**(3)**:** e17739.

318. Hirunpetcharat C, Finkelman F, Clark IA, Good MF. Malaria parasite-specific Th1-like T cells simultaneously reduce parasitemia and promote disease. *Parasite Immunol* 1999, **21**(6)**:** 319-329.

319. Olotu A, Moris P, Mwacharo J, Vekemans J, Kimani D, Janssens M, *et al.* Circumsporozoite-specific T cell responses in children vaccinated with RTS,S/AS01E and protection against P falciparum clinical malaria. *PloS one* 2011, **6**(10)**:** e25786.

320. Lumsden JM, Schwenk RJ, Rein LE, Moris P, Janssens M, Ofori-Anyinam O, *et al.* Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells. *PloS one* 2011, **6**(7)**:** e20775.

321. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, *et al.* Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. *The Journal of infectious diseases* 2009, **200**(3)**:** 337-346.

322. Artavanis-Tsakonas K, Tongren JE, Riley EM. The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clinical and experimental immunology* 2003, **133**(2)**:** 145-152.

323. Karyana M, Burdarm L, Yeung S, Kenangalem E, Wariker N, Maristela R, *et al.* Malaria morbidity in Papua Indonesia, an area with multidrug resistant Plasmodium vivax and Plasmodium falciparum. *Malaria Journal* 2008, **7**(1)**:** 148.

324. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* 2007, **8**(12)**:** 1380-1389.

325. Pot C, Apetoh L, Awasthi A, Kuchroo VK. Molecular Pathways in the Induction of Interleukin-27-Driven Regulatory Type 1 Cells. *Journal of Interferon & Cytokine Research* 2010, **30**(6)**:** 381-388.

326. Murugaiyan G, Mittal A, Lopez-Diego R, Maier LM, Anderson DE, Weiner HL. IL-27 Is a Key Regulator of IL-10 and IL-17 Production by Human CD4(+) T Cells. *Journal of immunology (Baltimore, Md : 1950)* 2009, **183**(4)**:** 2435-2443.

327. Bosmann M, Strobl B, Kichler N, Rigler D, Grailer JJ, Pache F, *et al.* Tyrosine kinase 2 promotes sepsis-associated lethality by facilitating production of interleukin-27. *Journal of Leukocyte Biology* 2014, **96**(1)**:** 123-131.

328. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, *et al.* Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol* 2012, **13**(2)**:** 188-195.

329. McDermott DS, Varga SM. Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. *Journal of immunology (Baltimore, Md : 1950)* 2011, **187**(11)**:** 5568-5576.

330. Park EJ, Peixoto A, Imai Y, Goodarzi A, Cheng G, Carman CV, *et al.* Distinct roles for LFA-1 affinity regulation during T-cell adhesion, diapedesis, and interstitial migration in lymph nodes. *Blood* 2010, **115**(8)**:** 1572-1581.

331. Berlin-Rufenach C, Otto F, Mathies M, Westermann J, Owen MJ, Hamann A, *et al.* Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *J Exp Med* 1999, **189**(9)**:** 1467-1478.

332. Rose DM, Han J, Ginsberg MH. Alpha4 integrins and the immune response. *Immunol Rev* 2002, **186:** 118-124.

333. Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegenerative diseases* 2008, **5**(1)**:** 16-22.

334. Zander RA, Obeng-Adjei N, Guthmiller JJ, Kulu DI, Li J, Ongoiba A, *et al.* PD-1 Co-inhibitory and OX40 Co-stimulatory Crosstalk Regulates Helper T Cell Differentiation and Anti-Plasmodium Humoral Immunity. *Cell host & microbe* 2015, **17**(5)**:** 628-641.

335. Bunn PT, Stanley AC, de Labastida Rivera F, Mulherin A, Sheel M, Alexander CE, *et al.* Tissue requirements for establishing long-term CD4+ T cell-mediated immunity following Leishmania donovani infection. *Journal of immunology (Baltimore, Md : 1950)* 2014, **192**(8)**:** 3709-3718.

336. Rai D, Pham NL, Harty JT, Badovinac VP. Tracking the total CD8 T cell response to infection reveals substantial discordance in magnitude and kinetics between inbred and outbred hosts. *Journal of immunology (Baltimore, Md : 1950)* 2009, **183**(12)**:** 7672-7681.

337. Sedegah M, Finkelman F, Hoffman SL. Interleukin 12 induction of interferon gamma-dependent protection against malaria. *Proceedings of the National Academy of Sciences of the United States of America* 1994, **91**(22)**:** 10700-10702.

338. Liew FY, Millott S, Li Y, Lelchuk R, Chan WL, Ziltener H. Macrophage activation by interferon-gamma from host-protective T cells is inhibited by interleukin (IL)3 and IL4 produced by disease-promoting T cells in leishmaniasis. *Eur J Immunol* 1989, **19**(7)**:** 1227-1232.

339. Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM, *et al.* Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 2006, **7**(5)**:** 466-474.

340. Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E, *et al.* Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 2006, **7**(5)**:** 457-465.

341. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010, **10**(3)**:** 170-181.

342. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, *et al.* Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007, **27**(4)**:** 670-684.

343. Workman CJ, Rice DS, Dugger KJ, Kurschner C, Vignali DA. Phenotypic analysis of the murine CD4-related glycoprotein, CD223 (LAG-3). *Eur J Immunol* 2002, **32**(8)**:** 2255-2263.

344. Koch K, Koch N, Sandaradura de Silva U, Jung N, Schulze Zur Wiesch J, Fatkenheuer G, *et al.* Increased Frequency of CD49b/LAG-3+ Type 1 regulatory T cells in HIV infected Individuals. *AIDS research and human retroviruses* 2015.

345. Kemp K, Kemp M, Kharazmi A, Ismail A, Kurtzhals JA, Hviid L, *et al.* Leishmania-specific T cells expressing interferon-gamma (IFN-gamma) and IL-10 upon activation are expanded in individuals cured of visceral leishmaniasis. *Clinical and experimental immunology* 1999, **116**(3)**:** 500-504.

346. Taylor PR, Seixas E, Walport MJ, Langhorne J, Botto M. Complement Contributes to Protective Immunity against Reinfection by Plasmodium chabaudi chabaudi Parasites. *Infection and Immunity* 2001, **69**(6)**:** 3853-3859.

347. Stevenson MM, Tam MF. Differential induction of helper T cell subsets during blood-stage Plasmodium chabaudi AS infection in resistant and susceptible mice. *Clinical and experimental immunology* 1993, **92**(1)**:** 77-83.

348. Su Z, Stevenson MM. Central Role of Endogenous Gamma Interferon in Protective Immunity against Blood-Stage Plasmodium chabaudi AS Infection. *Infect Immun* 2000, **68**(8)**:** 4399-4406.

349. Carvalho LH, Sano G, Hafalla JC, Morrot A, Curotto de Lafaille MA, Zavala F. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med* 2002, **8**(2)**:** 166-170.

350. Overstreet MG, Chen YC, Cockburn IA, Tse SW, Zavala F. CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites. *PloS one* 2011, **6**(1)**:** e15948.

351. Murray HW, Jungbluth A, Ritter E, Montelibano C, Marino MW. Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment. *Infect Immun* 2000, **68**(11)**:** 6289-6293.

352. Engwerda CR, Kaye PM. Organ-specific immune responses associated with infectious disease. *Immunology today* 2000, **21**(2)**:** 73-78.

353. Gorak PM, Engwerda CR, Kaye PM. Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection. *Eur J Immunol* 1998, **28**(2)**:** 687-695.

354. Murray HW, Delph-Etienne S. Roles of Endogenous Gamma Interferon and Macrophage Microbicidal Mechanisms in Host Response to Chemotherapy in Experimental Visceral Leishmaniasis. *Infection and Immunity* 2000, **68**(1)**:** 288-293.

355. Rocha FJ, Schleicher U, Mattner J, Alber G, Bogdan C. Cytokines, signaling pathways, and effector molecules required for the control of Leishmania (Viannia) braziliensis in mice. *Infect Immun* 2007, **75**(8)**:** 3823-3832.

356. Jankovic D. Conventional T-bet( )Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *The Journal of experimental medicine* 2007, **204**(2)**:** 273-283.

357. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993, **75**(2)**:** 263-274.

358. Pontoux C, Banz A, Papiernik M. Natural CD4 CD25(+) regulatory T cells control the burst of superantigen-induced cytokine production: the role of IL-10. *International immunology* 2002, **14**(2)**:** 233-239.

359. Berretta F, St-Pierre J, Piccirillo CA, Stevenson MM. IL-2 Contributes to Maintaining a Balance between CD4+Foxp3+ Regulatory T Cells and Effector CD4+ T Cells Required for Immune Control of Blood-Stage Malaria Infection. *The Journal of Immunology* 2011, **186**(8)**:** 4862-4871.

360. Abel S, Luckheide N, Westendorf AM, Geffers R, Roers A, Muller W, *et al.* Strong impact of CD4+ Foxp3+ regulatory T cells and limited effect of T cell-derived IL-10 on pathogen clearance during Plasmodium yoelii infection. *Journal of immunology (Baltimore, Md : 1950)* 2012, **188**(11)**:** 5467-5477.

361. Yao Y, Vent-Schmidt J, McGeough MD, Wong M, Hoffman HM, Steiner TS, *et al.* Tr1 Cells, but Not Foxp3+ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10-Dependent Mechanism. *Journal of immunology (Baltimore, Md : 1950)* 2015, **195**(2)**:** 488-497.

362. Torrado E, Fountain JJ, Liao M, Tighe M, Reiley WW, Lai RP, *et al.* Interleukin 27R regulates CD4+ T cell phenotype and impacts protective immunity during Mycobacterium tuberculosis infection. *J Exp Med* 2015, **212**(9)**:** 1449-1463.

363. Desreumaux P, Foussat A, Allez M, Beaugerie L, Hebuterne X, Bouhnik Y, *et al.* Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology* 2012, **143**(5)**:** 1207-1217.e1201-1202.

364. Dalton JE, Glover AC, Hoodless L, Lim EK, Beattie L, Kirby A, *et al.* The neurotrophic receptor Ntrk2 directs lymphoid tissue neovascularization during Leishmania donovani infection. *PLoS Pathog* 2015, **11**(2)**:** e1004681.

365. Suryaprasad AG, Prindiville T. The biology of TNF blockade. *Autoimmunity Reviews* 2003, **2**(6)**:** 346-357.

366. Chaudhari U, Romano P, Mulcahy LD, Dooley LT, Baker DG, Gottlieb AB. Efficacy and safety of infliximab monotherapy for plaque-type psoriasis: a randomised trial. *Lancet (London, England)* 2001, **357**(9271)**:** 1842-1847.

367. Braun J, Brandt J, Listing J, Zink A, Alten R, Golder W, *et al.* Treatment of active ankylosing spondylitis with infliximab: a randomised controlled multicentre trial. *Lancet (London, England)* 2002, **359**(9313)**:** 1187-1193.

368. Hanauer SB, Sandborn WJ, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh D, *et al.* Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology* 2006, **130**(2)**:** 323-333; quiz 591.

369. Maini RN, Breedveld FC, Kalden JR, Smolen JS, Davis D, Macfarlane JD, *et al.* Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis and rheumatism* 1998, **41**(9)**:** 1552-1563.

370. Rau R. Adalimumab (a fully human anti-tumour necrosis factor alpha monoclonal antibody) in the treatment of active rheumatoid arthritis: the initial results of five trials. *Annals of the rheumatic diseases* 2002, **61 Suppl 2:** ii70-73.

371. Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, *et al.* Infliximab for induction and maintenance therapy for ulcerative colitis. *The New England journal of medicine* 2005, **353**(23)**:** 2462-2476.

372. Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, *et al.* A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *The New England journal of medicine* 1997, **337**(15)**:** 1029-1035.

373. Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, *et al.* Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. *Lancet (London, England)* 2006, **367**(9504)**:** 29-35.

374. Nolte MA, Arens R, Kraus M, van Oers MH, Kraal G, van Lier RA, *et al.* B cells are crucial for both development and maintenance of the splenic marginal zone. *Journal of immunology (Baltimore, Md : 1950)* 2004, **172**(6)**:** 3620-3627.

375. Crowley MT, Reilly CR, Lo D. Influence of lymphocytes on the presence and organization of dendritic cell subsets in the spleen. *Journal of immunology (Baltimore, Md : 1950)* 1999, **163**(9)**:** 4894-4900.

376. Alexandre-Pires G, Pais D, Correia M, Pina JA. Leishmaniosis--a report about the microvascular and cellular architecture of the infected spleen in Canis familiaris. *Microscopy research and technique* 2006, **69**(4)**:** 227-235.

377. Gorak PMA, Engwerda CR, Kaye PM. Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection. *European Journal of Immunology* 1998, **28**(2)**:** 687-695.

378. Lohmann-Matthes ML, Luttig B, Hockertz S. Involvement of membrane-associated TNF in the killing of Leishmania donovani parasites by macrophages. *Behring Institute Mitteilungen* 1991(88)**:** 125-132.

379. Kiderlen AF, Kayser O, Ferreira D, Kolodziej H. Tannins and related compounds: killing of amastigotes of Leishmania donovani and release of nitric oxide and tumour necrosis factor alpha in macrophages in vitro. *Zeitschrift fur Naturforschung C, Journal of biosciences* 2001, **56**(5-6)**:** 444-454.

380. Mendez S, Reckling SK, Piccirillo CA, Sacks D, Belkaid Y. Role for CD4(+) CD25(+) Regulatory T Cells in Reactivation of Persistent Leishmaniasis and Control of Concomitant Immunity. *The Journal of Experimental Medicine* 2004, **200**(2)**:** 201-210.

381. Silva KL, de Andrade MM, Melo LM, Perosso J, Vasconcelos RO, Munari DP, *et al.* CD4+FOXP3+ cells produce IL-10 in the spleens of dogs with visceral leishmaniasis. *Veterinary parasitology* 2014, **202**(3-4)**:** 313-318.

382. Evans HG, Roostalu U, Walter GJ, Gullick NJ, Frederiksen KS, Roberts CA, *et al.* TNF-alpha blockade induces IL-10 expression in human CD4+ T cells. *Nature communications* 2014, **5:** 3199.

383. Oswald IP, Wynn TA, Sher A, James SL. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. *Proceedings of the National Academy of Sciences of the United States of America* 1992, **89**(18)**:** 8676-8680.

384. Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med* 1991, **174**(6)**:** 1549-1555.

385. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. *Journal of immunology (Baltimore, Md : 1950)* 2001, **166**(2)**:** 1141-1147.

386. O'Garra A, Vieira PL, Vieira P, Goldfeld AE. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. *The Journal of clinical investigation* 2004, **114**(10)**:** 1372-1378.

387. Filardy AA, Costa-da-Silva AC, Koeller CM, Guimaraes-Pinto K, Ribeiro-Gomes FL, Lopes MF, *et al.* Infection with Leishmania major induces a cellular stress response in macrophages. *PloS one* 2014, **9**(1)**:** e85715.

388. Lopes MF, Costa-da-Silva AC, DosReis GA. Innate immunity to Leishmania infection: within phagocytes. *Mediators of inflammation* 2014, **2014:** 754965.

389. Gottlieb AB. Tumor Necrosis Factor Blockade: Mechanism of Action. *J Invest Derm Symp P* 2007, **12**(1)**:** 1-4.

390. Goldenberg MM. Etanercept, a novel drug for the treatment of patients with severe, active rheumatoid arthritis. *Clinical therapeutics* 1999, **21**(1)**:** 75-87; discussion 71-72.

391. Moreland LW, Cohen SB, Baumgartner SW, Tindall EA, Bulpitt K, Martin R, *et al.* Long-term safety and efficacy of etanercept in patients with rheumatoid arthritis. *The Journal of rheumatology* 2001, **28**(6)**:** 1238-1244.

392. Keyser FD. Choice of Biologic Therapy for Patients with Rheumatoid Arthritis: The Infection Perspective. *Current Rheumatology Reviews* 2011, **7**(1)**:** 77-87.

393. Ali T, Kaitha S, Mahmood S, Ftesi A, Stone J, Bronze MS. Clinical use of anti-TNF therapy and increased risk of infections. *Drug, Healthcare and Patient Safety* 2013, **5:** 79-99.

394. Taylor AP, Murray HW. Intracellular antimicrobial activity in the absence of interferon-gamma: effect of interleukin-12 in experimental visceral leishmaniasis in interferon-gamma gene-disrupted mice. *J Exp Med* 1997, **185**(7)**:** 1231-1239.

395. Martins G, Calame K. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol* 2008, **26:** 133-169.

396. Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, *et al.* The Role of Interleukin (IL)-10 in the Persistence of Leishmania major in the Skin after Healing and the Therapeutic Potential of Anti–IL-10 Receptor Antibody for Sterile Cure. *The Journal of Experimental Medicine* 2001, **194**(10)**:** 1497-1506.

397. Martins GA, Cimmino L, Liao J, Magnusdottir E, Calame K. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. *The Journal of Experimental Medicine* 2008, **205**(9)**:** 1959-1965.

398. Kraal G, Rodrigues H, Hoeben K, Van Rooijen N. Lymphocyte migration in the spleen: the effect of macrophage elimination. *Immunology* 1989, **68**(2)**:** 227-232.

399. Lyons AB, Parish CR. Are murine marginal-zone macrophages the splenic white pulp analog of high endothelial venules? *Eur J Immunol* 1995, **25**(11)**:** 3165-3172.

400. Baluk P, Yao LC, Feng J, Romano T, Jung SS, Schreiter JL, *et al.* TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *The Journal of clinical investigation* 2009, **119**(10)**:** 2954-2964.

401. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature* 1987, **329**(6140)**:** 630-632.

402. Szekanecz Z, Koch AE. Mechanisms of Disease: angiogenesis in inflammatory diseases. *Nature clinical practice Rheumatology* 2007, **3**(11)**:** 635-643.

403. Szekanecz Z, Besenyei T, Paragh G, Koch AE. Angiogenesis in rheumatoid arthritis. *Autoimmunity* 2009, **42**(7)**:** 563-573.

404. Paleolog EM. Angiogenesis in rheumatoid arthritis. *Arthritis research* 2002, **4 Suppl 3:** S81-90.

405. Gonzalez-Lombana C, Gimblet C, Bacellar O, Oliveira WW, Passos S, Carvalho LP, *et al.* IL-17 Mediates Immunopathology in the Absence of IL-10 Following Leishmania major Infection. *PLoS Pathogens* 2013, **9**(3)**:** e1003243.

406. Lan Q, Fan H, Quesniaux V, Ryffel B, Liu Z, Guo Zheng S. Induced Foxp3+ regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? *Journal of Molecular Cell Biology* 2012, **4**(1)**:** 22-28.

407. Miyara M, Ito Y, Sakaguchi S. TREG-cell therapies for autoimmune rheumatic diseases. *Nature reviews Rheumatology* 2014, **10**(9)**:** 543-551.

408. Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, *et al.* Generation and Large-Scale Expansion of Human Inducible Regulatory T Cells That Suppress Graft-Versus-Host Disease. *American Journal of Transplantation* 2011, **11**(6)**:** 1148-1157.

409. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, *et al.* Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. *European Journal of Immunology* 2009, **39**(4)**:** 1088-1097.

410. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of immunology (Baltimore, Md : 1950)* 1986, **136**(7)**:** 2348-2357.

411. O'Garra A, Vieira P. TH1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* 2007, **7**(6)**:** 425-428.

412. Grazia Roncarolo M, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* 2006, **212**(1)**:** 28-50.

413. Minigo G, Woodberry T, Piera KA, Salwati E, Tjitra E, Kenangalem E, *et al.* Parasite-Dependent Expansion of TNF Receptor II–Positive Regulatory T Cells with Enhanced Suppressive Activity in Adults with Severe Malaria. *PLoS Pathog* 2009, **5**(4)**:** e1000402.

414. Sun T, Holowka T, Song Y, Zierow S, Leng L, Chen Y, *et al.* A Plasmodium-encoded cytokine suppresses T-cell immunity during malaria. *Proceedings of the National Academy of Sciences* 2012, **109**(31)**:** E2117–E2126.

415. Gwyer Findlay E, Villegas-Mendez A, de Souza JB, Inkson CA, Shaw TN, Saris CJ, *et al.* IL-27 Receptor Signaling Regulates CD4+ T Cell Chemotactic Responses during Infection. *The Journal of Immunology* 2013.

416. World-Health-Organisation. Preventing Chronic Diseases - a vital investment - Part two. Geneva, Switzerland; 2005.

417. American-Autoimmune-Related-Diseases-Association-Inc. Autoimmune statistics. 2015 [cited 2015 17th December 2015]Available from: http://www.aarda.org/autoimmune-information/autoimmune-statistics/

418. Cooper GS, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *Journal of autoimmunity* 2009, **33**(3-4)**:** 197-207.

419. Steinman L, Merrill JT, McInnes IB, Peakman M. Optimization of current and future therapy for autoimmune diseases. *Nat Med* 2012, **18**(1)**:** 59-65.

420. Regules JA, Cummings JF, Ockenhouse CF. The RTS,S vaccine candidate for malaria. *Expert review of vaccines* 2011, **10**(5)**:** 589-599.

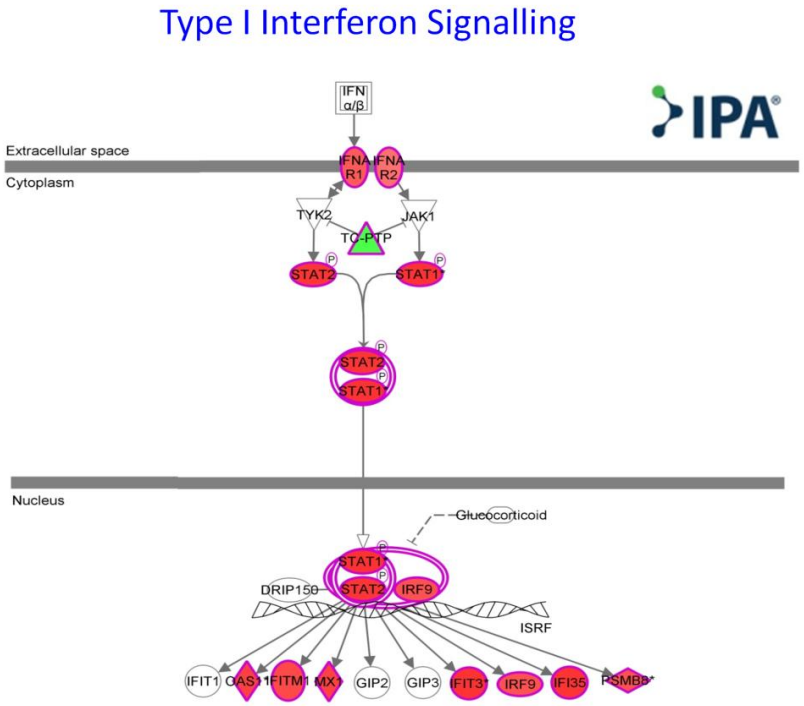
421. Stoute JA, Kester KE, Krzych U, Wellde BT, Hall T, White K, *et al.* Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. *The Journal of infectious diseases* 1998, **178**(4)**:** 1139-1144.

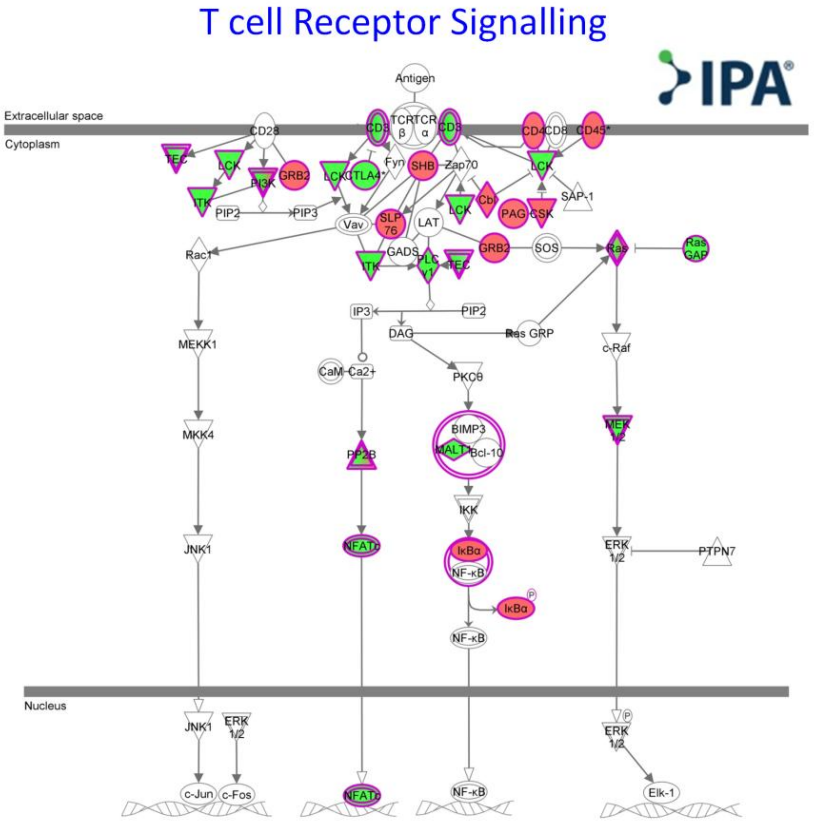
Appendices

Appendix 1: Composition of the RTS,S/AS01vaccine candidate against malaria

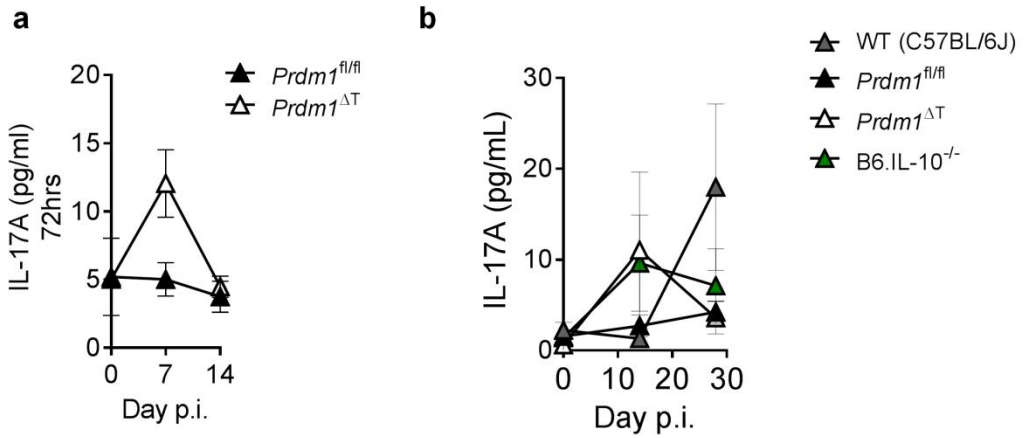
The 'R' stands for the central repeat region of *P. falciparum* circumsporozoite protein (CSP), the 'T' for the T-cell epitopes of the CSP; and the 'S' for hepatitis B surface antigen (HBsAg). These are combined in a single fusion protein ('RTS') and co-expressed in yeast cells with free HBsAg. The 'RTS' fusion protein and free 'S' protein spontaneously assemble into 'RTS,S' particles and the AS01 stands for adjuvant system 01[420, 421].

Appendix 2: Enlarged Ingenuity Pathway Analysis – Signalling pathways





Appendix 3



Appendix 3: In the absence of IL-10, IL-17A appears to play a minor role in contributing to the inflammation-induced pathology observed in the T cell specific Blimp1 deficient mice

a) Splenocytes from *L. donovani* infected *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were cultured in the presence of fixed *L. donovani* (4% PFA) amastigotes for 72 hours and culture supernatants were assessed for IL-17A levels using a BD Flex set kit at day 7 and day 14 p.i.. **b)** WT (C57BL/6J), IL-10 deficient (IL-10$^{-/-}$), *Prdm1*$^{\Delta T}$ and *Prdm1*$^{fl/fl}$ mice were infected with 2x10^{7} *L. donovani* amastigotes i.v. and serum IL-17A levels were measured using a BD Flex set kit at day 14 and 28p.i..