Investigating the Effect of *Plasmodium falciparum* Infected Red Blood Cells on Dendritic Cell Function

Rowland Sadia Osii

BSc. MSc.

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

Institute of Infection, Immunity, and Inflammation

College of Medical, Veterinary & Life Sciences

University of Glasgow

May, 2022

Abstract

Malaria remains a global health problem, that affects majority of the world's population living in tropical and subtropical areas of the world. Its impact, in terms of morbidity and mortality is highest in sub-Saharan Africa, and especially among children under the age of 5. While the intensive application of currently available malaria control tools has greatly reduced malaria transmission, the possibility of malaria elimination remains a distant goal. Thus, novel innovative methods are required to complement the existing control tools to push malaria further towards elimination. Vaccine induced immunity is one of the most cost-effective methods for controlling infectious diseases and has led to the elimination of some. Encouragingly, residents of highly endemic regions develop naturally acquired immunity that offers protection against clinical symptoms of malaria. However, naturally acquired immunity is incomplete as its permissive of asymptomatic infections and develops slowly after several rounds of repeated infections. This slow development of immunity may be attributed to the ability of the parasite to modify dendritic cell function, though neither the mechanisms of this alteration, nor its impact on the downstream cellular and antibody host responses are well understood. A better understanding of the basic biology of the interactions of malaria parasites with the human host is therefore, clearly warranted, and will be instrumental in the design and development of highly effective vaccines.

To investigate the cellular and molecular pathways underpinning the alteration of dendritic cell function upon exposure to parasite infected red blood cells (iRBC), I established an *in vitro* assay using monocyte-derived dendritic cells from adults, irrespective of prior exposure to malaria. Exposure of dendritic cells to iRBCs did not result in activation. However, when the iRBC-exposed dendritic cells were subsequently stimulated with lipopolysaccharide (LPS), they were unable to express key co-stimulatory molecules to the same extent as LPS stimulated control RBC exposed dendritic cells. Nonetheless, these iRBC treated dendritic cells were still capable of inducing upregulation of the early activation marker, CD69, in CD4 T cells in a mixed lymphocyte reaction. Single cell RNA sequencing of cells from the mixed lymphocyte

reaction between iRBC pre-treated dendritic cells and CD4 T cells initiated transforming growth factor beta driven signalling that altered dendritic cell function, resulting in expansion of the CD4 T regulatory cells.

Having delineated the molecular pathways elicited by the iRBC treatment of dendritic cell function *in vitro,* I wanted to see whether I would observe similar changes in cellular function *in vivo.* I took advantage of samples from a medical experiment involving a controlled human malaria infection (CHMI) study at the KEMRI-Wellcome Trust Research Programme in Kilifi, Kenya. The participants in this study were recruited from malaria endemic areas with varying levels of *P. falciparum* transmission intensity. Specifically, I compared changes in gene expression with CHMI between two groups using longitudinal samples: those that developed a productive infection with no symptoms ("chronic") and those that became infected and developed fever ("febrile"). Monocytes from the chronic participants exhibited an early production of type II interferon, while monocyte from the febrile participants upregulated type 1 interferons. Interestingly, type II interferons are thought to be involved in immune control of malaria infections, while type I interferons can hamper the establishment of an adequate immune response.

The work presented herein provides insight into the signalling pathways that are activated by dendritic cells and monocytes upon their interaction with iRBCs, and how adults recruited from malaria endemic area (grouped into febrile and chronic participants) can have varying abilities to control infection based on the type of the immune response induced following CHMI.

Acknowledgements

First, I would like to thank my supervisors James Brewer, Francis Ndungu, and Paul Garside for the giving me this learning opportunity and for the constant support that they have given me throughout the PhD project, and for the constructive discussions that we have had around the project. I also want to thank Thomas Otto for the bioinformatics training, and support that he offered me in data analysis.

I would also like to thank past and present LIVE-group members for making my PhD journey enjoyable. A big thank you, to Gavin and Hannah for all the technical support they offered me in the lab and especially in teaching me the various lab techniques critical to my research, helping me establish experiments, and for preparing and tagging the CHMI samples for sequencing on my behalf when I couldn't travel during the COVID-19 pandemic. Great thanks also to Josh, Shaima, Priscilla, and Lauriane for helping me out in lab, and for making me feel at home in Glasgow.

I would like to thank Jedida and Jennifer from KEMRI-Wellcome Trust Research Programme in Kilifi, who were instrumental in assisting me to locate all the CHMI samples and ensuring that they were shipped to UofG in time. Francis's group members Diana and Koko for the discussions and feedback on my project.

I am thankful to the Initiative to Develop African Research Leaders (IDeAL) under the leadership of Sam Kinyanjui for funding my PhD studies under. Thanks to the administrative support from Liz Murabu, David Ngui, Rita Baya, Florence Kirimi and Dorcas Mbuvi.

I would like to thank my parents for their constant support, love, and encouragement. Finally, I would like to thank my wife, Mercy, for supporting me throughout this project, and being patient with me during this time. Thank you for being my support system and ensuring that I always got enough food and rest during a busy writing period.

Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Name: Rowland Sadia Osii

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Abbreviations

Abbreviation	Definition
ADAM17	A Disintegrin And Metalloproteinase 17
ADC1	Antibody-Dependent Cell Inhibition
Ag	Antigen
AP1	Activator Protein 1
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BCL6	B-Cell Lymphoma 6 Protein
BIRC5	Baculoviral Iap Repeat-Containing Protein 5
BMDCs	Bone Marrow Derived Dendritic Cells
CCL	C-C Motif Ligand
CCR	C-C Chemokine Receptor
CD274	Programmed Cell Death 1 Ligand 1
CD36	Scavenger Receptor Class B Member 3
CD4 T CM	CD4 T Central Memory
CD4 T EM	CD4 T Effector Memory
cDCs	Conventional Dendritic Cells
CelTOS	Cell Traversal Protein For Ookinete And Sporozoite
CHMI	Controlled Human Infection Model
CIDR	Cysteine-Rich Inter-Domain Region
CLEC	C-Type Lectin Domain Family
CLIP	Class II-Associated Invariant Chain Peptide
CR1	Complement Receptor 1
CSA	Chondroitin Sulphate A
CSP	Circumsporozoite Protein
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CXCL	C-X-C Motif Ligand
CXCR	C-X-C Motif Receptor
cyclic AMP	Cyclic Adenosine Monophosphate
DAMP	Damage-Associated Molecular Patterns
DARC	Duffy Antigen Receptor For Chemokines
DBL	Duffy-Binding Like
DBP	Duffy Binding Protein
DC-SIGN	Dendritic Cell Specific Intercellular Adhesion Molecule3-Grabbing Non-Integrin
DCs	Dendritic Cells

DUSP	Dual-Specificity Phosphatases
EBI3	Epstein-Barr Virus Induced 3
EBP2	Erythrocyte Binding Protein
EPCR	Endothelial Protein C Receptor
ER	Endoplasmic Reticulum
ERAAP	ER Aminopeptidase Associated With Ag Processing
FCGR	Fc Gamma Receptors
FLT3	FMS-Like Tyrosine Kinase 3
FLT3L	FMS-Like Tyrosine Kinase 3 Ligand
FOXP3	Forkhead Box Protein P3
GAG	Glycosaminoglycan
GBP	Guanylate-Binding Proteins
GITR	Glucocorticoid-Inducible Tumour Necrosis Factor Receptor
GLURP	Glutamate-Rich Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPI	Glycosylphosphatidylinositol
GSH	Glutathione
GZMA	Granzyme A
HbAS	Sickle-Cell Trait
HbC	Haemoglobin C
HbE	Haemoglobin E
HBEGF	Proheparin-Binding EGF-Like Growth Factor
HLA-DM	Human Leukocyte Antigen, DM
HMGB	High-Mobility Group Box
ICAM-1	Intercellular Adhesion Molecule-1
ICOS	Inducible T-Cell Costimulator
ICOSL	Inducible T-Cell Costimulatory Ligand
IES	Inter-Endothelial Slits
IFIT	Interferon Induced Protein With Tetratricopeptide Repeats
IFITM	Interferon-Induced Transmembrane Proteins
IFN-α	Interferon Alpha
IFNγ	Interferon Gamma
IFNAR	Interferon Alpha/Beta Receptor 1
Ii	Invariant Chain
IL	Interleukin
iRBC	Parasite Infected Red Blood Cell
IRF	Interferon Regulatory Factor
IRS2	Insulin Receptor Substrate 2

ISG	Interferon-Stimulated Gene
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITGAX	Integrin Subunit Alpha X
ITGB1	Integrin Beta-1
iTreg	Induced T Regulatory Cells
JAK2	Janus Kinase 2
JUND	Transcription Factor Jun-D
KAHRP	Knob-Associated Histidine-Rich Protein
KC	Kupffer Cells
KLF	Kruppel-Like Factor
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cells
LTB	Lymphotoxin-Beta
MAdCAM1	Mucosal Addressin Cell Adhesion Molecule 1
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MIIC	MHC-II Compartment
MKI67	Proliferation Marker Protein Ki-67
moDC	Monocyte Derived Dendritic Cell
MS4A1	Membrane Spanning 4-Domains A1
MSP1	Merozoite Surface Protein-1
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAI	Naturally Acquired Immunity
NK Cells	Natural Killer Cells
NKG2D	Killer Cell Lectin Like Receptor K1
NKp30	Natural Cytotoxicity Triggering Receptor 3
NKp44	Natural Cytotoxicity Triggering Receptor 2
NKp46	Natural Cytotoxicity Triggering Receptor 1
NR4A1	Nuclear Receptor 4A1
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Patterns
PD-1	Programmed Cell Death-1
PD-L1	Programmed Death-Ligand 1
PDCD1	Programmed Cell Death Protein 1
PDCD1LG2,	Programmed Cell Death 1 Ligand 2
pDCs	Plasmacytoid Dendritic Cells
PEP	Phosphoenolpyruvate

PEXEL	*Plasmodium* Export Element
PfEMP-1	*Plasmodium* Falciparum Erythrocyte Membrane Protein 1
PGE2	Prostaglandin E2
PK	Pyruvate Kinase
PNEP	PEXEL-Negative Exported Proteins
PRF1	Perforin-1
PRR	Pattern Recognition Receptors
PTPRC	Protein Tyrosine Phosphatase Receptor Type C
PVM	Parasitophorous Vacuole Membrane
RANTES	Regulated Upon Activation, Normal T Cell Expressed And Presumably Secreted
RBC	Red Blood Cell
RIFIN	Repetitive Interspersed Family
SAP	Signalling Lymphocytic Activation Molecule-Associated Protein
SELL	L-Selectin
SET	Protein SET
SLO	Secondary Lymphoid Organs
SMAD	*Caenorhabditis Elegans* Sma And *Drosophila* Mad Proteins
SMC4	Structural Maintenance Of Chromosomes Protein 4
SOSC	Suppressor Of Cytokine Signalling
SPECT	Sporozoite Microneme Protein Essential For Cell Traversal
SPPL2A	Signal Peptide Peptidase-Like 2A
STAT1	Signal Transducer And Activator Of Transcription 1
STEVOR	Sub-Telomeric Variable Open Reading Frame
STMN1	Stathmin
TAB	Transforming Growth Factor-B-Activated Kinase-Binding Proteins
TAK	Transforming Growth Factor-B-Activated Kinase 1
TAP	Transporter Associated With Antigen Processing
TCR	T Cell Receptor
Tfh	CD4 T Follicular Helper
TGF-β	Transforming Growth Factor Beta
Th1	CD4 T Helper 1
Th17	CD4 T Helper 17
Th2	CD4 T Helper 2
TIMP-1	Tissue Inhibitor Of Metalloproteinases 1
TLP	TRAP-Like Protein
TLR	Toll-Like Receptor

TNF-α	Tumour Necrosis Factor Alpha
TNFAIP3	Tumour Necrosis Factor Alpha-Induced Protein 3
TNFSF	Tumour Necrosis Factor Superfamily
TNSF13B	Tumour Necrosis Factor Ligand Superfamily Member 13B
TNSF4	Tumour Necrosis Factor Superfamily 4
TOP2A	DNA Topoisomerase 2-Alpha
Tr1	Regulatory Type 1 Cells
TRAF	Tumour Necrosis Factor Receptor (TNFR)Associated Factor
TRAP	Thrombospondin-Related Anonymous Protein
Treg	CD4 T Regulatory Cells
TRIF	Toll/IL-1R-Containing Adaptor Inducing IFN-B
TSC22D3	Glucocorticoid-Induced Leucine Zipper
UBE2S	Ubiquitin-Conjugating Enzyme E2 S
VCAM-1	Vascular Cell Adhesion Molecule 1
VSA	Variant Surface Antigens
XAF1	XIAP-Associated Factor 1
YARS	Tyrosine-Trna Ligase
ZFP	Zinc Finger Protein

Chapter 1 Introduction

1.1 Overview

Malaria is a life-threatening disease that affects majority of the world's population. Annually, the disease is approximated to cause 228 million cases of infection, resulting in 405,000 deaths (W.H.O, 2019). Africa bears majority of the malaria burden as it accounts for about 93% of the reported cases and 94% of reported mortality with most of reported mortality cases occurring in children under the age of 5 (W.H.O, 2019). The causative agent of malaria is the Apicomplexan parasite of the genus *Plasmodium*. *P. falciparum* is the deadliest of the *Plasmodium* species and is responsible for majority of the global mortality and morbidity. While *P. vivax* is the most widespread of the *Plasmodium* species that can result in severe and even fatal infections that results in mortality.

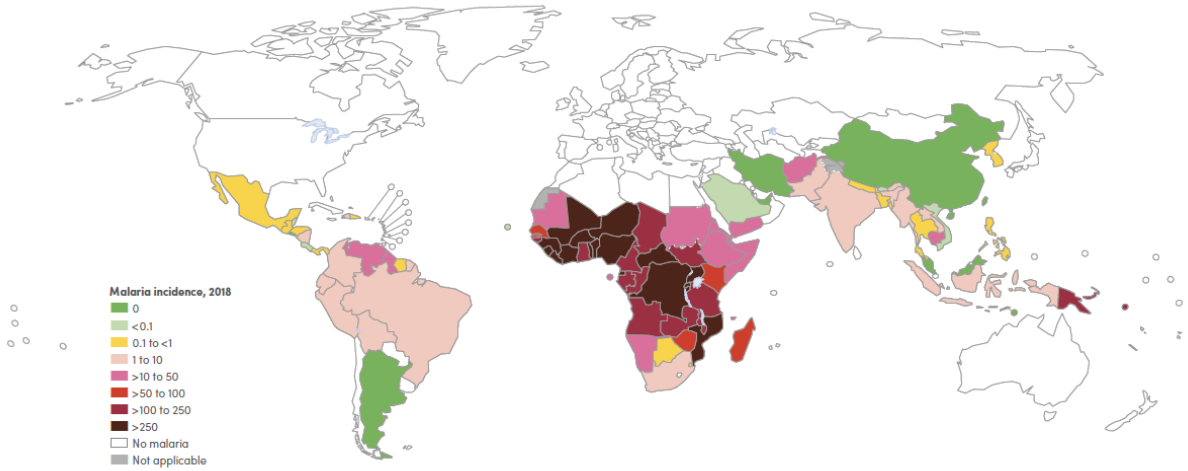


Figure 1-1: Map showing the global distribution of malaria

The map shows the global distribution of malaria throughout the world, with Sub-Saharan Africa bearing majority of the malaria burden. (W.H.O, 2019)

1.2 Malaria Species That Infect Humans

Majority of malaria mortality is caused by *P. falciparum*, the dominant species in the tropical parts of Africa, while in the Asian and South American continents, *P. vivax* is the prevalent species. Initially infection with *P. vivax* was thought to be a "benign" infection due to the low blood stage parasitaemia, but this is not the case as it has been shown to cause morbidity and has been associated with severe malaria and death (Baird, 2009, Baird, 2013, Price et al., 2007, Genton et al., 2008, Barcus et al., 2007, Dini et al., 2020). *P. vivax* was thought to be absent in Africa due to the high frequency of the Duffy negative phenotype (Miller et al., 1976, Howes et al., 2011) that is found is western, central, and eastern African. The Duffy negative phenotype is an inherited trait that results in a lack of expression of the Duffy receptor which is key in invasion of *P. vivax* parasite. Recent studies have shown that *P. vivax* can still infect Duffy negative red blood cells (Howes et al., 2015, Gunalan et al., 2018, Twohig et al., 2019, Zimmerman, 2017) as its transmission has been seen in Duffy negative populations of Angola and Equatorial Guinea (Mendes et al., 2011), Benin (Poirier et al., 2016), Botswana (Motshoge et al., 2016), Cameroon (Mbenda and Das, 2014, Russo et al., 2017), Ethiopia (Woldearegai et al., 2013, Gunalan et al., 2016), Western Kenya (Ryan, 2006), and Mauritania (Wurtz et al., 2011). Thus, raising the possibility of an alternative receptor/mechanism for invasion of *P. vivax* in Duffy negative red blood cells.

P. ovale is primarily found in Sub-Saharan Africa and the islands in the western Pacific (Collins and Jeffery, 2005) with the highest prevalence reported in Nigeria (May et al., 1999) and Papua New Guinea (Mehlotra et al., 2000). *P. ovale* infection is difficult to diagnose due to its low blood parasitaemia and its similar morphology to *P. vivax*, and this has led to underestimation of *P. ovale* cases (Faye et al., 2002). Two subspecies of *P. ovale* parasite exists, *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al., 2010) but little is known about their geographical distribution and severity of infection of the two subspecies.

P. malariae infections are often asymptomatic and rarely fatal. This species is known to cause a low-grade chronic infection that is associated with anaemia (Douglas et al., 2013, Langford et al., 2015). *P. malariae* is endemic to West Africa (Roucher et al., 2014), South America

(Camargo-Ayala et al., 2016, Scopel et al., 2004), Asia (Zhou et al., 1998) and the western Pacific region (Mueller et al., 2005).

P. knowlesi was thought to be predominantly a non-human primate parasite but there have been several demonstrations of human infections recently. Most *P. knowlesi* infection cases in humans were misdiagnosed as *P. malariae*, due to their similarity in morphology when observing blood smears using a microscope (Singh et al., 2004). *P. knowlesi* infection in humans results in hyper parasitaemia due the parasites relatively short intra-erythrocytic cycle that lasts 24hr (Knowles and Gupta, 1932, Coatney, 1971) and can result in severe disease (Seilmaier et al., 2014). The majority of human *P. knowlesi* infections have been reported in Malaysia and southeast Asia (Ahmed and Cox-Singh, 2015, Singh and Daneshvar, 2013), with some cases of infection rapidly progressing to severe malaria and resulting in mortality (Cox-Singh et al., 2008, Cox-Singh et al., 2010).

1.3 *Plasmodium* Life Cycle

Plasmodium parasites have a complex life cycle that occurs in two hosts: the female *Anopheles* mosquito (sexual reproductive stage) and a vertebrate host (asexual development stage). The latter stage begins when an infectious female *Anopheles* mosquito probes the dermis of a vertebrate host as it takes a blood meal, releasing a highly motile form of the parasite, sporozoites, from its saliva (Menard et al., 2013, Cowman et al., 2016). A single bite can release around 100 sporozoites in an experimental situation (Ponnudurai et al., 1991, Medica and Sinnis, 2005). Natural infection by mosquitoes normally inoculates fewer than 50 sporozoites per bite at a release rate of about 1-2.5 sporozoites per second (Frischknecht et al., 2004). Not all sporozoites manage to penetrate the blood vessel and those that remain in the dermis are either destroyed or drained into the lymphatic system where most are degraded but some can partially develop into exoerythrocytic form of the parasite (Cowman et al., 2016, Vaughan et al., 2008, Amino et al., 2006).

Those that manage to enter the bloodstream, then circulate and enter the liver through a process known as traversal. To gain access to a single liver hepatocyte, the sporozoite must traverse

through the liver sinusoid, a unique blood vessel that contains oxygen rich blood from the hepatic artery and nutrient rich blood from the portal vein (Yang and Boddey, 2017, Vaughan et al., 2008). The sinusoid is lined with endothelial cells and macrophage-like Kupffer cells, which the sporozoite traverses through with the aid of two proteins; sporozoite microneme protein essential for cell traversal 1 (SPECT1) and SPECT2, which are secreted by micronemes. SPECT2 contains a membrane attack complex/perforin-related domain which is key in allowing the sporozoite to traverse through Kupffer cells (Ishino et al., 2005, Ishino et al., 2004, Yang et al., 2017). Apart from SPECT1 and SPECT2 other proteins that play a role in cell traversal include, TRAP family member, TLP (TRAP-like protein) (Moreira et al., 2008), sporozoite-secreted phospholipase (Bhanot et al., 2005), and cell traversal protein for ookinete and sporozoite (CelTOS) (Kariu et al., 2006). Sporozoites highly express circumsporozoite protein (CSP), which is key in supressing the respiratory burst from Kupffer cells. CSP does this by stimulating adenyl cyclase activity which induces cyclic AMP activity and cyclic AMP inhibits assembly of NADPH oxidase and this blocks the generation of reactive oxygen species (Usynin et al., 2007). Once the sporozoite passes through the sinusoid it migrates through several hepatocytes before it takes residence in one of them. Traversal through the hepatocytes results in secretion of hepatocyte growth factor, which increases hepatocyte survival and susceptibility to infection, but is not essential for parasite invasion (Graewe et al., 2011, Vaughan et al., 2008, Prudêncio et al., 2006). During sporozoite invasion, the host's cell membrane invaginates to form a parasitophorous vacuole around the parasite. Majority of the host cell proteins are removed from the parasitophorous vacuole membrane (PVM) during this process and the sporozoites glides through the cell via a tight junction that prevents entry of substances from the outside (Yang and Boddey, 2017, Graewe et al., 2011). The PVM creates a physical barrier between the host cytoplasm and the parasite. In the PVM the sporozoite undergoes asexual development and numerous rounds of replications to become a pre-erythrocytic schizont which contains merozoites. A single sporozoite can generate up to 40,000 merozoites in the PVM, causing an expansion of the PVM to accommodate the merozoites (Cowman et al., 2016, Crompton et al., 2014, Ménard et al., 2013, Prudêncio et al., 2006). The accumulated merozoites then bud off the hepatocyte with part of the host cell membrane in structures known as merosomes which enter the blood stream and rupture releasing the encapsulated merozoites to infect red blood cells (Cowman et al., 2016, Graewe et al., 2011).

The incubation periods in the liver and the numbers of merozoites per schizont vary: 6-9 days and 40,000 merozoites for *P. falciparum* , 8-12 days and 10,000 merozoites for *P. vivax*; 10-14 days and 15,000 merozoites for *P. ovale*; and 15-18 days and 2,000 merozoites for *P. malariae* (Antinori et al., 2012). During the liver stages for *P. ovale* and *P. vivax*, some sporozoites remain in a dormant state known as hypnozoites. Hypnozoite can remain dormant for months, even after clearance of the initial infection (Hulden and Hulden, 2011).

In the blood, the free merozoites identifies a competent red blood cell (RBC) for invasion. Invasion into the RBC occurs in four phases. The first phase is adhesion where the merozoite binds to the surface of the RBC. The second phase is reorientation, which enables the parasite to bring its apical end containing the invasion enzymes in close contact with the RBC. After reorientation, the merozoite forms a tight junction. The tight junction allows for movement of the parasite into the RBC and also triggers the release of the rhoptry bulb, providing proteins and lipids that are required to form the parasitophorous vacuole, which surrounds the merozoite (Cowman and Crabb, 2006, Cowman et al., 2012, Gilson and Crabb, 2009).

Once inside the RBC, the merozoite matures in three morphologically distinct stages namely the ring, trophozoite and schizont stages. During the different stages of development, the merozoite digests haemoglobin for generation of energy and protein synthesis resulting in the formation of hemozoin crystals which are stored in the food vacuole (Mohandas and An, 2012). Parasite generated proteins are key in aiding the development of the parasite as well as remodelling the RBC for its survival. RBC remodelling is done by various parasite proteins that are exported to the RBC cytosol cytoskeleton and membrane (Cowman et al., 2016). To reach their target, the proteins must be transported through the parasite membrane to the parasitophorous vacuole, which restricts access to the host cell. Export of proteins through the parasitophorous vacuole membrane is mediated by either *Plasmodium* export element (PEXEL) motif or PEXEL-negative exported proteins (PNEPs) (Boddey et al., 2009, Boddey and Cowman, 2013). Once the proteins are in the host cell microenvironment, they are trafficked by a number of parasite derived vesicular structures such as electron-dense vesicles, J-dots and Maurer's clefts to their destination in the RBC (Spycher et al., 2006, Cowman et al., 2016, Boddey and Cowman, 2013, Hanssen et al., 2008).

PEXEL/PNEP are expressed immediately after invasion, allowing for the export of proteins expressed during the late ring/early trophozoite stage such as knob-associated histidine-rich protein (KAHRP), and *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1). These proteins are then trafficked to the RBC membrane via Maurer's cleft. KAHRP forms the knobs that are expressed on the RBC surface during the trophozoite stage. The knobs allow the Maurer's cleft to translocate PfEMP-1 to the outer surface of the RBC (Boddey and Cowman, 2013, Cowman et al., 2016). Expression of PfEMP-1 is encoded by the diverse var gene family and is key in antigenic variation and central to *P. falciparum* pathogenesis (Smith et al., 1995, Kyes et al., 2007, Claessens et al., 2014). At the late trophozoite and schizont stage, the RBC has undergone structural changes that allows it to evade splenic clearance and adhere to vascular endothelium, preventing destruction of *P. falciparum* infected RBCs (iRBCs) by the spleen. Apart from the structural changes that occur to the RBC, the parasite also undergoes nuclear division producing merozoites that fill the PV, this stage is termed the schizont stage. The merozoites egress from the RBC by a process that is tightly regulated by multiple proteolytic enzymes and invade other RBCs initiating another cycle for parasite replication **Figure 1-2**.

After rounds and rounds of replication, some of the merozoites commit themselves to sexual development and form gametocytes (Cowman et al., 2016, Farrow et al., 2011). The gametocytes undergo five stages of maturation while being sequestered in the bone marrow. Only stage five gametocytes re-enter circulation and are taken up by a mosquito during a blood meal (Ngotho et al., 2019). In the mosquito midgut, the male and female gametocytes fuse to form a zygote that differentiates into ookinetes, which crosses the midgut wall forming oocysts, in which haploid sporozoites develop. The oocysts rupture releasing sporozoites that travel through the haemocoel to invade the salivary gland, completing the parasites life cycle (Crompton et al., 2014).

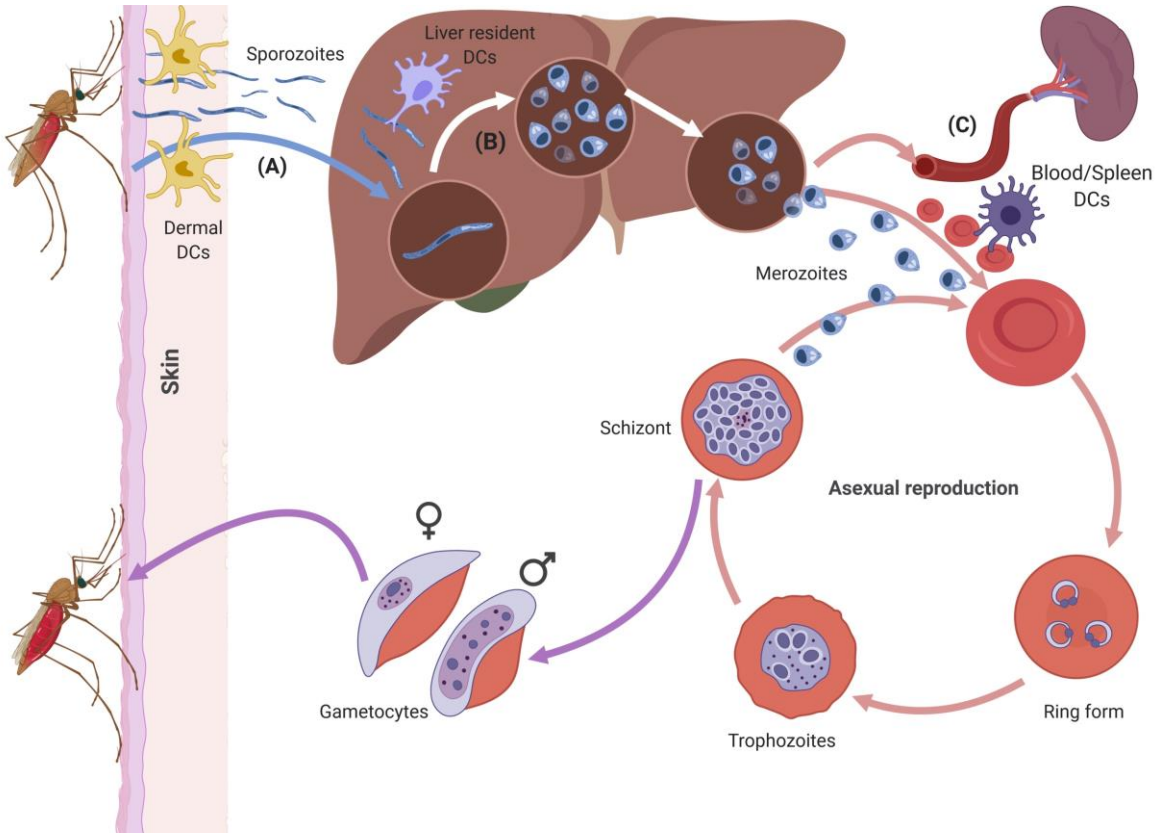


Figure 1-2 Life Cycle of *Plasmodium falciparum* and points of interaction with DCs.

The asexual stage of *P. falciparum occurs* in humans when an infected mosquito takes a blood meal releasing the motile form of the parasite (sporozoite) into the blood stream, the sporozoite traverses into the liver where they differentiate into merozoites, the merozoites are then released into the blood stream where they invade RBCs initiating round of replication within the RBC. Dendritic cells can interact with different forms of the parasite at the skin (A), the liver (B), the bold and spleen (C). Figure adapted from Osii et al. (2020)

1.4 Structural Changes in *Plasmodium* Infected RBCs

Structural changes that the RBC undergoes during the late stages of the parasites erythrocytic cycle (late trophozoite and schizont) enables them to express various parasite-derived variant surface antigens (VSAs), which are encoded by multigene families namely, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Howard et al., 1988, Chen et al., 1998), sub-telomeric variable open reading frame (STEVOR) proteins (Naing et al., 2014), and repetitive interspersed family (RIFIN) proteins (Goel et al., 2015). These VSAs enable the iRBC to bind and adhere to various endothelial cells, thus sequestering in the microvasculature of various

organs a process known as cytoadherence. Multiple adhesion domains have been identified on the extracellular region of PfEMP1 such as, the N-terminal segment (NTS), the C2 region, the Duffy-binding like (DBL) and the cysteine-rich inter-domain region (CIDR) (Smith et al., 2001). These domains bind to a number of host receptors which include CD36 (Hsieh et al., 2016, Robinson et al., 2003), chondroitin sulphate A (CSA) (Fried and Duffy, 1996, Khattab et al., 2001), intercellular adhesion molecule-1 (ICAM-1) (Bertonati and Tramontano, 2007, Smith et al., 2000) and endothelial protein c receptor (EPCR) (Turner et al., 2013). Rosetting is another phenomenon that has been observed in iRBC, where uninfected RBCs form clusters around an iRBC. This observation was first reported in monkeys infected with *Plasmodium fragile* (David et al., 1988), and has been seen in various species of malaria parasite. This cytoadherence property of late stage iRBC has been mapped to the DBL1α domain on PfEMP1 which contains glycosaminoglycan (GAG) binding motifs (Baruch, 1999, Baruch et al., 2002, Cooke et al., 2001).

Structural modification of the RBC by the parasite renders the iRBC susceptible to splenic filtration. Healthy RBCs with normal morphology and rheology are able to pass through the narrow inter-endothelial slits (IES) of the red pulp (Pivkin et al., 2016), whereas the abnormal cells are retained in the spleen and engulfed by the various immune cells that present in the red pulp. To avoid splenic clearance, iRBC tend to cytoadhere to vascular endothelium and sequester in capillary beds of less dangerous organs (Suwanarusk et al., 2004, Sosale et al., 2015). Rosetting on the other hand is thought to protect the iRBC from immune recognition by antibodies and phagocytic cells as the iRBCs are masked from the immune cells by uninfected RBCs (Wolofsky et al., 2012).

1.5 Clinical Presentation of Malaria

Infection with *P. falciparum* is normally followed with a variety of clinical manifestations. The manifestation of these clinical symptoms is mainly dependent on an individual's level of immunity. Non-immune individuals always develop clinical symptoms of malaria even at low parasite densities. The symptoms include intermittent fever and other non-specific symptoms such as headaches, malaise, abdominal pain, muscle aches and diarrhoea. The periodic fever

seen in these individuals occurs in three distinct stages (cold stage, hot stage, and sweating stage), and is synchronous to the egressing of merozoites from mature schizonts (Hirako et al., 2018). If treatment is not administered immediately, the disease can progress to a severe malaria where the parasite sequesters in capillary beds, causing microvascular obstruction, leading to decreased delivery of oxygen to tissues, anaerobic metabolism, lactic acidosis, hypovolemia and cerebral malaria (Maitland et al., 2003, Taylor et al., 1993, World Health, 2000). In areas with a high transmission of malaria, clinical symptoms of severe malaria are mostly seen in children under the age of 5 years and are also associated with severe anaemia and hypoglycaemia. The risk of developing severe malaria in these areas decreases with age due to the early development of immunity to severe malaria (Snow et al., 1997).

1.6 Genetically Based Resistance to Malaria

Constant interaction between the malaria parasite and the general population in endemic areas has resulted in selective pressure of genetic traits that confer protection against the parasite (Smith et al., 2002, Haldane, 1949, Yuthavong and Wilairat, 1993). These genetic traits include haemoglobinopathies, erythrocyte cell surface polymorphisms and enzymopathies.

1.6.1 Haemoglobinopathies

Haemoglobinopathies refer to genetic mutation or variants that alter the structure of haemoglobin, and this has been shown to confer resistance to malaria (Yuthavong and Wilairat, 1993, Williams, 2006a, Williams, 2006b). Haemoglobinopathies which have been associated with protection from malaria include thalassemia (α-thalassemia or β-thalassemia), sickle-cell trait (HbAS), haemoglobin C (HbC), and haemoglobin E (HbE). Some of the protection mechanisms include; reduced invasion of RBCs (Pasvol et al., 1978), enhanced phagocytosis of parasite-infected RBCs (Cappadoro et al., 1998, Ayi et al., 2004) and increased immune response against parasite-infected RBCs (Duffy and Fried, 2006).

1.6.1.1 Thalassemia

Thalassemia is a hereditary anaemia that results in the production of defective haemoglobin (Higgs et al., 2001). Haemoglobin consists of an iron containing heme ring and four globin chains: two α-chains and two β-chains and is responsible for transporting oxygen throughout the body. This haemoglobinopathy is classified according to the affected globin chain i.e., α-thalassemia, which is because of decrease in the synthesis of α-globin chain, or β-thalassemia which is because of decrease in the synthesis of β-globin chain.

α-thalassemia results from deletion or mutation of the α-globin chain on chromosome 16p13.3 (Yuthavong and Wilairat, 1993). There are two variants of α-thalassemia, α+ and α0 thalassemia. Heterozygous α+-thalassemia have lost one α-globin genes (−α/αα) whereas homozygous (−α/−α) develop mild anaemia with reduced levels of haemoglobin in erythrocytes. α+-thalassemia is common in malaria endemic areas as it confers protection from severe *P. falciparum* infection (Wambua et al., 2006). Individuals with this haemoglobinopathy have ineffective erythropoiesis, reduced erythrocyte survival resulting in a high turnover of erythrocytes, and many circulating young RBCs (Williams et al., 1996, Weatherall, 1997). The high turnover of RBCs in α-thalassemia limits *Plasmodium* invasion reducing parasite growth. α-thalassemia has been linked with complement receptor 1 (CR1) deficiency, which limits merozoite invasion (Tham et al., 2010) and rosetting of RBCs (Opi et al., 2016, Rowe et al., 1997).

β-thalassemia is a disorder that results in decreased synthesis of β-globin chain. This haemoglobinopathy is caused by either point mutation or short nucleotide insertions or deletions of a single β-globin gene on chromosome 11p15.5 (Yuthavong and Wilairat, 1993). The heterozygous state of β-thalassemia (β+-thalassemia) is associated with changes in the morphology of RBCs and mild anaemia (Roberts and Williams, 2003, Weatherall, 2000) while the homozygous state of β-thalassemia (β0-thalassemia) causes severe anaemia that is detrimental in the first two years of life if not properly managed (Weatherall, 2000). *Plasmodium* infected β-thalassemia RBCs have been described to be susceptible to phagocytosis

compared with normal RBCs (Luzzi et al., 1991) and the β-thalassemia trait shown to diminish parasite growth.

1.6.1.2 Sickle cell Trait

Sickle haemoglobin is a structural variant of normal haemoglobin, that has a single amino acid substitution at position 6 of the β-globin chain (β6Glu→Val) (Serjeant, 1997), producing Haemoglobin S (HbS). The homozygous form (HbSS) is fatal in early life while the heterozygous form (HbAS) has been shown to confer protection from malaria. Deoxygenation of HbAS RBCs results in the formation of paracrystalline needles that transform the RBCs into their characteristic sickle shape and alters the intracellular viscosity (Edelstein and Crepeau, 1979) and this hinders parasite growth and invasion (Friedman, 1978, Williams et al., 2005). Parasite infected HbAS RBCs have been shown to lose their discoid shape and form the sickle shape faster (because of deoxygenation and lower pH caused by the parasite) and are highly susceptible to phagocytosis (Weatherall, 1987, Pasvol et al., 1978, Roberts and Williams, 2003). *P. falciparum* infected HbAS RBCs have lower surface expression of PfEMP-1, which reduces cytoadherence and offers protection against severe malaria (Cholera et al., 2008).

1.6.1.3 Haemoglobin C and Haemoglobin E

Haemoglobin C (HbC) occurs as a result of a point mutation that replaces the amino acid glutamate with lysine at position 6 of the β-globin chain (β6Glu→Lys) (Itano and Neel, 1950). This haemoglobinopathy occurs at a high frequency in western Africa (Livingstone and Livingstone, 1985). The exact mechanism of protection from malaria offered by HbC has been contradictory. *In vitro* studies by Friedman et al. (1979) and Olson and Nagel (1986) showed that homozygous HbC (CC) infected RBC interfered with release of merozoites therefore reducing intra-erythrocytic parasite growth. Contrary to this, Agarwal et al. (2000), Modiano et al. (2001) and Duffy and Fried (2006) showed that *P. falciparum* parasites were able to replicate in homozygous HbC erythrocytes and offered no protection against mild malaria infection but protection was seen in severe malaria infection. HbC infected RBCs have also been shown to

display abnormal PfEMP-1 on their surface, thus affecting the ability of the infected RBCs to cytoadhere to microvasculature (Fairhurst et al., 2005).

Haemoglobin E (HbE) is produced when the glutamic acid in position 26 of the β-globin chain is replaced by a lysine (Nagel et al., 1981). This haemoglobinopathy is very common in South-eastern Asia (Chotivanich et al., 2002). HbE RBCs have reduced plasticity and hypochromic microcytic red cells with significant morphological abnormalities. This defects of HbE RBCs may offers protection from malaria by reducing erythrocyte invasion by merozoites, lowering intraerythrocytic parasite growth and enhancing phagocytosis of infected erythrocytes.

1.6.2 Red Blood Cell Polymorphisms

The asexual stage of *Plasmodium spp* life cycle is mostly spent replicating within RBCs. This creates a selection pressure perpetuating RBC associated gene polymorphisms that confer protection against the malaria parasite. These polymorphisms include Duffy antigen, Gerbich negative blood group, South-East Asian Ovalocytosis (SAO) and complement receptor 1 (CR1) polymorphism.

Ovalocytosis is an inherited RBC membrane disorder that is caused by heterozygosity for a 27-base pair deletion in the gene encoding the erythrocyte membrane protein band 3 on chromosome 17 (Jarolim et al., 1991). Only the heterozygous form of ovalocytosis is known to exist and is endemic in Southeast Asia (Jarolim et al., 1991). SAO RBCs have a modified cytoskeleton that makes the RBCs extraordinary rigid thus offering resistance to invasion by various *Plasmodium spp.* (Mohandas et al., 1984). SAO has been shown to offer protection against cerebral malaria (Genton et al., 1995), as *P. falciparum*-infected SAO RBCs sequester in vasculature that express high levels of CD36, which are mostly found in non-vital organs and tissues (Cortés et al., 2005).

Duffy antigen also known as DARC (Duffy antigen receptor for chemokines) is a receptor for a broad range of pro-inflammatory cytokines (Horuk et al., 1993, Neote et al., 1994) and is normally expressed on the surface of RBCs. This antigen is required for *P. vivax* invasion of

reticulocytes and a point mutation (T46C) in the GATA box of the gene's promoter region (Michon et al., 2001), results in a lack of DARC expression on reticulocytes. This Duffy negative phenotype Fy(a−b−) is known to confer protection against *P. vivax* infection (Hamblin and Di Rienzo, 2000, Tamasauskas et al., 2001) and this phenotype is common in Western Africa and Papua New Guinea (Zimmerman et al., 1999). However, it has been shown that *P. vivax* is capable of infecting Duffy negative individuals (Ménard et al., 2010, Ryan et al., 2006, Niangaly et al., 2017) suggesting that the parasite has evolved to infect Duffy negative individuals through an alternative pathway. The erythrocyte binding protein (EBP2), a member of the Duffy binding protein (DBP) family, has been identified as the alternative pathway for *P. vivax* invasion (Ntumngia et al., 2016), but the exact mechanism of invasion and ligands it interacts with on RBCs is still unknown.

Gerbich negative blood group (Ge-) occurs due to a deletion of exon 3 in the glycophorin C gene (Booth and McLoughlin, 1972, Serjeantson et al., 1994). Ge- occurs at a high frequency in the Melanesian population of Papua New Guinea, an area where malaria transmission is high (Patel et al., 2001). Glycophorin C is an important receptor for the *Plasmodium falciparum* erythrocyte-binding antigen 140 (EBA140) (Maier et al., 2003). EBA140 binds to glycophorin C, with a high affinity, on the surface of RBCs and this is key in initiating invasion of RBCs. EBA140 is unable to bind to glycophorin C in Ge- RBCs and this inhibits the process of parasite invasion (Maier et al., 2003).

Complement receptor 1 (CR1) protein is expressed on the surface of RBCs and is responsible and is responsible for complement regulatory processes such as removing of immune complexes from circulation (Stoute, 2005, Reinagel et al., 1997). CR1 protein has been linked with rosette formation, as uninfected RBCs with high levels of CR1 protein on the surface are more likely interact with PfEMP-1 on *P. falciparum* infected RBCs and form rosettes (Rowe et al., 1997, Cockburn et al., 2004). The CR1 gene displays a number of polymorphisms, such as size variation which occur as a result of unequal gene crossover (Ahearn and Fearon, 1989) and the Knops blood group antigens , Swain-Langley (Sl) and McCoy (McC), have been mapped to the CR1 protein long homologous regions (Rao et al., 1991, Moulds et al., 2001, Moulds et al., 1991). The absence of these antigens gives rise to the Sl2 and McCb allelic variants commonly

found in Africa and correlates with a low-CR1 copy number on the surface of RBCs (Thathy et al., 2005, Stoute, 2005). Low CR1 expression has been associated with protection from severe malaria in Papua New Guinea (Cockburn et al., 2004), India (Panda et al., 2012) and Kenya (Thathy et al., 2005).

1.6.3 Enzymopathies

Mutations in the RBC enzyme, glucose-6-phosphate dehydrogenase (G6PD) has been observed in malaria endemic areas (Allison, 1963), which may have arisen or maintained in frequency through natural selection by malaria (Ruwende and Hill, 1998, Ruwende et al., 1995).G6PD is an enzyme in the pentose phosphate pathway that catalyses the conversion of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form NADPH (Frank, 2005). NADPH is in turn responsible for maintaining glutathione (GSH) levels, an antioxidant, that protects RBCs from oxidative stress (Frank, 2005). G6PD-deficiency in RBCs causes a decrease in GSH levels, promoting oxidative stress which affects the integrity of the RBCs, resulting in haemolysis that limits the malaria parasites ability to survive and replicate (Vega-Rodríguez et al., 2009). Ring stage infected G6PD-deficiency RBCs are more susceptible to phagocytosis, and this seems to be a protective mechanism that arises from the increased sensitivity of the G6PD-deficiency RBCs to oxidative damage (Cappadoro et al., 1998, Ayi et al., 2004).

Pyruvate kinase (PK) is an enzyme that catalyses the irreversible conversion of phosphoenolpyruvate (PEP) to pyruvate an important step in producing ATP in the glycolytic route. PK is important in RBCs metabolism as the ATP produced is used to maintain RBCs structure and functionality (Min-Oo et al., 2003). PK-deficient RBCs have decreased levels of ATP and increased levels of glycolytic intermediates (Colombo et al., 1988). This causes changes in RBC membrane structure, making it more rigid and resistant to invasion but it also reduces the RBCs half-life and increases its susceptible to phagocytosis and damage from oxidative species (Min-Oo et al., 2003).

1.7 Immunity to Malaria

The residents of malaria endemic regions develop some form of immunity following repeated infection. This immunity is complex and varies depending on the malaria transmission intensity, epidemiological factors, genetics, host age, and parasite species (Doolan et al., 2009). Repeated infection with the parasite results in the development of clinical immunity, which reduces the risk of death and severity of the clinical symptoms of malaria, and later anti-parasitic immunity which reduces parasitaemia (Mohan and Stevenson, 1998) but does not protect the individual from concurrent infection. Both the innate and acquired immune responses play a role in development of immunity to malaria, and have been shown to reduce severity of disease and offer protection from malaria (Smith et al., 2002).

1.7.1 Innate Immunity

The innate immune system is the first line of defence against invading pathogens, but its role in malaria immunity has not been well studied. Key players in innate immunity during a malaria infection include monocyte/macrophages, natural killer cells (NK cells) and γδ T cells.

Monocyte/macrophages contain a number of Pattern Recognition Receptors (PRRs) that are capable of recognising molecules frequently found in pathogens (Pathogen-Associated Molecular Patterns—PAMPs), or molecules released by damaged cells (Damage-Associated Molecular Patterns—DAMPs) (Medzhitov and Janeway, 1997). Parasite infected RBCs undergo several transformations that lead to expression of parasite proteins on their surface, which can interact with receptors on monocyte/macrophages. Phagocytosis of iRBCs by monocytes/ macrophages is an effective way of reducing parasitaemia and can be enhanced in the presence of opsonins (Chan et al., 2012, Chan et al., 2017). Complement-mediated phagocytosis is key in protecting against early erythrocytic stages via complement receptor 1 (CR1 or CD35) (Silver et al., 2010). Antibody-mediated phagocytosis is important in opsonising iRBCs and merozoites that are phagocytosed by monocytes/macrophages via FcγRs (Zhou et al., 2015, Lambert et al., 2014, Mota et al., 1998). Monocyte/macrophages also control parasitaemia via antibody-dependent cell inhibition (ADCI) (Chimma et al., 2009). ADCI

requires antibodies (IgG1 and IgG3) against merozoites such as merozoite surface protein-1 (MSP-1) (Galamo et al., 2009) and glutamate-rich protein (GLURP) (Pratt-Riccio et al., 2011). The antibody-opsonised merozoites activate monocytes to release soluble mediators that inhibit the growth of the parasite in iRBCs (Galamo et al., 2009, Bouharoun-Tayoun et al., 1995).

Natural killer cells (NK cells) are a major source of IFNγ, as well as TNF-α, GM-CSF, and other cytokines and chemokines (Campbell and Hasegawa, 2013). Apart from cytokine production they are also able to detect and destroy damaged, dysfunctional or infected host cells that have downregulated MHC class 1 (missing self-hypothesis) (Karre, 2008). In mice infected with *Plasmodium chabaudi chabaudi,* NK cells have been shown to rapidly produce IFNγ, which activates macrophages to phagocytose merozoites and iRBCs in both an opsonization dependent (Mota et al., 1998) and independent manner (Su et al., 2002). IFNγ also induces the production of parasiticidal free radicals by macrophages which are effective at killing iRBCs (Fritsche et al., 2001). In humans, most of the studies on NK cells have been performed *in vitro*, with a few *ex vivo* studies looking at IFNγ production and cytotoxic responses in infected individuals (Agudelo et al., 2012, Hermsen et al., 2003, McCall et al., 2010b). *In vitro* studies using PBMCs from malaria naïve individuals has shown that iRBC can induce NK cells to produce IFNγ within 6 hours of coculture though the magnitude of the IFNγ production differed between individuals (Artavanis-Tsakonas and Riley, 2002, Korbel et al., 2005), and required direct contact between the NK cell and iRBC (Artavanis-Tsakonas et al., 2003, Dodoo et al., 2002). NK cells isolated from peripheral blood of experimentally infected malaria naïve volunteers and naturally infected individuals when cultured *in vitro* with hepatocytes infected with *Plasmodium* were seen to release cytotoxic mediators (Hermsen et al., 2003). Similar results were seen in Kenyan adults and children in response to iRBC (Orago and Facer, 1991) and NK cells have also repeatedly been observed forming stable conjugates with iRBCs *in vitro* (Korbel et al., 2005, Baratin et al., 2005). PfEMP1 is thought to be the key parasite molecule that interacts with NK cells but Baratin et al. (2007) showed that the interaction between PfEMP1 and NK cells does occur but is not necessary for activation of NK cells. Mavoungou et al. (2007) suggested that PfEMP1 binds to NKp30 receptors on NK cells, but Chen et al. (2014) later found that NKG2D, NKp30, NKp44, and NKp46 receptors on NK cells were not responsible for the

interaction seen with iRBCs. Thus, the exact receptors on NK cells and iRBCs that mediate these interactions are still unknown.

γδT cells are T cell that are generated in the thymus from double negative progenitor cells, which commit to the γδT cell lineage (Zarin et al., 2015). After immunisation with radiation attenuated sporozoites or live sporozoites under chloroquine prophylaxis, infected volunteers were found to have an expanded population of γδT cell which were detected in the peripheral blood (Zaidi et al., 2017, Seder et al., 2013, Teirlinck et al., 2011), indicating that γδT cells may play a role in vaccine induced malaria immunity. γδT cells have also been seen to proliferate and increase in number when adults with little or no previous exposure to malaria are infected with *P. falciparum* or *P. vivax* (Ho et al., 1994, Perera et al., 1994, Roussilhon et al., 1994, Schwartz et al., 1996). In mouse models of malaria, γδT cells are seen to expand on infection with rodent malaria (van der Heyde et al., 1993, Langhorne et al., 1993) and depletion or targeted deletion of γδT cells results in increase in parasitaemia and in some cases leads to lethal infection (Weidanz et al., 2010). γδT cells are known to produce large amounts of IFNγ after in-vitro stimulation with iRBCs (Troye-Blomberg et al., 1999, Goodier et al., 1995) and during early blood stage infection (Seixas and Langhorne, 1999). IFNγ is key in activating phagocytic cells that eliminate the blood stage of the parasite by either antibody dependent or independent mechanisms (Su et al., 2002). Activated γδT cells (human Vδ2+ and Vδ1+) when cultured *in vitro* with free merozoites, can degranulate, releasing granulysin, and kill the merozoites thus inhibiting *P. falciparum* replication (Troye-Blomberg et al., 1999, Costa et al., 2011). The effectiveness of γδT cells killing mechanism *in vivo* is a debate as free merozoites spend a very short time in the extracellular environment between egress and reinvasion. Overall γδT cells do have a role in innate immunity to malaria but this is yet to be clearly elucidated.

1.7.2 Naturally Acquired Immunity to Malaria

Historical and epidemiological observation of malaria endemic regions have shown that residents in this area are able to acquire some form of partial immunity to malaria after continuous exposure to the parasite. Robert Koch was able to see this phenomenon in children and adults in the highly endemic areas of Papua New Guinea and Indonesia. Koch observed that

children from the indigenous Melanesian populations were frequently infected by the malaria parasite and developed symptoms of disease whereas indigenous adults were less likely to develop symptoms of disease and had lower number of circulating parasites. This led to the conclusion that naturally acquired immunity (NAI) to malaria develops slowly after years of exposure and this immunity is not sterile (Ewers, 1972). Later, cross-sectional studies in endemic areas showed that the frequency of disease, parasite prevalence and their densities varied with age among populations within the same geographical area. Naturally acquired immunity can be described as (i) acquired at a rate that varies with transmission intensity, (ii) is strain/species specific and somewhat stage specific (iii) acquired slowly over time (iv) effective in adults after continuous heavy long-term exposure, (v) is lost to some extent when exposure to parasite ceases (Doolan et al., 2009). How NAI develops in this population is still an area that is yet to be well understood but NAI mainly involves T cells and antibodies.

1.7.3 Antibodies in Naturally Acquired immunity to Malaria

Antibodies play a role in naturally acquired immunity as passive transfer of immunoglobulins from malaria immune adults to non-immune children hospitalised with malaria resulted in a rapid drop in parasite density and fever (Cohen et al., 1961). Antibodies mediate protection against the parasites during the asexual blood-stages by various mechanisms such as opsonization, complement mediated lysis and antibody dependent cellular cytotoxicity (Teo et al., 2016, Fowkes et al., 2016). However, it is unknown which specific antigens induce protective immunity, and which antibody effector functions are the most important in protection from disease. Antibody production requires the coordinated efforts of dendritic cells, CD4 T cells and B cells to produce functional class switched antibodies that can target the parasite at various stages of its life cycle.

1.7.4 Dendritic Cells

DCs are mononuclear phagocytic cells that are found in the blood, lymphoid organs and all tissues. They are the most effective antigen presenting cells in the body due to their ability to capture, process and present antigen on either major histocompatibility complex (MHC) class I

or MHC class II molecules and activate naive CD8 or CD4 T cells (Dudziak et al., 2007, Banchereau and Steinman, 1998). DCs are central in initiating and regulating adaptive immune responses and act as a bridge between the innate and adaptive arms of the immune system. DCs differentiate from hematopoietic stem cells (HSC) (Stockwin et al., 2000) in the bone marrow to immature DCs, which circulate in blood and home to various peripheral tissues. Immature DCs recognize a range of danger signals such as pathogen-associated molecular patterns (PAMPS) which are found on pathogens and damage associated molecular patterns (DAMPS) which are released by injured host cells (Tang et al., 2012), through a number of pathogen recognition receptors (PRRs) (Kawai and Akira, 2005, Mogensen, 2009). Ligation of PRRs initiates DC phagocytosis, resulting in ingestion of the invading pathogen. PRRs can also initiate DC maturation and migration into the lymph node where they present antigens to naive T cells (Geissmann et al., 2010). The maturation process results in increased expression of MHC surface molecule coupled with pathogen antigens and costimulatory molecules (CD80, CD86, and CD40), which are essential for activation and differentiation of naive T cells into effector cells (De Smedt et al., 1996). DCs also secrete cytokines and chemokines that attract other immune cells to sights of infection/injury and influence the outcome of T and B cells responses (Blanco et al., 2008).

Dendritic cells provide three signals that are key in activating naive CD4 and CD8 T cells **(Figure 1-6).** The first signal is in the form of MHC-peptide (pMHC) complexes. The MHC class I molecule is composed of two chains, a polymorphic α-chain and β2 microglobulin (β2m) light chain that assemble in the ER (Peaper and Cresswell, 2008). The MHC class I peptide binding groove is closed at both ends by conserved tyrosine residues leading to a size restriction of the processed peptides to usually 8–10 amino acids (Zacharias and Springer, 2004, Matsumura et al., 1992, Bouvier and Wiley, 1994). Endogenously synthesized proteins of either self or pathogen origin are processed and presented on MHC class I via two presentation pathways, the vacuolar and the cytosolic pathway as shown in **Figure 1-3**. In the vacuolar pathway, antigens are internalised and degraded in the endolysosome by the protease Cathepsin S (Shen et al., 2004) and loaded onto MHC class I for presentation on the cells surface. In the cytosolic pathway, internalised antigens are transported from the endosome to the cytosol where they are degraded by a proteasome (Ackerman et al., 2003, Palmowski et al., 2006, Rock et al.,

1994, Michalek et al., 1993). The polypeptides generated from proteasome degradation are then transported to the lumen of the endoplasmic reticulum (ER) via transporter associated with antigen processing 1 (TAP1) and TAP2 (Reits et al., 2003). TAP molecules also act as an anchor for the peptide loading complex, by binding to MHC class I dedicated chaperone protein tapasin, while two other chaperone proteins, calreticulin and protein disulphide isomerase ERp57, help in stabilising the empty MHC class I molecule (Cresswell et al., 1999). In the ER, the N-termini of the polypeptides are trimmed by the ER aminopeptidase associated with Ag processing (ERAAP) to the required amino acids for loading onto the empty MHC class I molecule, after which the pMHC is transported to the plasma membrane of the DC (Firat et al., 2007).

Components of the ER, containing TAP and MHC class I complex and loading proteins, have been shown to fuse with the phagosome (Guermonprez et al., 2003). This fusion enabled TAP to reimport the peptides generated by the proteosome into the endosome and load them onto MHC class I (Burgdorf et al., 2008, Houde et al., 2003). pMHC Class I complex is normally presented to CD8 T cells and binds to the T cell receptor (TCR) this causes a cascade of signalling events that result in the activation of the CD8 T cells.

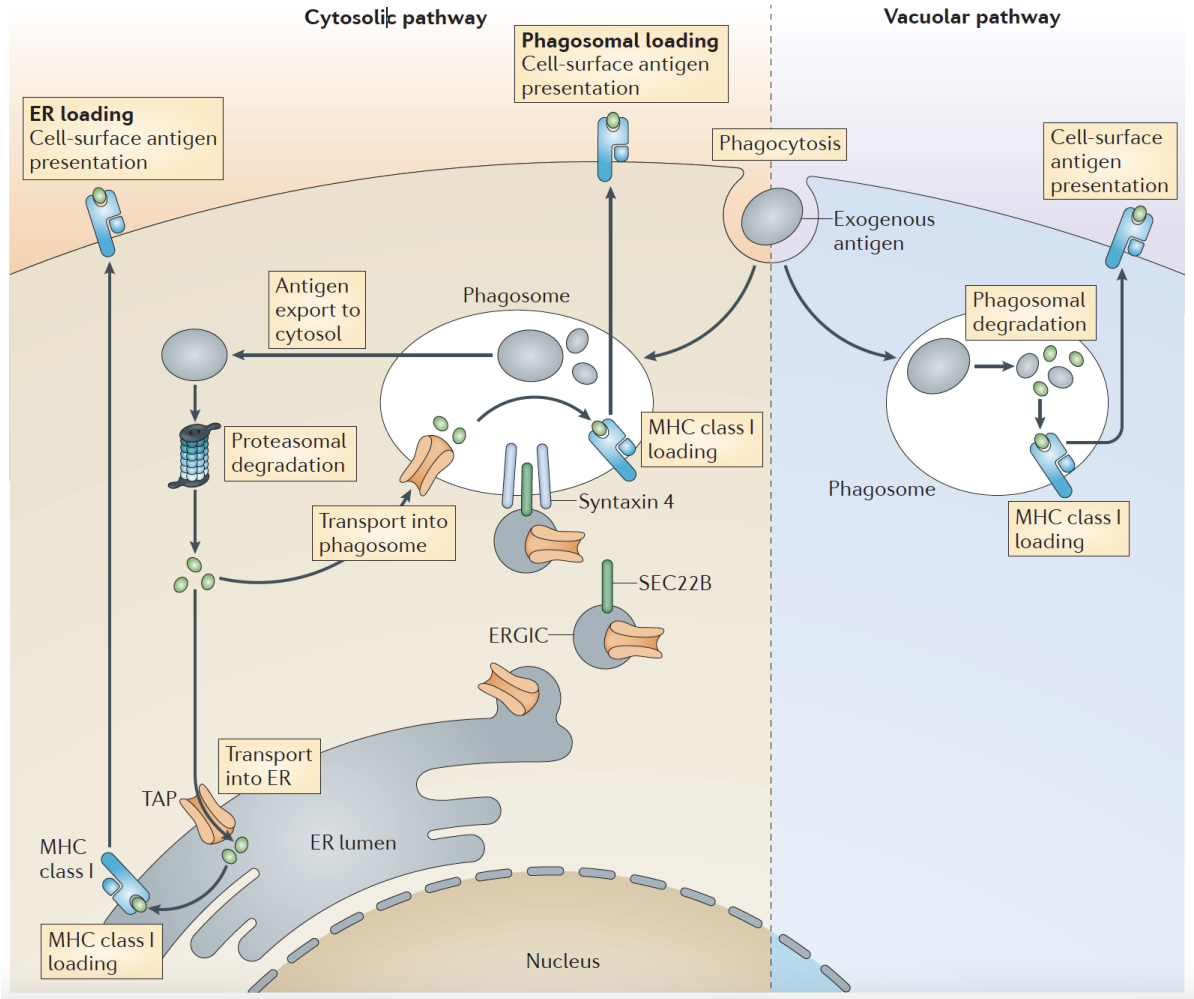


Figure 1-3 Antigen presentation pathway for MHC class I molecule

The various pathways that are used by dendritic cells during antigen processing. Figure obtained from Joffre et al. (2012).

MHC class II molecules are made up of two transmembrane proteins, an α chain and a β chain, which form an open-ended peptide binding groove (Stern et al., 1994). The open-endedness allows for binding of peptides with about 10-20 amino acids or more. Peptides presented on MHC class II molecules are normally derived from extracellular proteins that have been endocytosed. The MHC class II presentation pathway starts with the synthesis and assembly of

the MHC class II molecule in the ER and is associated with the invariant chain (Ii) for proper folding, trafficking and protection of the peptide binding groove from binding with other peptides in the ER (Bryant and Ploegh, 2004, Cresswell, 1996) Ii promotes exit of MHC class II-Ii complex from the ER and sorting in the trans-Golgi network to the late endosomal acidic compartment called the MHC-II loading compartment (MIIC) (Neefjes et al., 1990, Zhong et al., 1996, Peters et al., 1991). MHC-II–Ii complexes that fail to be sorted are transported to the cell surface, and later internalised and transported to the MIIC (Roche et al., 1993). Entry of the MHC-II–Ii complex into the late endosome, initiates the degradation of the Ii chain in a stepwise manner by a series of proteases which include cathepsin S (Villadangos et al., 1999) and Signal peptide peptidase-like 2A (SPPL2A) (Beisner et al., 2013). A small fragment of the Ii, which sits in MHC II peptide groove is protected from degradation by the proteases is called Class II-associated invariant chain peptide (CLIP) (Ghosh et al., 1995). CLIP is dissociated from MHC II by a late endosomal chaperon protein called HLA-DM , which has a similar structure to MHC class II molecule but does not bind peptides (Sherman et al., 1995, Mosyak et al., 1998). After dissociating CLIP, HLA-DM binds to MHC class II stabilising it while it exchanges peptides, ultimately editing peptides thus ensuring that peptides with a high binding affinity binds onto the empty MHC class II molecule (Denzin and Cresswell, 1995, Kropshofer et al., 1996). The pMHC class II molecule is then transported to the cell surface, where it interacts with the TCR on CD4 T cells, causing the activation of the CD4 T cells as shown in **Figure 1-4**.

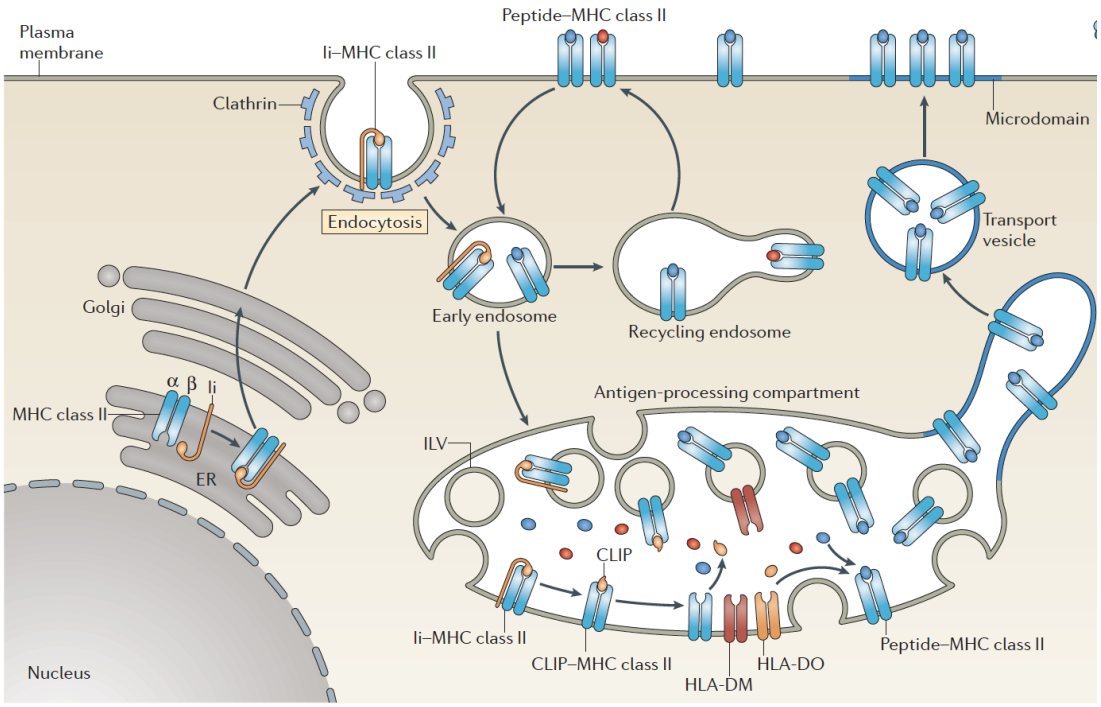


Figure 1-4 Antigen presentation pathway for MHC class II

The figure shows the different pathways used for antigen process and loading onto MHC class II. Figure modified from Roche and Furuta (2015)

A unique feature of dendritic cells is their ability to cross-present exogenous antigens on MHC I molecules (**Figure 1-5**). The MHC class I antigen processing pathway relies mainly on endogenous antigens that are presented to naïve CD8 T cells, limiting the MHC class I pathway to pathogens that can infect DCs but pathogen infected DCs are comprised and may not adequately present antigens (Engelmayer et al., 1999, Salio et al., 1999). To overcome this, DCs can present exogenous antigen, from pathogen internalised via phagocytosis can be diverted into the cytosol and processed through the cytosolic pathway, resulting in the antigens being presented on MHC class I. This enables the DC to the prime CD8 T cells that can detect and destroy pathogen infected cells.

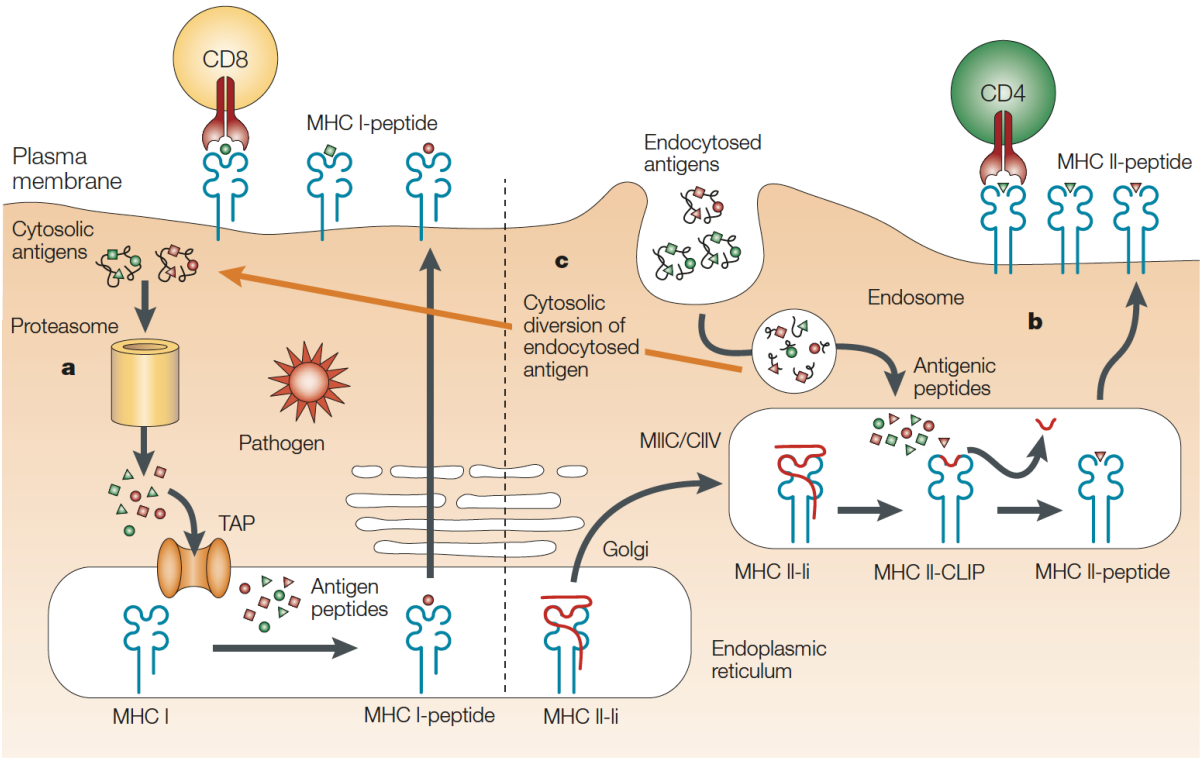


Figure 1-5 Cross presentation of antigens by dendritic cells.

Dendritic cells can process antigens and present them on either MHC Class I or MHC Class II by diverging antigens from the endosomal pathway to the cytosolic pathway. Figure modified from Heath and Carbone (2001)

The second signal that DCs provide to naïve CD4 and CD8 T cells is in the form of costimulatory molecules. The ligation of the TCR to pMHC class I or class II molecule initiates signalling by phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on the CD3 by Lck (Rossy et al., 2013). This activation signal needs to be sustained long enough for effective activation of the signal transduction pathway that results in activation of nuclear transcription factors. Costimulatory molecules offer additional activation signal that aid in amplifying and sustaining the TCR activation signal or hamper/dampen the TCR activation signal. Positive signals, such as binding of the costimulatory molecule CD28 on T cells to either B7-1 (CD80) and/or B7-2 (CD86) on DCs, enhances the TCR signal. While negative signals, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), binding to CD80/86 on DC, and

programmed cell death-1 ((PD-1), binding to PD-1 ligand (PD-1L) suppress the activation signal from TCR (Thangavelu et al., 2010, Okoye et al., 2017). CD40 is another costimulatory molecule that positive signals for T cell activation (Grewal and Flavell, 1996) and licensing of DCs (Guerder and Matzinger, 1992). Interaction of CD40 on DCs with its ligand CD40L on CD4 T cells results in upregulation of CD80 and CD86 expression, enhancement of cytokine production and cross-presentation of antigens in DCs (Ma and Clark, 2009, O'Sullivan and Thomas, 2003, Cella et al., 1996, Ridge et al., 1998). The CD40-CD40L interaction also activates CD4 and CD8 T cells (Grewal and Flavell, 1996). CD83 is another costimulatory molecule that expressed on DCs and is important in stabilising the surface expression of MHC class II and CD86 (Tze et al., 2011), and plays a role in T cell activation through interaction with CD83L (Prechtel et al., 2006).

The third signal that DCs provide to CD4 and CD8 T cells is in the form of cytokines. Cytokines dictate the effector phenotype of the activated T cell. In the case of CD4 T cells, different cytokines skew development towards distinct subsets of CD4 T cells including Th1 type (CD4 T cells exposed to the cytokine IL-12), Th2 (IL-4), Th-17 (IL-6, IL-23), Tfh (IL6, IL21), and iTreg (TGF-β). While in CD8 T cells, IL-12 has been shown to provide the third signal needed for strong proliferation and development of effector function (Curtsinger et al., 1999, Valenzuela et al., 2002)

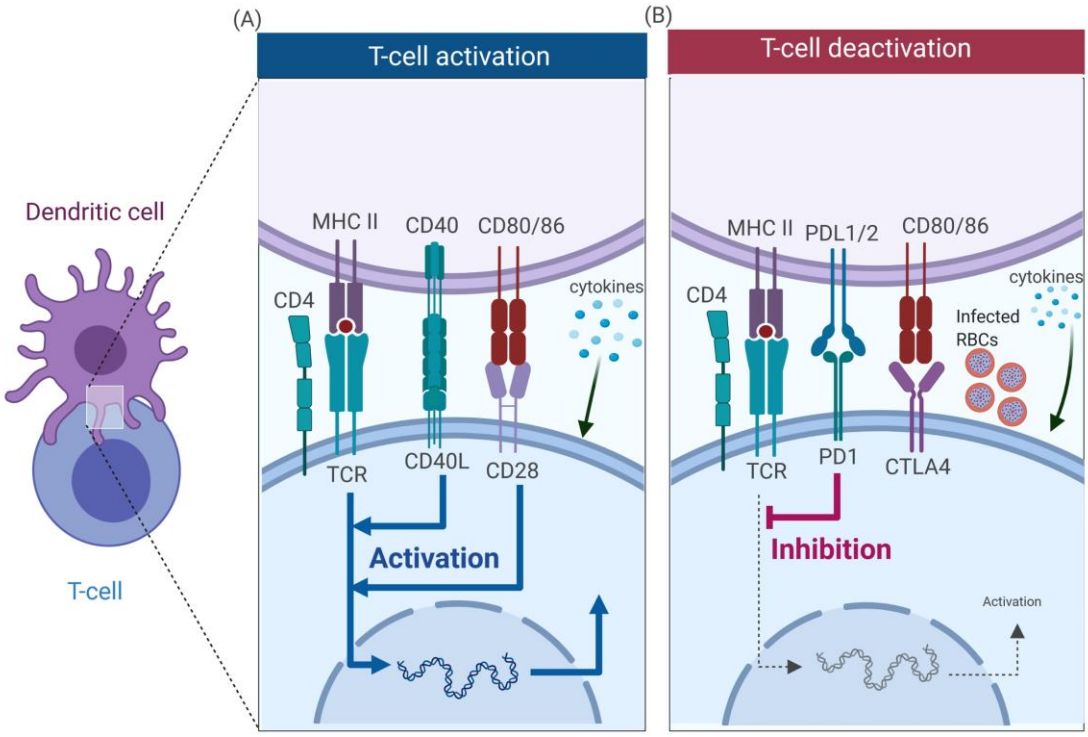


Figure 1-6 Signals provided by dendritic cells to activate T cells

Dendric cells provide three signals that are key in activating T cells. The first signal is in the form of MHC-antigen complex, the second is in the form of co-stimulatory molecules that can enhance (A) or inhibit (B) T cell activation an the third is in the form of cytokines. Figure modified from Osii et al. (2020)

DCs are broadly classified into either plasmacytoid DCs (pDCs) or conventional DCs (cDCs). In humans, pDCs are characterised by expression of CD123, CD303 (BDCA-2) and CD304 (Villani et al., 2017) and are known to produce large amounts of type I interferon in response to viruses (Palucka et al., 2010). This is enabled by the high expression levels of toll-like receptor 7 (TLR7) and TLR9, which recognise nucleic acids from viruses, bacteria, and dead cells (Collin and Bigley, 2018, Boltjes and van Wijk, 2014). cDCs specialise in priming and presenting antigen to T cells. They can be further classified into cDC1 and cDC2. cDC1 express BDCA-3/CD141, CLEC9A, and XCR1 and have enhanced ability to cross present antigen (Jongbloed et al., 2010) to CD8 T cells. cDC2 express BDCA-1/CD1c and have a wide variety of pattern recognition receptors (PRR's) and a good capacity to stimulate naive CD4 T cells but they have

a poor ability to cross-present antigens to CD8 T cells compared with cDC1 (Collin and Bigley, 2018, Villani et al., 2017).

During the life cycle of the *Plasmodium*, the parasite encounters DCs in the skin (**Figure 1-2 (A)**) (Amino et al., 2006, Hellmann et al., 2011), the liver (**Figure 1-2 (B))** (Renia et al., 1994, Kurup et al., 2019), and the blood and spleen (**Figure 1-2 (B)**) (Urban et al., 1999). Tissue resident DCs in each of the sites can phagocytose parasite components and initiate specific immune responses to the parasite. As discussed later, the parasite's numerous immune evasion mechanisms interfere with DC function, thus altering downstream immune effector functions and the course of the disease.

1.7.5 CD4 T cells

T cells develop in the thymus from the common lymphoid progenitors which originate from bone marrow derived hematopoietic stem cells (Stutman, 1978). After thymic education, naïve T cells exit the thymus and enter circulation expressing either CD4 or CD8 and an antigen-specific T cell receptor (TCR) on their surface. The naive T cells recirculate through secondary lymphoid organs (SLO) providing host immune surveillance, via interactions with DC.

CD4 T cells recognise antigens presented by MHC class II molecules, which are present on antigen presenting cells such as B cells, macrophages, and dendritic cells. CD4 T cells generally provide help to B cells in the germinal centre enabling class switching and production of high-affinity antibodies (He et al., 2013). They also aid in CD8 T cell activation by licensing DCs (Lanzavecchia, 1998, Bennett et al., 1998, Ridge et al., 1998) or directly signalling CD8 T cells via CD40 (Bourgeois et al., 2002). They also secrete cytokines such interferon gamma (IFNγ), C-X-C motif ligand 9 (CXCL9), CXCL10 (Nakanishi et al., 2009), Interleukin-2 (IL-2) (D'Souza et al., 2002, D'Souza and Lefrancois, 2003, Williams et al., 2006), and IL-21 (Kwon et al., 2009) that are key in shaping immune responses. The diverse range of CD4 T cell functions are handled by distinct subsets of cells.

Tfh cells have been a recent focus of interest in malaria immunology due to their role in supporting B cell antibody production (He et al., 2013). Differentiation of CD4 T cells to Tfh is a multistep step process that first begins with DC interacting with a naive CD4 T cell in the T cells zone of lymphoid organs. Tfh differentiation requires signal 1 in the form of antigen presented on MHC II by DCs (**Figure 1-7**). This interaction occurs at the T cell zone and involves the costimulatory molecules CD80, CD86, and inducible costimulatory ligand (ICOSL) on DC that interact with CD28 and ICOS to generate signal 2 in T cells. The CD28-CD80/86 interaction results in the upregulation of ICOS on T cells that interacts with ICOSL on DCs. The cytokine (signal 3) produced by DCs that helps in the initial process of Tfh differentiation is IL-12 (Schmitt et al., 2009). A combination of CD28-mediated signalling on T cells and IL-12 is adequate to upregulate the expression of Bcl-6, IL-12 also induces IL-21 production in T cells, which acts in an autocrine manner to ensure growth and survival of pre-Tfh. Bcl6 expression upregulates CXCR5 expression allowing the pre-Tfh cells to migrate to the T cell-B cell zone (Nurieva et al., 2009). At this zone, the Tfh cell interacts with B cells via ICOS-ICOSL, committing the cell to the Tfh lineage and further upregulating CXCR5 and SAP (Kerfoot et al., 2011). The CXCR5 and SAP expressing Tfh cells then move into the B cell follicle and form stable, long-lasting interactions with B cells forming germinal centre where Tfh cells aid in B cell class switching, affinity maturation and generation of long-lived plasma cells that secrete high levels of antibodies. Germinal centre Tfh cells are also involved in guiding B cells to form either long-lived plasma cells or memory B cells (Vinuesa and Cyster, 2011, Ma et al., 2012) (**Figure 1-7**).

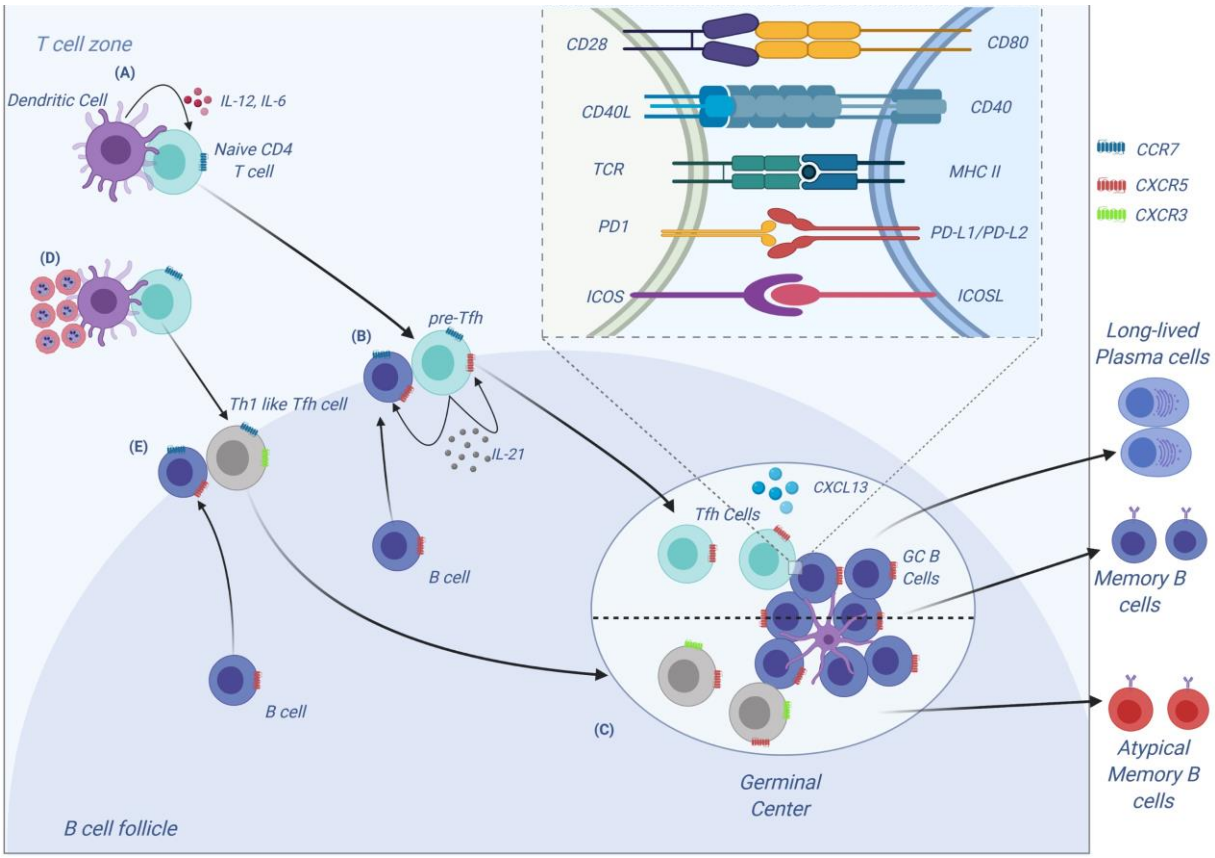


Figure 1-7 The process of Tfh development in health (B) and during a malaria infection (D, E).

Tfh differentiation is a multistep process that begins when an activated DC primes a naive CD4 T cell and requires well-coordinated and timed signals provided by B cells and DCs to fully develop to a Tfh cell (A-C). *Plasmodium*-induces polarization of T follicular helper (Tfh) cells to Th1 like phenotype that expresses Tbet PD-1, CXCR5, CXCR3 and contributes to the inefficient acquisition of humoral immunity to malaria (D-E) (Crotty, 2014). Figure modified from Osii et al. (2020)

CD4 Tfh cells are essential for promoting antibody response that aid in resolving malaria infection (Zander et al., 2015, Ryg-Cornejo et al., 2016). In malaria infected humans and mice, Tfh cells adopt a Th1 like phenotype that expresses Tbet+ PD-1+, CXCR5+, CXCR3+, and secretes IFNγ (Ryg-Cornejo et al., 2016, Obeng-Adjei et al., 2015). This Tfh phenotype is less effective in providing help to B cells resulting in suboptimal antibody responses. Dysfunctional DCs that are induced by malaria may play a role in initiating this Th1-like phenotype that compromises the humoral response.

1.7.6 CD8 T cells

Naive CD8 T Cells are activated by recognition of foreign antigens presented by MHC class I molecules on DCs in the secondary lymphoid organs. Additional co-stimulatory signals and cytokines from DCs and/or CD4 T cells help in differentiation and clonal expansion of the T cells (Curtsinger et al., 2005, Kolumam et al., 2005, Kalia et al., 2010, Joshi et al., 2007). The activated effector CD8 T cells migrate from the secondary lymphoid organs into the circulation and identify their target cells which express cognate antigens on the cell surface bound to MHC class I. MHC class I is expressed on all nucleated cells; therefore, it is absent on mature red blood cells. The target cells are killed by effector CD8 T cells (cytotoxic T cells; CTLs) through cell contact dependent cytolysis by releasing granzyme B and perforin (Yannelli et al., 1986, Peters et al., 1989, Stinchcombe et al., 2001). Perforin creates pores on the plasma membrane of the target cell; the pores allow granzyme B to enter the target cell and initiate apoptosis resulting in killing of infected cells. After clearing the invading pathogen, antigen specific effector CD8 T cells die off and a small number differentiate into memory CD8 T cells (Kaech and Wherry, 2007, Joshi et al., 2007).

Antigen specific CD8 T cells have been observed in the peripheral blood of residents from a malaria endemic area (Sedegah et al., 1992) and after vaccination of malaria naive individuals with irradiated sporozoites (Doolan et al., 2000). In experimental mouse models of malaria, CD8 T cells specific for sporozoite antigens, liver stage antigens, and blood stage antigens were observed when mice were challenged with radiation attenuated sporozoites (Epstein et al., 2011). It is believed that the priming of CD8 T cells against the pre-erythrocytic stages of

Plasmodium occurs in the skin draining lymph nodes when sporozoites are injected into the skin by an infected mosquito (Chakravarty et al., 2007, Radtke et al., 2015). These CD8 T cells may offer protection against subsequent *Plasmodium* infections as incubation time in the liver offers a short window of opportunity for the effector CTLs CD8 T to mount an effective response.

1.8 *P. falciparum* Immune Evasion and Suppression of Immunity

P. falciparum is equipped with multiple mechanisms which it uses to evade the host's immune system. These mechanisms include antigenic variation of surface antigens (VSA) expressed on iRBCs, sequestration and cytoadherence or rosetting. Antigenic variation of surface antigens (VSA) includes PfEMP1 which is encoded by the *var* genes (Smith et al., 1995), sub-telomeric variable open reading frame (STEVOR) encoded by the *stevor* genes (Niang et al., 2009, Lavazec et al., 2007) and repetitive interspersed repeats (RIFIN) encoded by the *rif* genes (Fernandez et al., 1999, Lavazec et al., 2006). Antigenic variation of VSAs normally occurs when the parasite is under intense immune pressure from the host to avoid recognition by various immune cells (Bachmann et al., 2019, Gilbert et al., 1998). The expression of different VSAs on iRBCs allows the parasite to establish new infections (Marsh and Howard, 1986). VSAs are key in sequestration and cytoadherence of maturing parasites (trophozoite and schizonts) and rosetting (Smith et al., 2013, Rowe et al., 1997). Merozoite surface protein (MSP) polymorphism (Pacheco et al., 2007, Tanabe et al., 1987, Polley et al., 2003) and complement evasion by surface proteins PfMSP3.1 (Kennedy et al., 2017), Pf92 (Kennedy et al., 2016) and PfGAP50 (Simon et al., 2013) expressed on merozoites and gametes are other mechanisms used by the parasite to escape elimination by the immune system.

Apart from immune evasion, ongoing *Plasmodium* infections have been shown to cause a generalised immune suppression that affects immunogenicity of vaccines in children. Antibody responses to non-typhoidal salmonella and tetanus vaccines were greatly reduced in malaria infected children compared to healthy control and children with other acute illnesses (Greenwood et al., 1972). Adults with previous exposure to *P. falciparum*, showed no response to malaria antigen, regardless of disease severity, and reduced response to non-specific antigens (Ho et al., 1986). Infection of influenza-immune mice with *P. chabaudi* resulted in a decrease

in influenza specific antibodies and plasma cells resulting in a loss of protective immunity against influenza (Ng et al., 2014), which recovered several weeks after parasite clearance. This indicates that malaria infections somehow suppress immune function by interfering with the development of adaptive immunity. Ongoing malaria infection reduces immunogenicity to heterologous vaccines and malaria derived antigens. The exact mechanism used to induce this suppression is yet to be uncovered.

The suppression of immune function seen in malaria infection could be attributed to DC/iRBC interaction which alter the maturation state and function of DC in both humans (Urban et al., 1999, Elliott et al., 2007, Gotz et al., 2017) and mice (Millington et al., 2006, Millington et al., 2007). DCs exposed to iRBC *in vitro* and *in vivo* have reduced expression of MHC on the surface and are unable to form stable interactions with CD4 helper T cells (Millington et al., 2007). The DCs also downregulate key costimulatory molecules, such as CD86, CD80, CD40, and secrete IL-10 (Urban and Roberts, 2002), providing a suppressive environment for CD4 T cell development. This hampers their ability to activate naive CD4 T cells and a failure to generate Tfh cells that are critical in the formation of germinal centre and generation of protective antibodies against malaria infection (Ryg-Cornejo et al., 2016, Millington et al., 2007). In contrast to this, Clemente et al. (2013) showed that monocyte derived dendritic cells (moDCs) exposed to low concentrations of soluble *P. falciparum* schizont extract can successfully activate CD4 T cells causing them to differentiate to Th1 CD4 T cells and T reg. Gotz et al. (2017), later showed that myeloid derived DCs exposed to *P. falciparum* infected RBCs are able to activate naïve CD4 T cells to Th-1 like effector cells despite the DCs secreting low levels of cytokines.

1.8.1 Dendritic Cells Interaction with *Plasmodium* Parasite

During the *Plasmodium* parasite life cycle, different forms of the parasite interact with resident DCs in various organs as it establishes infection. Sporozoites from infectious mosquitoes that are injected into the dermis interact with resident DCs in the skin (Amino et al., 2007). The sporozoites reach the liver interact with Kupffer cells, hepatocyte, and liver sinusoidal endothelial cells and resident DCs in the liver (Frevert et al., 2005). The blood stage of the

parasite interacts with DCs in the blood (Urban et al., 1999) and spleen (Cadman et al., 2008, Achtman et al., 2003, Helmby et al., 2000).

1.8.1.1 DC Interaction with *Plasmodium* Sporozoites in the Skin and Liver

The skin is the first point sporozoites encounter DC as they are inoculated by infected mosquitoes (**Figure 1-2 A**). Studies conducted in mice have shown that only a small percentage of inoculated sporozoites leave the site of injection as most end up trapped in the dermis or enter the lymphatic system rather than the blood vessel (Amino et al., 2006). Other sporozoites infect keratinocytes, hair follicles, and develop into exoerythrocytic forms of the parasite (Gueirard et al., 2010). Sporozoites that are trapped in the dermis are phagocytosed by resident DCs which migrate to the skin-draining lymph node and can prime CD4 (Bijker et al., 2014, Roestenberg et al., 2009) and CD8 (Chakravarty et al., 2007) T cell responses. The immune response towards the sporozoite stage of the parasite may protect against subsequent challenges from infected mosquitoes (Roestenberg et al., 2009).

The sporozoites that manage to enter blood circulation move to the liver and must traverse the sinusoidal barrier to access hepatocytes (**Figure 1-2 B**) (Ishino et al., 2004). The liver environment is tolerogenic due to the presence of IL-10 and TGF-β which are secreted by Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC) (Racanelli and Rehermann, 2006). These cytokines reduce expression levels of MHC class II and costimulatory molecules on the surface of liver resident DCs compared to resident DCs in lymphoid organs and those circulating in the blood (Goddard et al., 2004) thus reducing their capability to activate T cells (Bamboat et al., 2009, Pillarisetty et al., 2004). The tolerogenic environment of the liver could play a role in sporozoite immune evasion as DCs and other immune cells in the liver act to suppress adaptive immune responses which would lead to the elimination of sporozoites (Crispe, 2009, Racanelli and Rehermann, 2006).

Apart from DCs, the liver has other potential APCs that can present antigens to the adaptive immune system; this includes Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatocytes. LSECs are scavenger cells that express MHC class I and II molecules, low levels

of CD86, and the adhesion molecules ICAM-1, VCAM-1, and dendritic cell specific intercellular adhesion molecule3-grabbing non-integrin (DC-SIGN). In mice, these cells have the ability to cross present antigens in the liver and activate CD8 T cells, but the T cells are generally tolerised due to the secretion of IL10 and PGE2 by LSECs (Limmer et al., 2000, Knolle et al., 1998). KCs are resident tissue macrophages found in the liver that express MHC class I and class II molecules, ICAM-1, CD86, CD80, and can activate naive CD4 and CD8 T cells *in vitro* (Ebrahimkhani et al., 2011, Bertolino et al., 2002). The role of KC as an APC is controversial as *in vitro* experiments show that they inhibit T cell activation by secreting IL-10 (Knoll et al., 1995), but activation of KCs via TLR3 increased the expression of MHC class II and their APC function (You et al., 2008). Kuniyasu et al. (2004) showed that the liver had the ability to retain adoptively transferred T cells. The T cells proliferated and expanded in the liver, but the expansion was followed by apoptosis, which was initiated by KCs (Kuniyasu et al., 2004). It was later shown that KCs induce T cell apoptosis via the FAS-FAS-L signalling pathway (Chen et al., 2008).

Hepatocytes express MHC class I and ICAM-1 in their steady state and during inflammation they have been shown to express MHC class II CD40L, CD80 and CD86 and are capable of activating CD8 T cells. (Bertolino et al., 1998). Their role in generation of malaria liver immunity has been controversial with different studies using mouse models drawing different conclusions of their role in the generation of pre-erythrocytic immunity. Intrasplenic injection of parasite infected hepatocytes in mice resulted in T cell mediated immunity against *P. yoelii* and *P. berghei* infections (Renia et al., 1994), thus suggesting that hepatocytes are capable of activating T cells. Another study demonstrated that parasite infected hepatocytes undergo apoptosis, thus providing liver DCs with a source of *Plasmodium* antigens for initiating the adaptive immune response (Leiriao et al., 2005). This idea has been challenged and it has been suggested that DCs could obtain *Plasmodium* antigens directly from viable infected hepatocytes. This is supported by the fact that DCs have the ability to acquire antigens from other live cells and cross present to CD8 T cells (Harshyne et al., 2001).

Chakravarty et al. (2007) showed that cross presentation of *Plasmodium* antigens by DCs was key in CD8 T cell activation, and this occurred in the skin draining lymph node, not in the liver.

These activated T cells recirculated to the liver (Chakravarty et al., 2007), indicating that DCs in the skin that encounter sporozoite play a crucial role in generating T cell mediated liver immunity. Recently Kurup et al. (2019) showed that during a malaria infection, a subset of monocyte derived CD11c+ APC infiltrate the liver after hepatocyte infection by *Plasmodium* parasite and acquire *Plasmodium* antigens. The monocyte derived CD11c+ APC present the antigens to naive CD8 T cells in the liver draining lymph node, priming them, and initiating T cell mediated immunity against *Plasmodium* infection (Kurup et al., 2019).

While there are still some gaps into how the generation of liver immunity against *Plasmodium* infection is acquired, APCs, and especially DCs, play a central role. Hepatocytes may play a part in the generation of liver immunity by providing parasite antigens to DCs but the exact mechanism of this is yet to be uncovered. A better understanding of DC and hepatocyte involvement in the generation of liver immunity is required and the roles played by KCs and LSECs. The use of humanised mice might provide an opportunity to further investigate skin and liver immunity against *P. falciparum* (Tyagi et al., 2018, Sack et al., 2017).

1.8.1.2 DCs interaction with *Plasmodium* in the Spleen

The spleen is a secondary lymphoid organ that can induce both innate and adaptive immune responses. It also has an efficient blood filtration system that is important for removal of both old and damaged RBCs and sensing of blood borne pathogens (Steiniger and Barth, 1999). The spleen is divided into three distinct regions, the white pulp, the red pulp, and the marginal zone.

1.8.1.3 The Red Pulp

Majority of the spleen is made up of the red pulp, which has a unique architectural and microcirculatory feature which is composed of cords and venous sinuses. Splenic cords or cords of Billroth lack an endothelial lining but are populated with a larger number of macrophages together with reticular fibres and reticular cells (Chadburn, 2000). Red blood cells from arteriole blood are collected from the splenic cords through the inter-endothelial slits (IESs) of venous sinuses, which are parallel to the blood flow and have a narrow opening of between 0.2-2 μm

(Bowdler, 2001). The narrow opening allows for normal RBC to deform and squeeze through but abnormal and old RBCs with low deformability are trapped in the spaces and phagocytosed by the resident splenic macrophages (Mohandas and Gallagher, 2008). Squeezing of the RBCs through the slits can leave behind rigid cytoplasmic particulate matter, including oxidized hemozoin (Heinz bodies), nuclear remnants (Howell Jolly bodies) and malarial parasites, preserving RBC viability, a process named 'pitting' (Schnitzer et al., 1972).

Blood flow in the red pulp is divided into two paths. One path is a fast closed circulation which goes around the white pulp and follows a specific path from arterioles to venules and is guided by specific Mucosal Addressin Cell Adhesion Molecule 1 (MAdCAM1)-positive cells in the perifollicular zone (Groom et al., 2002). The other path is a slow open circulation in the red pulp, through the cords of Billroth, and the IES to join venous sinuses upstream from venules. Crossing the IES is the most stringent biomechanical challenge for RBCs to exit the spleen and return to the circulation.

1.8.1.4 The White Pulp

The white pulp is the spleen's lymphoid tissue and contains T- and B- cells, each with its own specific compartment around the arterial vessels. The cells are organised into compartments and chemokines attract and maintain the cells in their specific compartment/zones. In the T cell zone, T cell interact with DCs and passing B cells , and in the B cell zone, clonal expansion, class switching and somatic hypermutation of B cells occurs (Bowdler, 2001).

1.8.1.5 The Marginal Zone

The marginal zone is transit area for cells that are leaving the blood stream and entering the white pulp, a process that requires signalling trough the G-protein-coupled receptors (Cyster and Goodnow, 1995). The marginal zone consists of a marginal sinus that is lined by reticular fibroblast and a specific subset of macrophages that are important in sensing blood borne pathogens (Geijtenbeek et al., 2002). The two macrophages that facilitate this function are; (i) marginal zone macrophages (MZM), adjacent to the red pulp (Geijtenbeek et al., 2002, Elomaa

et al., 1995), and (ii) the marginal metallophilic macrophages (MMM), situated at the white pulp border (Munday et al., 1999). Between the two macrophage subsets are the marginal-zone B cells and a subset of DCs (Martin and Kearney, 2002, Yu et al., 2002)

1.8.1.6 Role of the spleen in Malaria

The importance of the spleen in malaria has been studied through the observation of responses of splenectomised humans and rodents to infection. There have been various reports on asplenic *P. falciparum*-infected patients with varying levels of natural immunity (Buffet et al., 2011). In splenectomised immune patients, the severity and fatality of malaria infection is increased, and there is a significant increase in parasitaemia compared to patients with an intact spleen (Bach et al., 2005). In splenectomised patients, antibody mediated clearance of iRBC is done by the liver sinus and other organs though they are less efficient at parasite clearance than the spleen. This highlights the importance of the spleen in controlling parasite load in the blood. Splenectomised malaria naïve patients are at a higher risk of mortality and had high parasitaemia with mature forms of iRBCs being seen in circulation more frequently than in non-splenectomised patients. During antimalaria treatment, asplenic patients showed a delay in parasite clearance compared to patients with a function spleen (Guerin et al., 1996, Thu et al., 1997), further highlight the importance of the spleen in parasite clearance (**Figure 1-8**).

In splenectomised mice, the findings were like those in humans, with the mice showing a reduction in iRBC clearance. Splenectomised mice infected with *Plasmodium chabaudi AS* and *P. yoelli* showed higher peak parasitaemia, prolonged waves of parasitaemia and impaired parasite clearance (Yap and Stevenson, 1994, Sayles et al., 1991) compared to mice with intact spleens. In *P. yoelli* infected mice, the genetic background of the mice seemed to play a role in response to the infection after removal of the spleen. DBA/2 mice were not adversely affected by splenectomy, while C57BL/6 and BALB/c mice failed to resolve infection (Sayles et al., 1991).

1.8.1.7 Immunity in the Spleen

During a malaria infection, the spleen can sense parasite infected RBCs as they pass through the spleen's microcirculation. The *Plasmodium* parasite alters the surface and structure of RBCs, making it more rigid, and this affects the RBCs ability to pass through the IES of the red pulp in the open circulation (Cranston et al., 1984, Nash et al., 1989). Therefore, the spleen retains many ring-infected RBCs in addition to other mature forms of parasite infected RBCs (Safeukui et al., 2008). The retention of the iRBC in the spleen results in splenomegaly, decreased flow of blood in the cord and an increase in macrophage numbers which are responsible for destruction of the iRBCs (Wyler and Gallin, 1976). The spleen is also adept at 'pitting', of iRBCs, this results in removal of the parasite from the RBC, which is then returned back into circulation (Angus et al., 1997). The process of pitting provides antigens to the resident MZMs and DCs which are captured with high efficiency (Kraal et al., 1989) (**Figure 1-8**). .

In mice, plasmacytoid DCs (pDCs) appear unable to take up iRBC and isolated pDCs from *P. chabaudi*-infected spleens do not present malaria antigens (Voisine et al., 2010). However, the classical DCs in mice can take up malaria antigen and present them to CD4 T cells (Sponaas et al., 2006, Lundie et al., 2010) but at different stages of infection. During early *P. chabaudi* infection CD8+ DCs are effective antigen presenting cells that produce IL-12 and induce proliferation of IFN-γ producing CD4 T cells and NK cells (Ing and Stevenson, 2009, Sponaas et al., 2006). These responses are short lived as this population of DCs die off and at peak parasitaemia CD8- DCs are the major population of DCs antigen presenting cells. These DCs induce CD4 T cells to produce IL-10 and IL-4 (Sponaas et al., 2006). At peak parasitaemia splenic DCs have been shown to be unable to efficiently activate CD4 T cells and this impairs antibody production by B cells (Millington et al., 2006)

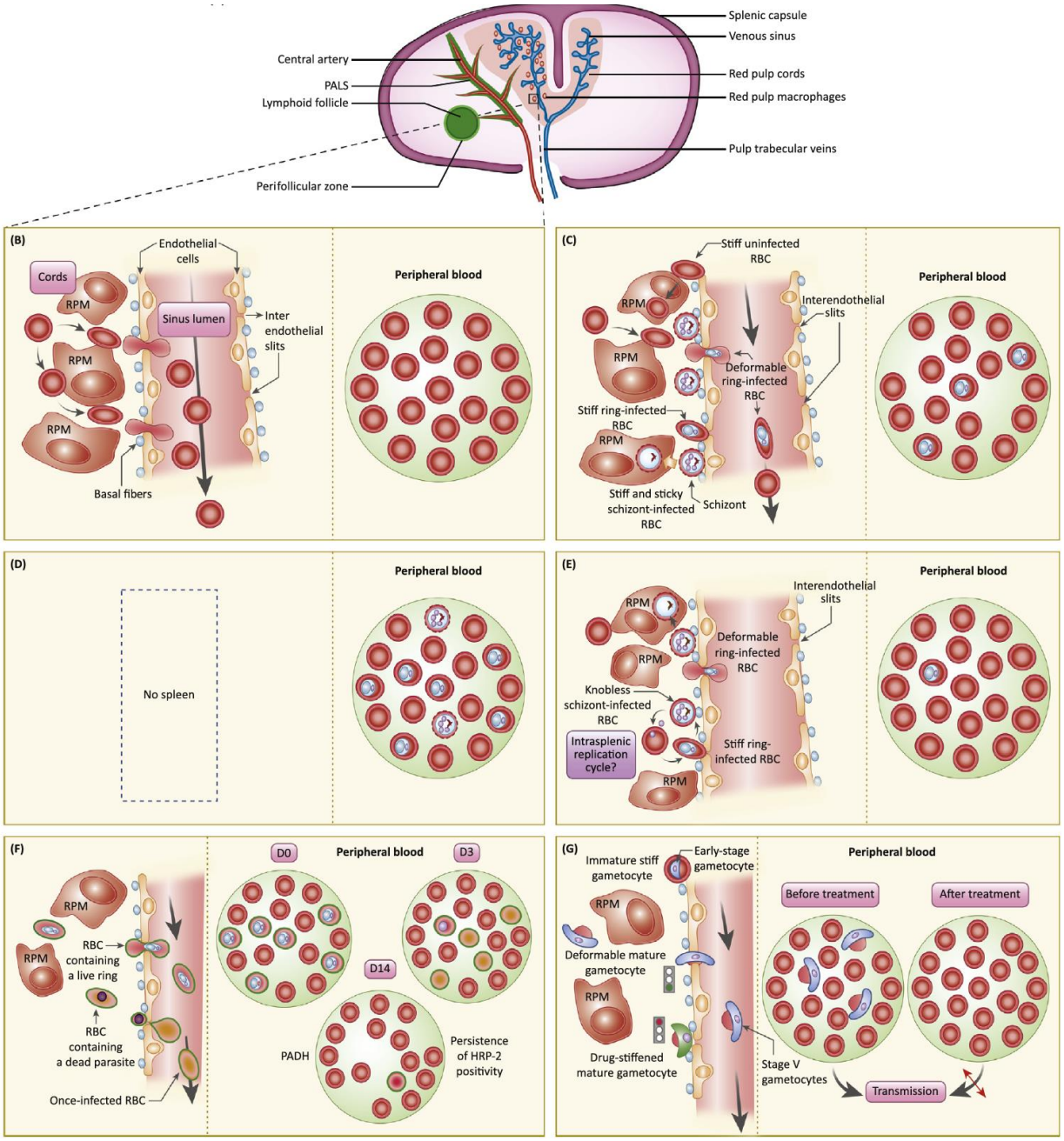


Figure 1-8 Structure and function of the spleen during a malaria infection.

(A) Schematic view of the spleen PALS, periarteriolar lymphoid sheath (B) The slow open circulation in the red pulp, where RBCs pass through the cords of Billroth and squeeze through inter-endothelial slits (IESs) to join venous circulation. (C) During an acute malaria infection iRBCs are maintained in the spleen and destroyed by red pulp macrophages (RPMs). (D) In splenectomised patients mature forms of the parasite are seen in peripheral circulation while in spleen-intact patients mature forms of the parasite are normally

sequestered in the spleen. (E) During chronic malaria infection, many infected and uninfected RBCs are maintained in the spleen, leading to splenomegaly and this could lead to a fully intrasplenic replication cycle of the parasite. (F) Pitting process of RBCs occurs in the spleen after treatment with antimalaria drugs. Pitting is the removal of dead parasites from infected RBC, without lysing the RBC. (G) gametocytes, retention of immature forms (stages I to IV) occurs in the spleen due to their reduced deformability, while the mature forms can cross IESs and circulate in the peripheral blood. Figure obtained from Henry et al. (2020)

A major feature of malaria infection is the alteration of the splenic structure, and this interferes with the development of an adequate immune response. Malaria infection causes an expansion of the red pulp which is vital for haematopoiesis in mice, temporary loss of the marginal zone and disorganisation of the B and T cell zones in the white pulp (Urban et al., 2005, Achtman et al., 2003). Theses structural change impedes the movement of T cells into the B cell zones, hence B cells are not adequately activated and are unable to move out of the white pulp. This affects antibody production and generation of long-lived plasma cells, which are vital in mounting an effective immune response against the parasite.

1.8.2 DC Interaction with *Plasmodium* During the Blood Stage of Malaria

The blood stage of the malaria parasite life cycle provides several opportunities for DC in the blood and spleen to interact with infected RBC (**Figure1-5**). This stage requires remodelling of the RBC to enable the parasite to survive (Boddey and Cowman, 2013) and results in the expression of parasite antigens on the RBC surface. One of these antigens, PfEMP1, play a key role in immune evasion and vascular sequestration/cytoadherence to avoid splenic clearance (Smith et al., 1995, Baruch et al., 1995). It has been suggested that PfEMP1 may be involved in modulation of DC function via interaction with CD36 (Urban et al., 2001b, Urban et al., 1999).

Maturation of iRBCs (schizont stage) results in lysis of the iRBCs, releasing merozoites into circulation and the contents of the PV such as the parasites digestive vacuole which contains hemozoin and waste products. The free merozoites have a short window to invade new RBCs (Gilson and Crabb, 2009) and those that fail to invade remain in circulation where they are

cleared in the spleen. Parasite waste products and hemozoin do interact with DCs but their overall effect on DC function is contradictory. The effect of hemozoin on DCs has yielded varying results with some studies showing that hemozoin is capable of activating DCs (Coban et al., 2002) while others showed that DC maturation and function was inhibited by hemozoin (Skorokhod et al., 2004, Millington et al., 2006). The varying results could be due to the different methods that were used to generate hemozoin with contamination by parasite DNA being a potentially confounding factor (Gazzinelli et al., 2014).

Overall, the blood stage has an abundance of parasite antigens that DCs can use to mount an immune response. However various immune evasion mechanisms, such as antigenic variation of VSAs (Escalante et al., 1998, Wilson et al., 2016) and sequestration of mature schizont and trophozoites in blood capillaries (Kraemer and Smith, 2006) thus avoiding splenic clearance (Bachmann et al., 2009, Demar et al., 2004) could slow the acquisition of immunity. DCs at this stage are critical in maintaining an immunological balance between parasite burden and a sufficient immune response. Immune evasion by the parasite could cause an increase in parasite burden resulting in severe pathology, while an excessive and uncontrolled immune response may lead to the development of a severe life threating cerebral malaria (Gowda and Wu, 2018, deWalick et al., 2007, Piva et al., 2012).

Studies of blood stage infections with DC have largely employed DCs prepared from peripheral blood monocytes or isolated from peripheral blood of uninfected individuals (Urban et al., 1999, Elliott et al., 2007). Fewer studies have analysed the phenotype and function of peripheral blood DCs from individuals who are currently undergoing a malaria episode (Gotz et al., 2017, Arama et al., 2011, Urban et al., 2001a). In this context, *in vivo* mouse models of malaria have been particularly helpful to understand the tissue responses of DC, for example splenic DC and allow temporal analysis of how *Plasmodium* infection changes DC phenotype (Millington et al., 2006, Millington et al., 2007).

1.8.2.1 *In Vitro* DC Interaction with *Plasmodium*

In vitro studies have been used to identify the mechanisms used by the parasite to modulate DC function. These studies have either used human monocyte derived dendritic cells (moDCs) or bona fide DCs to assess DC- *P. falciparum* interactions.

Urban et al. (1999) showed that when moDCs were co-cultured with iRBC, at a ratio of 1:100, and later stimulated with lipopolysaccharide (LPS), they exhibited a decreased expression of key maturation markers (CD40, MHC Class II, CD80, CD86 and CD83) (Urban et al., 1999). Once moDCs were exposed to iRBC, they lacked the capacity to activate allogeneic T cells (Urban et al., 1999). This modulation of DC maturation may result from an interaction between CD36 on DCs with PfEMP-1 on iRBC (Urban and Roberts, 2002). A subsequent study found that a ratio of 1 DC: 100 iRBC inhibited LPS induced moDCs activation, cytokine production, and allogeneic T cell activation regardless of CD36-binding (Elliott et al., 2007). The high ratio of DC to iRBC coincided with an increase in apoptotic and necrotic cells, which was observed in both PfEMP1-deficient and PfEMP1-expressing iRBCs, this could account for the failure of DCs to respond (Elliott et al., 2007). At low iRBC to moDC ratio (10:1), moDCs made a modest response to LPS induced maturation and retained their ability to secrete cytokines and activate T cells (Elliott et al., 2007). Elliot et al. (2007) were unable to point out the mechanism used by *P. falciparum* to modulate moDC function, although they found that hemozoin, from iRBC lysate, did not inhibit LPS maturation of moDCs (Elliott et al., 2007). The studies show that a dose-dependent relationship exists between iRBC and moDCs inhibition and dose range experiments are an essential part of ensuring experimental reproducibility in the future.

Another study found that at a low ratio of 10 iRBC per moDCs did not trigger the upregulation of HLA-DR, CD83, or CCR7 on moDCS (Giusti et al., 2011), contradicting the study by Elliot et al. (2007). At a ratio of 100 iRBCS per moDCs, moDCs were able to secrete IL-1β, IL-6, IL-10, TNF-α, and upregulate the chemokine receptor CXCR4 (Giusti et al., 2011). Exposure of moDCs to schizont lysate resulted in an increase in the expression levels of CD86 while CD80 and HLA-DR levels remained unaffected even at high concentration of schizont extract (Clemente et al., 2013). Exposure to schizont lysate, followed by LPS stimulation, did not affect

the maturation of moDCs. The schizont lysate exposed moDCs maintained their ability to differentiate allogeneic T cells into Th1 and regulatory T cells (Treg) that secrete large amounts of IFNγ. Additionally, the generated Tregs also secreted IL-10 and TGF-β (Clemente et al., 2013).

The different *in vitro* studies looking at the effect of *P. falciparum* on moDCs have yielded varying results. This could be attributed to the use of the *Plasmodium* parasite at different stages of development in the RBC. Another explanation could be that the studies used different experimental methods in the isolation of the *Plasmodium* infected red blood cells and in the generation of moDCs.

Few studies have examined the effect of *P. falciparum* on cDCs and pDCs due to their low numbers in peripheral blood. One study examined the effect of *P. falciparum* on cDC2 and pDCs (Gotz et al., 2017). The co-culture of cDC2 with *P. falciparum* at a ratio of 1:3 resulted in the upregulation of maturation markers (CD80, CD86, CD40, and HLA-DR) and inflammatory chemokines CCL2, CXCL9 and CXCL10 but did not induce secretion of inflammatory cytokines. Exposure of cDC2 to iRBC did not inhibit cytokine secretion in response to LPS, which was contrary to what was observed with moDCS (Gotz et al., 2017). The low ratio of iRBC to DC may account for this observation as the study did not use a higher ratio of iRBC to cDC2. The cDC2 exposed to iRBC maintained their ability to present antigens and activate naive T cells to polarise them towards a Tfh1 phenotype that secretes IFNγ (Gotz et al., 2017). The study also found that crosstalk between pDCs and cDC2 was important in shaping immune responses against malaria. The co-culture of pDCs and cDCs resulted in the upregulation of HLA-DR, CD86, and CD40 on pDCs and CD80 and CD86 on cDC2. There was also an increase in the secretion of interferon alpha (IFN-α) by pDCs and chemokines CXCL9 and CXCL10 by cDC2. This crosstalk between these two DCs was contact dependent, suggesting cell to cell interaction is necessary to initiate chemokine secretion (Gotz et al., 2017). The study highlighted the importance of cell-to-cell interaction which is crucial in trying to understand immune responses in malaria.

In mouse studies using bone marrow derived dendritic cells (BMDCs), *P. chabaudi* schizonts were shown to be able to activate BMDCs to produce the pro-inflammatory cytokines IL-12 and TNF-α. The *P. chabaudi* exposed DCs did not inhibit LPS activation, contrary to what was observed with *P. falciparum* exposed human DCs (Seixas et al., 2001). Millington et al. (2006), later showed *in vitro*, that DCs exposed to *P. chabaudi* infected RBC were unable to upregulate expression of MHC II and co-stimulatory molecules CD40, CD80, and CD86, and on LPS stimulation of these DCs, they were unable to increase their expression these key activation markers. This was similar to what was seen in the *in vitro* human moDC studies.

1.8.2.2 *Ex Vivo* DC Interaction with *Plasmodium*

Several studies have compared peripheral blood DCs in varying malaria transmission settings and different at-risk groups. In Kenya, children hospitalised with either mild or severe malaria were found to have a lower number of DC expressing HLA-DR and a lower number of circulating DCs compared with healthy children (Urban et al., 2001a). A follow up study revealed that the expression levels of HLA-DR was reduced on monocytes and cDC but not on pDC and that DC modulation continued during convalescence. An increase in the frequency of BDCA3+ cDC1 in the peripheral circulation was also observed during the course of the malaria infection (Urban et al., 2006).

A similar study was conducted in Mali looking at the function of DCs in children with severe malaria from the Dogon and Fulani community. The two communities reside in the same geographical region and are exposed to the same intensity of *P. falciparum* transmission yet the Fulani are less suspectable to *P. falciparum* infection (Dolo et al., 2005). DCs from malaria infected children of the Dogon community expressed lower levels of HLA-DR and CD86 on their DCs, while the frequency of BDCA-2+ pDCs and BDCA-3+ cDC1 increased compared to uninfected counterparts. Infected children from the Fulani community exhibited higher levels of HLA-DR and CD86 on their DCs but had a lower number of circulating BDCA-2+ pDCs and BDCA-3+ cDC1 compared to their uninfected counterparts (Arama et al., 2011). The study also showed that infected children from the Fulani community retained their ability to produce IFNγ after their PBMC were stimulated with specific TLR ligands at levels that were similar to those

of uninfected children. The Dogon children, on the other hand, had low levels of cytokine produced due to TLR impairment which increased parasite burden and development of malaria symptoms (Arama et al., 2011). This showed that *P. falciparum* infection resulted in altered DC activation with reduced response to TLR agonists in Dogon children, while in the Fulani children, DC activation and TLR responses were unaffected.

The increase in the number of circulating BDCA-2+ pDCs and BDCA-3+ cDC1 during malaria infection has been attributed to an increase in the amounts of FMS-like tyrosine kinase 3 (Flt3) ligand (Flt3-L) (Guermonprez et al., 2013). Flt3 is highly expressed on hematopoietic progenitor cells, but the expression is lost as cells commit to lymphoid and myeloid progenitor cells, which gives rise to the various cell lineages but its expression on DCs remains. Flt3 receptor tyrosine kinase and its ligand Flt3-L are known to be key in the development of dendritic cells and maintenance of their numbers (Karsunky et al., 2003, Waskow et al., 2008). Flt3-L production increases during a malaria episode as mast cells become activated and release membrane bound Flt3-L into circulation resulting in an increase in the number of pDCs and CD1c (Guermonprez et al., 2013).

A few studies have looked at the function of DCs in adults during a malaria episode. A study in Thailand found that adults with both severe and mild malaria had a decreased number of TLR2 expressing cDCs circulating in the periphery and a lower surface expression of TLR9 on pDCs but an increase in the surface expression of TLR2 on cDCs compared with healthy controls (Loharungsikul et al., 2008). There was also a marked reduction in the number of circulating pDCs, this could be attributed to their migration to the secondary lymph nodes, and an increase in serum levels of IFN-α (Pichyangkul et al., 2004b). A study conducted in Papua found that adults with acute *P. falciparum* malaria had a reduced number of circulating pDCs and cDCs, but higher numbers of immature DCs that were HLA-DR+CD11c−CD123− (Pinzon-Charry et al., 2013). Interestingly both pDCs and cDCs from infected participants were apoptotic as seen by Annexin-V binding. The DCs also expressed low levels of HLA-DR and costimulatory molecules and were unable to adequately capture antigen, resulting in reduced ability to prime naive CD4 T cells (Pinzon-Charry et al., 2013). These studies are therefore consistent with a

role for malaria infection in reducing the number of circulating DCs and their function in antigen presentation and T cell activation.

Controlled human infection models (CHMI) have also been used to assess the function of BDCA-1+ cDC2 and pDCs at varying doses of *P. falciparum* (Loughland et al., 2016). Healthy volunteers were enrolled into two cohorts; one cohort was inoculated with 150 iRBCs and the other 1800 iRBCs, participants were treated once parasitaemia reached ≥ 1,000 parasites/ml (Loughland et al., 2016, Loughland et al., 2017). The expression levels of HLA-DR on BDCA-1+ cDC2 and pDCs in both cohorts were significantly reduced at peak parasitaemia and this effect was still evident on BDCA-1+ cDC2 24 hours after anti-malarial treatment. The cohort inoculated with a higher dose of iRBC had a reduced number of circulating BDCA-1+ cDC2 which was attributed to apoptosis of the DCs during the infection, this was evident by the upregulation of caspase-3 (Loughland et al., 2016). The BDCA-1+ cDC2 from this cohort had a defective phagocytic capacity and there was a positive association between HLA-DR expression and phagocytic capacity (Loughland et al., 2016). pDCS on the other hand expressed low levels of CD123 at peak parasitaemia in both cohorts which persisted 24 hours after anti-malarial treatment. The number of pDCs in circulation significantly reduced in the 1800 iRBC cohort, this was due to apoptosis of pDCs during infection (Loughland et al., 2017). At peak parasitaemia DCs from the 1800 iRBC cohort were restimulated ex vivo with TLR ligands and their response measured. On re-stimulation with TLR1/2, TLR4, and TLR7, BDCA-1+ cDC2 failed to upregulate HLA-DR and CD86 but increased TNF secretion (Loughland et al., 2016). While re-stimulation of pDCS with TLR7 and TLR9 resulted in upregulation of HLA-DR, CD123, CD86 on their surface and an increased secretion of IFN-α (Loughland et al., 2017). This shows that malaria infection in naive individuals results in impairment of cDC function but not pDCs function. Indicating that pDCs may play a role during malaria infection and further studies are needed to deduce its role. The altered BDCA-1+ cDC2 also contributed to hampering effector T cells functions, allowing an increase of parasite burden (Loughland et al., 2016).

The various studies above have shown that DC phenotype is altered during a malaria episode resulting in impaired ability to upregulate HLA-DR and the costimulatory molecules CD86 (Arama et al., 2011, Urban et al., 2006, Loughland et al., 2016). This altered DC phenotype has

a reduced phagocytic capacity which impairs its ability to process antigens (Loughland et al., 2016) and adequately stimulate allogeneic T cells (Urban et al., 2006, Pinzon-Charry et al., 2013). The parasite also modulates TLR signalling thereby affecting cytokine secretion (Arama et al., 2011, Loughland et al., 2016) resulting in severe pathology. In children, there seems to be a notable increase in the number of circulating BDCA-3+ cDC1s during a malaria episode (Arama et al., 2011, Urban et al., 2006, Guermonprez et al., 2013), which was attributed to increases in serum levels of Flt3-L (Guermonprez et al., 2013), but this effect was not observed in children from Papua New Guinea (Kho et al., 2015). In both children and adults, there was a decrease in the number of circulating DCs which was attributed to increased DC apoptosis (Pinzon-Charry et al., 2013, Pichyangkul et al., 2004b, Loughland et al., 2017) but also increased DC migration to secondary lymphoid organs may also play a role in reduction on peripheral blood DC numbers. The decrease in peripheral numbers of DCs also corresponded with an increase in IL10 and TNF-α (Urban et al., 2001a, Urban et al., 2006, Pinzon-Charry et al., 2013), which may play a role in DC loss of function and suppression of T cell function. In these studies, DC function was altered regardless of the severity of malaria infection. The DC phenotype seen in the acute infection in the CHMI study (Loughland et al., 2016), was similar to those seen in naturally exposed individuals, and repeated infection, in naturally exposed individuals, could lead to sustained downregulation of DC function that may impact negatively on the immunity of an individual.

1.8.2.3 *In Vivo* Mouse Models of Malaria

Mouse models have been extensively used to study DC-*Plasmodium* interaction. *In vitro* interaction of *P. chabaudi* schizonts with mouse bone marrow derived DCs resulted in an increase in the secretion of tumour necrosis factor-α (TNF-α), IL-6, and IL-12p40 and IL-12p70 (Seixas et al., 2001). In mice injected with *P. chabaudi*, DCs had fully functional cytokine production 6 days after challenge with *Plasmodium* parasite (Ing et al., 2006). Further studies demonstrated DCs were able to upregulate co-stimulatory molecules CD40, CD54, CD86 (Perry et al., 2004) during acute infection, and were able to migrate into T cell areas in the spleen (Leisewitz et al., 2004). Other studies with *P. chabaudi* show that during initial stages of murine erythrocyte infection, CD8+ DCs are activated by infected erythrocytes as they expressed high

levels of MHC II and costimulatory molecules and initiated a Th1 type of response. This response is short lived as the CD8+ DCs undergo apoptosis and are soon replaced by CD8- DCs with lower expression levels of costimulatory molecules and MHC II (Sponaas et al., 2006).

Consistent with the studies above, Millington et al. (2006) showed that DCs isolated from the spleen of mice 4 days after *P. chabaudi* infection were moderately activated as they upregulated surface expression of CD40, CD80, and CD86. However, during convalescence (day 12 and day 21 post-infection), DC did not upregulate costimulatory molecules and were refractory to stimulation with LPS or CD40L. When mice infected with *P. chabaudi* were immunised with ovalbumin (OVA) antigen and LPS, they produced significantly lower levels of OVA-specific IgG compared with uninfected immunised mice, however, this effect was only seen when immunised at days 12 and 21 post infection (not day 4). Thus, initial malaria infection in mice does seem to cause DC activation; however, DCs enter a refractory state following the initial peak of parasitaemia. Similar to convalescent DCs, *in vitro* bone marrow derived DCs pre-exposed to *P. chabaudi* were unable to increase expression levels of MHC II and co-stimulatory molecules CD40, CD80, and CD86, and LPS stimulation of these DCs was unable to increase their expression (Millington et al., 2006).

Further work suggested that hemozoin could also modulate DC function which resulted in impairment of T cell and B cell function. Hemozoin treated DCs retained their capacity to process antigen and present them on MHC class II to naive CD4 T cells. Thus, providing the essential signal 1 (peptide-MHC complex) via the T cell receptor (TCR) but these DCs were unable to form stable long-lasting clusters with naive T cells, resulting in the generation of dysfunctional T cells (Millington et al., 2006, Millington et al., 2007). These dysfunctional T cells failed to proliferate and produce adequate amount of effector cytokines (IL-2, IL-5, IL-10, IFNγ) (Millington et al., 2006), and were unable to migrate to B cell areas in the lymph nodes to aid in B cell proliferation and antibody production (Millington et al., 2006, Millington et al., 2007). The short interactions and lack of large clustering observed are known to interfere with the generation of Tfh cells as long sustained DC-T cells interaction is required for commitment of naive CD4 T cells to Tfh cells (Benson et al., 2015). It is possible the dysfunctional DCs can lead to the generation of exhausted T cells, as a result of the short time of antigen presentation

to the T cells in the absence of adequate co-stimulation. The dysfunctional T cells could also lead to the generation of atypical memory B cells which are normally associated with malaria episodes.

Dendritic cells have been shown to play a vital role in the survival of mice during a lethal infection with *P. yoelii*. Wykes et al. (2007) showed that DCs from mice infected with nonlethal *P. yoelii* infection were fully functional APC and maintained their ability to stimulate T cells, unlike DCs from lethal *P. yoelii* infection which were not functional. DCs from mice infected with the nonlethal parasite were adoptively transferred into naive mice, which were then infected with lethal infection *P. yoelii*. These DCs were able to control parasitaemia and aid in survival of the mice by secreting IL-12 (Wykes et al., 2007). This could in part explain the difference in malaria outcomes observed in natural infections.

1.9 Aims of project

The public health burden caused by malaria in Sub-Saharan Africa remains unacceptably high, and especially in children under the age of five years, despite the implementation of various malaria control interventions over many decades with the goals of reducing the devastation and more recently, eradication. History has taught us that vaccines are the most cost-effective preventative tools against infectious diseases. Although significant advancements have been made in the development of a malaria vaccine, only RTS,S/AS01 has been approved for control, and this is despite the vaccine showing only partial and short lived protection in children living in endemic areas (Olotu et al., 2016, Ndungu et al., 2019). The push for vaccine development has been justified by the observation that constant exposure to malaria can lead to naturally acquired immunity, but the exact mechanism of how this immunity develops remains unknown. A clearer understanding of these mechanisms could lead to the development of more efficacious anti-malarial vaccines. Various studies have shown that *Plasmodium* infections affect dendritic cell function, but the exact mechanism of disruption remain unknown. For this thesis, I comprehensively characterised the molecular and functional changes in DC after co-incubation with iRBC *in vitro* assays. Subsequently, the occurrence of these changes, and their consequences in downstream activation of T cells, were tested in the immune response to

controlled human malaria infections in adults with different levels of pre-existing immunity to malaria. The study provides new insight on the mechanism and role of DC dysregulation in malaria and will inform the development of more efficacious vaccines and their accompanying delivery systems.

Chapter 2 Materials and Methods

2.1 Parasite culture

I grew NF54 and ItG strains of *P. falciparum* (Ockenhouse et al., 1991) for co-culturing with monocyte derived DC. ItG strain of *P. falciparum* was used due to ability to highly express PfEMP1 on the surface of infected RBC, as well as its ability to bind to both ICAM-1 and CD36 (Ockenhouse et al., 1991; Baruch et al., 1996). NF54 strain of *P. falciparum* was used as it resembles the whole organism malaria vaccine based on *P. falciparum* aseptic, purified, cryopreserved sporozoites (PfSPZ), the Sanaria® PfSPZ vaccine which was used for the CHMI challenge in Kilifi. *P. falciparum* was cultured as described previously (Trager and Jensen, 1976; Madkhali et al., 2014). Briefly, the cultures were grown in RPMI-1640 supplemented with 24mM sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA), 1M HEPES buffer (Gibco™, Waltham, MA USA) (incomplete media) and 10% human serum (Interstate Blood Bank Inc., Memphis, TN, USA) (complete media). They were maintained under 5% CO_2/1% O_2 and 94% N_2 mixture and at 37°C.

2.1.1 Isolation of *P. falciparum* Trophozoites

Parasite cultures were routinely synchronized to early stages (rings) by using 5% (w/v) D-sorbitol (Sigma). For this, mixed parasite cultures of rings and early trophozoites were transferred into 50ml Falcon tube and centrifuged at 300xg for 3 minutes. Subsequently, after the removal of the supernatant, 5% (w/v) D-sorbitol (1.5 times the pellet volume) was added slowly and with gentle mixing. The mixture was incubated at 37°C for 20 minutes. During the sorbitol incubation, a gentle hand agitation (two times during incubation) was performed. The sorbitol treated culture was centrifuged at 300xg for 5 mins and supernatant discarded. The synchronized parasites, which were mainly at the ring stage, were washed with incomplete media and centrifuged at 300xg for 5 minutes to remove sorbitol. After washing, the parasites were resuspended in complete medium and maintained under 5% CO_2/1% O_2 and 94% N_2 mixture and at 37°C for 26 to 34hr to allow the early stages of the parasite (rings) to develop to early or mid-trophozoites.

Trophozoites were magnetically isolated using CS columns (Miltenyi-Biotech, Bergisch Gladbach, Germany) in a SuperMACS II separator (Miltenyi-Biotech, Bergisch Gladbach, Germany) as per manufacturer's instructions. Columns were incubated with 3% BSA for 15 minutes. One 50mm x 9mm petri dish containing 25mL of parasites, maintained at 8-10% parasitaemia at 5% haematocrit was centrifuged at 300xg for 5 minutes and resuspended in 80% of its original volume in serum free RPMI and added to the CS column. The parasites were allowed to flow through the column at a slow rate to allow the trophozoites to bind onto the column. Once the eluent had run though the column, it was then washed with incomplete medium to remove unbound RBC. The column was detached from the magnet and the bound parasites were eluted from the column with 50mL of serum free RPMI, into a 50mL falcon tube and centrifuged at 250xg for 5 minutes. A smear was made to determine the purity of the isolated trophozoites. One petri dish produced approximately $6x10^8$ infected RBCs at a purity of 80-90%. The parasites were ready to use at the early/mid trophozoites stages.

2.2 Monocyte Derived Dendritic Cell Generation

Whole blood was either obtained from the National Blood Transfusion services in Edinburg (for experiments that were conducted in Glasgow) or from healthy volunteers living in a malaria endemic area (for experiments conducted at KEMRI-Wellcome Trust in Kilifi). Monocyte derived dendritic cells were generated at both labs as described below with the same reagents.

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation over Ficoll-Paque Plus (Sigma-Aldrich, St Louis, MO, USA). Monocytes were isolated from PBMC by positive selection using human anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions. The monocytes were cultured for 6 days at a concentration of $1x10^7$ cells in 10mLs (1×10^6 cells/mL) of RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U per ml) and streptomycin (100 ng/mL) (Invitrogen) granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (1000 IU/mL), and interleukin 4 (rhIL-4) (1000 IU/mL) (Immunotools, Friesoythe, Germany) in a T75 vented culture flask. On day 6 of the culture, the non-adherent monocyte derived DCs in the T75 flask were harvested by pipetting the cultured

cells out of the flask into a 50mL falcon tube. 10mL of ice cold $Ca^{2+/}Mg^{2+}$ free PBS was added to the flask and pipetted up and down to release the remaining loosely adherent monocyte derived dendritic cells and added to the 50mL falcon tube. The monocyte-derived dendritic cells (moDCs) were then centrifuged at 300xg for 5 minutes and supernatant discarded. 1mL of RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U per mL) and streptomycin (100 ng/mL) (Invitrogen) was added, and cell numbers and viability determined by trypan blue exclusion.

2.2.1 moDC-*P. falciparum* co-culture.

After day 6 the harvested non-adherent, immature monocyte-derived DCs (moDCs) were resuspended in RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U per mL) and streptomycin (100 ng/mL) (Invitrogen) at 5 x 10^5 cells/mL in 24-well plates (2 mL per well). Magnetically purified parasites at the trophozoite stage were added at iRBC/DC ratio of 100:1. Similarly, uninfected RBCs (RBCs) that were put under the same culture conditions as iRBCs and were later added to moDCs at the same ratio of 100:1 (RBCs:moDCs), this served as the experimental control. Additionally, moDCs were left in culture on their own and served as the negative control. I choose this ratio for comparability with other published data (Urban et al., 1999, Elliott et al., 2007, Giusti et al., 2011). The co-cultures of moDC-*P. falciparum* and moDC-uninfected RBCs were incubated for 16 hours after which LPS (100 ng/mL; InvivoGen Ultrapure *S. Minnesota*) was added to both co-cultures and incubated further for 24 hours. After 24 hours of incubation, the cells were stained with fluorescent antibodies for phenotyping by flow cytometry as described in Section 2.3.1. This experiment was initial setup at the University of Glasgow and later replicated at KEMRI-Wellcome Trust labs in Kilifi.

2.3 CD4 Isolation and moDC-T cell co-culture

Whole blood was either obtained from the National Blood Transfusion services in Edinburg (for experiments that were conducted in Glasgow) or from healthy volunteers living in a malaria endemic area (for experiments conducted at KEMRI-Wellcome Trust in Kilifi). PBMCs were

separated from the whole blood by density centrifugation over Ficoll-Paque Plus (Sigma-Aldrich, St Louis, MO, USA). After monocytes were isolated from PBMC by positive selection using human anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), CD4 T cells were subsequently isolated from the resultant flow through (from monocyte isolation) by negative selection using a human CD4 T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the manufacturer's instructions. The isolated CD4 T cells were resuspended in freeing medium (10% dimethyl sulfoxide and 90% heat-inactivated foetal calf serum) and stored in liquid nitrogen until when required.

After 16 hours of incubation the moDC-*P. falciparum* and moDC-uninfected RBCs co-cultures were each transferred from the 24-well plate into separate 15 mL flacon tube and spun at 260g for 5 mins and supernatant discarded. Both co-cultures were incubated with 1x RBC lysis buffer (Invitrogen, Carslbad, CA, USA) for two minutes at room temperature after which 10 mL of Dulbecco's phosphate-buffered saline (DPBS) was added and the cells spun at 400g for 5 mins. The supernatant was discarded, and cells were then resuspended in 5 mL of RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U per mL) and streptomycin (100 ng/mL) (Invitrogen). Cell numbers and viability determined by trypan blue exclusion.

Previously isolated CD4 T cells that were stored in liquid nitrogen were thawed a day before co-culture with moDCs. Once removed from liquid nitrogen, CD4 T cells were resuspended in 10 mL of PBS with 2% FCS, 2mM ETDA and spun at 400g for 5 minutes. The supernatant was discarded, and the cells resuspended in 20 mL of RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U per mL) and streptomycin (100 ng/mL) (Invitrogen) incubated overnight in a T75 culture flask. The CD4 T cells were later pipetted out of the T75 flash into a 50 mL flacon tube and spun at 400g for 5 minutes. The cells were then resuspended in 1mL of RPMI 1640 medium supplemented with L-Glutamine, 10% heat-inactivated foetal calf serum after which cell numbers and viability were determined by trypan blue exclusion. moDC that had been previously exposed to *P. falciparum* infected RBCs or uninfected RBCs were cocultured with the allogenic CD4 T cells at a ratio of 1:10 (moDC:CD4 T cell) for 72hrs, after which the cells were taken for single cell sequencing and

phenotyping using flow cytometry This experiment was initial setup at the University of Glasgow and later replicated at KEMRI-Wellcome Trust labs in Kilifi.

2.3.1 Staining of Cells for Flow Cytometry

2.3.1.1 moDC

The phenotype of moDCs was characterised using the monoclonal antibodies listed in **Table 2-1**. Cells were harvested and washed using cold $Ca^{2+/}Mg^{2+}$free PBS with 2 mM EDTA and 1% foetal calf serum. RBCs were lysed using 1x RBC lysis buffer (Invitrogen, Carslbad, CA, USA), and cells were then washed. The cells were suspended in $Ca^{2+/}Mg^{2+}$free PBS containing e780 viability dye (1:1000) (eBiosciences, Waltham, MA, USA) and incubated for 20 minutes at 4°C. Human Fc Block (Clone Fc1.3216; BD Biosciences, San Jose, USA) was added to the moDCs, and later a cocktail of the monoclonal antibodies in **Table 2-1** was added to the samples. The moDCs were washed and analysed using either Becton Dickinson LSRII or LSRFortessa flow cytometer. Analysis of flow cytometry data was performed using FlowJo 10 (FlowJo LLC, Ashland, OR, USA).

Table 2-1: List of antibodies used for phenotyping moDC

Marker	Fluorochrome	Clone	Concentration in Sample	Manufacturer	Cat. No.
CD14	PE/Cy7	M5E2	1:40	Biolegend	301814
CD1c	PerCP/CY5.5	L161	1:40	Biolegend	331514
CD80	FITC	2D10	1:40	Biolegend	305206
CD86	BV605	IT2.2	1:40	Biolegend	305430
CD83	APC	HB15e	1:40	Biolegend	305312
CD40	PE	5C3	1:40	Biolegend	334308
HLA-DR	BV421	L243	1:40	Biolegend	307636
Viability	e780	-	1:1000	eBioscience	65-0865-14

2.3.1.2 CD4 T Cells

The phenotype of CD4 T cells was characterised using the monoclonal antibodies listed in Table 2-2. Cells were harvested and washed using cold $Ca^{2+/}Mg^{2+}$ free PBS (with 1 mM EDTA and 1% foetal calf serum) and suspended in $Ca^{2+/}Mg^{2+}$ free PBS containing e450 viability dye (1:1000) (eBiosciences, Waltham, MA, USA) and incubated for 20 minutes at 4°C. Human Fc Block (Clone Fc1.3216; BD Biosciences, San Jose, USA) was added to the T cells, and later the appropriate monoclonal antibodies were added to the samples. The T cells were washed and analysed using LSRFortessa flow cytometer. Analysis of flow cytometry data was performed using FlowJo 10 (FlowJo LLC, Ashland, OR, USA).

Table 2-2: List of antibodies used for phenotyping moDC

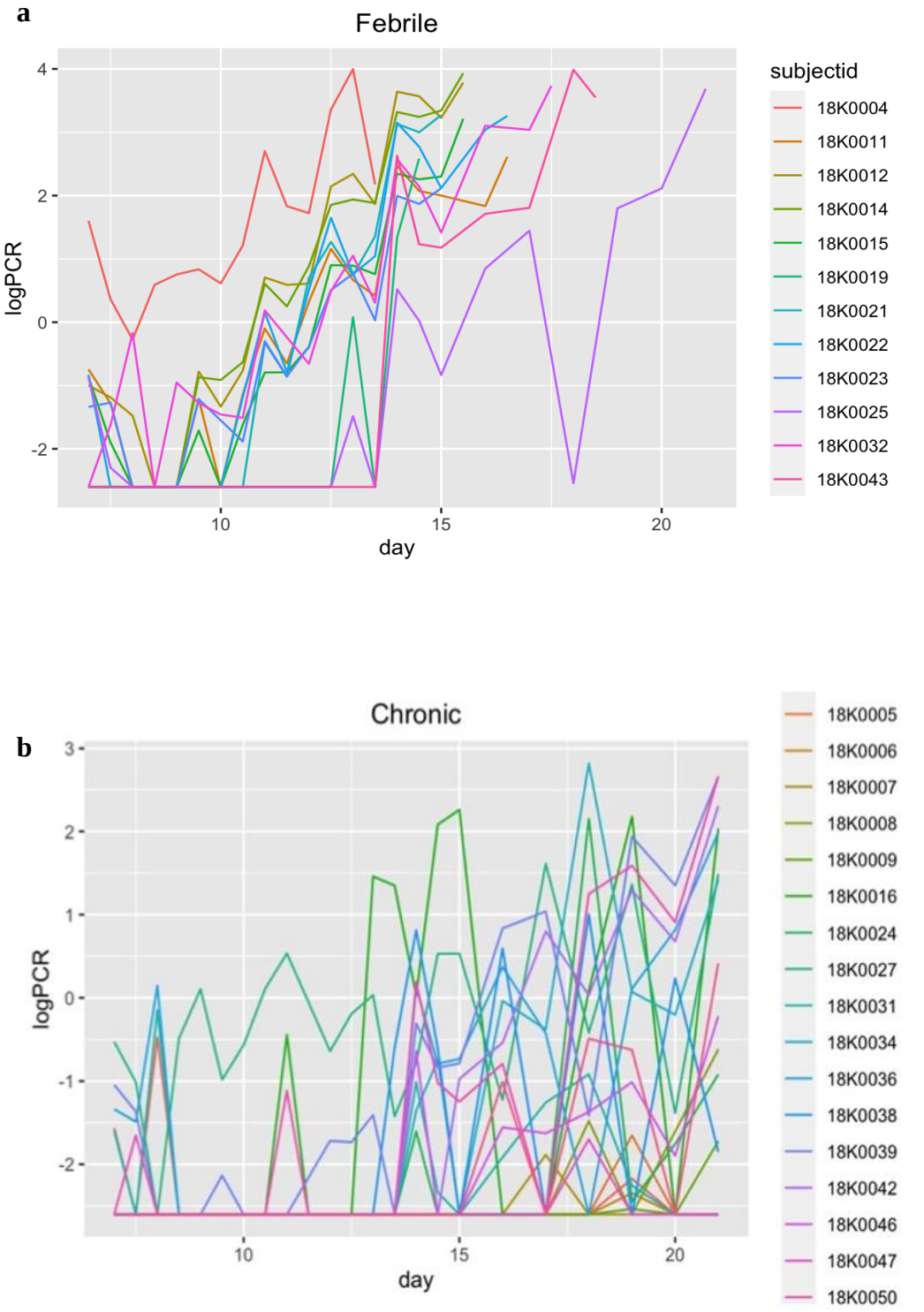
Marker	Fluorochrome	Clone	Concentration in sample	Manufacturer	Cat. No.
CD4	PerCP/Cy5.5	OKT4	1:40	Biolegend	317427
CD45RO	APC-Cy7	UCHL1	1:40	Biolegend	304227
CD45RA	APC	HI100	1:40	Biolegend	304111
CD62L	PE-Cy7	DREG-56	1:40	Biolegend	304821
CD197 (CCR7)	PE	G043H7	1:40	Biolegend	353203
CD25	FITC	BC96	1:40	Biolegend	302603
CD69	BV785	FN50	1:40	Biolegend	310931
CD40L	BV605	24-31	1:40	Biolegend	310825
PD1	BV711	EH12.2H7	1:40	Biolegend	329927

2.4 Controlled Human Malaria Infection (CHMI) Study Sample Selection criteria

Various *in vitro* studies have shown that infected RBC downregulate activation markers on moDCs or make them refractory to activating stimuli (Urban et al., 1999, Elliott et al., 2007, Millington et al., 2006). We therefore took advantage of a CHMI study to investigate the effect of exposure to *P. falciparum* infected RBC (*Pf*iRBC) on DC and CD4 T cells *in vivo*. The rationale behind using the CHMI study instead of using individuals from natural infection cohorts is that CHMI studies can control for confounding factors such as co-infection and co-morbidities, through screening participants before enrolment thus ensuring that these confounding factors do not affect the outcome of the infection.

The CHMI study setup in Kilifi, Kenya (Kapulu et al., 2018) was to investigate how individuals with previous exposure to the malaria parasite respond when inoculated with the Sanaria® PfSPZ. The participants from this study were recruited from areas with varying malaria transmission intensities and informed consent was obtained from each participant; this was after ethical approval of the study protocol from the KEMRI Scientific and Ethical Review Unit (SERU/029/3190).

The idea was to determine the cellular changes in malaria-exposed adult volunteers during a natural malaria infection (CHMI). The levels of exposure were determined during recruitment of the participants by measuring antibody levels against the schizont protein extract. The patient samples were selected according to their clinical phenotypes, which were assigned after analyses of the CHMI parasite growth PCR data and fever data. The different clinical phenotypes which were found were, febrile, chronic, susceptible and PCR negative (**Figure 2-1**).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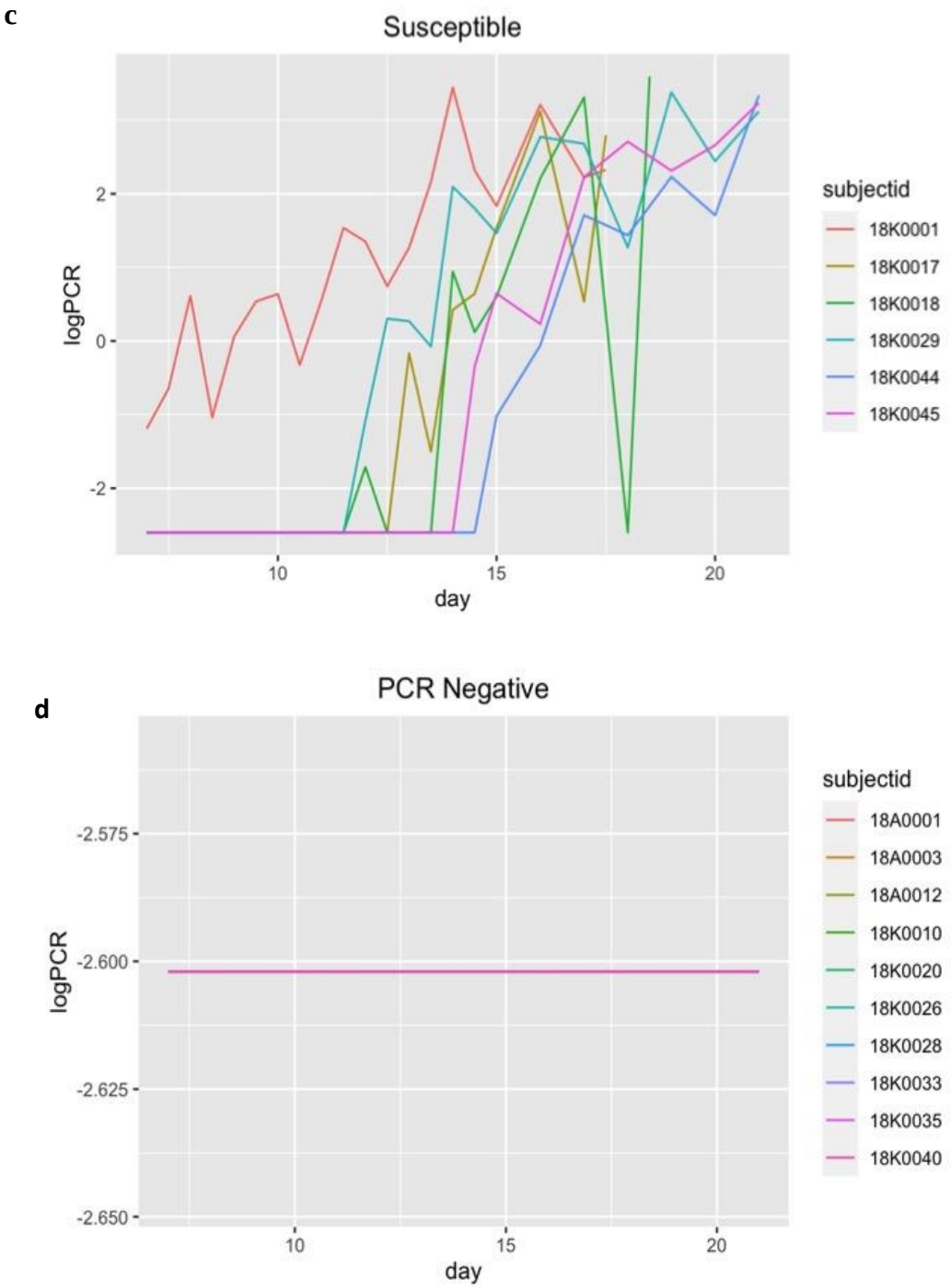


Figure 2-1 Graphs of the various clinical phenotypes identified in CHMI

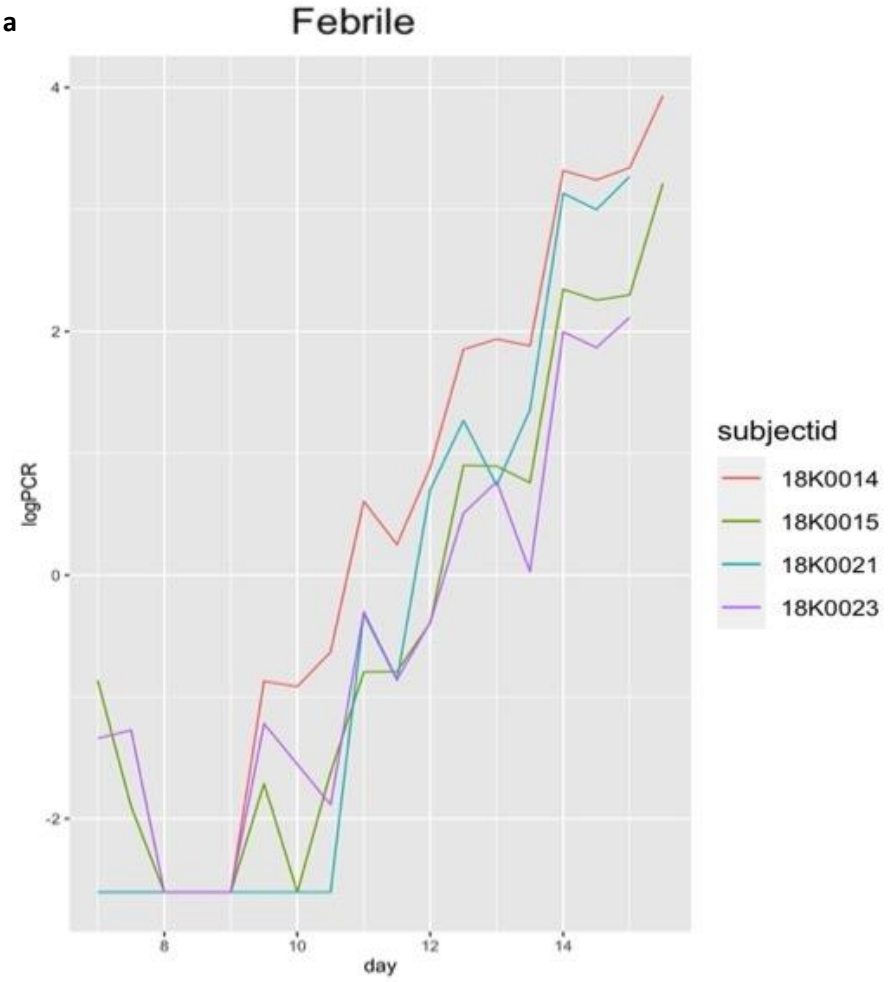
The daily CHMI parasite growth curves were used to classify the various patient samples to either (**a**) febrile, (**b**) chronic, (**c**) susceptible or (**d**) PCR negative.

From the study, two phenotypes were chosen for comparison. The febrile individuals, who were unable to control parasitaemia and developed clinical symptoms of malaria. These individuals had detectable parasites on blood film examination or reached the threshold of 500 parasites per μl (which was the threshold for treatment) and were treated after day 14 post challenge. While the chronic individuals were able to control parasitaemia, had detectable parasites (by PCR) but the parasites did not reach the threshold for treatment (500 parasites per μl), nor did they exhibit clinical symptoms of malaria and reached the endpoint of the CHMI study when they were treated with antimalaria drugs.

The selected samples were from febrile participants (18K0014, 18K0015, 18K0021, 18K0023) and chronic participants (18K0016, 18K0039, 18K0027, 18K0034). These samples were from two areas of Kilifi. Kilifi North, an area that has a low malaria transmission intensity, where most of the febrile participants originated from, and Kilifi South - an area where malaria transmission is high and most chronic participants were from this region (**Figure 2-2**).

The time points selected were, day C-1 (day before challenge with sporozoites), C9 (9 days after sporozoite challenge, day at which parasitaemia was at a similar level for majority of the samples, before divergence and development of different levels of parasitaemia), C14 (14 days after sporozoite challenge, at this timepoint the patients showed varied immunity, with some being able to control the parasite while others were not). The selected patient samples were not treated with anti-malarial drugs before day 14.

a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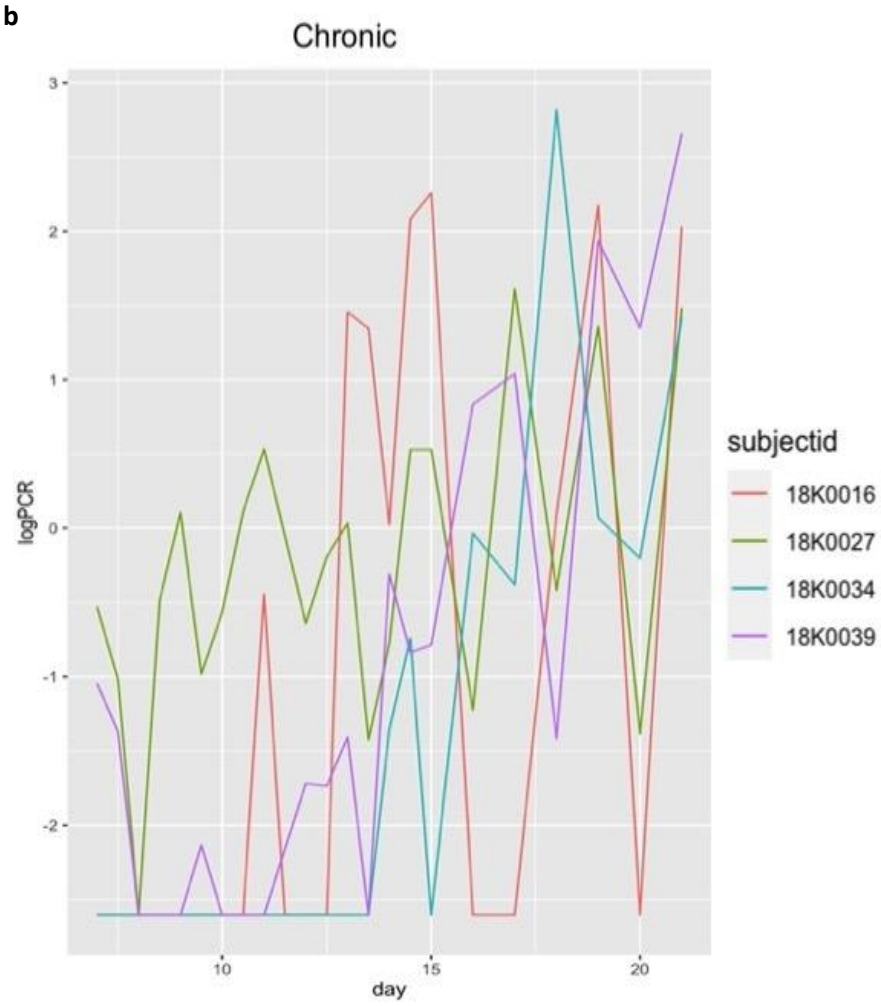


Figure 2-2 Selected CHMI patient samples.

The graphs of the daily CHMI parasite growth curves of the patient samples that I had selected for single cell sequencing. Two phenotypes that were chosen for sequencing were (**a**) febrile participants and (**b**) chronic participants.

2.5 Single Cell RNA-seq

2.5.1 moDC-CD4 T cells Co-culture

Single-cell suspensions of DC (iRBC exposed)-T cell co-culture and DC (RBC exposed)-T cell co-culture were loaded onto a 10x chromium single cell microfluidics chip (10x Genomics, Pleasanton, CA, USA) according to the manufacturer's instructions for co-encapsulation with barcoded Gel Beads (**Figure 2-3**). Libraries were constructed using Chromium Single Cell 3′ Solution (10x Genomics, Pleasanton, CA, USA) according to the manufacturer's instructions and sequenced using Ilumina Nextseq by Glasgow Polyomics. Sequence data was analysed using the Seurat package on R statistical software.

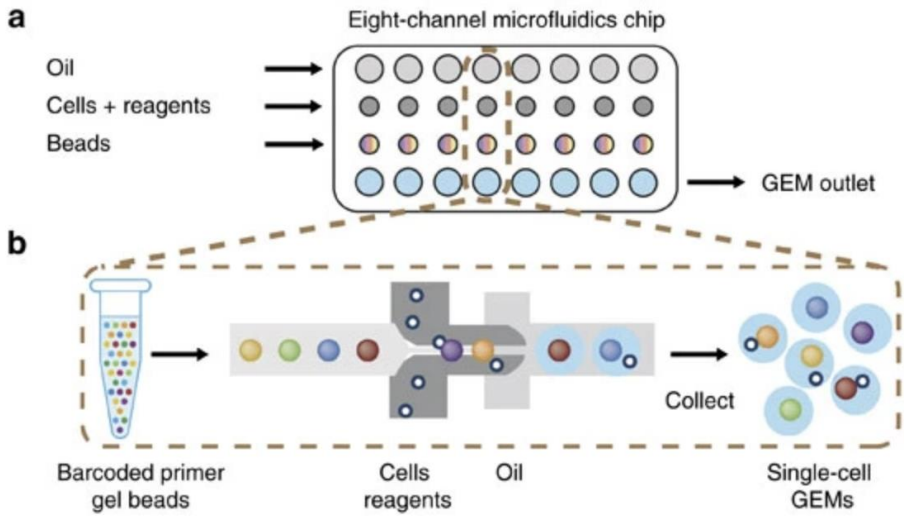


Figure 2-3 Workflow for generation of single suspension cells co-encapsulated with barcoded gel beds.

The 10x 8-channel microfluidic chip enables for the encapsulation of barcoded primer gel cells with single cells from our samples (a). Each well on the plate contains different reagents and cells from the samples combined with the barcoded primer gel and oil to form Gel bead in EMulsion (GEM). Each gel bead is functionalised with barcoded oligonucleotides that consists of: (i) sequencing adapters and primers, (ii) a 14 bp barcode drawn from ~750,000 designed sequences to index GEMs, (iii) a 10 bp randomer to index molecules (unique molecular identifier, UMI) and (iv) an anchored 30 bp oligo-dT to prime polyadenylated RNA transcripts (Zheng et al., 2017). Image modified from Zheng et al. (2017).

2.5.2 Cell Hashing and CITE seq of CHMI samples

Whole blood was collected from participants enrolled in the CHMI study. PBMCs were isolated from the whole blood by density centrifugation over Ficoll-Paque Plus (Sigma-Aldrich, St Louis, MO, USA). Ice cold Hanks' Balanced Salt Solution (HBSS) with 1% FCS was added to the separated PBMC and the mixture centrifuged at 400xg for 10 minutes and supernatant discarded. Freezing medium (90% FCS and 10% DMSO) was added to the pellet after centrifugation, and aliquots of the PBMC added into 1mL cryovials. The cryovials were placed in Mr. Frosty™ Freezing Container (ThermoScientific, Waltham, MA, USA) containing 100% isopropyl alcohol and placed in -80°C freezer and later transferred into liquid nitrogen. The selected samples were then shipped from Kilifi to the University of Glasgow after getting ethical approval from KEMRI Scientific and Ethical Review Unit (SERU/029/3190).

In order to identify our cell population of interest, which were: CD4 T cells, plasmacytoid dendritic cells, and myeloid dendritic cells (CD1c DCs and CD303 DCs) within the CHMI PBMCs and still perform single cells sequencing of the entire PBMC population, a Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) was performed on the PBMCs. CITE-seq enables tagging of the cells of interest with antibodies against surface receptors that are unique to the cell population. This will enable for easy identification and analysis of the cell population of interest. Each sample had PBMCs for 3 timepoints, which would be distinguished by addition of a hashtag antibody. Hence the timepoints (day C-1, day C9 and day C14) had a unique tag (Cell Hashing Antibodies) to enable for identification of the timepoint.

The frozen CHMI samples were thawed and transferred into a 15mL falcon tube. 10mL of RPMI medium (supplemented with 10% FCS+ Glut + Pen&Strep) was slowly added to the falcon tube and centrifuged at 400xg for 10 minutes and supernatant discarded. The resultant pellet was suspended in staining buffer (2%BSA/0.01%Tween, PBS). Fc block was added to the samples, after which the TotalSeq™ CITE-seq antibodies (Biolegend, San Diego, CA, USA) listed on **Table 2-3** and cell hashing antibodies were added to each sample. The samples were then incubated for 30 minutes at 4 °C, after which they were washed 3 times by adding 1mL with

staining buffer followed by centrifugation at 350xg for 5 minutes. The samples were pooled accordingly, and dead cells removed using a dead cell removal kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The samples were then processed for 10x Chromium sequencing by Julie Galbraith at Glasgow Polyomics.

Table 2-3: List of CITE-seq Antibodies used for Sequencing

TotalSeq™ CITE-seq antibody	Clone	Amount	Manufacturer	Cat no
A0370 anti-human CD303 (BDCA-2) Antibody	201A	1 µg	Biolegend	354239
A0207 anti-human CD370 (CLEC9A/DNGR1) Antibody	8F9	1 µg	Biolegend	353807
A0922 anti-human CD4 Antibody	OKT4	1 µg	Biolegend	317451
A0160 anti-human CD1c Antibody	L161	1 µg	Biolegend	331539

Table 2-4 List of Cell Hashing Antibodies used for Sequencing

Cell Hashing Antibody	Clone	Amount	Manufacturer	Cat no
TotalSeq™-A0252 anti-human Hashtag 2	LNH-94; 2M2	0.5 µg	Biolegend	394603
TotalSeq™-A0257 anti-human Hashtag 7	LNH-94; 2M2	0.5 µg	Biolegend	394613
TotalSeq™-A0260 anti-human Hashtag 10	LNH-94; 2M2	0.5 µg	Biolegend	394619

2.6 Statistical Analysis

The statistical analysis was done using GraphPad Prism Software (version 8.4.0, San Diego, California USA). Since the sample were paired samples, I used the Wilcoxon matched-pairs signed-ranks test for comparisons between the samples.

Chapter 3 Effect of *Plasmodium falciparum* Infected Red Blood Cells on Dendritic Cell Function

3.1 Introduction

Parasitic infections often trigger immune responses that ultimately determine the outcome of the infection. The parasite-dendritic cell interactions are key in determining how the host responds to parasitic infections, as they not only initiate cellular immunity or tolerance but also direct the cellular response to either a T helper cell 1, T helper cell 2, T helper cells 17 or T regulatory response. Parasites can regulate dendritic cell function, thus preventing effective immune responses. This regulation is beneficial to the parasite as it provides a conducive environment for its survival while ensuring that any host inflammatory response is controlled to limit tissue damage and to maintain host fitness (Medzhitov et al., 2012). Therefore there is a delicate balance during parasitic infections as immunomodulation may also lead to increase in parasite burden and an uncontrolled inflammatory response resulting in host tissue damage (Medzhitov et al., 2012).

The *Plasmodium falciparum* parasite is one such parasite that has been shown to modulate dendritic cell function. During the blood stage of the parasite's life cycle, DCs are able to sense pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) through pattern recognition receptors (PRRs) (Brubaker et al., 2015). The toll like receptors (TLR) family of PRRs is responsible for sensing the *Plasmodium* parasite through TLR2-TLR6 or TLR1-TLR2 heterodimers and to a lesser extent by TLR4 homodimers (Durai et al., 2013, Krishnegowda et al., 2005, Nebl et al., 2005). These TLRs are expressed by myeloid DCs in humans, and they recognise *P. falciparum* glycosylphosphatidylinositol (GPI) which anchors various proteins (that are involved in erythrocyte invasion) onto the plasma membrane of merozoites (Sanders et al., 2005, Gilson et al., 2006, Gowda et al., 1997). Another TLR that recognise parasites PAMPs is TLR9 which is expressed in pDCs and senses malaria parasite DNA coated on the crystallized form of heme that is hemozoin which is released when merozoites egress from the mature form of the parasite infected RBC (schizonts) during the intraerythrocytic cycle (Coban et al., 2005, Parroche et al., 2007). While these TLR are key in

sensing the parasite it has been shown that the parasite is cable of modulating TLR signalling and this affects cytokine secretion and maturation of DCs (Loughland et al., 2016, Loughland et al., 2017).

Apart from TLR, another receptor that has been shown to sense parasite infected RBCs is the CD36 receptor. CD36 is a class B scavenger receptor that also functions as a PRR in innate immune cells. CD36 has been shown to play a role in the controlling parasitaemia through phagocytic clearance of parasite infected RBC by macrophages and DCs (El Khoury et al., 2003, Erdman et al., 2009, Febbraio et al., 2001, Patel et al., 2004, Gowda et al., 2013). CD36 binds to *Pf*EMP1 that is expressed on the surface of iRBCs and this initiates internalisation by DCs (Gowda et al., 2013). The binding of *Pf*EMP1 to CD36 is believed to modulate DC function causing them to be less responsive to LPS stimulation (Urban et al., 2001b, Urban et al., 1999). Elliott et al. (2007), showed that DC modulation does not require CD36 binding to PfEMP1, or contact between DCs and infected RBCs. Using RBCs infected with a parasite line that expressed a variant of *Pf*EMP1 that binds chondroitin sulfate A (CSA). They were able to show that the variant *Pf*EMP1-iRBC were phagocytosed by DCs and were able to inhibit LPS induced phenotypic maturation and cytokine secretion. Indicating that other receptors on DCs may be responsible for binding and internalising iRBC and that CD36 was not involved in modulating DC activation and function.

Once exposed to iRBCs, DCs have been shown to alter their function and this affects their ability to interact with CD4 T cells. In mouse models of malaria, it has been shown that exposure of DCs to the malaria parasite hinders their ability to form stable clusters with CD4 T cells, and this impairs CD4 T cell activation (Millington et al., 2007). Similarly in humans, once DCs are exposed to iRBC, they are unable to adequately stimulate CD4 T cells (Urban et al., 1999, Elliott et al., 2007).

The ability of the malaria parasite to suppress DC maturation could be responsible for the generalised immune suppression that is seen in *P. falciparum* infected children that leaves them suspectable to non-typhoid salmonella infection (Greenwood et al., 1972). This immune suppression has been shown to reduce responses to heterologous antigens such as vaccines (Ho

et al., 1986), and may account for the reduced efficacy seen in malaria vaccines administered to individuals living in a malaria endemic area (Ho et al., 1986, Sissoko et al., 2017, Bejon et al., 2008).

To understand how DC phenotype is affected by malaria parasite I exposed moDCs to the ItG strain of *P. falciparum*. This strain was used due to ability to highly express PfEMP1 on the surface of infected RBC, as well as its ability to bind to both ICAM-1 and CD36 (Ockenhouse et al., 1991, Baruch et al., 1996). moDCs were also exposed to NF54 strain of *P. falciparum* as it resembles the Sanaria® PfSPZ vaccine that was used for the CHMI challenge in Kilifi.

Once moDCs were exposed to the malaria parasite, they were co-cultured with allogenic CD4 T cells. Allogenic responses initiate polyclonal T cell activation as the T cell receptor (TCR) would recognise polymorphic amino acids present on the allogeneic MHC molecule of the DC, independent of the bound peptide on the MHC molecule (Rogers and Lechler, 2001). This interaction results in the activation of T cells, but if the DCs were dysregulated would they maintain their ability to initiate the allogeneic response and activate the CD4 T cell.

3.2 Chapter aims

While malaria is known to alter the function and maturation status of DCs the exact mechanism involved and signalling pathways affected are still unknown. This chapter investigates the ability of moDCs to respond to LPS after exposure to RBC infected with either NF54 or ItG parasite strains of *P. falciparum*. Subsequently we investigate whether DCs exposed to iRBC maintain the ability to interact with and stimulate allogenic CD4 T cells.

3.3 Results

3.3.1 Expression of Activation Markers on Immature and LPS Stimulated Monocyte Derived Dendritic Cells

The phenotype of monocyte derived DCs (moDCs) was confirmed using flow cytometry (**Figure 3-1**). DCs were defined as $CD1c^{+}$, $CD14^{-}$ (**Figure 3-1 (d)**). After defining the population of DCs, the percentage of cells positive expressing the key maturation/activation markers such as, CD80, CD86, CD83, HLA-DR and CD40 were assessed in LPS stimulated moDCs (**Figure 3-2**). A high percentage of the LPS stimulated cells were positive for the markers being assessed and thus opted to use the mean fluorescence intensity (MFI) to evaluate expression levels of the markers (**Figure 3-3)**. The expression levels of CD80, CD86, CD83, HLA-DR and CD40 were assessed in the experimental control sample that is moDCs that were exposed to uninfected RBCs and later stimulated with LPS (**Figure 3-4**). A comparison of surface expression levels of the markers between immature unstimulated DCs and LPS stimulated DCs showed that immature DCs expressed lower levels of the markers (CD80, CD86, CD83, HLA-DR and CD40) compared with LPS stimulated DCs. LPS stimulation of the DCs resulted in upregulation of the key markers but the level of upregulation between the donors varied **(Figure 3-5)**. moDCs generated from volunteers that came from a malaria endemic region, that is Kilifi, (donors from Kilifi) and malaria naïve individuals (donors from Glasgow) demonstrated similar upregulation of activation markers in response to LPS stimulation (**Figure 3-5**).

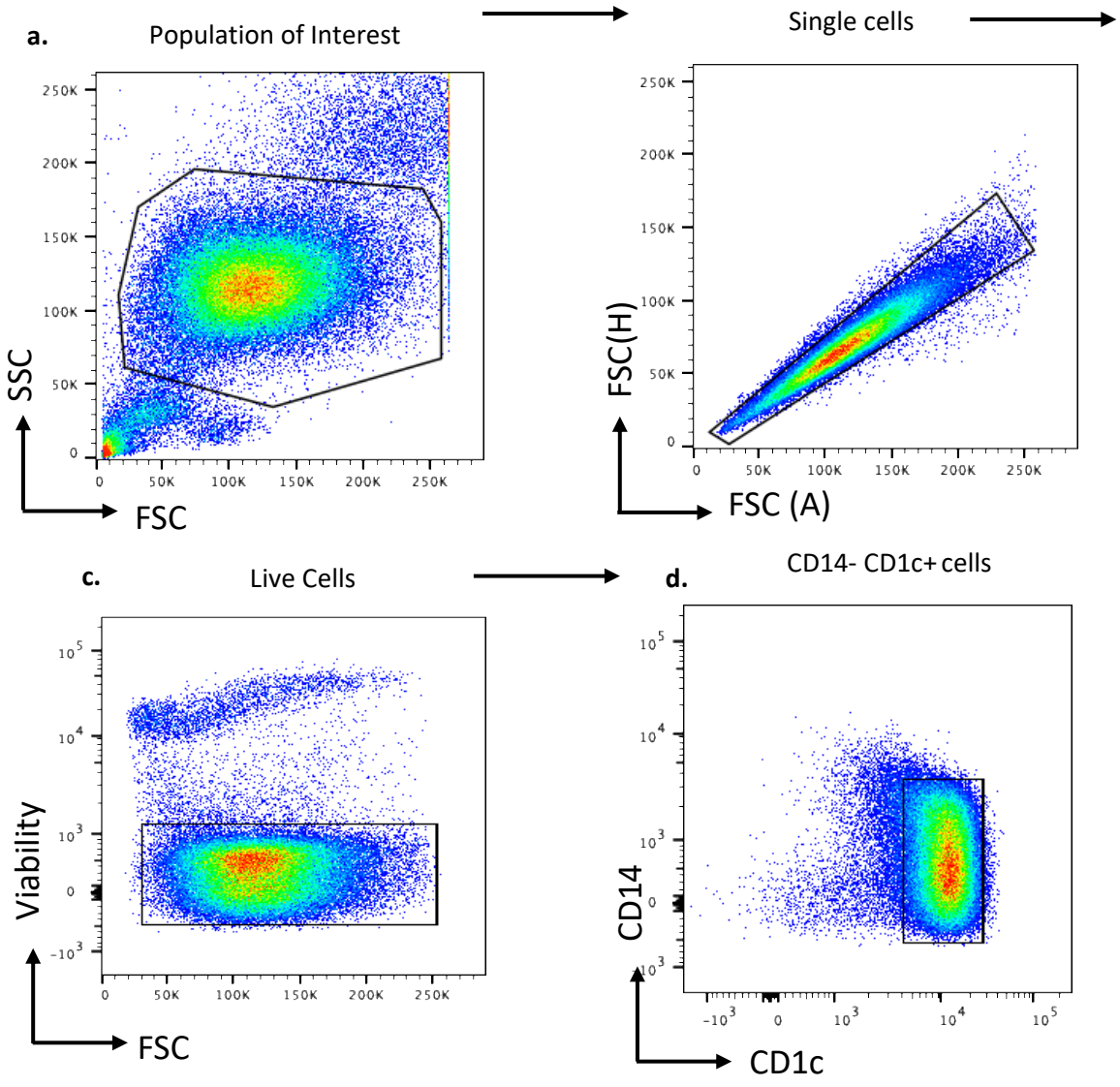


Figure 3-1 Gating strategy for identifying monocyte derived DC population.

(a). Forward versus side scatter (FSC vs SSC) was used to identify cells of interest based on size and granularity (complexity). **(b)** A forward scatter height (FSC-H) vs. forward scatter area (FSC-A) density plot was used to exclude doublets**.** (**c**) Live cells were gated using viability dye (e780) vs FSC. (**d**) Dendritic cell population was defined as $CD14^{-}$ $CD1c^{+}$

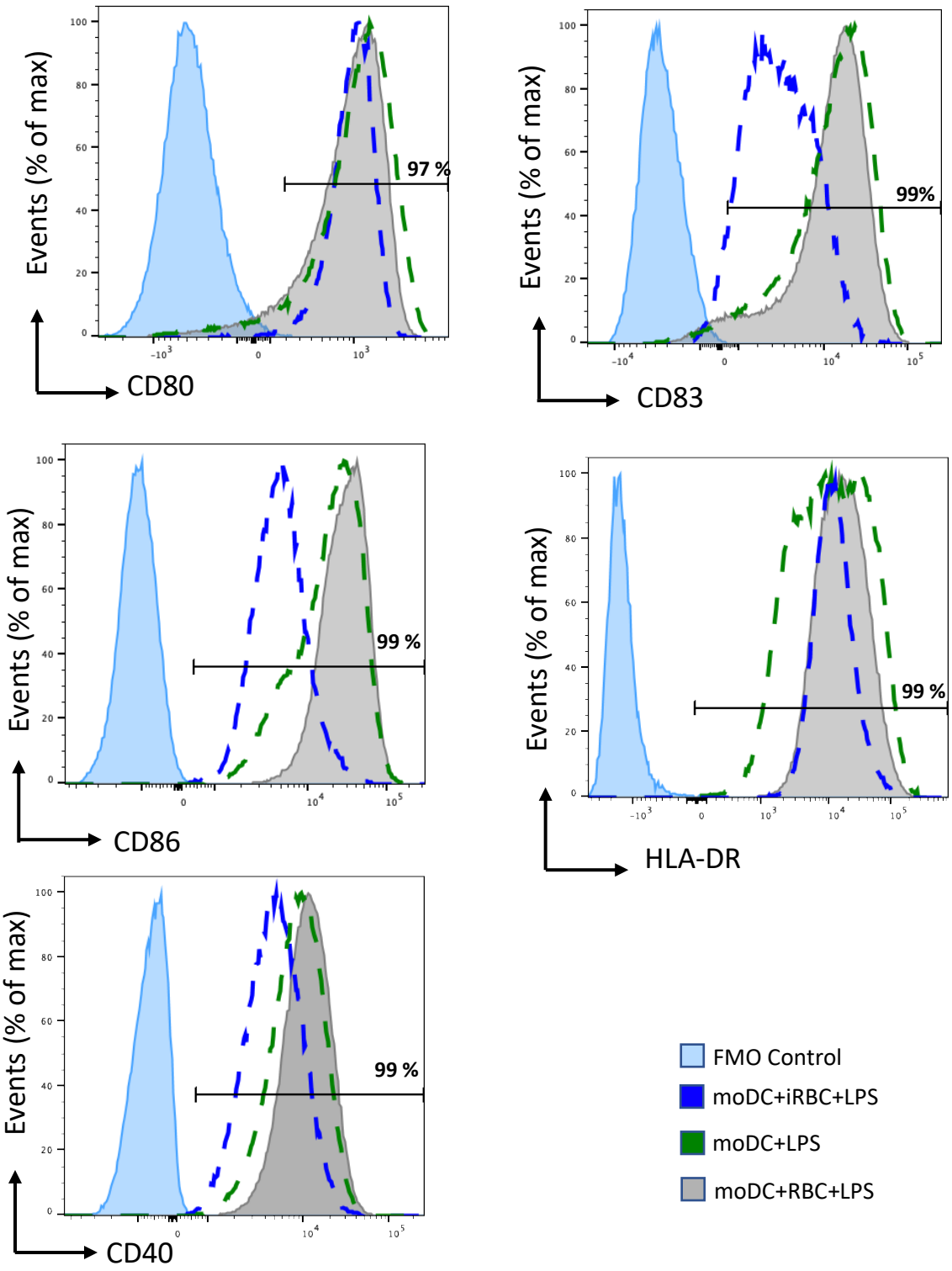


Figure 3-2 Percentage Positive Cells Expressing key moDCs markers.

After identification of the mDC population (**Figure 3-1 (d)**), The percentage of cells expressing the key markers that are upregulated on DCs activation were analysed. CD80, CD83, CD86, CD40, and HLA-DR markers were assessed in moDC stimulated with LPS (green dashed line), moDCs exposed to RBC and stimulated with LPS (grey shaded histogram), moDCs exposed to iRBC and stimulated with LPS (blue dashed histogram)

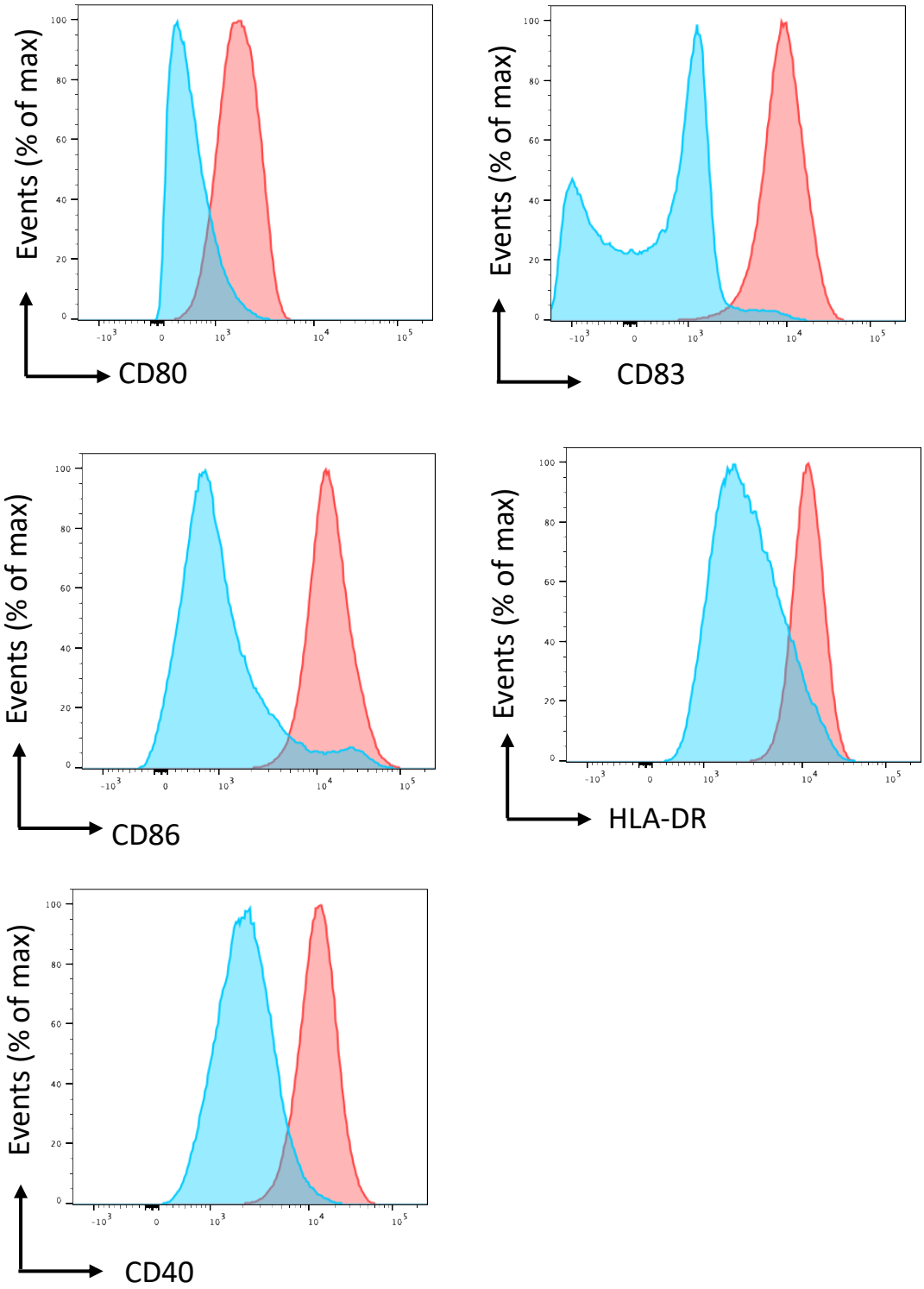


Figure 3-3 Relative expression of key moDC markers between immature and LPS stimulated dendritic cells.

After identification of the moDC population (**Figure 3-1 (d)**), key markers that are upregulated in LPS stimulated DCs (red shaded histogram) which are CD80, CD83, CD86, CD40, and HLA-DR were assessed and compared to unstimulated DCs (blue shaded histogram). The figure shows the expression levels of DCs markers from one malaria naive donor.

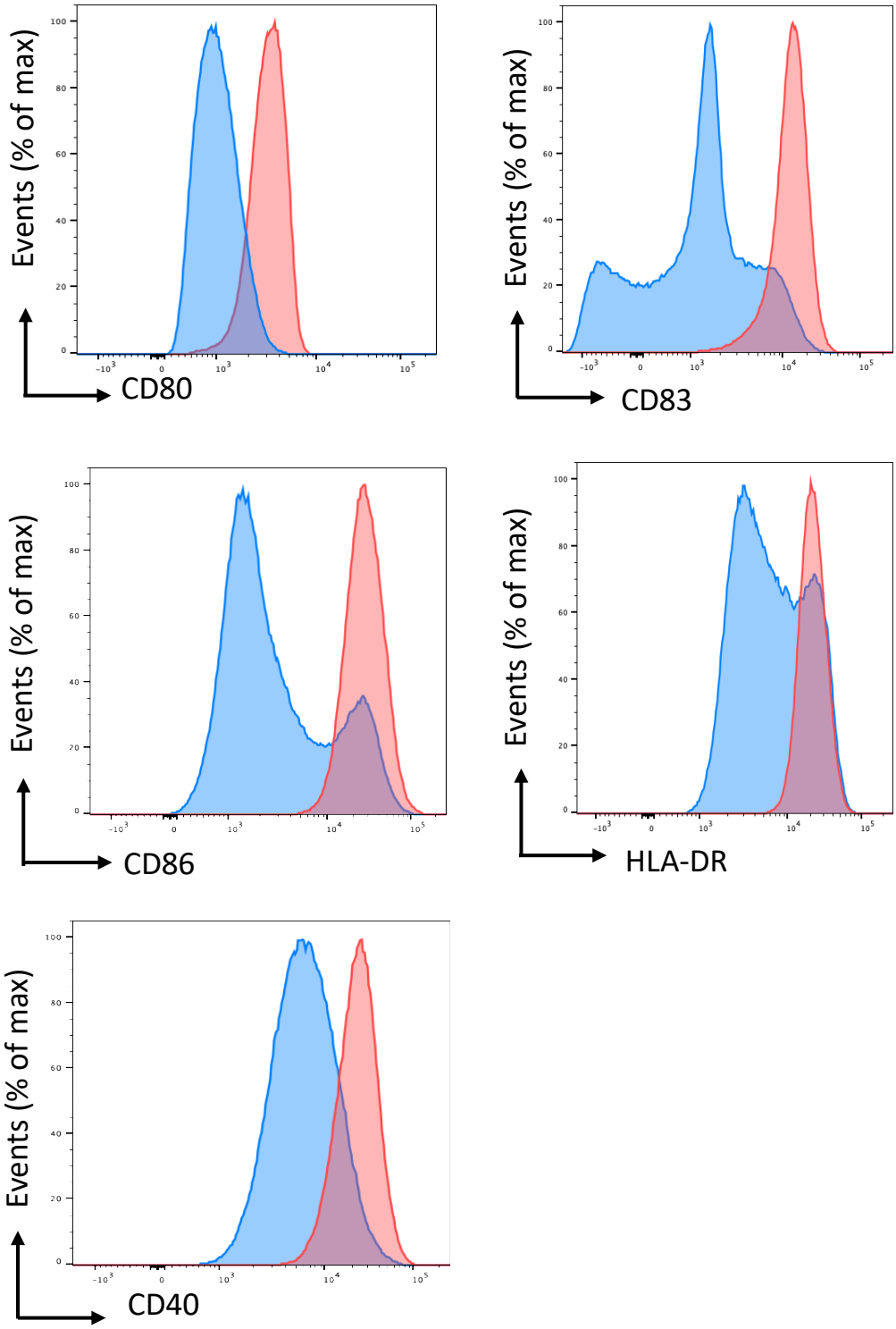


Figure 3-4 Relative expression of key dendritic cell markers in RBC exposed samples.

After identification of the moDC population (**Figure 3-1 (d)**), key markers that were upregulated in moDCs exposed to RBC prior to LPS stimulated DCs (red shaded histogram) which are CD80, CD83, CD86, CD40, and HLA-DR were assessed and compared to unstimulated moDCs exposed to RBCs (blue shaded histogram). The figure shows the expression levels of DCs markers from one malaria naive donor.

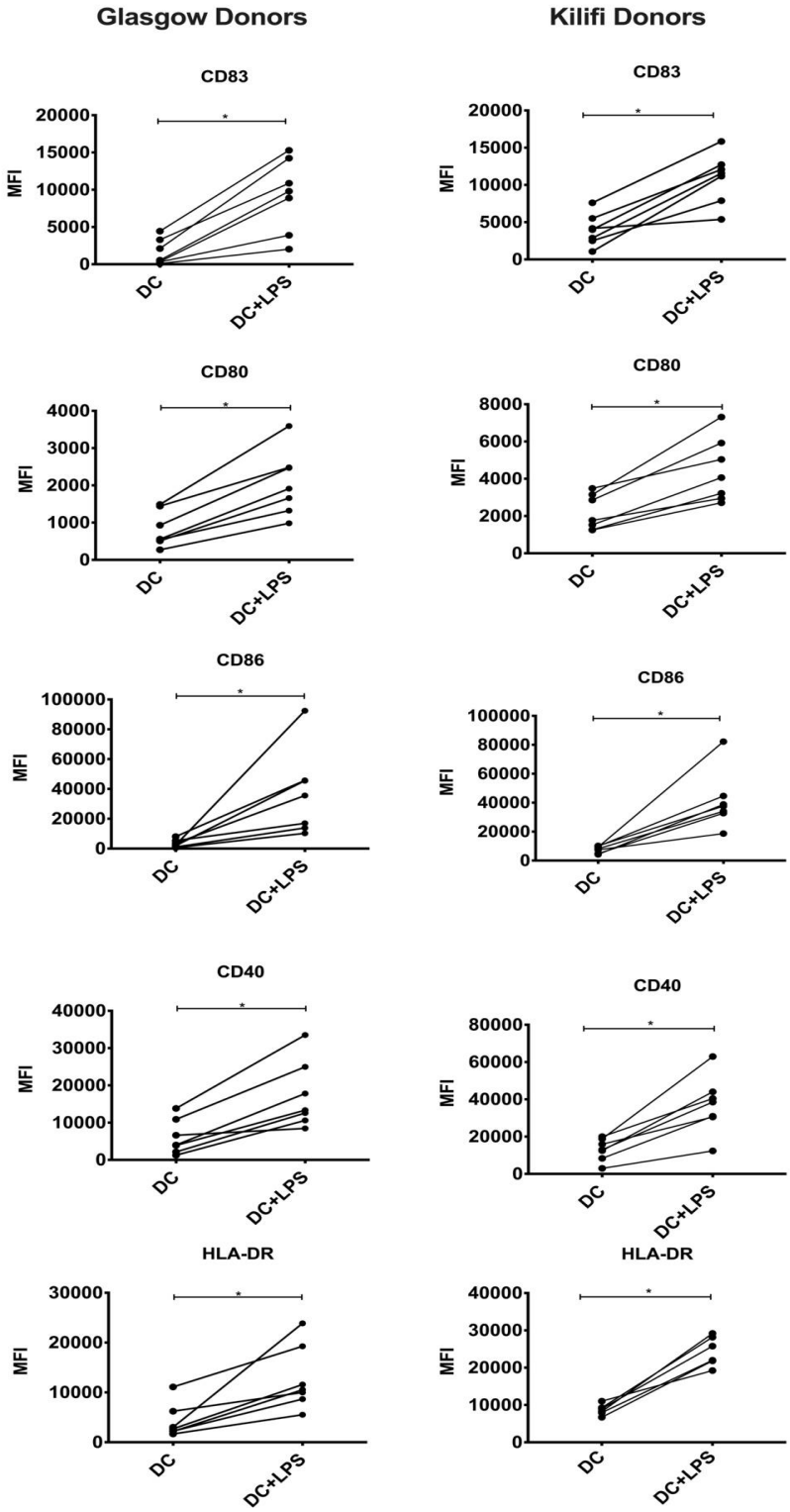


Figure 3-5 Comparison of moDC response to LPS.

Unstimulated and LPS stimulated moDCs generated from malaria naïve donor (Glasgow Donors) and donors living in a malaria endemic region (Kilifi Donors). Both set of samples responded in a similar manner to LPS stimulation the only difference was the levels of expression between the donors which varied. The graph shows overall analysis of seven donor matched samples for both Kilifi and Glasgow donors. Groups were compared using a paired Wilcoxon test. * p value of ≤ 0.05.

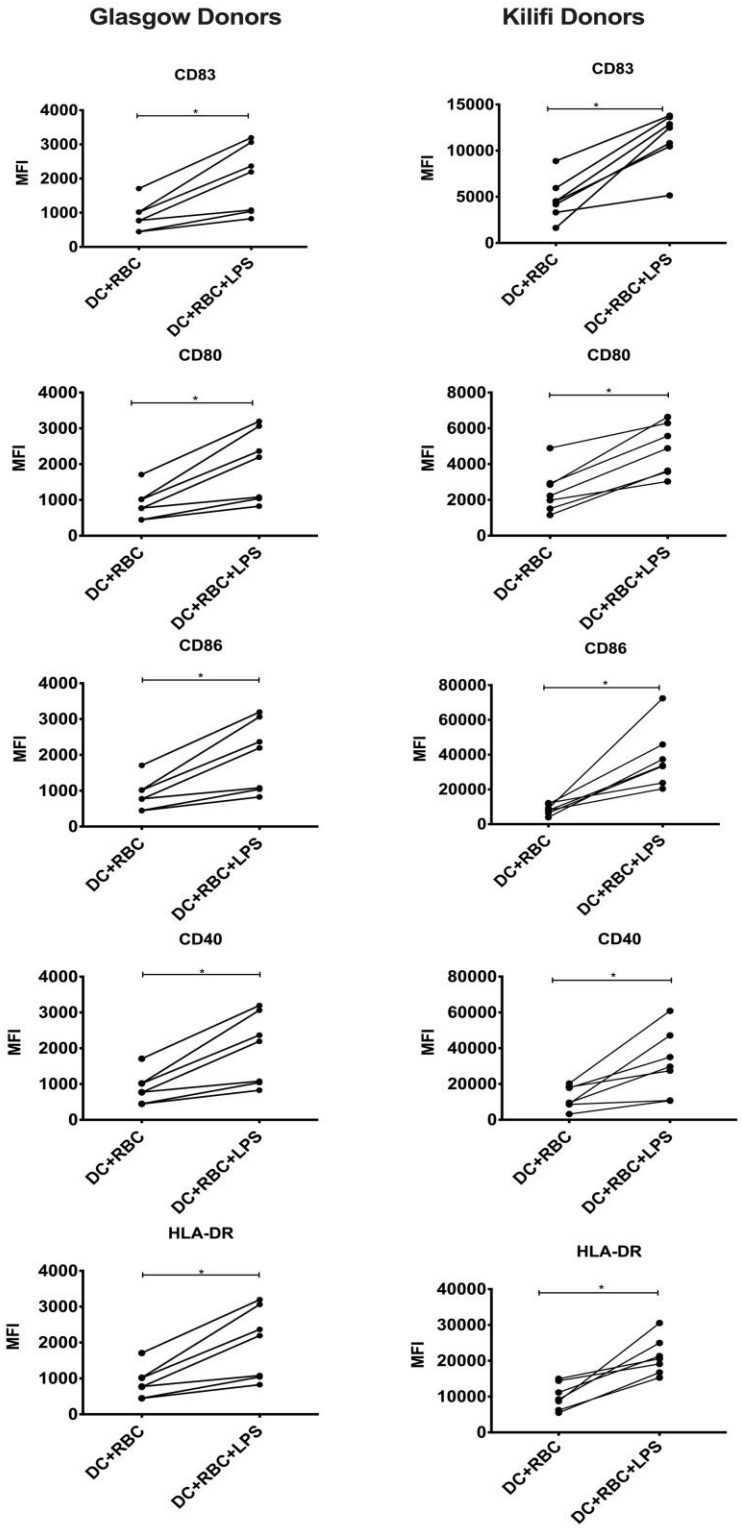


Figure 3-6 Comparison of moDC exposed to RBC response to LPS.

Unstimulated and LPS stimulated moDCs generated from malaria naïve donor (Glasgow Donors) and donors living in a malaria endemic region (Kilifi Donors). Both the Kilifi and Glasgow donors had seven participants recruited to each. Both set of samples responded in a similar manner to LPS stimulation the only difference was the levels of expression between the donors which varied. The graph shows overall analysis of seven donors matched samples for both Kilifi and Glasgow donors. Groups were compared using a paired Wilcoxon test. * p value of ≤ 0.05

3.3.2 Effect of iRBC on DC Activation

After validating the method used to generate moDCs, I investigated the effect of iRBCs on moDCs. Two *P. falciparum* isolates were used for this experiment; ItG and NF54. moDCs generated from malaria naïve donors in Glasgow were exposed to ItG strain of *P. falciparum* and moDCs from donors in Kilifi were exposed to NF54 strain of *P. falciparum*.

Exposure of moDCs from malaria naïve donors to ItG infected RBC (DC+iRBC) did not result in upregulation of the activation markers CD83, CD86, CD40 and HLA-DR (**Figure 3-7, Figure 3-8**) as their expression levels were similar to moDCs which were left in culture medium alone (**Figure 3-8 a, e, g**). moDCs that were exposed to uninfected RBC (DC+RBC) showed a slight reduction in the expression levels of the activation markers CD83, CD86 and CD40 compared with DC alone and DC+iRBC, though the reduction was not statistically significant (**Figure 3-8 a, e, g**).

When exposed to NF54, moDCs generated from donors living in a malaria endemic area showed varying responses (**Figure 3-8**), with the iRBC exposed moDCs showing signs of moderate upregulation of CD86 and CD40 **(Figure 3-8 f, h**), while downregulating CD83 and HLA-DR **(Figure 3-8 b, j**).

The data shows that exposure of moDCs to the ItG strain of *P. falciparum* did not result in the upregulation of activation markers, indicating that DCs from malaria naïve individual fail to respond to PAMPs and CD36 interaction with the parasite. The moDCs that were exposed to NF54 strain of *P. falciparum* showed varied responses to the parasite. While some donors were able to respond to the parasite by upregulating CD86, and CD40 others failed to respond to the parasite. Although overall these changes were not significant, these results suggest moDC isolated from volunteers residing in a malaria endemic region have variable responses to parasites.

3.3.3 Inhibition of LPS Induced Maturation by *Plasmodium falciparum*–infected Red Blood Cells.

After exposing moDCs to iRBC, I investigated if exposure to iRBCs affected moDCs ability to respond to LPS. moDCs from donors living in a malaria endemic area or from malaria naïve donors were exposed to NF54 and ItG strains of *P. falciparum* respectively before being stimulated with LPS. moDCs exposed to ItG prior to LPS stimulation (DC+iRBC+LPS) showed a reduction in the expression levels of CD83, CD80, CD86, CD40 and HLA-DR compared with moDCs exposed to RBC prior to LPS stimulation (DC+RBC+LPS) (**Figure 3-9, Figure 3-10**). Similarly, moDCs exposed to NF54 prior to LPS stimulation (DC+iRBC+LPS) also showed a reduction the expression levels of CD83, CD80, CD40 and HLA-DR when compared to moDCs exposed to RBC prior to LPS stimulation (DC+RBC+LPS) (**Figure 3-11, Figure 3-12**).

While majority of the moDC exposed to iRBC showed a reduction in the expression of the activation markers, some donors, especially in the NF54 exposed moDCs, showed an increase in the activation markers CD86 and CD40. A look at the expression levels of each of the activation marker per individual donor showed that some donors were able to upregulate the markers CD86 and CD40 on stimulation with LPS (**Figure 3-13**). Indicating that the moDCs from some of the donors are able to respond to LPS even after exposure to the parasite, while others are not.

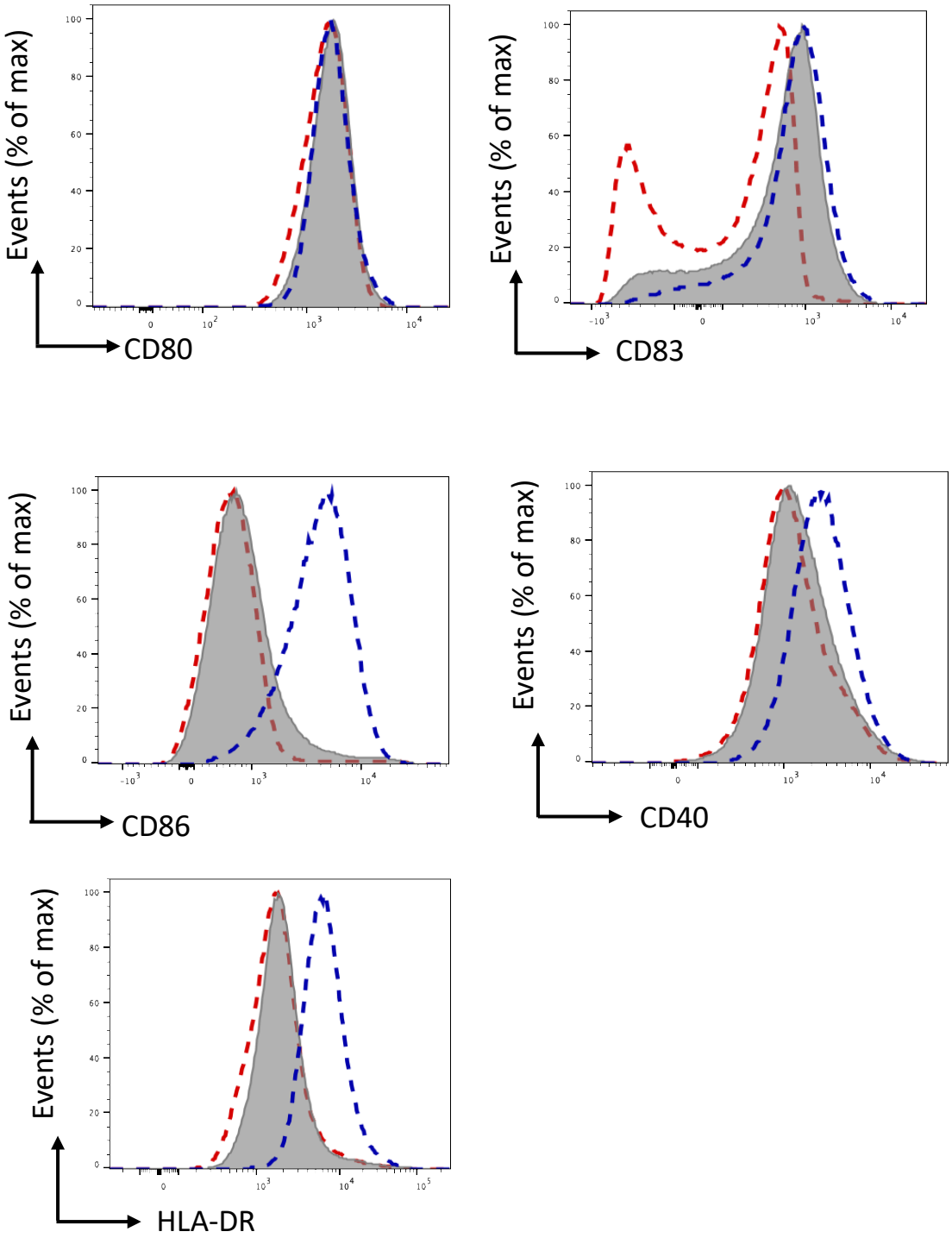


Figure 3-7 moDC response to ItG iRBC.

moDCs from malaria naïve donors were exposed to the trophozoite stage of ItG infected RBC for 20 hours after which the moDCs were accessed for the expression levels of the markers CD80, CD83, CD86, CD40 and HLA-DR. moDCs exposed to iRBC (blue dashed line), were compared to moDC in medium alone (red dashed lined) and moDCs exposed to uninfected RBC (grey shaded histogram).

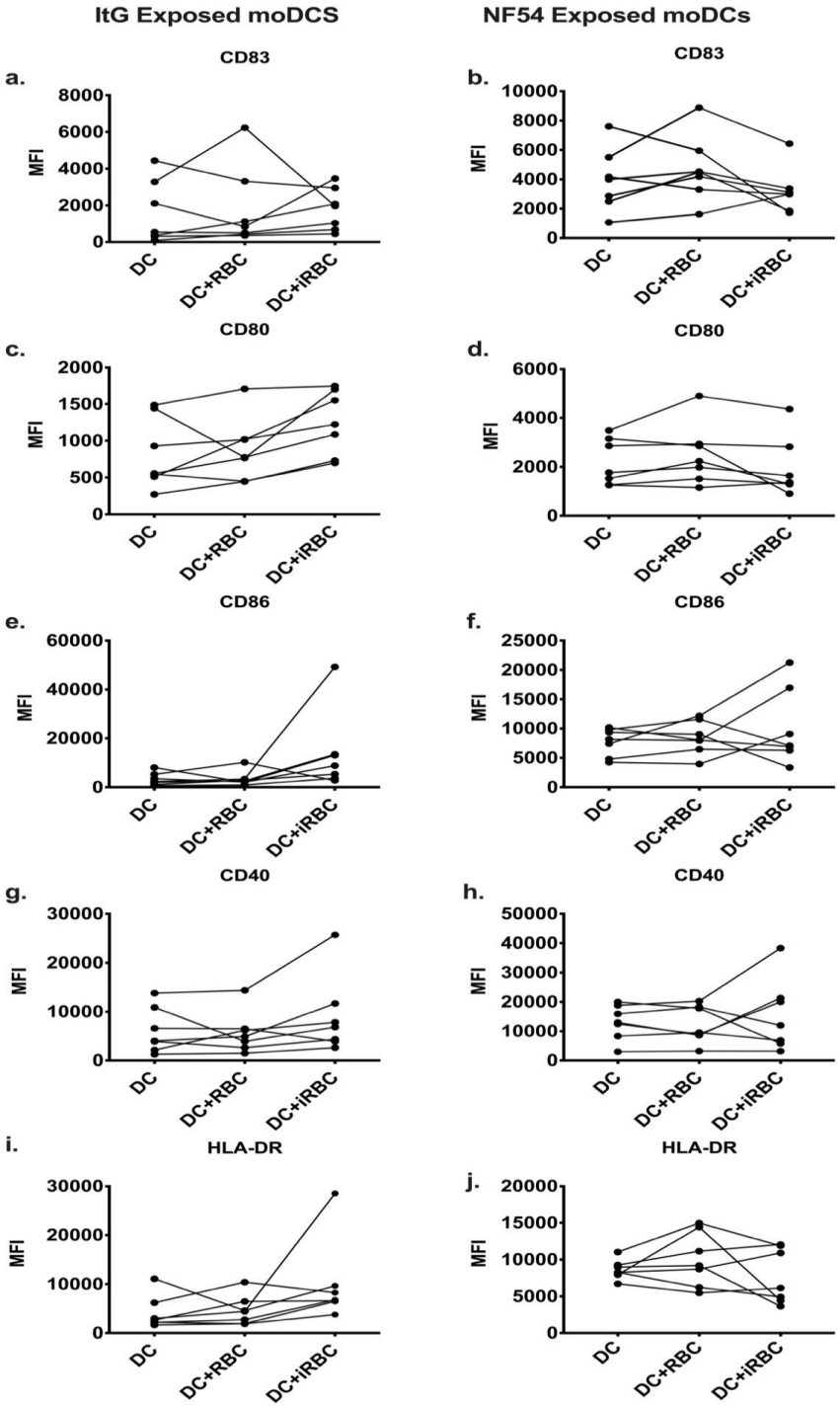


Figure 3-8 Comparison of activation markers on moDCs from malaria naïve donors exposed to ItG and moDCs from donors living in a malaria endemic area exposed to NF54.

moDCs from malaria naïve donors were either left alone in medium (DCs), exposed to ItG iRBC (DC+iRBC) or uninfected RBCs (DC+RBC). The expression levels of the marker CD83 (**a**), CD80 (**c**), CD86 (**e**), CD40 (**g**) and HLA-DR (**i**) were measured by flow cytometry. moDCs from donors living in a malaria endemic region were left alone in medium or exposed to either NF54 iRBC or uninfected RBCs and the expression levels of the marker CD83 (**b**), CD80 (**d**), CD86 (**f**), CD40 (**h**) and HLA-DR (**j**) measured by flow cytometry. DCs in medium (DC) and uninfected RBC treated DCs (DC+RBC) were used as controls. The graph shows overall analysis of seven donor matched samples for both ItG and NF54 exposed moDCs. There was no statistically significant difference between the experimental conditions.

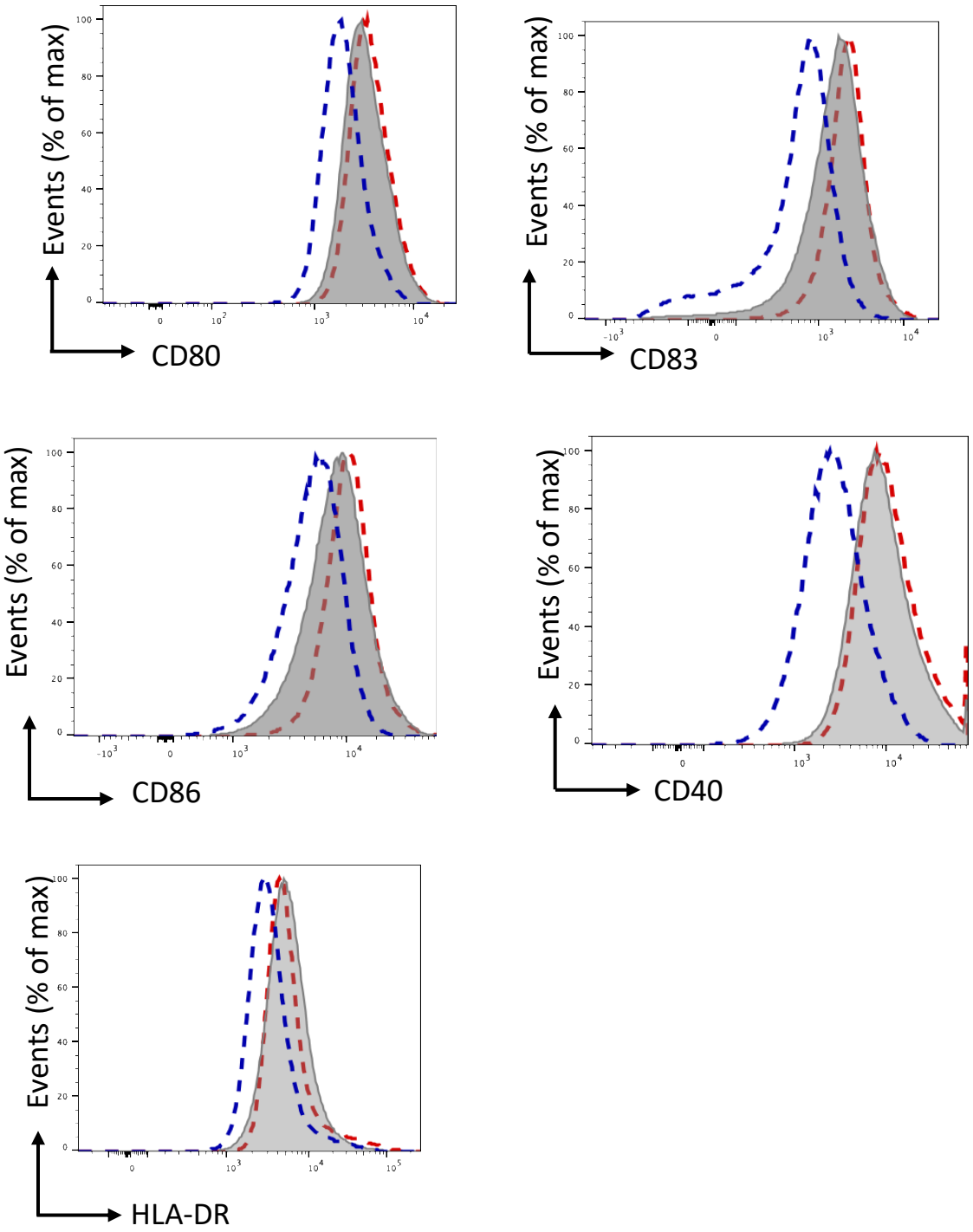


Figure 3-9 LPS stimulation of moDCs pre-exposed to ItG iRBC

moDCs from malaria naïve donors were exposed to the trophozoite stage of ItG infected RBC for 20 hours after which the moDCs were stimulated with LPS for 24 hours and the expression levels of the markers CD80, CD83, CD86, CD40 and HLA-DR were accessed using flow cytometry. moDCs pre-exposed to iRBC prior to LPS stimulation (blue dashed line), were compared to moDC stimulated with LPS (red dashed lined) and moDCs pre-exposed to uninfected RBC prior to LPS stimulation (grey shaded histogram).

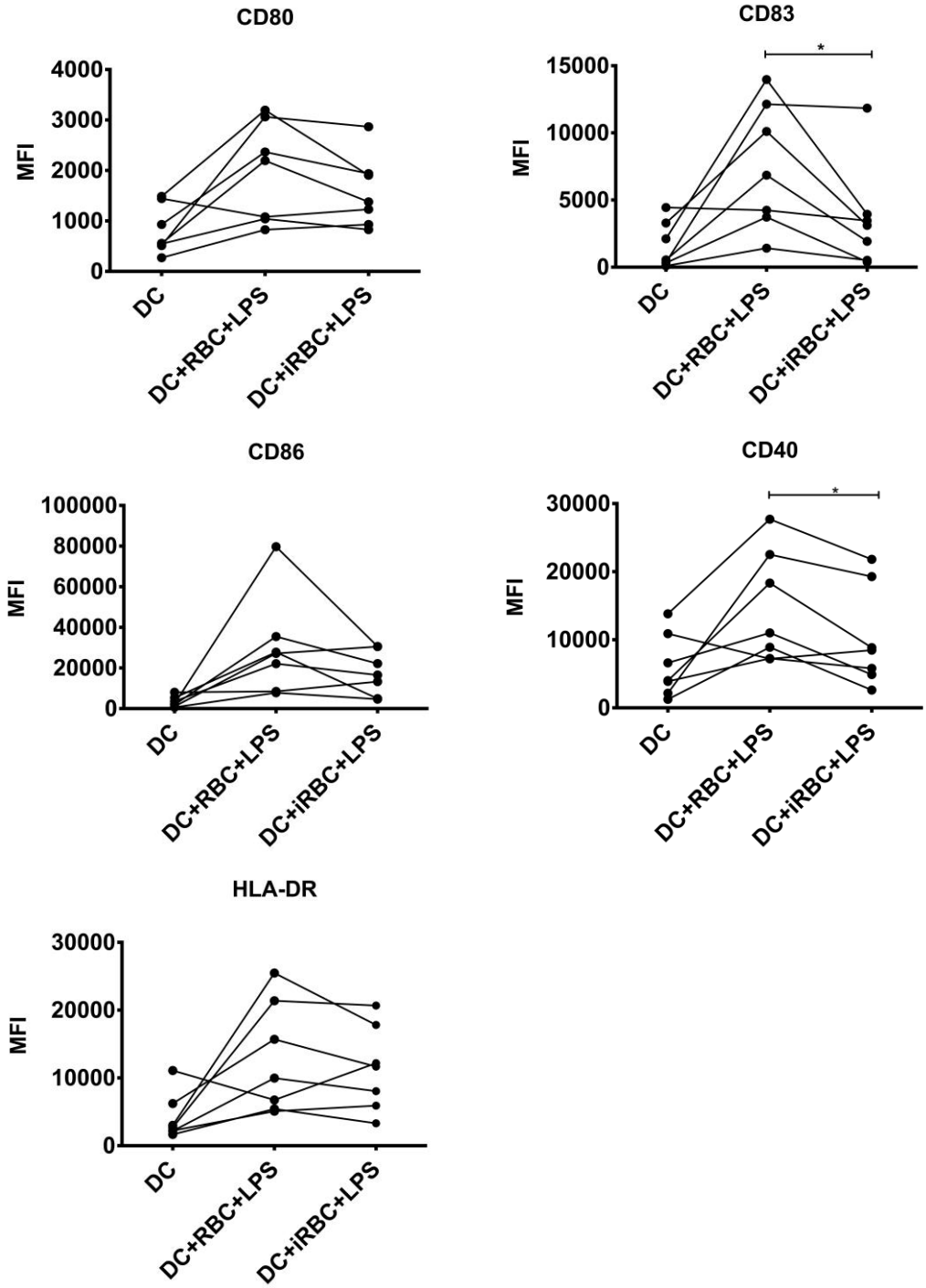


Figure 3-10 LPS stimulation of moDCs pre-exposed to ItG iRBC

moDCs were either left alone (DCs), incubated with uninfected RBCs (DCs+RBCs), or incubated with iRBCs (DC+iRBC) for 20 hours prior to LPS stimulation. The levels of the activation marker CD80, CD83. CD86, CD40 and HLA-DR were accessed by flow cytometry and relative expression of the markers quantified by MFI. The graph shows overall analysis of seven donors matched samples. Groups were compared using a paired Wilcoxon test. * p value of ≤ 0.05

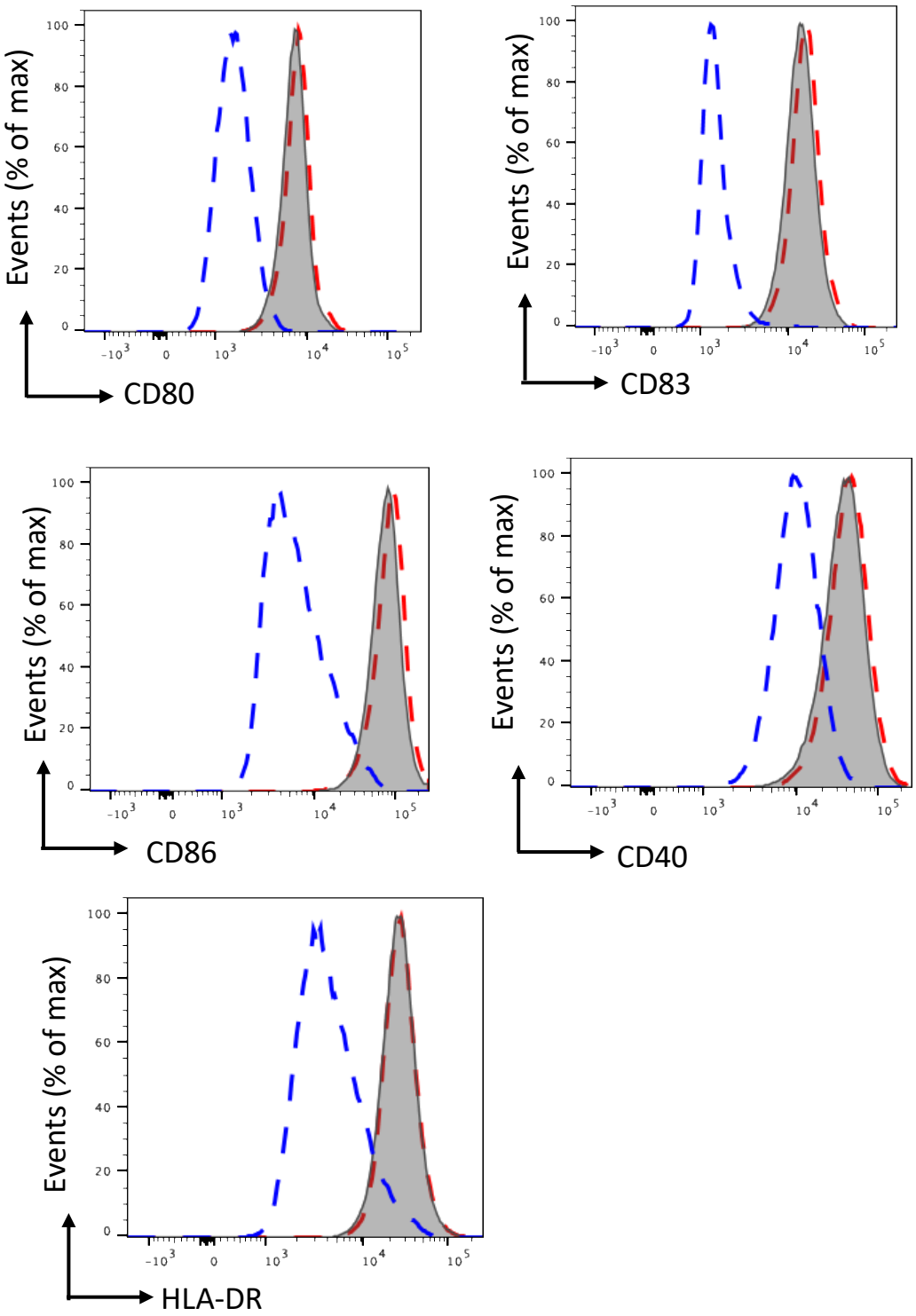


Figure 3-11 LPS stimulation of moDCs pre-exposed to NF54 iRBC.

moDCs from malaria naïve donors were exposed to the trophozoite stage of NF54 infected RBC for 20 hours after which the moDCs were stimulated with LPS for 24 hours and the expression levels of the markers CD80, CD83, CD86, CD40 and HLA-DR were accessed using flow cytometry. moDCs pre-exposed to iRBC prior to LPS stimulation (blue dashed line), were compared to moDC stimulated with LPS (red dashed lined) and moDCs pre-exposed to uninfected RBC prior to LPS stimulation (grey shaded histogram).

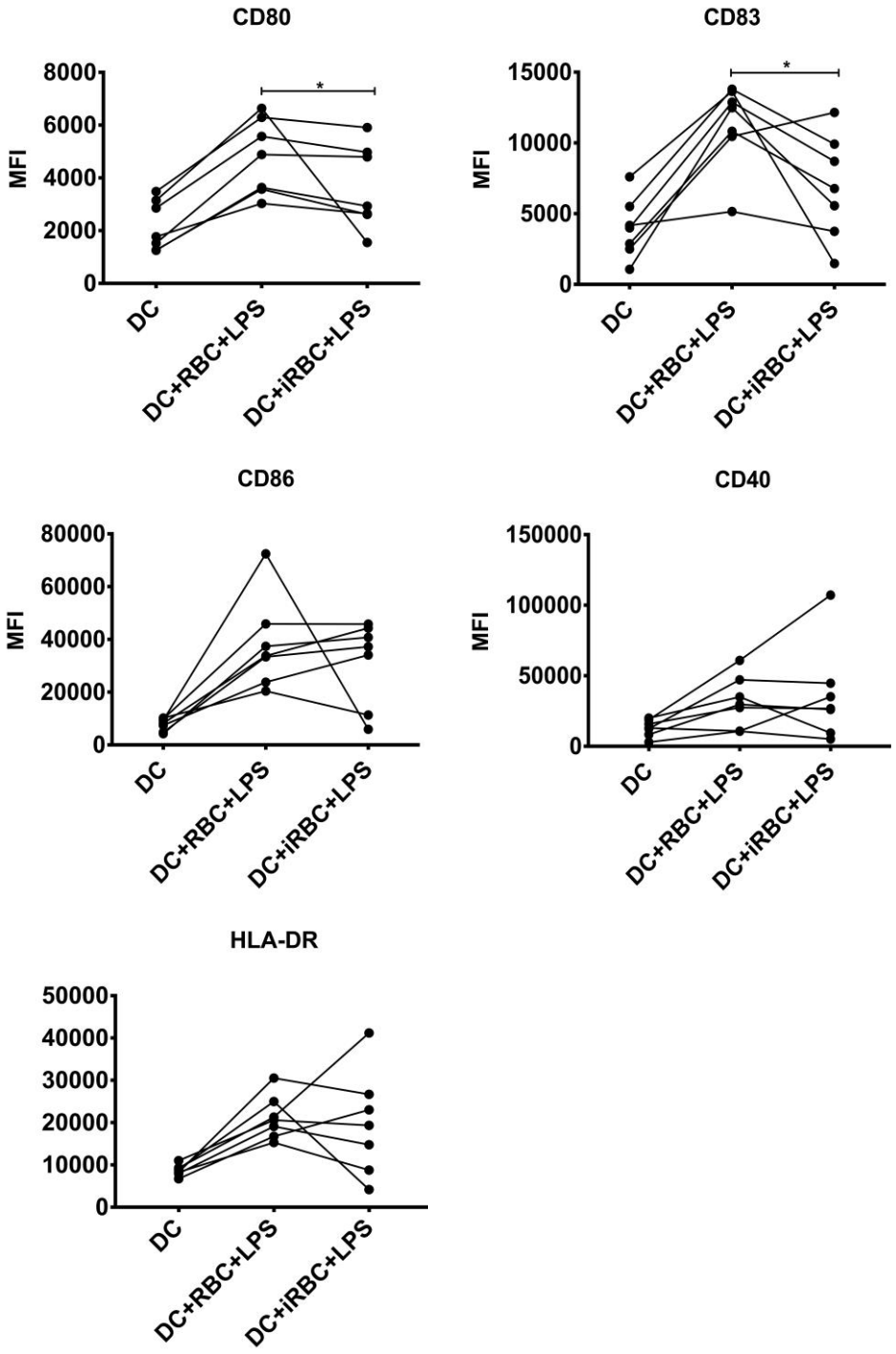


Figure 3-12 LPS stimulation of moDCs pre-exposed to NF54 iRBC

moDCs were either left alone (DCs), incubated with uninfected RBCs (DCs+RBCs), or incubated with iRBCs (DC+iRBC) for 20 hours prior to LPS stimulation. The levels of the activation marker CD80, CD83. CD86, CD40 and HLA-DR were accessed by flow cytometry and relative expression of the markers quantified by MFI. The graph shows overall analysis of seven donors matched samples. Groups were compared using a paired Wilcoxon test. * p value of ≤ 0.05

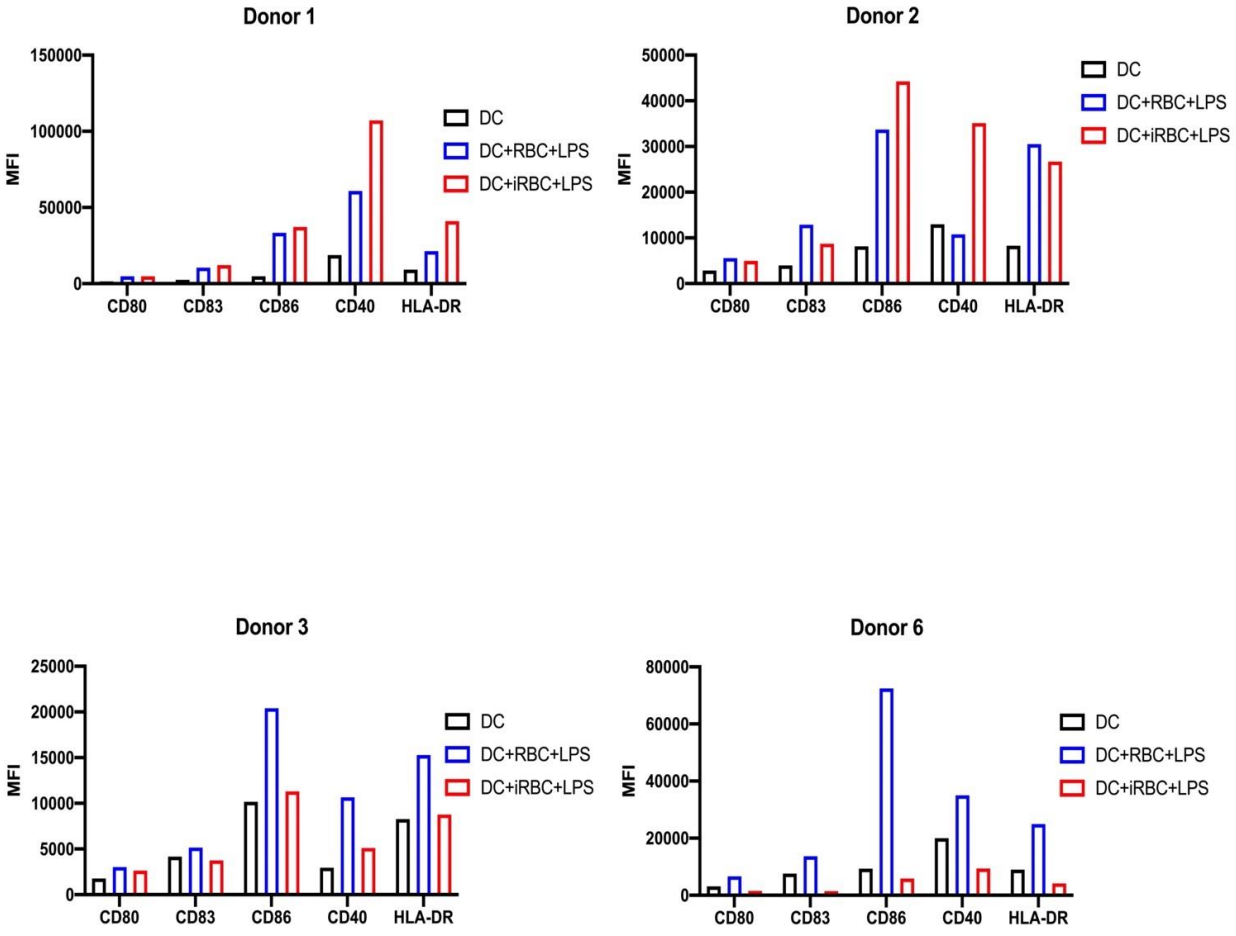


Figure 3-13 LPS stimulation of moDCs pre-exposed to NF54 iRBC

moDCs from each individual donors were assessed to determine the expression levels of the markers CD80, CD83, CD86, CD40 and HLA DR. It was evident that donors 1 and 2 were able to upregulate the markers CD86, and CD40 on LPS stimulation, while donors 3 and 6 downregulated the markers CD80, CD83, CD86, CD40 and HLA DR.

3.3.4 Allogeneic CD4 T Cell Response to DC

Mature DCs are known to activate CD4 T cells leading to their proliferation and differentiation (Sallusto and Lanzavecchia, 2002). The data presented in the previous sections shows that DCs exposed to iRBCs show a reduction in key co-stimulatory molecules and are refractory to LPS stimulation. Here I examined if DCs pre-exposed to iRBC maintain their ability to stimulate allogeneic CD4 T cells and cause them to express CD69 (an early activation marker), CD25 (a late activation marker), PD1 (its expression is induced upon TCR activation and transiently decreases in the absence of TCR signalling), and CD154 (CD40L, a key costimulatory molecule).

DC exposed to either iRBC or RBC were co-cultured with CD4 T cells and the resultant phenotype of the CD4 T cells in the co-culture were analysed using flow cytometry (**Figure 3-14**). I identified 3 distinct CD4 populations: namely naïve CD4 T cells (CD45RA+, CCR7+, CD62L+), central memory T cells (CD45RO+, CCR7+, CD62L+) and effector memory T cells (CD45RO+, CCR7-, CD62L-) (**Figure 3-14**). After identification of the different populations, I then determined the expression levels of the markers PD-1, CD69, CD25, and CD40L in each of the different CD4 populations (**Figure 3-15**).

Naïve CD4 T cells that interacted with moDCs pre-exposed to the iRBC (ItG strain of *P. falciparum*), showed no change in the expression levels of PD-1, CD25 and CD154, but upregulated CD69 compared with CD4 T cells from moDCs that were exposed to uninfected RBC (RBC) (**Figure 3-16**). A similar trend was seen in the central memory CD4 population, with CD69 expression being upregulated by some donors, while PD-1, CD25 and CD154 showed a slight downregulation in expression levels (**Figure 3-16 a**). This shows that the parasite exposed moDCs are capable of moderately upregulating CD69 in both central memory and naïve CD4 T cells but are unable to fully activate the CD4 T cells. This may be due to the low levels of costimulatory molecules expressed on the surface of moDCs.

moDCs pre-exposed to NF54 strain of the parasite prior to co-culture with CD4 T cells (**Figure 3-17**), showed a similar trend in the expression of CD69 as observed with the ItG pre-exposed

moDCs. CD69 was upregulated in all the CD4 T cell populations that is the naïve (**Figure 3-17 c.**), central memory (**Figure 3-17 a.**), and effector memory (**Figure 3-17 b.**). However, PD-1, CD25 and CD154 showed a slight downregulation in expression levels in all the CD4 populations (from the iRBC group) when compared to CD4 T cells from the RBC group.

From the data presented it is evident that moDCs pre-exposed to either ItG or NF54 do induce initial activation of CD4 T cells, evident from an increase in CD69 expression. However, this activation is transient as later activation markers, CD25, and PD-1 levels remain low.

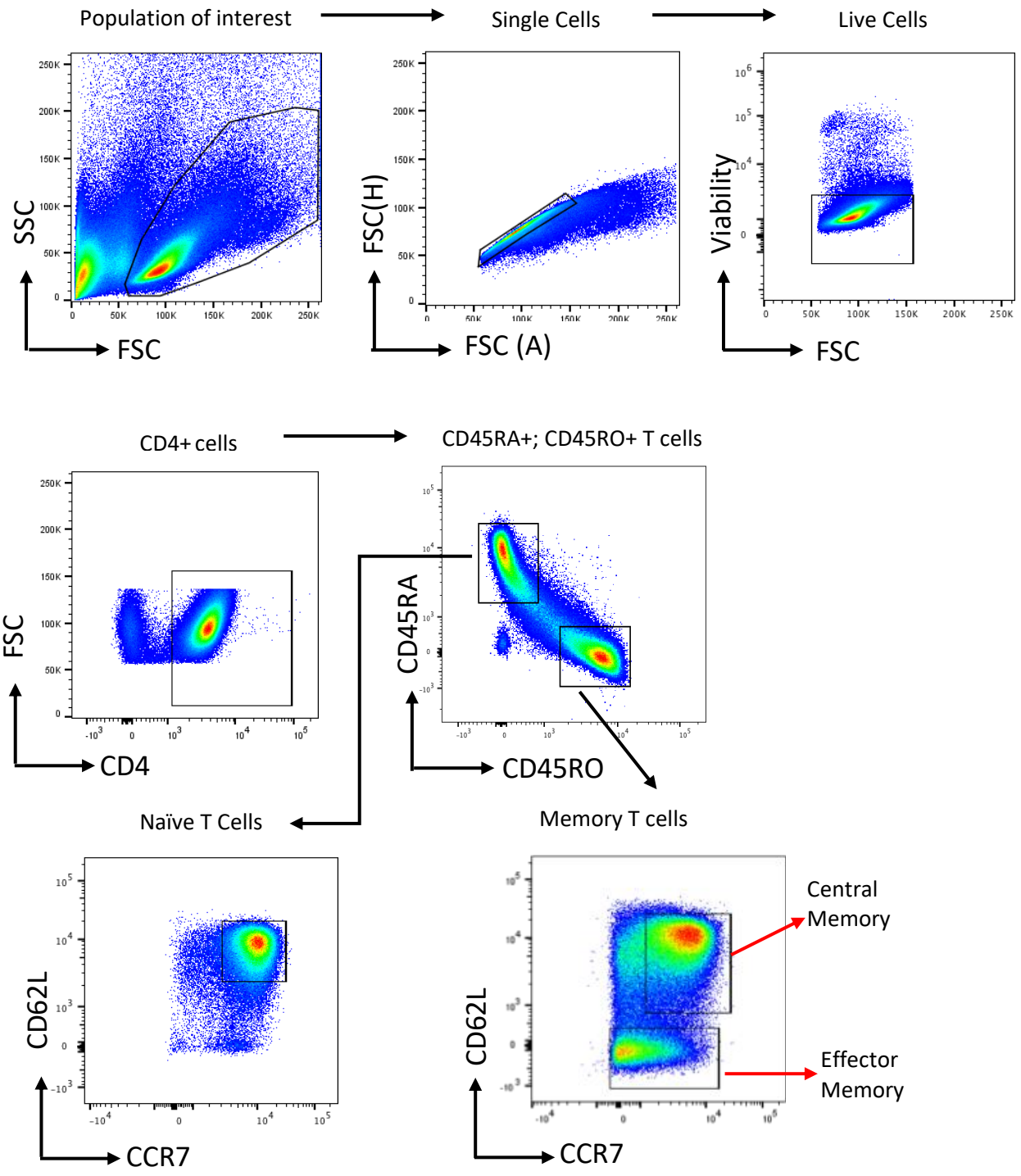


Figure 3-14 Gating strategy for identifying the different CD4 populations

Forward versus side scatter (FSC vs SSC) was used to identify cells of interest based on size and granularity (complexity). Forward scatter height (FSC-H) vs. forward scatter area (FSC-A) density plot was used to exclude doublets**,** and live cells were gated using viability dye (e450) vs FSC. After gating on live cells, CD4 positive cells were identified by plotting FSC vs CD4. The CD4 positive cells were then split by expression of CD45RA and CD45RO. CD45RA positive naïve T cells were identified by the expression of CD62L and CCR7. CD45RO positive cells were split into two population, CD62L+ and CCR7+ central memory T cell: CD62L- and CCR7- effector memory cells

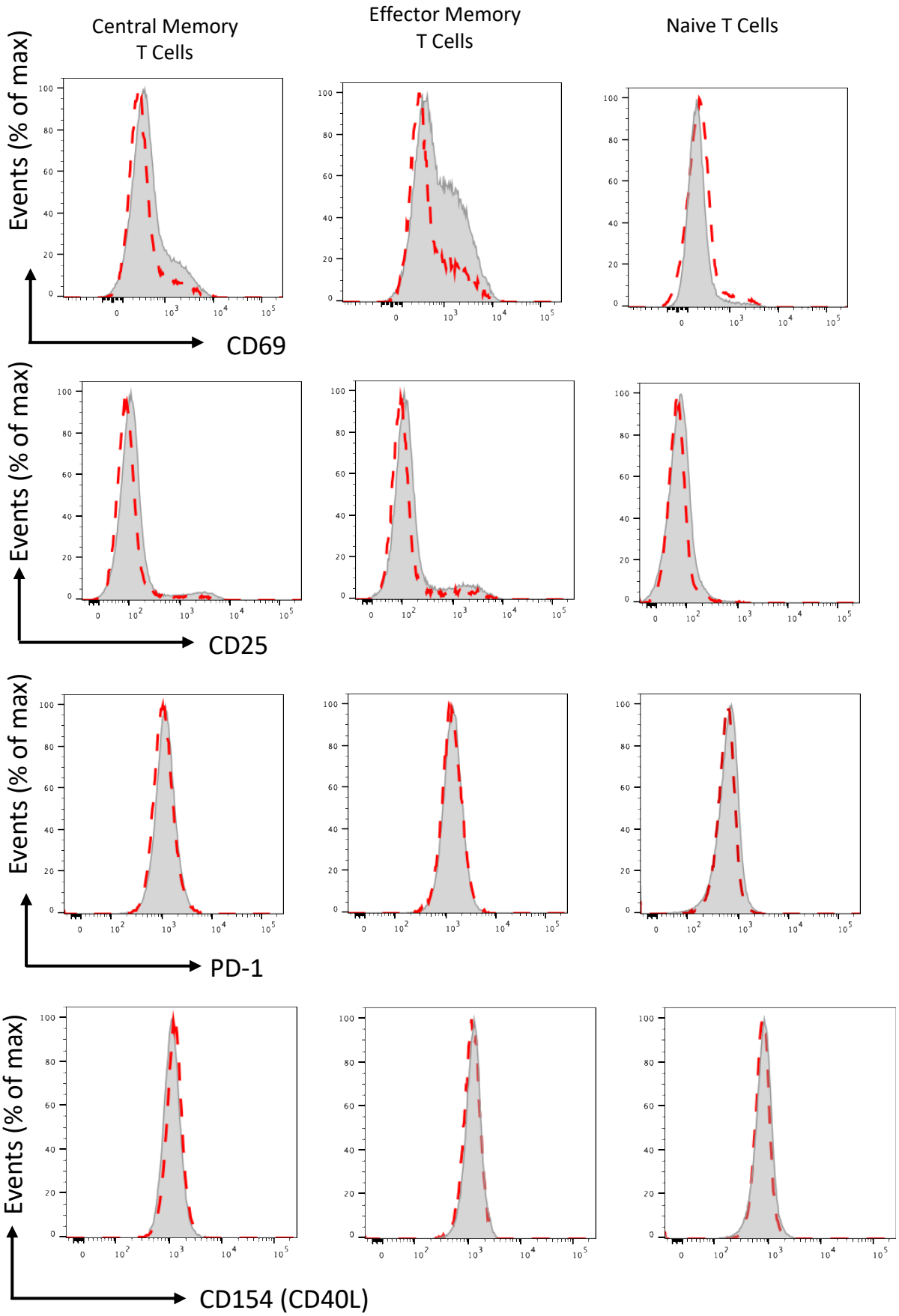

Central Memory T Cells
Effector Memory T Cells
Naive T Cells
Events (% of max)
CD69
Events (% of max)
CD25
Events (% of max)
PD-1
Events (% of max)
CD154 (CD40L)

Figure 3-15 Relative expression of CD4 activation markers in the different CD4 population

The expression levels of PD-1, CD69, CD25 and CD154 were measured in all the three identified CD4 population. The grey shaded histogram is of CD4 T cells interacting with moDCs that were pre-exposed to uninfected RBC and the red dashed histogram represent CD4 T cells that had interacted with moDCs that were pre-exposed to iRBCs.

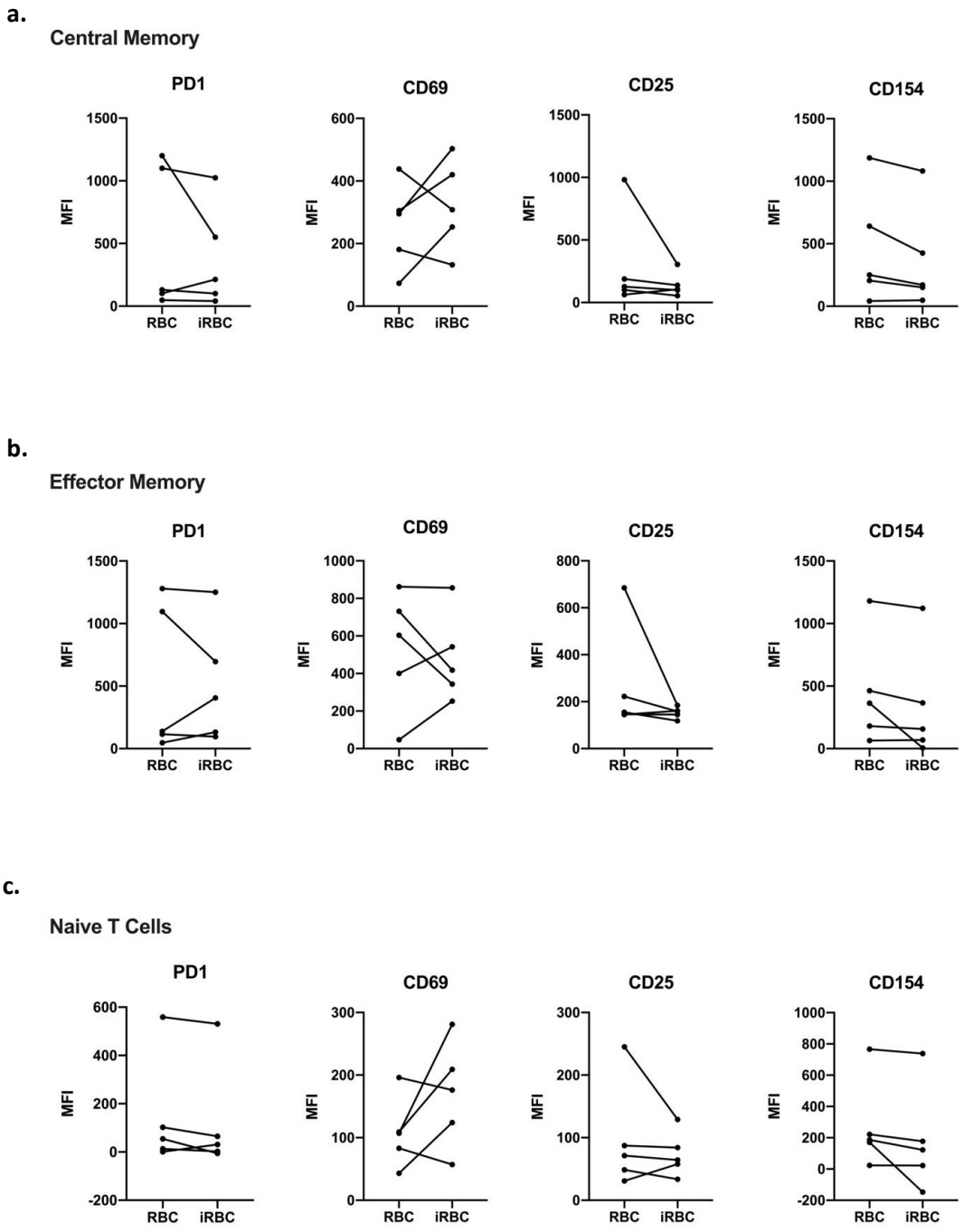


Figure 3-16 Relative expression of activation markers on CD4 T cells in malaria naïve donors

After identification of the different CD4 T cell population (**Figure 3-12**), the expression levels of the activation markers PD-1, CD69, CD25 and CD154 were measured in all the three identified CD4 populations. The graph shows analysis of five donor matched samples. There was no statistically significant difference between the experimental conditions.

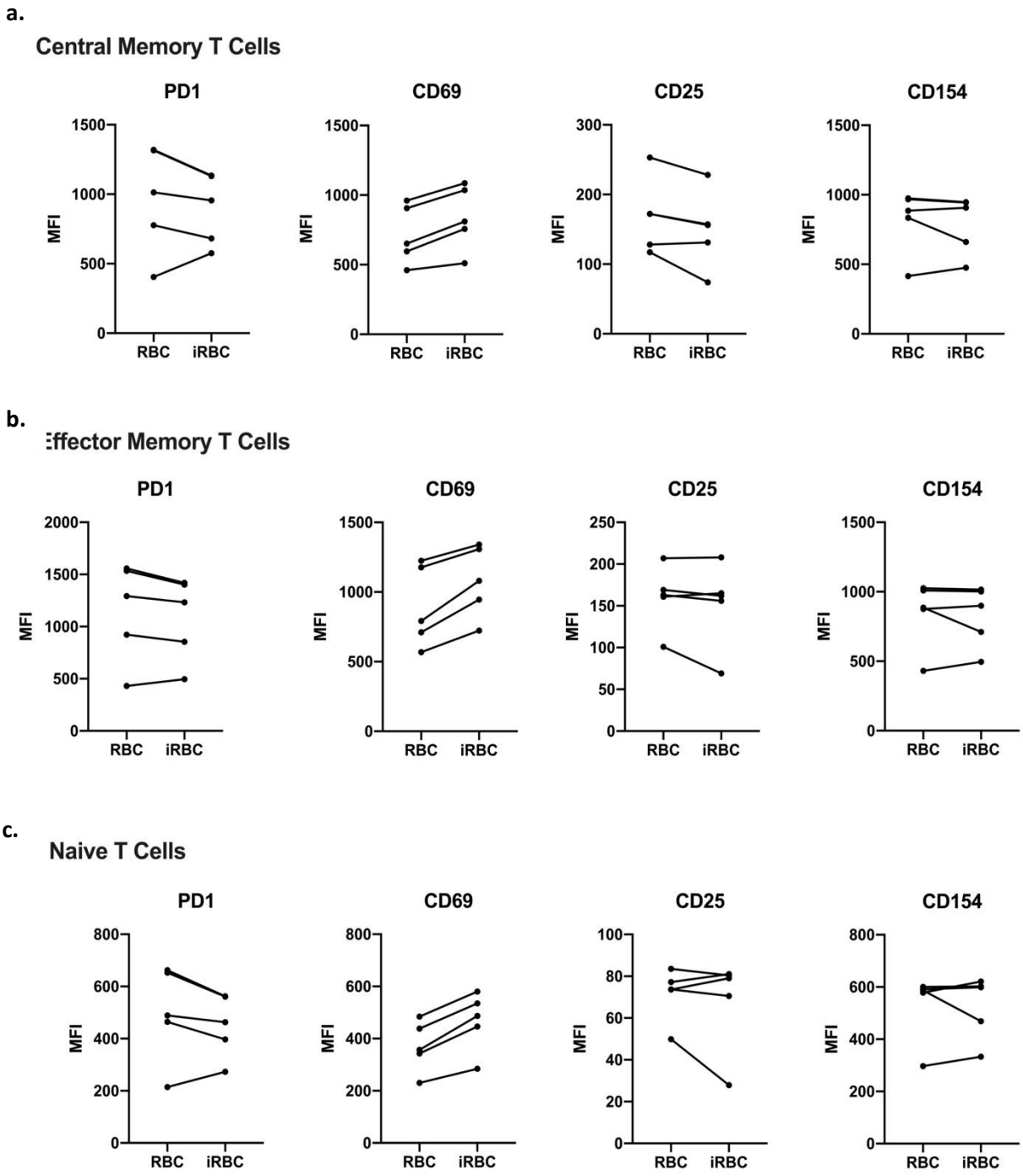


Figure 3-17 Relative expression of activation markers on CD4 T cells from donors residing in a malaria endemic region

After identification of the different CD4 T cell population (**Figure 3-12**), the expression levels of the activation markers PD-1, CD69, CD25 and CD154 were measured in all the three identified CD4 population. The graph shows analysis of five donor matched samples. There were no statistically significant differences between the experimental conditions.

3.4 Discussion

Previous studies have demonstrated that iRBCs can interact with dendritic cells through binding of PfEMP1 (which is expressed on the surface of iRBC) to the CD36 scavenger molecule on DC (Urban et al., 1999, Urban et al., 2001b). In this chapter I first investigated the ability of the trophozoite stage of the parasite, which is known to express PfEMP1 on its surface, to activate moDCs. From the data presented it was clear the parasite infected RBC were unable to activate moDCs from malaria naïve donors, consistent with previous studies by Giusti et al. (2011) and Gotz et al. (2017), despite the two previous studies using the parasite at the schizont stage. This observation was not seen in some of the donors living in a malaria endemic area when their moDCs were exposed to parasite infected RBC, as some donors were able to upregulate CD86, and CD40.

Binding of PfEMP1 onto the CD36 scavenger molecule on dendritic cells has been shown to initiate phagocytosis of iRBC. This CD36 mediated phagocytosis does not induce inflammatory cytokine secretion (Erdman et al., 2009, McGilvray et al., 2000). My data showed that LPS stimulation of moDC pe-exposed to iRBC resulted in a downregulation of costimulatory molecules, consistent with a previous study by Urban et al. (1999). Elliott et al. (2007) showed that inhibition of moDC response to LPS occurred at a high ratio of iRBC to moDCs, 100:1, and that a lower ratio of 10:1, induced a modest moDCs activation. In my experiments I used the higher of iRBC to moDCs (100:1) and found similar observations to Elliott et al. (2007), in that at a high ratio iRBC there was an inhibition of LPS response. A study by Mukherjee and Chauhan (2008), used a ratio of 25 iRBC per moDCs and this resulted in the upregulation of HLA-DR, CD40, CD80, and CD83 and an increase in the secretion of the cytokines of TNF-α, IL-6, and IL-10 when moDCs were stimulated with soluble CD40L (Mukherjee and Chauhan, 2008). Gotz et al. (2017) used a ratio of 3 iRBC per moDC and found that the low concentration inhibited moDCs upregulation of cytokines and costimulatory molecules. The varying results could be attributed to the use of the parasite at different stages of its intraerythrocytic life cycle and the different methodologies used in generation of moDCs and co-culture techniques. The dose dependent relationship between iRBC and moDCs may be physiologically relevant in the spleen during a malaria infection. The spleen is able to trap a large number of iRBC leading to

their accumulation within the red pulp causing an expansion of the red pulp, temporary loss of the marginal zone (Beattie et al., 2006) and disorganisation of the B and T cell zones in the white pulp (Urban et al., 2005, Achtman et al., 2003). The structural changes to the spleen impact the immune response as DCs in the white pulp are exposed to high levels of iRBCs, which affects DC function and this alters T cells function, rendering them unable to migrate to the B cell zone to provide help to B cells, this impedes the formation of germinal centres for B cell activation and maturation. Thus, impairing the development of immunity (Achtman et al., 2003, Urban et al., 2005, Keitany et al., 2016).

CD36-PfEMP1 interaction is thought to play a role in inhibiting LPS response of moDCs (Urban et al., 1999, Urban et al., 2001b). Co-incubation of moDCs with a parasite strain that binds to CSA, or PfEMP1 deficient parasites were shown to inhibit LPS-induced DC maturation and cytokine production (Elliott et al., 2007). However, CD36-PfEMP1 interactions are vital in controlling parasitaemia during the early stage of *P. falciparum* infection, but as the infection progresses, and parasite burden increases other scavenger receptor take over the role of CD36 in initiating phagocytosis and controlling parasitaemia (Gowda et al., 2013). The interaction between PfEMP1 with CD36 does not induce the production of cytokines, but CD36 may play a passive role by presenting PAMPs to TLR ligands (Gowda et al., 2013). CD36 can associate with TLR2 (Triantafilou et al., 2006) and the coordinated efforts of CD36 and TLR2 could be responsible for production of pro-inflammatory cytokines (Erdman et al., 2009). TLR2 can also be recruited to the phagosome where it senses for DAMPs or PAMPs. For example, CD36 mediated phagocytosis of *Staphylococcus aureus* results in translocation of TLR2 to the phagosome where it senses the bacteria's lipoteichoic acid and initiates a cytokine response (Ozinsky et al., 2000, Underhill et al., 1999, Stuart et al., 2005). In mice infected with *P. chabaudi,* DCs were moderately activated at day 4 of infection, but as the infection progressed DC did not upregulate costimulatory molecules and were refractory to stimulation with LPS or CD40L (Millington et al., 2006). This supports the idea that during the early during infection with malaria, DCs are partially activated but as infection progresses and parasite burden increases, DCs become dysfunctional, rendering them refractory to either TLR or CD40L stimulation. This indicates that a common/shared pathway used by costimulatory molecules to initiate cytokine secretion is hampered by the malaria when phagocytosed by DC.

After ascertaining that exposure of moDCs to parasite infected RBC resulted in the downregulation of costimulatory molecules, I then investigated the ability of the iRBC exposed moDCs to stimulate allogenic CD4 T cells. The data presented in this chapter showed that there was a slight increase in CD69 expression in the iRBC exposed moDCs though it was not statistically significant, while the other markers of T cell activation were slightly downregulated. This observation was similar to previous work by Elliott et al. (2007) and (Urban et al., 1999) as they found that moDCs exposed to iRBC were poor stimulators of allogeneic responses, despite stimulating their iRBC exposed moDCs with LPS. The downregulation of MHC class II could have contributed to this phenomenon as in the allogeneic response, the TCR may not receive adequate stimulation for activation.

Collectively the data presented in this chapter show that iRBC are capable of downregulating costimulatory molecule expression on moDCs, and LPS stimulation of these moDCs does not cause an upregulation of the costimulatory molecules. This altered moDC phenotype is unable to adequately stimulate both naïve and memory T cells, this can lead to generation of regulatory T cells (Kho et al., 2016) or result in a failure of T cell activation leading to T cell anergy (Alves et al., 2015). This dysregulation of immune cells favours the parasite as it is able to avoid elimination by the host immune system, enhancing its chances of survival. Apart from interacting with DCs, other parasite products also modulate DC function such as hemozoin which impairs DC-T cell clustering (Millington et al., 2006, Millington et al., 2007) and uric acid (Gallego-Delgado et al., 2014, van de Hoef et al., 2013) all which have an effect on DC function.

Hemozoin, is a by-product of heme detoxification that accumulates in the food vacuoles of *Plasmodium* parasite (Arese and Schwarzer, 1997) and is released into the environment when merozoites egress from the mature schizont. Hemozoin on its own is unable to illicit an immune response but when released during merozoite egress it is contaminated with parasite DNA which enables it to be recognized by the intracellular TLR9 (Parroche et al., 2007). Hemozoin can also bind to host fibrinogen and activate DCs via TLR4 (Barrera et al., 2011), but at a suboptimal level when compared to LPS activated DCs and hemozoin treated DCs failed to respond to LPS

stimulation (Millington et al., 2006, Millington et al., 2007, Giusti et al., 2011, Bujila et al., 2016). This shows that hemozoin can partially activate DCs as well as modulate their function.

Uric acid is another parasite by-product that has also been shown to have inflammatory properties. Uric acid is a DAMP released by injured or dying cells that initiates a cell death-induced inflammatory response *in vivo* (Kono et al., 2010, Johnson et al., 2011). During the intra-erythrocytic cycle, the parasite accumulates uric acid precipitates in the cytosol and is released into the environment (van de Hoef et al., 2013). The released uric acid has been shown to upregulate the expression of co-stimulatory molecules CD80, CD86 and the integrin CD11c while downregulating HLA-DRA (van de Hoef et al., 2013). Uric acid therefore plays a key role in triggering an inflammatory response and activating DCs.

A better understanding of the molecular events that lead the dysregulation of DC signalling pathways is needed to identify possible ways of restoring their capacity to activate naïve T cells. In work presented in chapter 4, moDC-CD4 T cell co-cultures from malaria naïve donors were sent to Glasgow Polyomics for sequencing to shed some light on the transcriptional pathways involved in the dysregulation of DC and T cell function, upon iRBC-DC interaction. Subsequently (Chapter 5), I will describe the molecular pathways activated in semi-immune adults with varying levels of immunity, upon controlled human malaria infection, and discuss how they relate to the *in vitro* data presented here and DC function. This will provide a better insight onto how the parasite modulates DCs function, the pathways that are active and how natural immunity to malaria develops.

Chapter 4 Investigating the Effects of iRBCs on moDCs-CD4 T cell Interaction and Function

4.1 Introduction

DC play an essential role in the activation of CD4 T cells. Furthermore, costimulatory signals provided by the DCs and the cytokine milieu in the environment control T cell differentiation into different subsets (Luckheeram et al., 2012). The combination of these signals results in the differentiation of naïve CD4 T cells into different phenotypes like Th1, Th2, Th17, Tfh, Tr1, and Treg, where each subset has a specific effector function dependent on the inducing DC phenotype (Luckheeram et al., 2012).

CD4 T follicular helper cells (Tfh) are crucial in generation of humoral immunity, as they promote the development of memory and class-switched, affinity-matured antibody-producing B cells (Vinuesa et al., 2016). Tfh cell differentiation is a multistep, progressive developmental process, that is shaped by various spatiotemporal regulated extracellular cues within secondary lymphoid (Qi, 2016, Weinstein et al., 2016, Liu et al., 2012). The differentiation of Tfh results in generation of different subsets which include Th1-Tfh (CXCR3+, CCR6-), Th2-Tfh (CXCR3-, CCR6-), and Th17-Tfh (CXCR3-, CCR6+) (Morita et al., 2011). In humans, malaria has been shown to skew the development of Tfh towards a Th1-Tfh phenotype, which has a reduced capacity to activate both naïve and memory B cells as seen in *P. falciparum* infected Malian children (Obeng-Adjei et al., 2015), and *P. vivax* infected Brazilian adults (Figueiredo et al., 2017). This Th1-Tfh phenotype is also associated with an increase in the expression of Tbet, a transcription factor that is highly expressed in atypical memory B cells (Obeng-Adjei et al., 2017). In mice, *P. chabaudi* AS infection resulted in activation of CD4 T cells and bifurcation of the cells into Tfh and Th1 cell linages (Lonnberg et al., 2017). Tfh development and function in mice requires IL-6 (Sebina et al., 2017), and ICOS (Wikenheiser et al., 2016), but ICOS driven Tfh responses are hampered by type I interferon signalling (Sebina et al., 2016) and negatively regulated by interferon regulatory factor 3 (IRF3) (James et al., 2018).

Apart from Tfh cells, Th1 cells are thought to be critical in controlling the blood stage of *Plasmodium* infection though direct evidence of this is lacking. Th1 cells are thought to be the main source of interferon gamma (IFNγ) during a *Plasmodium* infection, but other cells have been shown to be sources of IFNγ, such as CD8+ T cells (Ong'echa et al., 2003), NK cells (Artavanis-Tsakonas et al., 2003), and γδ T cells (Hviid et al., 2001). IFNγ is a pro-inflammatory cytokine that has been shown to have antiparasitic effect. The presence of IFNγ producing γδ T cells have been associated with protection against the blood stage of malaria infection (D'Ombrain et al., 2008). Mice treated with IFNγ neutralizing antibodies failed to control the parasitaemia of both lethal (Yoneto et al., 1999) and non-lethal (Stevenson et al., 1990) forms of *Plasmodium.* IFNγ deficient mice are unable to control the parasitaemia when infected with non-lethal *Plasmodium* species and these mice also produced less parasite-specific IgM, IgG2a, and IgG3 when compared to wild type control mice (Su and Stevenson, 2000). Blocking IFNγ in immune mice also resulted in increased parasitaemia following a second challenge (da Silva et al., 2013), These Th1 responses in mice are suppressed by type I IFN signalling and its transcription factor interferon regulatory factor 7 (Haque et al., 2011, Haque et al., 2014, Edwards et al., 2015). In humans naturally exposed to *Plasmodium* and those experimentally infected with *Plasmodium*, IFNγ producing CD4 Th1 cells coincided with a reduction in parasitaemia and disease severity (Reece et al., 2004), though the exact immune mechanism of control was not detailed. Malaria naive volunteers, enrolled in CHMI, under chloroquine chemoprophylaxis prior to inoculation with *P. falciparum* sporozoites, showed a reduction in parasite burden. This was associated with CD4+ T cells exhibiting characteristics of cytotoxic Th1-like cells (Bijker et al., 2014). While Th1 cells have been described in mice and CHMI, they are yet to be seen in individuals who have been naturally exposed to malaria and their exact mechanism and role in controlling blood stage or liver stage *Plasmodium* is yet to be well examined.

The anti-parasitic effect of Th1 cells and their cytokines are important in controlling malaria but if not kept in check, the activity of Th1 cells and their cytokines can lead to disease progression and development of severe malaria syndromes. Th1 cytokines, namely IFNγ and TNF, stimulate vascular endothelial cells to express integrins and adhesion molecules causing iRBCs to sequester and accumulate on them. IFNγ and TNF also interfere with the differentiation and

development of Tfh cells by stimulating the expression of Tbet and upregulation of the chemokine receptor CXCR3, leading to the development of Th1 like Tfh cells (Ryg-Cornejo et al., 2016). In mice infected with non-lethal *Plasmodium*, IFNγ promoted the depletion of antigen specific CD4 T cells (Xu et al., 2002, Villegas-Mendez et al., 2011). This shows that Th1 cells in malaria can contribute to controlling malaria but if left unchecked can also contribute the progression of the disease.

CD4 T regulatory cells (Tregs) are key in maintaining a balance during an inflammatory response by repressing inflammatory signals thereby modulating immune-mediated pathology. Tregs express a number of inhibitory receptors including the cytotoxic T lymphocyte–associated molecule 4 (CTLA-4) (Salomon et al., 2000) and the glucocorticoid-inducible tumour necrosis factor receptor (GITR) (McHugh et al., 2002) and produce a number of anti-inflammatory cytokines such as TGF-β (Nakamura et al., 2001) and IL-10 (Asseman et al., 1999). These immunomodulatory cytokines and receptors supress the function of a number of cells in a bid dampen inflammation and to restore immune homeostasis. In malaria naïve adults undergoing CHMI, *P. falciparum* infections resulted in an increase in the Treg population, and this was directly associated with an increase in *P. falciparum* parasite load (Walther et al., 2005, Minigo et al., 2009). This association is further supported by the observation that Treg levels transiently increase during acute malaria infection and decrease following anti-malarial drug treatment (Minigo et al., 2009). In mice infected with *P. yoelii*, a similar observation was seen with T regs increasing transiently early on during infection but did not correlate with parasitaemia (Hisaeda et al., 2005) as Tregs numbers decreased before peak parasitaemia in both lethal and non-lethal *P. yoelii* infections. In *P. berghei* ANKA infected mice, Treg levels are high before parasitaemia is detectable and decrease as parasitaemia increases (Long et al., 2003). As discussed above Th1 cells are important in controlling parasitaemia but can also contribute to immunopathology if left unchecked. Tregs inhibit the protective and pathogenic roles of Th1 cells as seen in cerebral malaria resistant BALB/c mice, which have higher number of Tregs compared to C57BL/6 mice, both in the naïve state (Chen et al., 2005) and during acute infection (Griffith et al., 2007). Treg depletion in *P. berghei* ANKA-infected mice resulted in an increase in cerebral malaria scores (Nie et al., 2007).

Induction of naïve CD4 T cells to Tregs during a malaria infection is dependent on the availability of the cytokines IL-10 and transforming growth factor beta (TGF-β) (Levings et al., 2002, Shevach et al., 2008). These cytokine also enhance Treg mediated suppression and decrease disease severity during a malaria infection. In C57BL/6 mice infected with *P. yoelii* 17XL-infected showed an early upregulation of TGF-β but failed to control parasitaemia as it inhibited Th1 responses. Inhibition of IL-10 and TGF-β increased the survival of the mice (Omer et al., 2003). BALB/c mice infected with lethal *P. berghei* had lower levels of TGF-β between day 8 and day 20 post infection compared to baseline levels, but were higher in resolving infections of *P. chabaudi* or *P.yoelii* (Omer and Riley, 1998b). This shows that these immunomodulatory cytokines can be either protective, or pathogenic depending on the timing of their induction.

4.2 Chapter aims

In Chapter 3, I demonstrated a reduction in the expression levels of key costimulatory molecules in moDCs pre-exposed to iRBC, resulting in the inability of the DC to adequately stimulate CD4 T cells. In this chapter, moDC pre-exposed to either iRBCs or RBC were co-incubated within a mixed lymphocyte reaction with CD4 T cells to examine the effects of iRBC on T cell function, at the gene expression level. The influence of iRBC on the signalling pathways and genes that are expressed because of this interaction were assessed by single cell RNA sequencing.

4.3 Methods

4.3.1 Sample Preparation

moDC's exposed to either *P. falciparum* infected RBCs or uninfected RBCs and later co-cultured with allogenic CD4 T cell as described on Chapter 2 (section 2.3 and section 2.5) were taken to Glasgow Polyomics for sequencing. The two samples were loaded into a 10X chromium 8 channel microfluids chip and sequenced using Ilumina Nextseq. A total of 15000 cells were loaded into the microfluids chip.

4.3.2 Analysis of Data on R

Monocyte derived dendritic cells (moDCs) were exposed to either uninfected RBC or parasite infected RBC as described in Chapter 2 (Section 2.2 and 2.3). The co-cultures were taken for single cell RNA sequencing, which was done by Glasgow Polyomics (as described in Chapter 2), after which the data was pre-processed and a gene count matrix file with the sequenced data generated. The data file was analysed on R statistical software using Seurat (Satija et al., 2015) and NicheNet (Browaeys et al., 2020) pipelines.

4.3.3 Seurat Analysis

To identify the different cell populations that were in the sequenced data, the Seurat package was installed on R and gene count matrix was loaded into R using the code.

```
rbc.data<-Read10X(data.dir=
"TDC.Rowland/Plus/filtered_feature_bc_matrix/GRCh38")
```

After loading the data, I investigated the quality of the data and filtered out low read cells using the code:

```
rbc <- CreateSeuratObject(counts = rbc.data, project = "IMMUNE_CTRL",
min.cells = 5)
rbc$irbc<- "RBC"
rbc <- subset(rbc, subset = nFeature_RNA > 500)
rbc <- NormalizeData(rbc, verbose = FALSE)
rbc <- FindVariableFeatures(rbc, selection.method = "vst", nfeatures = 2000)
```

I then proceeded to identify the cell clusters that were in the sequenced data using the code:

```
DimPlot(immune.combined, reduction = "umap")
```

In order to identify the cell types that were represented by the different clusters, I used the code,

```
Find.markers <- FindConservedMarkers(immune.combined, only.pos = TRUE, ident.1 = 0, grouping.var = "irbc", verbose = FALSE)
```

This enabled me to identify the gene markers that had a similar expression level in both the iRBC and RBC experimental conditions and aided me in identifying the cell types in the different clusters using the list of genes that was generated. After identifying the different cell types and assigning them to their respective cell cluster the following code was used to generate **Figure 4-1 a:**

```
DimPlot(immune.combined, label = FALSE)
```

For determining the cells frequencies per samples, **Figure 4-1 b and c**, I used the code

```
DimPlot(immune.combined, reduction = "umap", split.by = "irbc")
freq_table_sample<-as.data.frame(prop.table(x = table(Idents(immune.combined), immune.combined@meta.data$irbc),margin=2))
ggplot(data=freq_table_sample, aes(x=Var1, y=Freq, fill = Var2)) + geom_bar(stat="identity", color="black", position = position_dodge2(reverse = TRUE)) + labs(x="Cluster", y="Percentage of Total Cells", fill="Sample", title = "Cell Frequencies") +
  scale_x_discrete(limits = levels(freq_table_sample$Var1)) +
  scale_y_continuous(labels = scales::percent) +
  theme(plot.title = element_text(hjust = 0.5))
```

I then proceeded to check the differential expressed genes in the between the iRBC and RBC for each identified cell population samples using the code,

```
moDC1.parasite.response <- FindMarkers(immune.combined, ident.1 = "moDC_1_iRBC", ident.2 = "moDC_1_RBC", test.use = "MAST", verbose = FALSE)
moDC1.parasite.response$genes <- row.names(moDCs1.parasite.response)
```

For visualisation of the differentially expressed genes using volcano plots, I set the fold change (FC) limit to 0.7 and the adjusted p value to 10e-3 and used the code below to generate **Figure 4-2**,

```
FC <- 0.7
p <- 10e-3
res2<-moDC2.parasite.response
keyvals <- rep('grey75', nrow(res2))
names(keyvals) <- rep('NS', nrow(res2))

keyvals[which(abs(res2$avg_log2FC) > FC & res2$p_val_adj > p)] <- 'grey50'
names(keyvals)[which(abs(res2$avg_log2FC) > FC & res2$p_val_adj > p)] <-
'log2FoldChange'

keyvals[which(abs(res2$avg_log2FC) < FC & res2$p_val_adj < p)] <- 'grey25'
names(keyvals)[which(abs(res2$avg_log2FC) < FC & res2$p_val_adj < p)] <- '-
Log10Q'

keyvals[which(res2$avg_log2FC < -FC & res2$p_val_adj< p)] <- 'blue2'
names(keyvals)[which(res2$avg_log2FC < -FC & res2$p_val_adj < p)] <-
'Signif.down-regulated'

keyvals[which(res2$avg_log2FC > FC & res2$p_val_adj < p)] <- 'red2'
names(keyvals)[which(res2$avg_log2FC > FC & res2$p_val_adj < p)] <-
'Signif.up-regulated'

unique(keyvals)
unique(names(keyvals))

EnhancedVolcano(moDC2.parasite.response,
                lab = moDC2.parasite.response[,'genes'],
                x = 'avg_log2FC',
                y = 'p_val_adj',
                xlim = c(-6.5,6.5),
                xlab = bquote(~Log[2]~ 'fold change'),
                ylab = bquote(~-Log[10] ~ italic(P)),
                title = 'Differentially Expressed Genes moDC_2',
                subtitle = NULL,
                pCutoff = 10e-3,
                FCcutoff = 0.7,
                pointSize = 2.5,
                labSize = 4,
                colCustom = keyvals,
                colAlpha = 0.75,
                legendPosition = 'right',
                legendLabSize = 15,
                legendIconSize = 5.0,
                max.overlap = 20,
                drawConnectors = T,
                widthConnectors = 0.5,
                colConnectors = 'grey50',
                gridlines.major = TRUE,
                gridlines.minor = FALSE,
                border = 'partial',
                borderWidth = 1.5,
                borderColour = 'black')
```

I then proceeded to check the expression levels of the key activation markers on DCs and CD4 T cells using violin plots **Figure 4-3**, using the code

```
plots <- VlnPlot(immune.combined, features = c("CD80", "CD83"), split.by =
"irbc", idents = c("moDC_1", "moDC_2"),
                 pt.size = 0, combine = TRUE, ncol=1)
plots
```

4.3.4 NicheNet Analysis

I used NicheNet to be able to infer intercellular communication between moDCs and CD4 T cells and how theses intercellular communication influences gene expression in CD4 T cells. NicheNet uses curated data from cell signalling and gene regulatory networks to infer active ligands and their effect on gene expression. The data sources from which the cell signalling (ligand-receptor interaction and downward signalling) were aggregated into a weighted ligand-signalling network and the data from the gene regulatory networks were also aggregated into a weighted gene regulatory network (Browaeys et al., 2020). These networks were then integrated into a single model to infer regulatory potential scores between ligands and target genes allowing me to study how ligands affect gene expression in interacting cells. To do this NicheNet uses Pearson's correlation for scoring ligand activity. The Pearson correlation is calculated for each ligand separately between 1) vector of ligand-target regulatory potential scores for all genes and 2) vector that indicates for each whether it belongs to the geneset of interest or not. Higher correlation values mean that genes with more evidence of being a target of a ligand are enriched in the geneset of interest.

The NicheNet pipeline analysis can be summarized as follows:

1. Define a "sender/niche" cell population and a "receiver/target" cell population present in your expression data and determine which genes are expressed in both populations
2. Define a gene set of interest: these are the genes in the "receiver/target" cell population that are potentially affected by ligands expressed by interacting cells (e.g. genes differentially expressed upon cell-cell interaction)

3. Define a set of potential ligands: these are ligands that are expressed by the "sender/niche" cell population and bind a (putative) receptor expressed by the "receiver/target" population
4. Perform NicheNet ligand activity analysis: rank the potential ligands based on the presence of their target genes in the gene set of interest (compared to the background set of genes)
5. Infer top-predicted target genes of ligands that are top-ranked in the ligand activity analysis. (Browaeys et al., 2020)

For this analysis, I used the data generated using Seurat (Section 4.3.1.1), RBC experimental condition was used as reference condition and the iRBC experimental condition was the condition of interest. Throughout the analysis the iRBC condition was compared to the RBC condition.

I first checked for the ligand activity in the moDCs populations as they primarily influence the activity of CD4 T cells. The fold change in the ligands was represented by a heatmap in **Figure 4-4 a** which was generated using the code,

```
ligand_pearson_matrix = ligand_activities %>% select(pearson) %>% as.matrix()
%>%magrittr::set_rownames(ligand_activities$test_ligand)

rownames(ligand_pearson_matrix)    =    rownames(ligand_pearson_matrix)    %>%
make.names()
colnames(ligand_pearson_matrix)    =    colnames(ligand_pearson_matrix)    %>%
make.names()

vis_ligand_pearson    =    ligand_pearson_matrix[order_ligands,    ]    %>%
as.matrix(ncol = 1) %>% magrittr::set_colnames("Pearson")
p_ligand_pearson = vis_ligand_pearson %>% make_heatmap_ggplot("Prioritized
ligands","Ligand activity", color = "darkorange",legend_position = "top",
x_axis_position    =    "top",    legend_title    =    "Pearson    correlation
coefficient\ntarget gene prediction ability)") + theme(legend.text =
element_text(size = 9))
p_ligand_pearson
```

I then proceeded to check the expression levels of the active ligands between the two experimental conditions. This was visualised using a heatmap **Figure 4-4 b**, the code used to generate the heatmap was,

```
DE_table_all = Idents(seuratObj) %>% levels() %>% intersect(sender_celltypes)
%>% lapply(get_lfc_celltype, seurat_obj = seuratObj, condition_colname =
"aggregate", condition_oi = condition_oi, condition_reference =
condition_reference, expression_pct = 0.10) %>% reduce(full_join)
DE_table_all[is.na(DE_table_all)] = 0

ligand_activities_de = ligand_activities %>% select(test_ligand, pearson) %>%
rename(ligand = test_ligand) %>% left_join(DE_table_all %>% rename(ligand =
gene))
ligand_activities_de[is.na(ligand_activities_de)] = 0

lfc_matrix = ligand_activities_de %>% select(-ligand, -pearson) %>%
as.matrix() %>% magrittr::set_rownames(ligand_activities_de$ligand)
rownames(lfc_matrix) = rownames(lfc_matrix) %>% make.names()

order_ligands = order_ligands[order_ligands %in% rownames(lfc_matrix)]
vis_ligand_lfc = lfc_matrix[order_ligands,]

colnames(vis_ligand_lfc) = vis_ligand_lfc %>% colnames() %>% make.names()

p_ligand_lfc = vis_ligand_lfc %>%
make_threecolor_heatmap_ggplot("Prioritized ligands","LFC in Sender",
low_color = "midnightblue",mid_color = "white", mid = median(vis_ligand_lfc),
high_color = "red",legend_position = "top", x_axis_position = "top",
legend_title = "LFC") + theme(axis.text.y = element_text(face = "italic"))
p_ligand_lfc
```

To check which genes in CD4 T cells the active ligands identified in moDCs were regulating, NicheNet compared the genes and ligands in my sequenced data to a curated databases of known ligands and the genes that they regulated. This was visualised as a matrix in Figure 4-5, using the code,

```
vis_ligand_target = active_ligand_target_links[order_targets,order_ligands]
%>% t()
p_ligand_target_network = vis_ligand_target %>%
make_heatmap_ggplot("Prioritized ligands","Predicted target genes", color =
"purple",legend_position = "top", x_axis_position = "top",legend_title =
"Regulatory potential") + theme(axis.text.x = element_text(face = "italic"))
+ scale_fill_gradient2(low = "whitesmoke", high = "purple", breaks =
c(0,0.0045,0.0090))
p_ligand_target_network
```

I then checked on the ligands that were interacting between the moDCs and CD4 T cells and generated the matrix **Figure 4- 6,** using the code

```
vis_ligand_receptor_network = lr_network_top_matrix[order_receptors,
order_ligands_receptor]
```

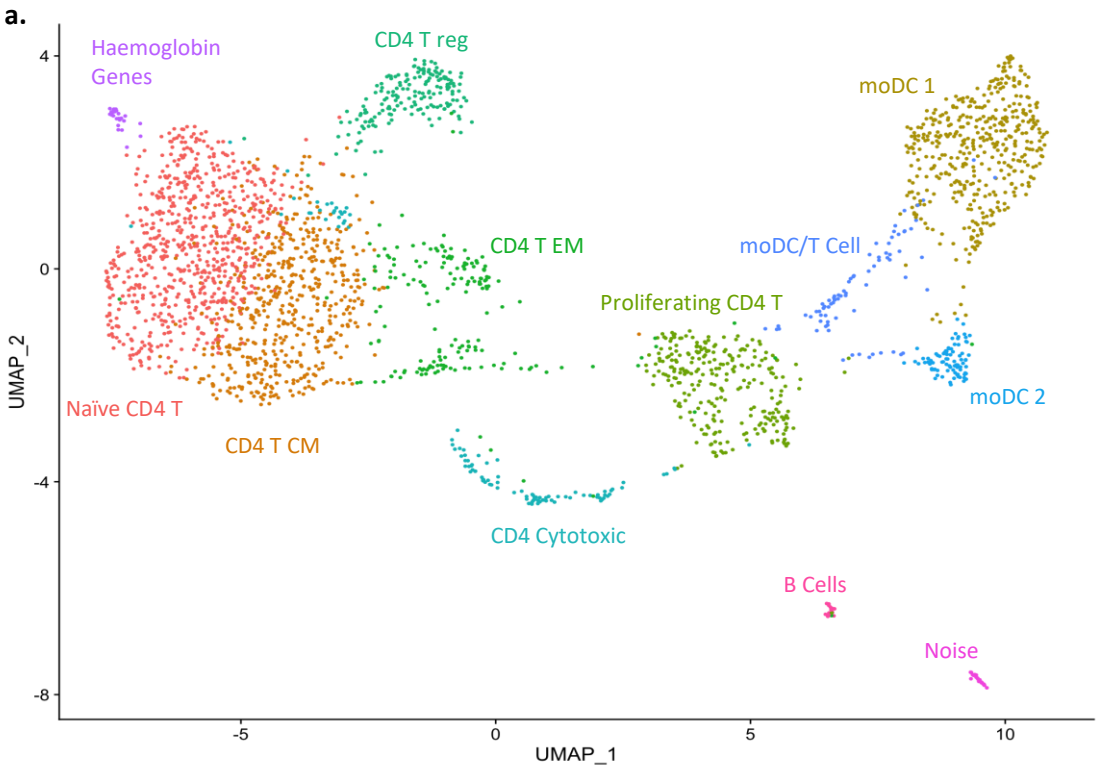
```
rownames(vis_ligand_receptor_network) = order_receptors %>% make.names()
colnames(vis_ligand_receptor_network)     =     order_ligands_receptor     %>%
make.names()
p_ligand_receptor_network   =   vis_ligand_receptor_network   %>%   t()   %>%
make_heatmap_ggplot("Ligands","Receptors",   color   =   "mediumvioletred",
x_axis_position = "top",legend_title = "Prior interaction potential")

p_ligand_receptor_network
```

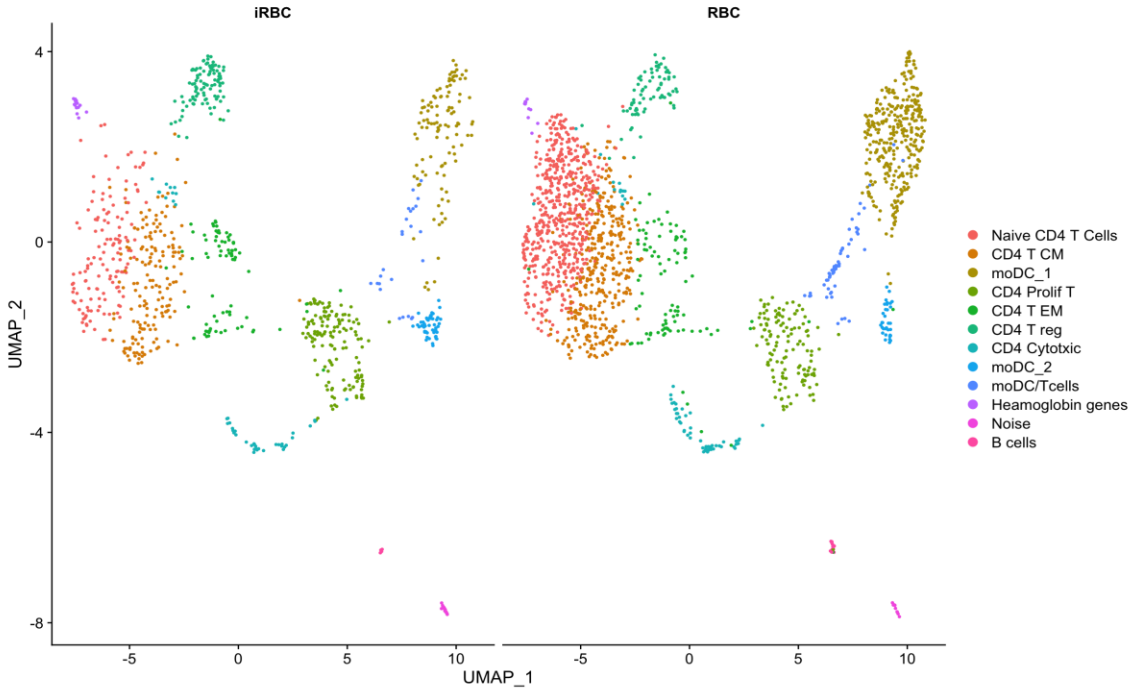
4.4 Results

4.4.1 Identification of Cell Clusters and Cell Distribution Between the Sequenced Samples.

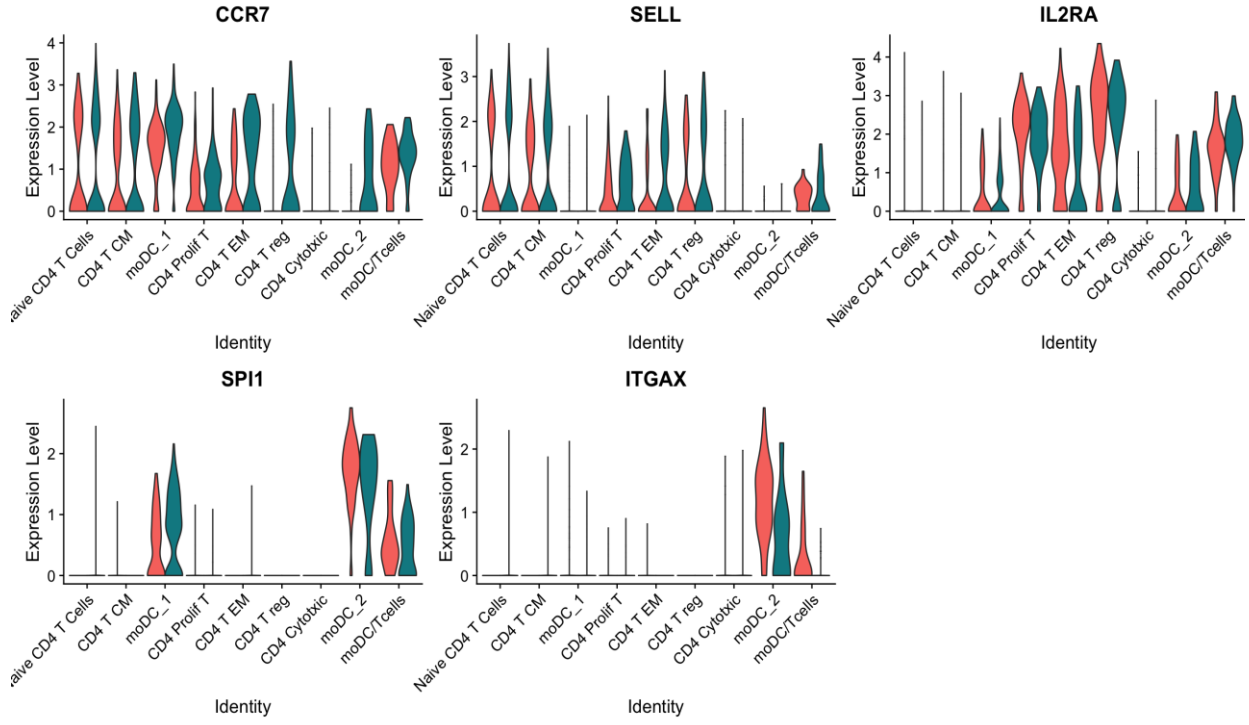
moDC's exposed to either *P. falciparum* infected RBCs or uninfected RBCs and later co-cultured with allogenic CD4 T cell were sent to Glasgow Polyomics for sequencing. The sequenced data was then loaded onto R and I first identified the cell populations in data set using Seurat (Satija et al., 2015). **Figure 4-1** shows the different cell populations that could be identified from the gene count matrix.

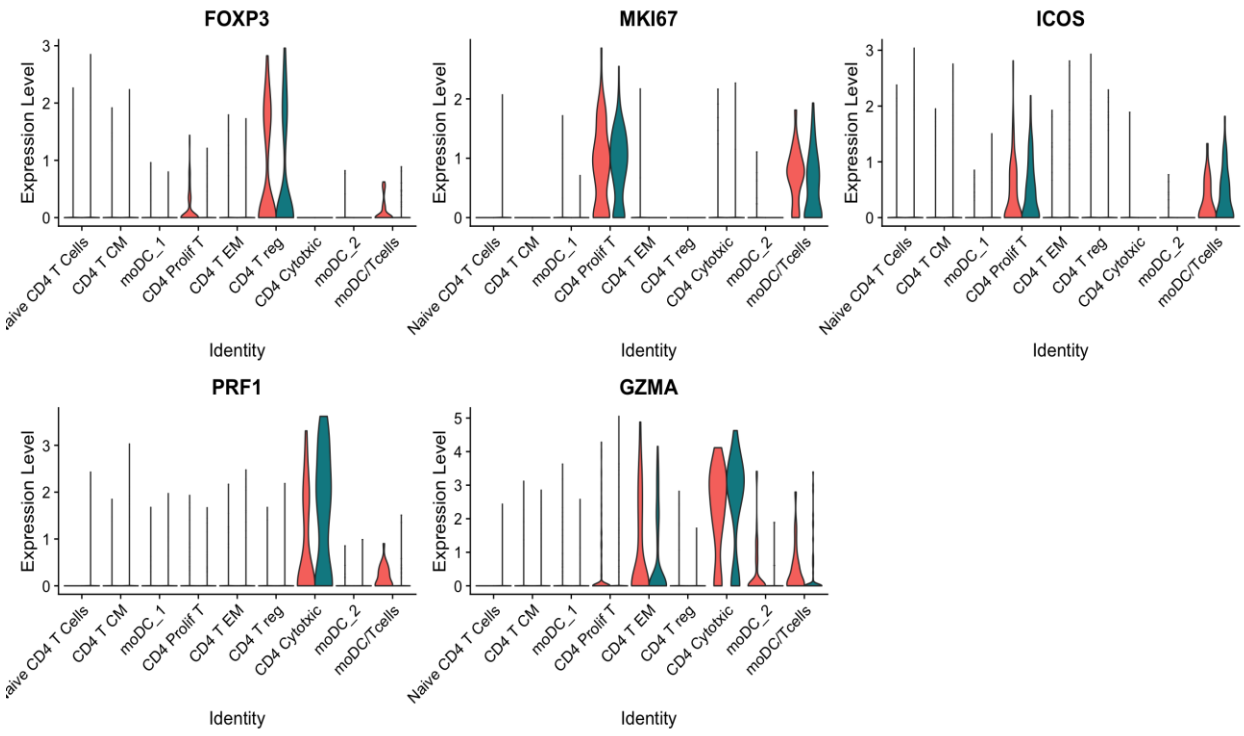


b.


iRBC
RBC
UMAP_2
UMAP_1
Naive CD4 T Cells
CD4 T CM
moDC_1
CD4 Prolif T
CD4 T EM
CD4 T reg
CD4 Cytotxic
moDC_2
moDC/Tcells
Heamoglobin genes
Noise
B cells

c.


CCR7
SELL
IL2RA
SPI1
ITGAX
Expression Level
Identity
Naive CD4 T Cells
CD4 T CM
moDC_1
CD4 Prolif T
CD4 T EM
CD4 T reg
CD4 Cytotxic
moDC_2
moDC/Tcells


FOXP3
MKI67
ICOS
PRF1
GZMA
Expression Level
Identity
Naive CD4 T Cells
CD4 T CM
moDC_1
CD4 Prolif T
CD4 T EM
CD4 T reg
CD4 Cytotxic
moDC_2
moDC/Tcells

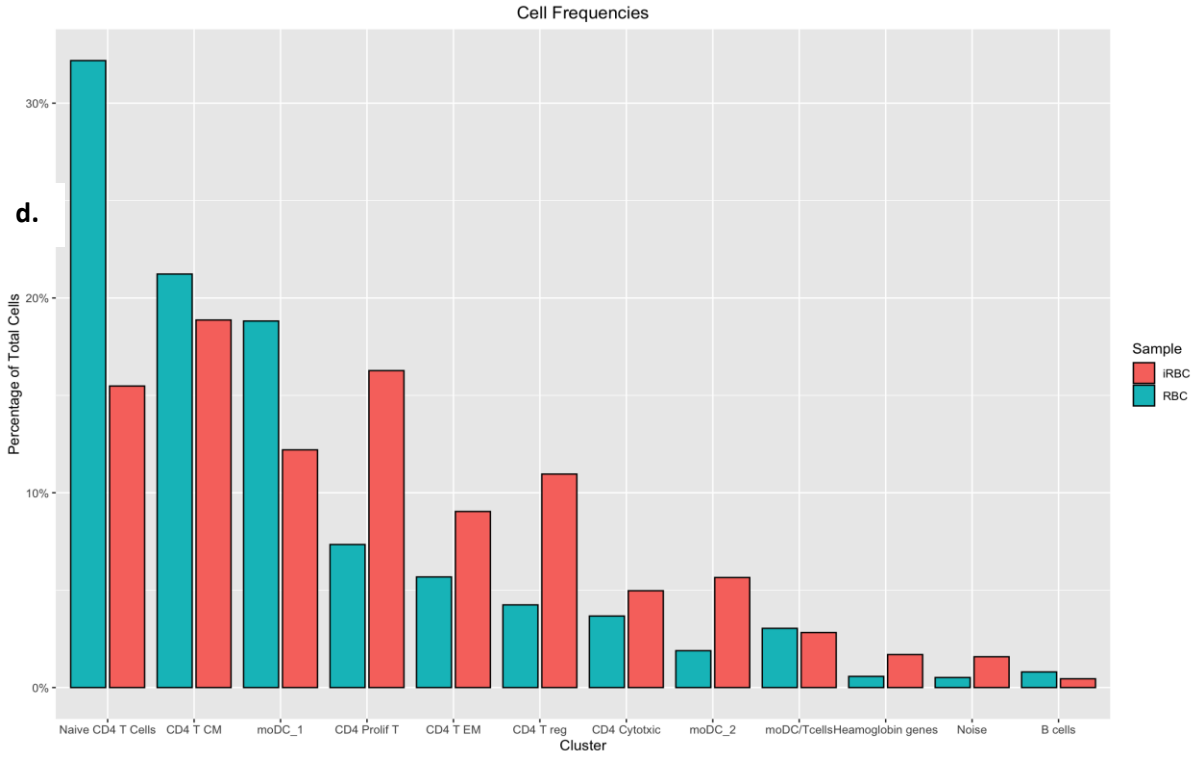

d.
Cell Frequencies
Percentage of Total Cells
30%
20%
10%
0%
Naive CD4 T Cells
CD4 T CM
moDC_1
CD4 Prolif T
CD4 T EM
CD4 T reg
CD4 Cytotxic
moDC_2
moDC/Tcells
Heamoglobin genes
Noise
B cells
Cluster
Sample
iRBC
RBC

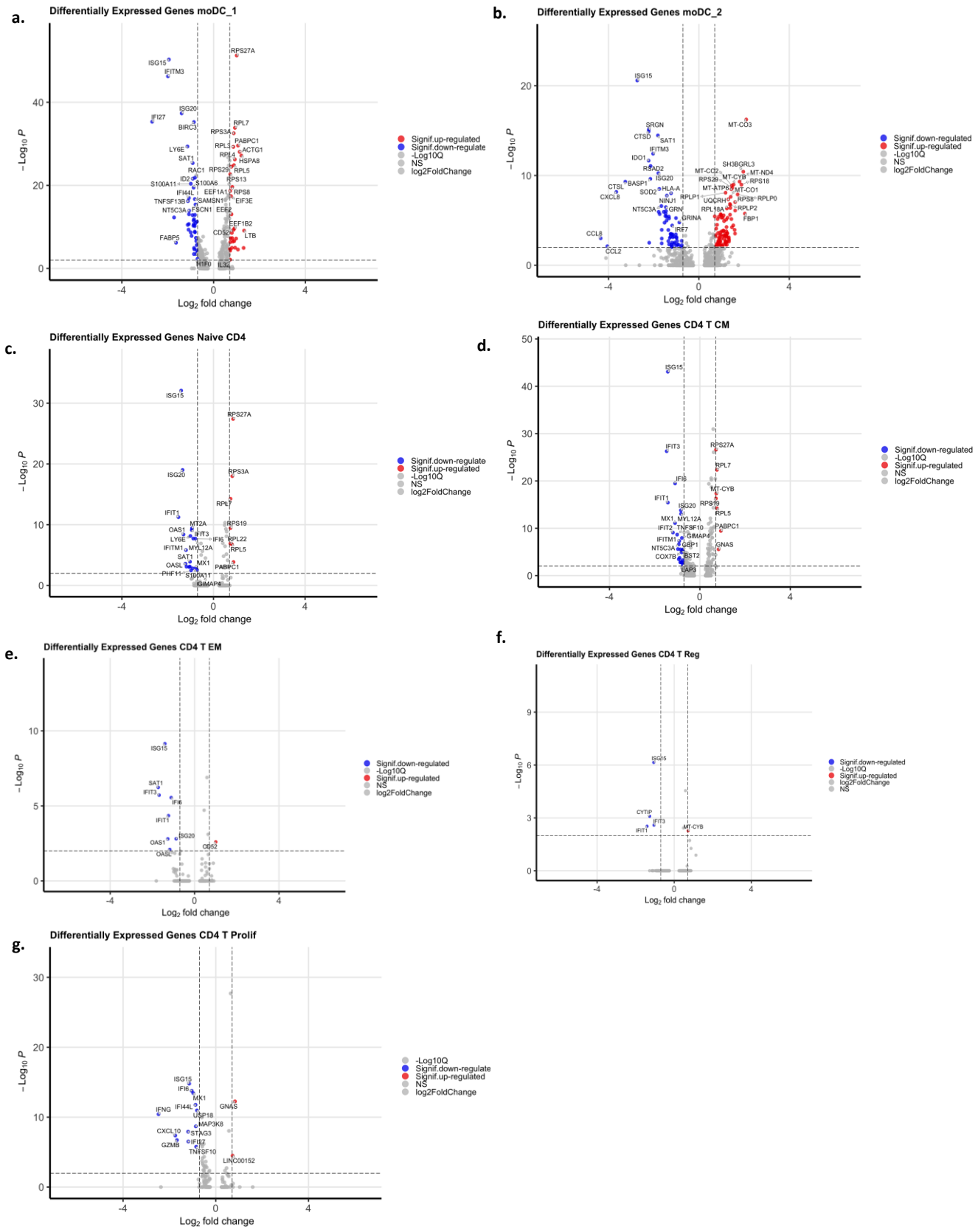
Figure 4-1 Identification of cell cluster and distribution of cells across experimental conditions in scRNA sequenced data.

The data was processed and visualised in R and the identification of each cluster determined based on conserved markers across both samples (**Figure 4-1 c**). I was able to identify two populations of moDCs (moDC_1 and moDC_2), naïve CD4 T cells, CD4 T Central Memory (CD 4 T CM), CD4 T regulatory cells (CD4 T reg), CD4 T Effector Memory (CD4 T EM), Cytotoxic CD4 T cells (CD4 Cytotoxic), Proliferating CD4 T cells (Proliferating CD4 T), and a moDC/T CD4 T cell population. **Figure 4-1 d** shows the distribution of the cells across the different experimental conditions.

After identifying the cell populations using the markers shown in **Figure 4-1 c**, I then proceeded to check the cell numbers that were in the two experimental conditions that I had set up. **Figure 4-1 b and d,** show the distribution of cells between the moDC pre-exposed to RBC (RBC) prior to CD4 co-culture and moDCs pre-exposed to iRBC (iRBC) prior to co-culture with CD4 T cells. There was a higher percentage of CD4 T reg, CD4 proliferating cells, CD4 cytotoxic T cells and CD4 T effector memory (CD4 T EM) in the iRBC exposed samples compared with the RBC exposed samples (**Figure 4-1 d**).

4.4.2 Differentially Expressed Genes in the Identified Clusters

For each cell population identified, I checked the differential expressed genes by comparing the iRBC exposed samples to the RBC exposed samples. The volcano plots (**Figure 4-2**) show that majority of the significantly downregulated genes in all the cells were interferon stimulated genes such as ISG15, ISG20, IFIT3 and IFITM3. Indicating that the type I interferon signalling pathway is inhibited in the malaria exposed samples.


a.
Differentially Expressed Genes moDC_1
b.
Differentially Expressed Genes moDC_2
c.
Differentially Expressed Genes Naive CD4
d.
Differentially Expressed Genes CD4 T CM
e.
Differentially Expressed Genes CD4 T EM
f.
Differentially Expressed Genes CD4 T Reg
g.
Differentially Expressed Genes CD4 T Prolif
−Log10 P
Log2 fold change
Signif.up-regulated
Signif.down-regulate
Signif.down-regulated
-Log10Q
NS
log2FoldChange

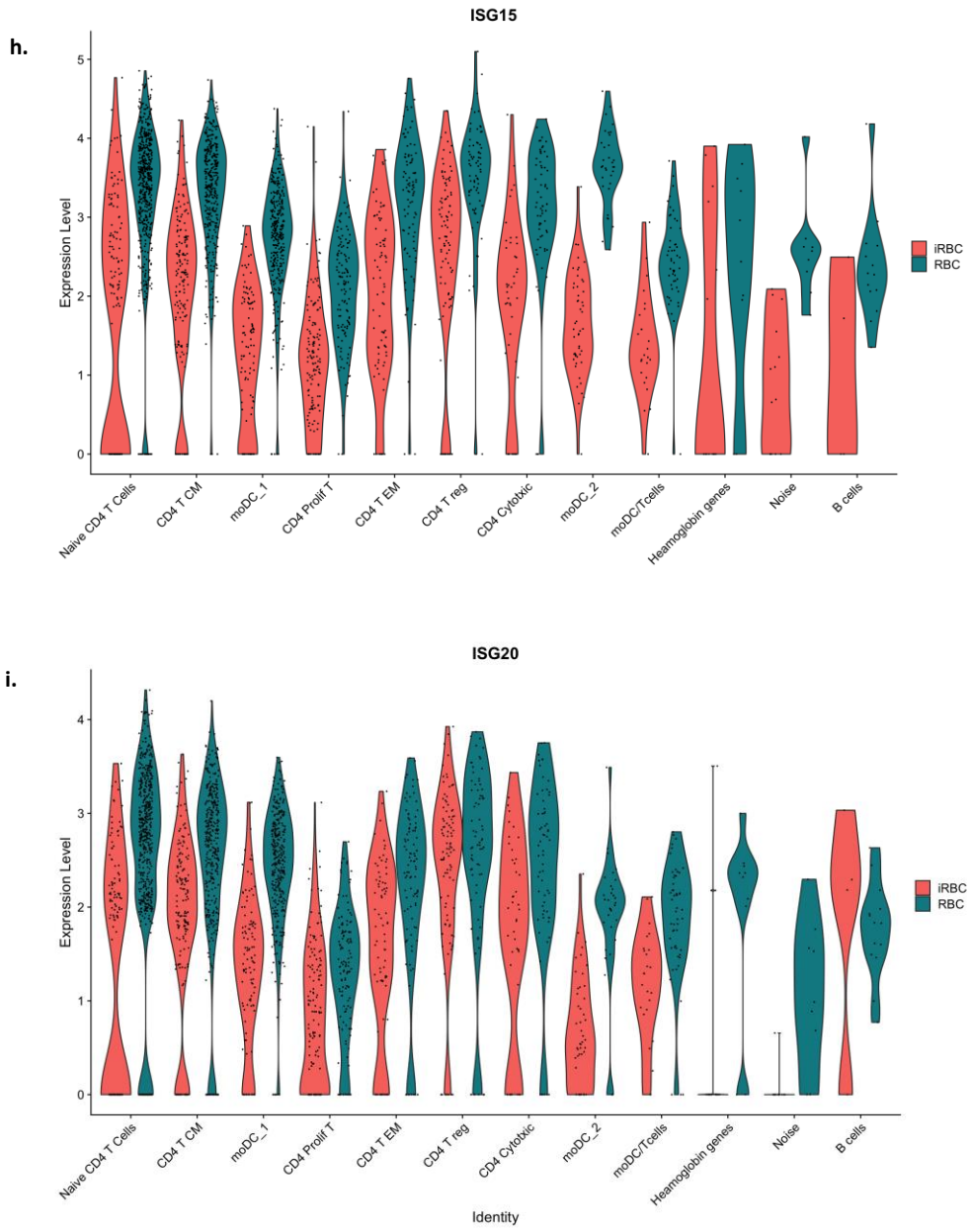


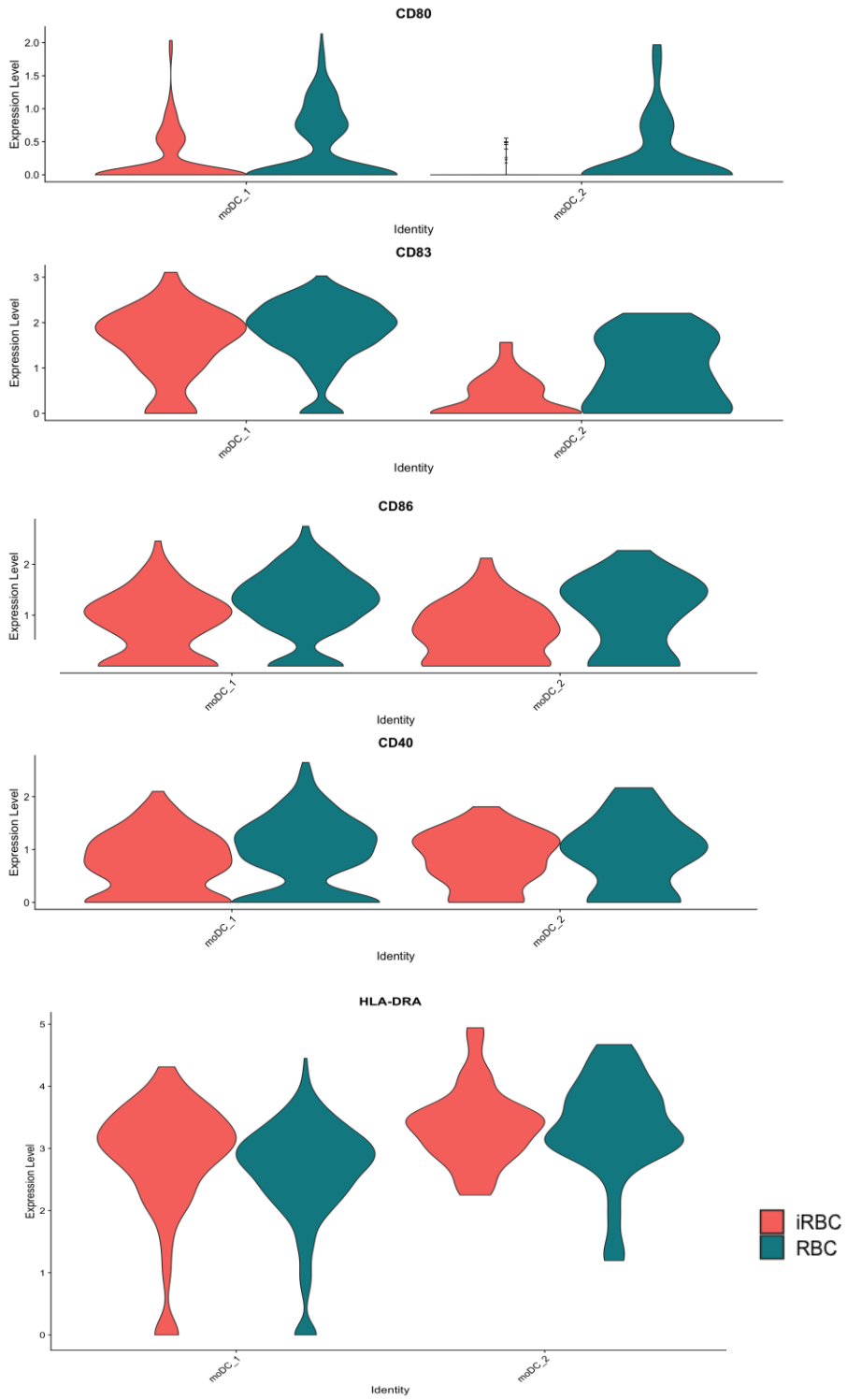
Figure 4-2 Differentially expressed genes the moDC and CD4 T Cell population

Differentially expressed genes were compiled by comparing the iRBC exposed samples to RBC exposed samples in R software and the volcano plots generated using a p value of 10e-3 and fold change limit of 0.7 (**Figure 4-2 a-g**). To identify the distribution of the two topmost significant genes in the dataset, violin plots of the raw data were plotted and this showed that indeed the genes ISG20 and ISG15 were downrefulated in the iRBC exposed cells (**Figure 4-2 h-i**)

4.4.3 Expression Levels of Activation Markers in DCs and CD4 T Cells populations

The activation status of moDCs was checked by looking at the expression levels of CD80, CD83 CD86, CD40 and HLA-DRA in the single cell data, **Figure 4-3 a**. The expression levels of CD80, CD83, CD86 and CD40 were downregulated in both moDC populations that were exposed to iRBCs. For CD4 T cells, I checked the expression levels of the activation markers CD69, CD25, PD1 (PDCD1) and CD154 (CD40LG). CD69 was upregulated in CD4 T central memory (CD4 T CM) that were exposed to RBC, while in the CD4 effector memory T cells (CD4 T EM) both RBC and iRBC samples showed similar expression levels of CD69. Naïve CD4 T cells in both samples did not upregulate CD69. CD25 (IL2RA) was only expressed in the CD4 T EM, with the iRBC sample having a higher level of expression. PD1 was not expressed in any of the CD4 T cell populations and CD40L was only expressed in CD4 T EM cells that were exposed to iRBC. This confirms the observation seen in Chapter 3, that iRBC causes a downregulation of co-stimulatory molecules on moDCs. The iRBC exposed moDCs are then unable to stimulate naïve CD4 T cells and CD4 T CM but were capable of partially stimulating CD4 TEM.

a.


CD80
CD83
CD86
CD40
HLA-DRA
Expression Level
Identity
moDC_1
moDC_2
iRBC
RBC

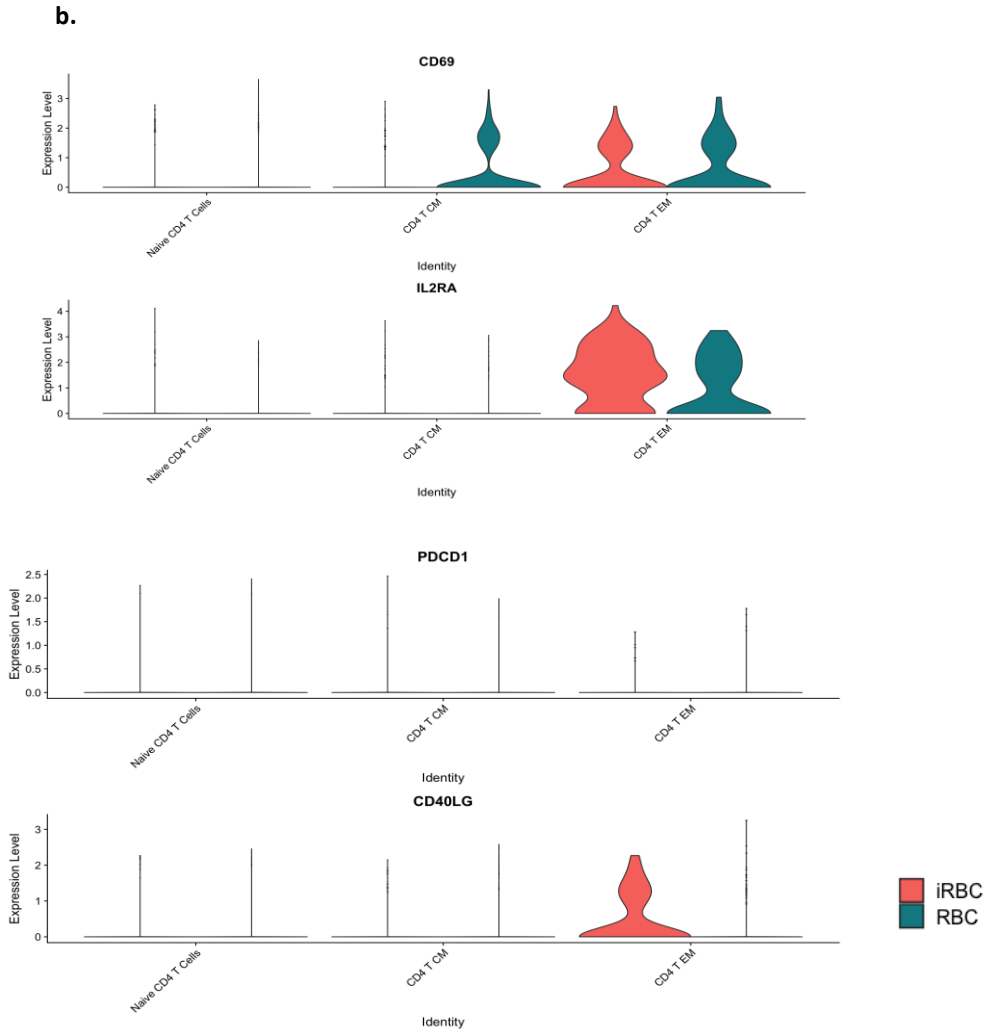


Figure 4-3 Expression levels of key activation markers in moDCs and CD4 T cells.

a. moDCs expression levels of the activation markers CD80, CD83, CD86, CD40, and HLA-DR were determined in the sequenced data.

b. CD4 T cells activation status was checked by looking at the expression levels of CD69, IL2RA (CD25), PDCD1 (PD-1), and CD40LG

4.4.4 Intercellular communication between moDCs and CD4 T cells

In order to map out the interacting ligands between moDCs and CD4 T Cells that were driving responses to malaria, I used NicheNet analysis (as described in Section 4.3.1.2) to infer these interactions.

NicheNet works by inferring interactions from several complementary ligand-receptor, signalling and gene regulatory data from public data sources and repositories. This data is aggregated in respective integrated networks from which ligand-target regulatory potential scores are calculated. This model of prior information on potential ligand-target links can then be used to infer active ligand-target links between interacting cells. NicheNet prioritises ligands according to their activity (i.e., how well they predict observed changes in gene expression in the receiver cell) and looks for affected targets with high potential to be regulated by these prioritised ligands (Browaeys et al., 2020). For my data, the sender cells were moDCs as the signals they were providing through their ligands to drive CD4 T cell responses (receiver cells)

A Pearson's correlation coefficient score was used to rank the order of the ligands in moDCs as described in the methods (Section 4.3.1.2). **Figure 4-4 a** shows the rank of the ligands that were prioritised in both moDC populations. The ligands were prioritised after NicheNet determined the differentially expressed genes between the parasite exposed cells and the cells exposed to RBCs. These ligands are not all equally expressed in the two moDCs populations, hence I then checked for their expression levels in the two populations (**Figure 4-4 b).** In the moDC_1 group the expression of the ligands TNSF13B (BAFF) and CXCL10 genes was suppressed, while IL27, ICAM-1, HMBG2, IL15, CD40, TNSF4 and CD274 were not expressed. Upregulated genes in this population include TGF-β1, PTPRC, ADAM17, TNF and ITGB1.

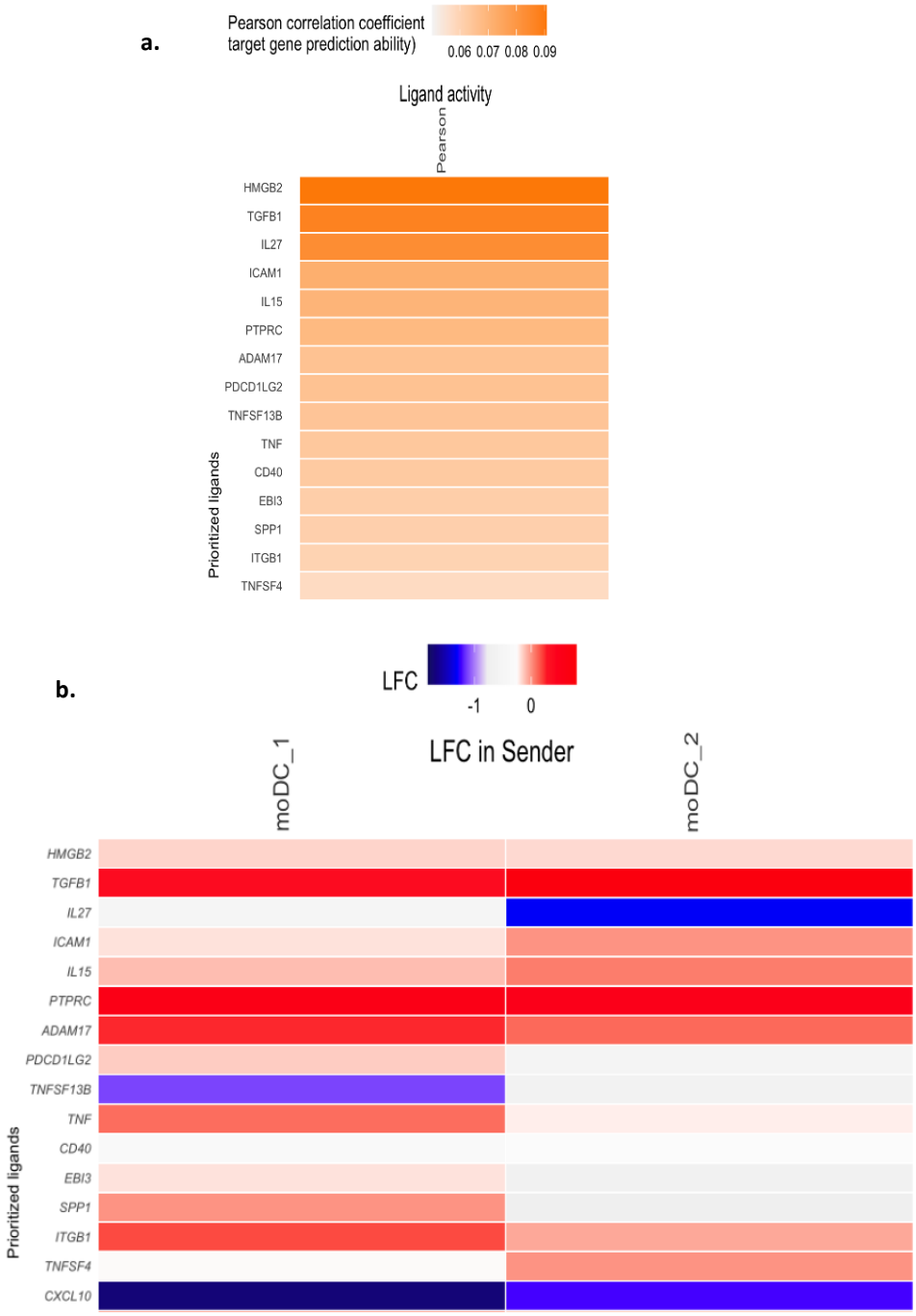


Figure 4-4 Top ligands that were involved in driving DCs responses

a. The top ligands ranked by Pearson's correlation coefficient score that are seen to be driving DCs responses and are key in supporting interactions between DCs and CD4 T cells

b. The expression levels of the ranked ligands varied in both moDC populations. This graph shows the log fold change (LFC) of each top ranked ligand and their expression levels in each moDC population.

In the moDC_2 population, the expression of IL27 and CXCL10 genes was suppressed, while PDCD1LG2, TNFSF13B, TNF, CD40, EBI3, SPP1, HLA.DMA and CD274 were not expressed. Upregulated ligands in this population included TGF-β1, PTPRC, ADAM17, DUSP18 HBEGF and YARS.

To infer which target genes in CD4 T cells are modulated by the top ranked ligands in moDCs, NicheNet compared the top ranked ligands to known curated databases of ligand-target genes and generated the ligand-target gene matrix in **Figure 4-5.**

The common ligands that were upregulated in both moDCs populations were TGFB1, PTPRC, and ADAM17. The **Figure 4-5** shows that TGFB1 regulates the genes FOXP3, TNFSF10 (TRAIL), and TNSFSF13B (BAAF). It also regulates ATP5L, CXCL10, IFIT3, KLF6 but to a lesser extent. The other upregulated genes, PTPRC and ADAM17 did not seem to influence gene expression in the CD4 T cells as their regulation potential scores were quite low. TNF was upregulated only in moDC_1 population (**Figure 4-4 b**), and negatively regulates interferon regulated genes (IRF7, STAT1, and XAF1), chemokines (CCR7and CXCL10), and the genes JUND, ITGB2, IL7R and LTB. IL15 ligand expression was higher in moDC_2 population (**Figure 4-4 b**) and regulated the transcription factor FOXP3 and the receptor IL2RA on CD4 T cells. HMGB2 was not expressed in either moDC population and this ligand was seen to regulate genes involved in cell proliferation and activation (BIRC5, GZMA, MKI67, SET, SMC4 STMN1, TOP2A, UBE2S) in CD4 T cells. IL27 expression was suppressed in moDC_2 population, meaning that the genes regulated by this cytokine were not being expressed by CD4 T cells.

To visualise the ligand-ligand interaction between moDCs and CD4 T cells that result in the expression of genes depicted in **Figure 4-5**. NicheNet generated a list of differentially expressed genes by comparing all CD4 T cells and moDCs that were exposed to iRBC to those that were exposed to uninfected RBCs. The top ranked ligands were paired with receptors that they could potentially interact with and were expressed in the CD4 T cells populations. Using curated

databases, NicheNet was able to infer the interaction potential of ligands between moDCs and CD4 T cells and generate the matrix, **Figure 4-6.** Most ligands on moDCs were linked to their receptors expressed on CD4 T cells.

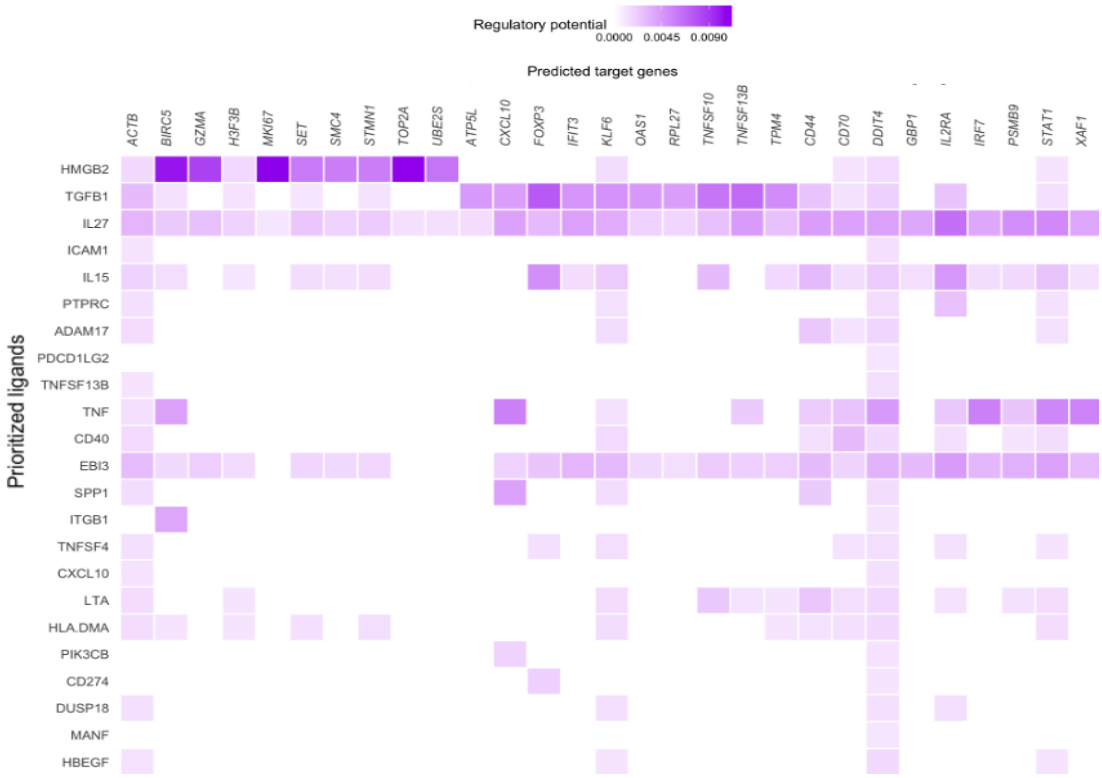


Figure 4-5 The target genes in CD4 T cells affected by the ligands expressed in moDCs

The matrix shows the ranked prioritised ligands identified by NicheNet analysis in moDCs and the predicted target gene that they influence in CD4 T cells.

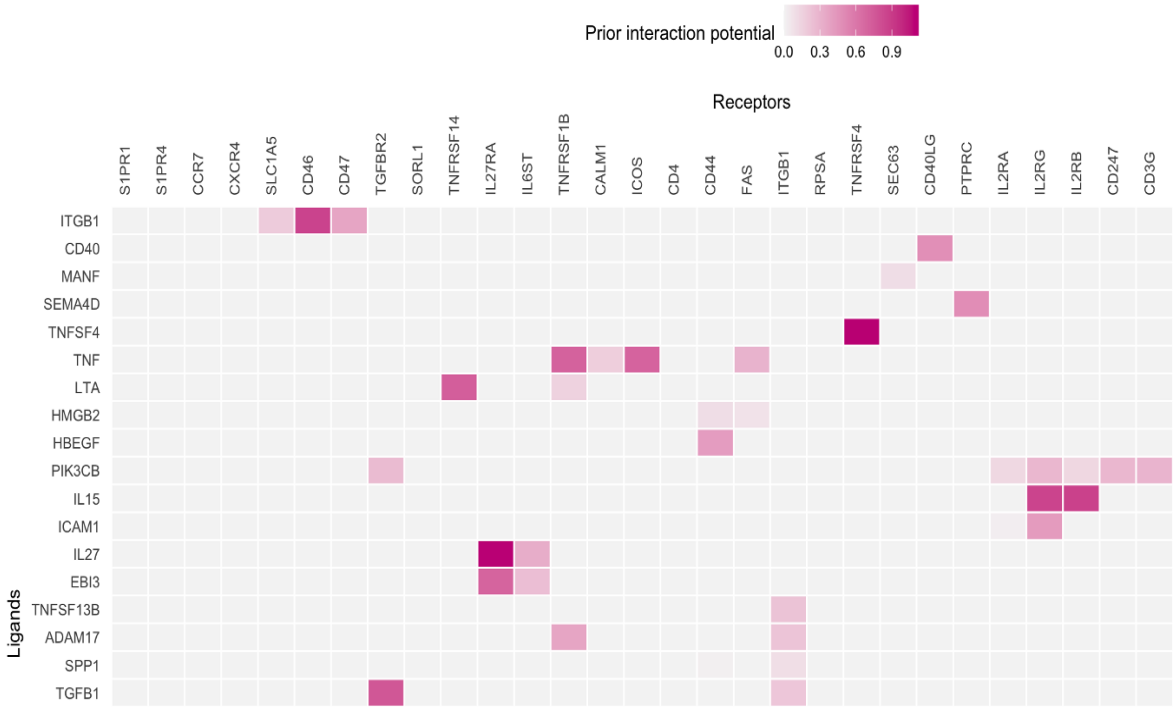
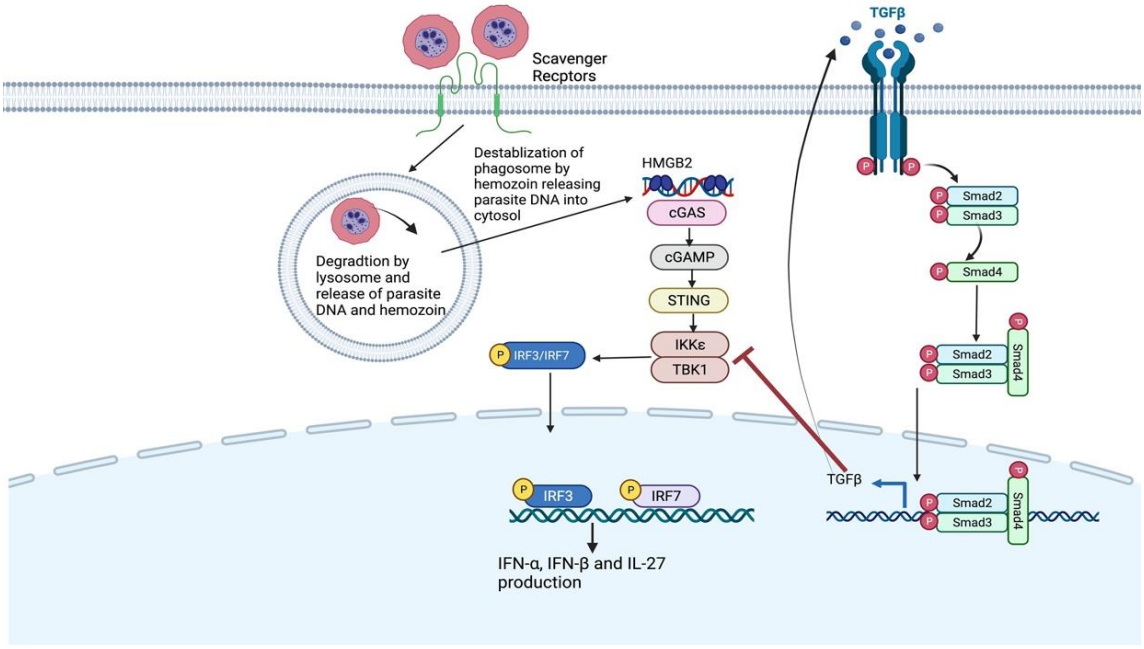


Figure 4-6 The ranked ligands in moDCs and their corresponding receptor they interact with on CD4 T cells

The matrix shows the interaction of the ligands expressed in moDCs and the receptors that they interact with in CD4 T cells

a.



b.

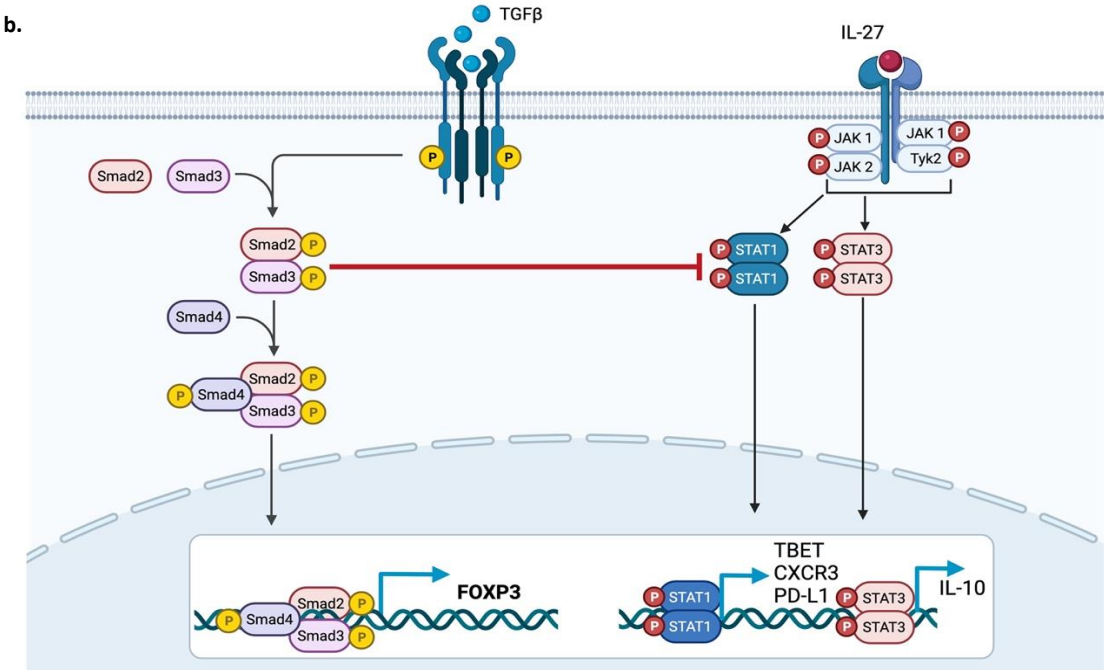


Figure 4-7 Summary of signalling events in moDC (a) and CD4 T Cells (b).

a. CD36 mediated phagocytosis of iRBCs by moDC leads to degradation of the iRBC in the phagosome, leading to accumulation of hemozoin, which destabilizes the phagosome leading to release of parasite DNA into the cytosol, which is recognised by HMGB2. HGMB2 in turn initiates **s**ignalling via the cGAS-STING-TBK1 pathway that leads to IFN-I production**.** TGF-β signalling on the other hand inhibits this pathway, thereby inhibiting IFN-I production

b. TGF-β released by moDCs inhibits IL-27 signalling in CD4 T cells and increase the expression of FOXP3 leading to generation of CD4 T regs. Images were created with BioRender.com

4.5 Discussion

The single cell RNA sequencing of the mixed lymphocyte reaction between moDC and CD4 T cells showed several changes in the cell populations that resulted from this interaction (**Figure 4-1**). Interestingly, the percentage of cells in the different populations varied, with cytotoxic CD4 T cells, CD4 Tregs, and CD4 T effector memory increasing in the iRBC condition compared to the RBC exposed cells. The increase in the number of cytotoxic CD4 T cells has been seen in a number of clinical studies (Moreno et al., 1991, Burel et al., 2016, Bijker et al., 2014). Burel et al. (2016), showed that the expanded population of cytotoxic CD4 T cells were poor producers of IFN-γ, an effector cytokine that has been linked to protective immunity in malaria (McCall and Sauerwein, 2010). Similarly, CD4 T regs have been shown increase during malaria infection (Walther et al., 2009, Walther et al., 2005, Minigo et al., 2009). The percentage of CD4 proliferating cells were also high in the iRBC exposed group compared to the RBC exposed group but determining if these proliferating cells originated from either from CD4 memory or naïve T cells was challenging. Nevertheless, this indicates that moDC exposed to iRBC can cause proliferation of allogeneic CD4 T cells despite having a lower expression level of co-stimulatory molecules. This result differs from the observations seen by Urban et al. (1999), where moDCs exposed to iRBC failed to stimulate allogenic T cells.

Looking at the differentially expressed genes between the two groups, it was clear that in the iRBC group, the majority of the suppressed genes in all the cells were genes that are induced by type I and type II interferon signals. Interferons are known to play a major role in protection against malaria, especially IFN-γ which is known to be crucial in controlling the blood stage of malaria infection. In a study where human volunteers were repeatedly injected with ultra-low doses of blood stage parasites, and subsequently treated with anti-malarial drugs before they got ill, the volunteers, produced strong IFN-γ-producing CD4 T cell responses, which were associated with protection against consequent blood stage challenge (Pombo et al., 2002). Several field studies have shown that IFN-γ responses correlate with protection from clinical symptoms of malaria (D'Ombrain et al., 2008, Robinson et al., 2009, McCall et al., 2010a). In mice, IFN-γ has been shown to play a protective role against the blood stage of malaria infection as mice lacking IFN-γ have a higher and longer blood stage parasitaemia compared to IFN-γ-

competent mice when infected with *P. yoelii* or *P. chabaudi adami* (van der Heyde et al., 1997). Early production of IFN-γ in mice has been shown to be protective against experimental cerebral malaria (ECM) as onset of ECM is accompanied by a reduction in the peripheral levels of IFN-γ (Mitchell et al., 2005) and this may be similar in humans (Prakash et al., 2006).

Apart from IFN-γ, type I IFNs (IFN-I), mainly IFN-α and IFN-β, have also been shown to play a role during malaria infection. The earliest evidence of IFN-I protection during a malaria infection was observed in mice, when exogenous mouse serum IFN was able to protect mice against the liver stage of sporozoite-induced *Plasmodium berghei* malaria, but not the blood stage (Jahiel et al., 1970). The mouse serum IFN was obtained from mice infected with Newcastle disease virus and the serum contained other cytokines that may have played a part in control of the liver stage of *P. berghei* malaria (Nüssler et al., 1991, Van Damme et al., 1989). A subsequent study by Vigário et al. (2001) used a hybrid form of human IFN-α that was able to bind to and signal through mouse IFNAR receptors *in vivo* (Vigário et al., 2001, Vigario et al., 2007). The hybrid human IFN-α was able to reduce the parasite burden during the blood stage of malaria infection in mice infected with *P. yoelii* 265 BY or *P. yoelii* 17XNL (Vigário et al., 2001) but was ineffective in controlling liver-stage infection. They also saw that the hybrid human IFN-α could prevent the onset of experimental cerebral malaria caused by *P. berghei* ANKA (Vigario et al., 2007). IFN-β has also been shown to play a role in murine models of malaria as it increases host survival and downregulates the expression of CXCL9 , ICAM-1 and TNF-α, which are associated with onset of cerebral malaria in mice infected with *P. berghei* ANKA (Morrell et al., 2011).

While these papers show the IFN-I signalling could be beneficial during blood stage infection, other groups have shown that IFN-I signalling played no role in controlling parasitaemia as observed in IFNAR1 deficient mice (129Sv background) infected with non-lethal *P. chabaudi chabaudi* AS (Voisine et al., 2010). Later studies using IFNAR1 and IRF7 knockout mice (C57BL/6 background), showed that IFN-I signalling via IFNAR1/IRF7 impaired early parasite control in both *P. chabaudi chabaudi* AS (Edwards et al., 2015, Sebina et al., 2016) and lethal

P. yoelii 17XNL (James et al., 2018, Sebina et al., 2016). These results suggest that the host genetic background could affect IFN-I signalling via IFNAR-I.

IFN-I have also been shown to impair DC function and development of IFN-γ producing CD4+ T cells for parasite control during *P. berghei* ANKA and *P. chabaudi chabaudi* AS infections (Haque et al., 2011, Haque et al., 2014). IFN-I and IFN-γ have been shown to induce cDC death in mice infected with *P. berghei* ANKA (Tamura et al., 2015). IFN-I also has the ability to expand the population of T regulatory 1 (Tr1) cells in mice infected with non-lethal *P. yoelii* 17XNL (Zander et al., 2016), and these Tr1 cells suppress innate immune responses, IFN-γ production by CD4 T cells and restrict the development of Tfh cells reducing parasite specific antibody responses (de Oca et al., 2016).

In humans, IFN-α, has been associated with protection against *P. falciparum* infections, as children with severe malaria had significantly lower levels of IFN-α than those with mild malaria (Luty et al., 2000). A similar observation was seen in Kenyan children where polymorphism in the IFN-α promoters [IFN-α2 (A173T) and IFN-α8 (T884A)] lead to a reduction in IFN-α production and are associated with increased susceptibility to severe malaria anaemia and mortality (Kempaiah et al., 2012). Malawian children recovering from severe malaria were seen to have a high expression of genes involved in IFN-I signalling (Krupka et al., 2012). IFN-I signalling has also been associated with immune suppression and increase in severity of disease in humans. Polymorphism in IFN-α/β receptor (IFNAR) have been linked to progression of cerebral malaria (Ball et al., 2013, Aucan et al., 2003), but the exact mechanism of how IFN-I signalling leads to development of cerebral malaria is not well understood.

My data shows that IFN-I signalling was suppressed in moDCs exposed to iRBC and in one of the moDC populations, Interferon Regulatory Factor 7 (IRF7) was also suppressed. IRF-7 is an interferon stimulated gene that is required for the expression of both IFN-α and IFN-β, and is key in the IFN-I amplification loop (Honda et al., 2006, Paun and Pitha, 2007b, Paun and Pitha, 2007a, Sato et al., 1998). Expression of IRF-7 is dependent on the expression of IRF-3, which triggers IFN-β transcription and IFN-β initiates a positive feedback mechanism loop that increase IRF-7 expression (Yoneyama et al., 1998, Yie et al., 1999, Wathelet et al., 1998). This

could point out to a possible inhibition of signalling via cytosolic DNA, RNA or TLR receptors (Wu and Chen, 2014) through the Toll/IL-1R-containing adaptor inducing IFN-β (TRIF) dependent pathway, which is required for the activation of IRF-3 and IRF-7 (Honda et al., 2005). It has been shown that hemozoin released by the parasite during egress from iRBC is contaminated with parasite DNA (Parroche et al., 2007). Hemozoin ingested by phagocytic cells has been shown to destabilise the phagosome, allowing parasitic DNA to move into the cytosol, where it is detected by cytosolic DNA sensors, initiating immune responses (Sisquella et al., 2017, Gallego-Marin et al., 2018)

I then looked at the expression of cell surface makers in the scRNA seq data for both moDCs and the CD4 T cell population for comparison to the flow data collected. The expression levels of CD86, CD83, CD40 and HLA-DR were downregulated in the iRBC exposed moDCs similar to what was seen when moDCs pre-exposed to iRBC were stimulated with LPS in Chapter 3, and also in line with observation by Urban et al. (1999) and Elliott et al. (2007).

Cellular interactions drive immune response, and in this case, I used NicheNet to be able to identify ligands in the moDCs that were influencing responses in CD4 T cells (moDC-CD4 T cell interaction). NicheNet analysis identified a number of ligands based in their Pearson coefficient corelation score (**Figure 4-4 a**), but these ligands driving the response were differentially expressed in both moDC populations (**Figure 4-4 b**). The highest ranked ligand according to the Pearson coefficient corelation score was the high-mobility group box 2 (HMGB2). HMGB2 is a nucleic acid sensing receptor that specifically senses only immunogenic DNA and is responsible for nucleic acid-mediated activation of innate immune responses (Yanai et al., 2009). Three HMGB proteins exist in humans, HMGB1, HMGB2, and HMGB3. A number of *in vivo* and *in vitro* studies have highlighted the role of HMBG proteins in immune activation. Stimulation of mouse embryonic fibroblasts (MEFs) from HMBG1 deficient mice with B form of DNA (classic, right-handed double helical structure of DNA) or poly(I:C), resulted in a defect in mRNA induction of type I IFN, IL-6 and RANTES (Yanai et al., 2009, Yanai et al., 2012). The same observation was seen in MEFs deficient in HMBG2 but only in response to the B form of DNA, as the reduction in mRNA was not seen when the cells were stimulated with poly(I:C) which is consistent with the observation that HMGB2 only binds

to DNA (Yanai et al., 2009), and the suppression of all three HMGB proteins in MEFs also coincided with inhibition of IRF3 (Suhara et al., 2000), NF-κB and extracellular signal-regulated kinase (ERK) activation (Yanai et al., 2009). Indicating that HMBGs function as a co-ligand for nucleic-acid-sensing cytosolic receptor signalling pathways (Yanai and Taniguchi, 2014). HMGBs are seen to be essential co-ligands that are required in nucleic acid sensing and immune activation. My data shows that HMGB2 expression was suppressed and this in turn affects IRF3 activation and this could account for the suppression of IRF7, leading to suppression of IFN-I cytokine production in the moDC population.

Transforming growth factor beta 1 (TGF-β1) was the second highest ranked ligand and this cytokine had been shown to restrict parasite growth as well as modulate immune responses during a malaria episode. In mouse models of experimental cerebral malaria, susceptible CBA/J mice infected with *P. berghei* ANKA were seen to have lower levels of TGF-β and more IFNγ than resistant BALB/c mice (de Kossodo and Grau, 1993). TGF-β levels were low in mice infected with lethal *P. berghei* NK65 and higher in mice recovering from infections with *P. chabaudi* or *P. yoelli* 17XNL (Omer and Riley, 1998b), indicating that TGF-β is key in limiting disease severity in resolving infections. The timing and magnitude of TGF-β is also vital in limiting disease severity. In mice infected with lethal *P. yoelli* 17XL, TGF-β was upregulated within the first 24hrs of infection. This early burst of TGF-β, reduced the production of pro-inflammatory cytokines IFNγ and TNF-α, compared to mice infected with non-lethal *P. yoelli* 17XNL (Omer et al., 2003). This early production of TGF-β favours the parasite as it limits inflammatory responses that are key in controlling parasitaemia. In experimental cerebral malaria using *P. berghei* ANKA infected mice, TGF-β response occurs later during the infection and at a low concentration and this fails to moderate the anti-parasitic inflammatory response, leading to disease severity and mortality (Omer and Riley, 1998b).

In humans, challenge of malaria naïve participants in a CHMI study with *P. falciparum* sporozoites produced variably TGF-β responses. Participants with a strong TGF-β response had a reduction in disease severity, but this was accompanied with high parasitaemia (Walther et al., 2006, de Jong et al., 2020), suggesting an inverse relationship between infection control and disease severity. In malaria endemic regions in Thailand, adults treated for symptomatic *P.*

falciparum malaria had low serum levels of TGF-β and high levels of TNF-α (Wenisch et al., 1995) when serum samples were compared before and after anti-malaria treatment. In Uganda TGF-β serum levels were significantly reduced in children with cerebral malaria when compared with healthy controls and uncomplicated malaria (Hanisch et al., 2015). In studies conducted in Tanzania and Thailand they found that TGF-β secretion was inhibited in cases of severe malaria compared to uncomplicated malaria (Chaiyaroj et al., 2004) and the same observation was seen in Mozambican children (Rovira-Vallbona et al., 2012). It still remains unclear how TGF-β production moderates disease severity, and my data showed TGF-β was upregulated in both moDC population and this is in line with the observation seen by malaria naïve CHMI participants who had a strong TGF-β response (Walther et al., 2006, de Jong et al., 2020).

IL27 was another cytokine that was highly ranked by the Persons corelation score as driving immune responses. IL27 is a heterodimer made up of IL-27p28 and EBI3 subunits (Pflanz et al., 2002) and is known to have both pro-inflammatory and anti-inflammatory properties during an immune response. IL-27 has been shown to promote differentiation of naïve CD4 T cells to Th1 cells by inducing the expression of T-bet as well as increasing their responsiveness to IL-12 (Takeda et al., 2003, Pflanz et al., 2002). IL-27 also limits the expansion of Foxp3+ regulatory T cells (Cox et al., 2011, Huber et al., 2008, Neufert et al., 2007). However, IL-27 has also been shown to limit Th1 responses. Although IL-27R deficient mice infected with *Toxoplasma gondii* (Villarino et al., 2003) could control parasite replication, the mice succumb to a lethal Th1 CD4 T cell mediated immune pathology within two weeks of challenge (Villarino et al., 2003). A similar observation was seen in malaria infected mice, as IL-27R deficient mice were highly vulnerable to *Plasmodium berghei* NK65 infection and developed uncontrolled Th1-mediated immune responses despite being able to effectively clear the parasite. The uncontrolled Th1-mediated immune responses lead directly to severe liver pathology (Findlay et al., 2010).The suppressive effect of IL-27 on CD4 inflammation is due to its ability to limit IFN-γ production by CD4 T cells (Hamano et al., 2003, Holscher et al., 2005, Rosas et al., 2006). IL-27 also inhibits the development of Th17 cells by limiting the expression of retinoid-related orphan receptor (ROR)c (Batten et al., 2008, Diveu et al., 2009, Stumhofer et al., 2006), and stimulates production of IL-10 in a number of effector CD4 T cells (Fitzgerald et al., 2007). My

data shows that IL-27 was suppressed in moDCs (**Figure 4-4 b**), and this favours the expansion of Foxp3+ CD4 T regulatory cells.

Protein Tyrosine Phosphatase Receptor Type C (PTPRC) encodes for the CD45 antigen. CD45 is essential for T and B cell signalling, and is known to dephosphorylate Lck, the Src family kinase which in turn phosphorylates CD3 leading to initiation of the T cell receptor signalling cascade (Mee et al., 1999, Byth et al., 1996, Kishihara et al., 1993). CD45 also regulates the phosphorylation state of Hck and Lyn (Roach et al., 1997) and had been implicated in regulating Jak-mediated signalling (Irie-Sasaki et al., 2001). In DCs, CD45 has been shown to both positively and negatively regulate the production of cytokines in response to TLR stimulation. CD45 inhibits TLR signalling via the MyD88 dependent signalling pathway (TLR2 and TLR9), and this affects production of pro-inflammatory cytokines that are induced via the MyD88 signalling pathway. On the other had CD45 positively regulated the secretion of cytokines stimulated via the MyD88 independent pathway/ TRIF-dependent pathway (TLR4 and TLR3), leading to the upregulation of IFN-β and costimulatory molecules (Cross et al., 2008). While CD45 is upregulated in both moDCs population, there is a general inhibition of IFN-I signalling in both moDCs population as seen by the suppression of the IRF-7 gene and multiple interferon stimulated/response genes. Therefore, it seems that CD45 upregulation may inhibit signalling via the MyD88 dependant pathway leading to a suppression of IFN-I signalling and interferon stimulated/response genes.

A disintegrin and metalloproteinase 17 (ADAM17), is a membrane bound enzyme that cleaves off surface proteins such as cytokines (Moss et al., 1997, Black et al., 1997), cytokine receptors (Mullberg et al., 1993, Reddy et al., 2000), ligands and adhesion molecules (Garton et al., 2003, Venturi et al., 2003). Malaria infection is normally characterised by proinflammatory cytokines particularly, tumour necrosis factor α (TNFα), a cytokine that is associated with the clinical symptoms of malaria (Wajant et al., 2003, Sundgren-Andersson et al., 1998). TNFα is released by ADAM17 where it is cleaved of from the surface, initiating a proinflammatory signal. ADAM17 also cleaves tumour necrosis factor receptor I (TNFRI), cleaving of this receptor reduces the sensitivity of TNFα in cells (Bell et al., 2007, Santos et al., 1999, Chanthaphavong et al., 2012). ADAM17 was upregulated in moDC_1 population together with TNFα, indicating

that the ADAM17 was initiating a proinflammatory response, through the cleaved TNFα, initiating paracrine signalling via the soluble TNFα.

The ranked ligands on moDCs were linked to specific receptors expressed on CD4 T cells These interacting ligands between moDC and CD4 T cells can be linked to activation of specific genes in CD4 T cells that drive their activation. The major ligands that influenced CD4 activity were HMGB2, TGF-β1, and IL27. HMGB2 acts as a co-ligand that enhances the production of IFN-I cytokines. IFN-I enhances DC function (Luft et al., 2002, Padovan et al., 2002) leading to efficient activation of CD4 T cells. IFN-I is also important in the differentiation and clonal expansion of CD4 T cells (Havenar-Daughton et al., 2006). My data shows that HMGB2 was suppressed and hence the production of IFN-I by moDCS was impaired and this affected CD4 T cells activation, thus the target genes in CD4 T cells especially MKI67 gene (KI67), an indicator of T cell activation and proliferation, was suppressed. While TGF-B1 is associated with control of clinical symptoms of malaria in malaria naïve individuals (Walther et al., 2006, de Jong et al., 2020), it is also known to induce the expression of FOXP3 in CD4 T cells leading to the generation, expansion, and maintenance of CD4 T regulatory cells (CD4 Tregs) (Figure 4-7) (Fu et al., 2004, Bommireddy and Doetschman, 2007). TGF-BR2 activation initiates signalling through *Caenorhabditis elegans* Sma and *Drosophila* Mad proteins (SMAD) pathway, leading expression of FOXP3 (Zheng et al., 2006, Fantini et al., 2004). This could account for the increase in CD4 Tregs that was seen in the iRBC exposed sample. IL-27 on the other hand inhibits the expression of TGFβ-mediated FOXP3. This is achieved through signalling via its receptor IL27RA (**Figure 4-7**), leading to downstream activation of STAT1 and STAT3 (**Figure 4-7**) (Huber et al., 2008). But my data shows that TGF-β1was upregulated, and this inhibits the IL-27 mediated suppression of FOXP3 expression and possibly inhibition of IL-27 production in moDCs.

Overall, the data shows that in malaria naïve individuals, the initial response is to control inflammation as seen by the suppression of type I and type II interferon, and expansion of CD4 T regs that limit inflammatory response. The focus on limiting inflammation is beneficial to host as it limits clinical symptoms associated with malaria and any inflammatory mediated pathology.

While NicheNet provides a method to understanding cell-cell communication it does have some limitation. NicheNet depends on curated data from various database and as such there tends to be over-representation of secreted ligands, mostly cytokines and growth factors, while membrane bound ligands tend to be under-represented. Thus, the model might miss out on some membrane bound receptors that may initiate signalling. Another limitation is that some response to extracellular signals depend on the cell type and state. This is not considered in the general model of NicheNet.

The data shown here was from an *in vitro* experiment, while during a malaria infection *in vivo*, there are multiple cellular interactions and cues that determine the outcome of the infection. To have a better understanding of the cellular processes that occur during a malaria episode, I took advantage of the CHMI study that was conduct in Kilifi, Kenya at the KEMRI-Wellcome Trust Research Programme. The next chapter looks at tracking the cellular changes that occur during a malaria infection in the CHMI study. Through this we will be able to see the pathways and cellular cues that drive gene expression DCs and CD4 T cells in the course of the infection and how it influences the outcome of a malaria infection.

Chapter 5 Using CHMI as an *in vivo* Model to Assess Cellular Changes During a Malaria Infection

5.1 Introduction

Controlled human malaria infection (CHMI) studies involve deliberate infection of healthy individuals with *Plasmodium* parasites. CHMI has historically been used to assess the efficacy of malaria drugs and vaccines (McCarthy et al., 2011, Sauerwein et al., 2011). CHMI studies are typically conducted in a controlled environment to monitor parasite growth rates and host response to infection. Most CHMI studies have been undertaken in malaria naïve populations (Sauerwein et al., 2011), as researchers in malaria-endemic areas lack the appropriate insectary facilities. Such CHMI studies administer sporozoites to participants via mosquito bites, also known as the 5-bite CHMI models, which mimic malaria infections that occur in the field (Hodgson et al., 2014).

To overcome the challenge of insectary facilities, Sanaria Inc. developed an aseptic, purified, cryopreserved *P. falciparum* sporozoites (NF54 strain) for injection (PfSPZ Challenge). This has facilitated CHMI studies assessing the efficacy of malaria vaccines to be conducted in malaria exposed populations, while also allowing for assessment of the mechanisms of immune control in previously exposed adults. Thus far, several CHMI studies have been conducted in endemic areas using PfSPZ challenge. The first one was in Tanzania (Shekalaghe et al., 2014) followed by another in Kenya (Hodgson et al., 2014). In Kenya, one of the CHMI studies was conducted in Kilifi at the Kenya Medical Research Institute Wellcome Trust Research Programme (KEMRI-WTRP). The CHMI study was setup to investigate how pre-existing immunity influenced the *in vivo* parasite growth rate of *P. falciparum*. Study participants were recruited from areas with moderate and high malaria transmission intensities, with the hypothesis that they would exhibit varying levels of pre-existing immunity (Kapulu et al., 2018).

The work presented in this chapter is based on the analysis of cellular changes in a subset of the participants in this study. For this analysis, I chose to focus on participants from Kilifi North and Kilifi South, which are 20 kilometres apart separated by the Kilifi Indian Ocean Creek.

Although the two regions are culturally similar, Kilifi South experiences moderately high levels of malaria exposure, while malaria transmission in Kilifi North has tailed off to very low levels during the last 20 years. Malaria transmission in Kilifi is bi-annual with the main season occurring between May and July and the second in November (Olotu et al., 2010, Mwangi et al., 2005), which corresponds to the long and short rains, respectively. This means that the selected samples were from the same communities with similar environmental conditions, but different levels of malaria exposures. The levels of exposure to malaria were determined during recruitment of the participants by measuring antibody levels against the schizont protein extract. The CHMI participants were stratified according to their clinical phenotypes, which were assigned after analyses of the CHMI parasite growth PCR data and fever data discussed in Chapter 2 (Section 2.4). Of interest to me were two phenotypes, the febrile and chronic participants. Febrile participants were unable to control parasitaemia and developed clinical symptoms of malaria and were mainly from Kilifi North. They also had detectable parasites on blood film examination or reached the threshold of 500 parasites per µl (which was the threshold for treatment) and were treated after day 14 post challenge. While the chronic participants, who were from Kilifi South, were able to control parasitaemia, had detectable parasites (by PCR) but the parasites did not reach the threshold for treatment (500 parasites per µl) nor did they exhibit clinical symptoms of malaria and reached the endpoint of the CHMI study when they were treated with antimalarial drugs.

The rationale for using samples from the CHMI samples was to investigate how the host immune cells respond during a natural *P. falciparum* infection. This *in vivo* system would complement the *in vitro* data collected from the malaria naïve population (discussed in Chapter 4). Although the set up did not offer a direct comparison, it would give me the opportunity to assess whether the molecular pathways elicited by the iRBC modulated DCs and CD4 T cells from malaria naïve adults in the *in vitro* experiment, would also be stimulated by *in vivo* exposure in malaria exposed adults.

While the samples collected from volunteers were PBMCs, I chose to focus specifically on CD4 T cells, monocytes, and dendritic cells. This this was because monocytes are heavily involved in controlling parasitaemia through their phagocytic capacity (Zhou et al., 2015) and can also

differentiate into moDCs that can activate the adaptive immune system. Monocytes can be divided into three distinct subsets depending on the expression levels of CD14 and CD16. Classical monocytes which expressed CD14 (CD14+CD16-), intermediate monocytes that express both CD14 and CD16 ($CD14^{+}CD16^{+}$) and non-classical monocytes that express CD16 ($CD14^{-}CD16^{+}$) (Ziegler-Heitbrock et al., 2010). Finally, dendritic cells are the primary APC and are vital in driving adaptive immunity through activating CD4 T cells and driving them to differentiate towards various phenotypes as described in Chapter 1 (section 1.6.3.2), although this is dependent on the signals provided by the DCs and the cytokine milieu.

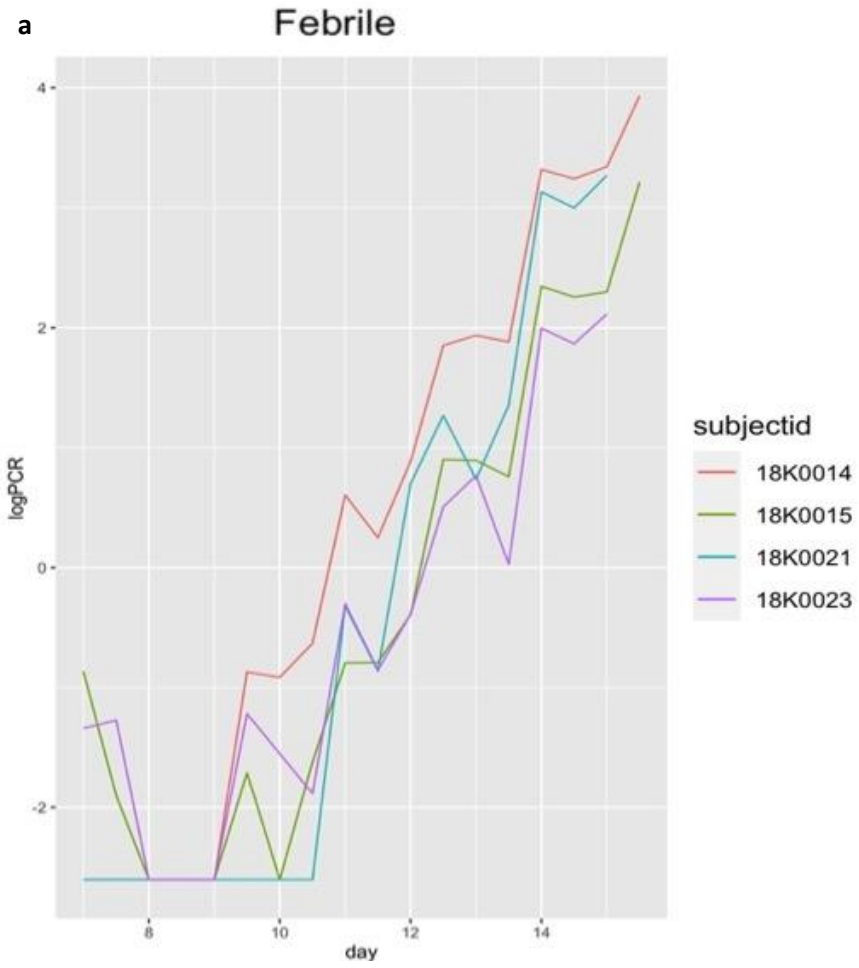
5.2 Chapter Aims

This chapter aims to give a detailed comparison of the immune responses of adults with different disease outcomes that is, febrile participants versus chronic participants, following challenge with *P. falciparum* sporozoites in a CHMI study. This will be determined through single cell RNA sequencing of selected samples at specific time points of the CHMI study to identify the pathways associated with the immune response to the parasite. These responses can lead to some individuals controlling parasite growth and expression of clinical symptoms better (chronic participants) than others (febrile participants).

5.3 Material and Methods

CHMI samples were obtained from a previous study (Kapulu et al., 2018). Criteria for inclusion into the CHMI study and ethical considerations have been well described by Kapulu et al. (2018). CHMI sample processing and selection have been described in Chapter 2 (section 2.5). The selected samples from the febrile participants were 18K0014, 18K0015, 18K0021, 18K0023, and the sample selected from the chronic participants were, 18K0016, 18K0039, 18K0027, 18K0034. These samples were selected as they had detectable parasites throughout the CHMI study as shown by the daily PCR parasite growth curve (**Figure 5-1**) and all the selected febrile participants, reached the threshold for treatment (which was 500 parasites per μl) after day C14.

a


Febrile
4
2
0
-2
logPCR
8
10
12
14
day
subjectid
18K0014
18K0015
18K0021
18K0023

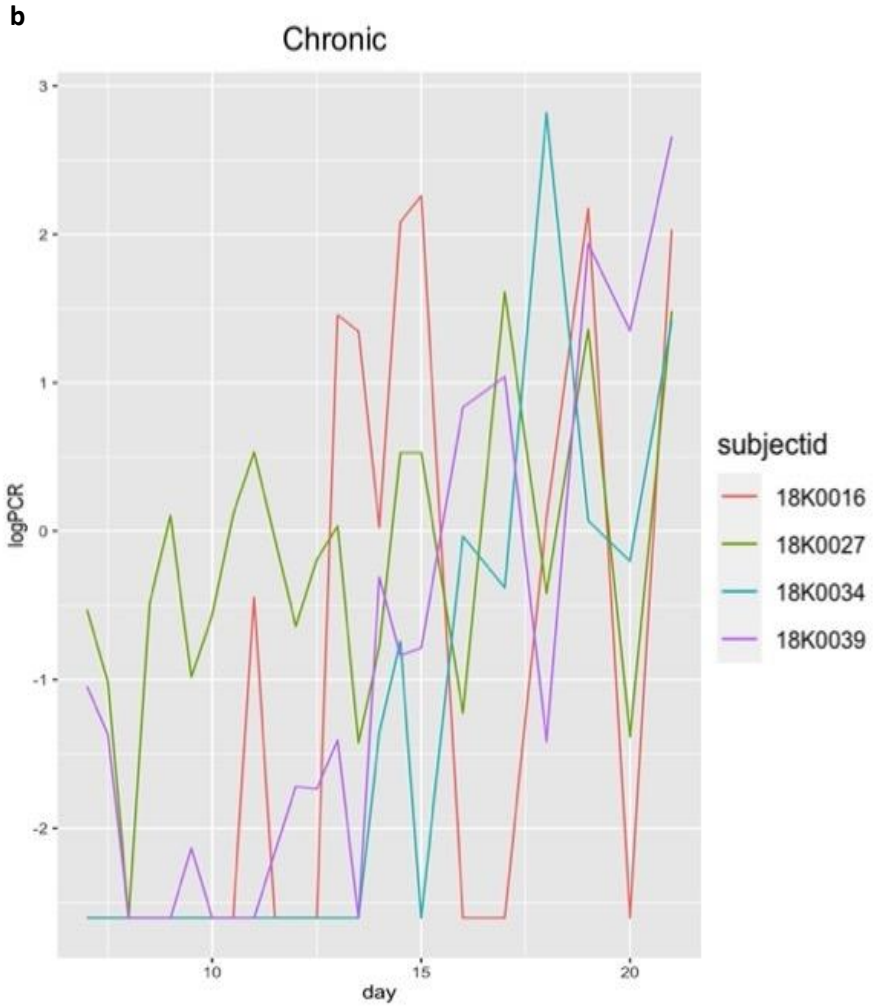


Figure 5-1 CHMI parasite growth curves for selected samples.

a. shows the daily PCR growth curve for the selected samples from febrile participants. The participants showed a steady increase in parasitaemia as days progressed up to 14 days after challenge with *P. falciparum*, after which most participants reached the threshold for treatment with antimalarial drugs.

b. shows the daily PCR growth curve for samples from the chronic participants. These participants were able to control parasitaemia below the threshold for treatment until study endpoint

Twenty-four PBMC samples (4 from febrile participants and 4 from chronic participants) at three different timepoints, were shipped to the University of Glasgow for sequencing. The samples were pooled into 3 different 10x sequencing runs, with each timepoint assigned a unique hashtag (cell hashing antibody; Biolegend, San Diego, CA), which are described in Chapter 2 (Section 2.5.2), for easy identification of samples during the analysis of the sequenced data. **Table 5-1** show the various runs and pools of samples.

Table 5-1: Pooling of CHMI samples for the various sequencing runs

Run 1			
Cell Hashtag Antibody (HTO)	Run 1 - Pool 1	Run 1 - Pool 2	
Hash10	18K0016 - Day C+14	18K0023 -Day C+14	
Hash2	18K0016 - Day C-1	18K0023 -Day C-1	
Hash7	18K0023 - Day C+9	18K0016 - Day C+9	
Run 2			
Cell Hashtag Antibody (HTO)	Run 2 - Pool 1	Run 2 - Pool 2	Run 2 - Pool 3
Hash10	18K0015 - Day C+14	18K0014 -Day C+14	18K0027 - Day C+14
Hash2	18K0014 - Day C-1	18K0027 -Day C-1	18K0015 - Day C-1
Hash7	18K0027 - Day C+9	18K0015 - Day C+9	18K0014 - Day C+9
Run3			
Cell Hashtag Antibody (HTO)	Run 3 - Pool 1	Run 3 - Pool 2	Run 3 - Pool 3
Hash10	18K0034 - Day C+14	18K0039 - Day C+14	18K0021 - Day C+14
Hash2	18K0021 - Day C-1	18K0034 - Day C-1	18K0039 - Day C-1
Hash7	18K0039 - Day C+9	18K0021 - Day C+9	18K0034 - Day C+9

After sequencing of the samples by Glasgow Polyomics, the sequences were aligned by Dr. Thomas Otto to generate a count matrix which I loaded onto R software for further analysis. Due to a miscommunication with Glasgow Polyomics, the HTO that would enable us to separate

the samples by timepoint in Run 1 was not sequenced. Thus Run 1 samples could not be distinguished by the different timepoints and were not included in this analysis.

5.3.1 Data Analysis on R

The count matrix of the aligned sequences contained three different types of sequenced data. The first was the RNA sequences for each of the cells with the unique molecular identifier (UMI) barcode. The second was the CITE-seq antibodies (ADTs) that were used to tag cells population of interest. The ADT tagged cells were: CD4 T cells (tagged with A0922 anti-human CD4 Antibody, Biolegend, San Diego, CA), myeloid cDC1 (tagged with A0207 anti-human CD370 (CLEC9A/DNGR1) Antibody, Biolegend, San Diego, CA), myeloid cDC2 (tagged with A0160 anti-human CD1c Antibody, Biolegend, San Diego, CA) and plasmacytoid dendritic (tagged with A0370 anti-human CD303 (BDCA-2) Antibody, Biolegend, San Diego, CA) (described in section 2.5). The third data was the cell hashtag antibodies (HTO) that were used to tag the different timepoints in each of the runs as shown in **Table 5-1**.

5.3.2 Normalisation of HTO data

The HTO data, which contained the different tags for each timepoint was normalised using cellhashR package on R. The code below shows the process of HTO normalisation.

First the cellhashR package was loaded onto R followed by the sequenced data containing the HTO.

```
library(cellhashR)
barcodeData.RA <- ProcessCountMatrix(rawCountData = "HTO.PoolC-
1/umi_count/", minCountPerCell = 5)
```

Quality of the HTO data was checked using the code: -

```
PlotNormalizationQC(barcodeData.RA)
```

The data was normalised using a consensus of two algorithms, bff_cluster and mutliseq, using the code:

```
calls.pool1 <- GenerateCellHashingCalls(barcodeMatrix = barcodeData.RA,
methods = c("bff_cluster", "multiseq"))
SummarizeCellsByClassification(calls = calls.pool1, barcodeMatrix =
barcodeData.RA)
```

The normalised HTO data was then saved as a data table using the code,

```
Pool1.HTO <- data.frame(row.names = calls.Run1$cellbarcode)
Pool1.HTO$HTO <- calls.pool1$consensuscall
Pool1.HTO$HTO_status <- calls.pool1$consensuscall.global
Pool1.HTO
write.table(Pool1.HTO,file ="Pool1_HTO.umi.txt",sep="\t")
```

This process of HTO normalisation was repeated for each pool in Run 2 and Run 3 and generated the **Figure 5-2**

5.3.3 Seurat Analysis

The Seurat package was loaded on R software, after which I loaded the gene count matrix for the RNA sequence, ADT and HTO.

```
library(Seurat)
data_dir <- 'PoolC.Full_Pool-1-10X'
data_ADT<- 'ADT.PoolC-1/umi_count'

pbmc.rna <- Read10X(data.dir = data_dir,
                    unique.features = TRUE,
                    strip.suffix = T)
pbmc.adt <- Read10X(data.dir = data_ADT,gene.column = 1,
                    unique.features = TRUE,
                    strip.suffix = T)
pbmc.htos <- read.table("Pool1.umi.txt", header=TRUE, sep = "\t",
                        row.names = NULL,
                        as.is=TRUE)
HTO<-pbmc.htos

rna_bcs <- colnames(pbmc.rna)
adt_bcs <- colnames(pbmc.adt)

names <- make.unique(HTO$row.names)
row.names(HTO) <- names
```

```
HTO <- HTO[,-1] # get rid of old names
HTO
```

The data from the RNA sequencing, ADT and HTO had different numbers of cell barcodes. I used the code below to ensure that the merged data containing all the different assays had equal number of the common cell barcodes between the 3 assays.

```
merged_bcs <- intersect(rna_bcs, names) %>%
  intersect(adt_bcs)

length(merged_bcs)

RNA <- pbmc.rna[, merged_bcs]
ADT <- as.matrix(pbmc.adt[, merged_bcs])
```

Later, after ensuring that the different assays had an equal number of cells, I then created a Seurat object and added the three assays

```
sobj <-  CreateSeuratObject(RNA,
                            min.cells = 5)
# Add CITE-seq data to Seurat object
sobj[["ADT"]] <-  CreateAssayObject(ADT)

#add it to QC HTO data Seurat object
sobj <- AddMetaData(sobj, metadata = HTO)

#Check to ensure the data is correct and add HTO names to the metadata
table
head(sobj@meta.data)
rownames(sobj@meta.data$HTO)
table(sobj$HTO_status)
features = sobj@meta.data$HTO
```

After I had created the Seurat object with all the data merged, I then checked the allocation of the HTO tags for each pool using the code below which generated **Figure 5-3**

```
# Create bar graphs comparing cell count for each sample
HTO_bars_1 <- sobj@meta.data %>%
  rownames_to_column("cell.id") %>%

  ggplot(aes(HTO_status, fill = HTO_status)) +
  geom_bar() +
  labs(y = "Cell Count") +
  cowplot::theme_cowplot() +
  theme(
```

```
    legend.position = "none",
    axis.title.x    = element_blank(),
    axis.text.x     = element_text(hjust = 1, angle = 45)
  )

# Create stacked bar graph showing fraction of cells
HTO_bars_2 <- sobj@meta.data %>%
  rownames_to_column("cell_id") %>%
  group_by(HTO) %>%
  dplyr::summarise(hash_frac = n() / nrow(.)) %>%

  ggplot(aes("HTO_status", hash_frac, fill = HTO)) +
  geom_bar(stat = "identity", color = "white", size = 0.5) +
  labs(y = "Fraction of Cells") +
  cowplot::theme_cowplot() +
  theme(
    legend.title = element_blank(),
    axis.title.x = element_blank(),
    axis.text.x  = element_blank(),
    axis.ticks.x = element_blank()
  )

# Combine plots
plot_grid(
  HTO_bars_1, HTO_bars_2,
  nrow        = 1,
  rel_widths  = c(0.5, 0.5),
  rel_heights = c(1, 0.8)
)
```

I then assessed the quality of the data by checking the gene expression data and the mitochondrial gene percentage, after which I filtered the data to remove cells with less than 250 detectable genes and greater than 3000 detectable genes (these could be two cells rather than single cells). Cells with a mitochondrial percentage of greater than 15% were and cells whose HTO classification were not singlets were also removed (Ilicic et al., 2016). The code below was used for filtering the cells,

```
# Add mitochondrial percentage to meta.data table
sobj <- sobj %>%
  PercentageFeatureSet(
    assay    = "RNA",
    pattern  = "^MT-",
    col.name = "percent_mito"
  )

plot1 <- FeatureScatter(object = sobj, feature1 = "nCount_RNA", feature2
=,"percent_mito")
```

```
plot2 <- FeatureScatter(object = sobj, feature1 = "nCount_RNA", feature2
=,"nFeature_RNA")
CombinePlots(plots = list(plot1, plot2))


# Filter cells based on HTO class, number of genes, and percent mito UMIs
filt_so1 <-  subset(sobj, nFeature_RNA > 250 &    # Remove cells with <
250 detected genes
                    nFeature_RNA < 3000 &   # Remove cells with > 3000
detected genes (could be doublets)
                     percent_mito < 15 &   # Remove cells with > 0.15
mito/total reads
                     HTO_status == "Singlet")
```

Once I had subset the data, I then renamed each of the assigned hashtags with the corresponding sample phenotype, ID and timepoint. The new labels were added to the metadata table using the code:

```
# Rename cell identities with sample names
filt_so1 <- filt_so1 %>%
  RenameIdents(
    "Hash2" = "FEB_18K21_C-1",
    "Hash7" = "CHR_18K39_C9",
    "Hash10" = "CHR_18K34_C14"
  )

# Add sample names to meta.data table
filt_so1 <- filt_so1 %>%
  AddMetaData(
    metadata = Idents(filt_so1),
    col.name = "sample"
  )
```

This entire process of Seurat analysis from adding and merging of the different sequenced data assays to the renaming of the hashtags with sample phenotype, ID and timepoint was repeated for each pool in Run 2 and Run 3. Next, I then merged the pools in their respective runs using the code,

```
RUN2 <- merge(filt_s1, y = c(filt_s2, filt_s3), add.cell.ids = c("SP_1",
"SP_2", "SP_3"), project = "RUN2")
RUN2

RUN3 <-  merge(filt_so1,  y  =  c(filt_so2,  filt_so3),  add.cell.ids  =
c("Pool1", "Pool2", "Pool3"), project = "RUN3")
RUN3
```

And subsequently merged the two runs into one Seurat object

```
CHMI_Data <- merge(RUN2, y=RUN3, add.cell.ids = c("RUN2", "RUN3"), project
= "CHMI",  merge.data = TRUE)
```

Once the data was all merged into one, I rearranged the sample IDs and added labels for the sample phenotype and timepoint to the metadata table for downstream analysis. This was done using the code:

```
filt<-CHMI_Data
filt$sample  <-  factor(x  =  filt$sample,  levels  =  c("CHR_18K27_C-1",
"CHR_18K27_C9",   "CHR_18K27_C14",     "CHR_18K34_C-1",   "CHR_18K34_C9",
"CHR_18K34_C14",
"CHR_18K39_C-1",   "CHR_18K39_C9",   "CHR_18K39_C14",     "FEB_18K14_C-1",
"FEB_18K14_C9", "FEB_18K14_C14",
"FEB_18K15_C-1",   "FEB_18K15_C9",   "FEB_18K15_C14",     "FEB_18K21_C-1",
"FEB_18K21_C9", "FEB_18K21_C14"))

filt <- filt %>%
  RenameIdents(
    "CHR_18K27_C-1" = "CHR_C-1",
"CHR_18K27_C9" = "CHR_C9",
"CHR_18K27_C14" = "CHR_C14",
"CHR_18K34_C-1"= "CHR_C-1",
"CHR_18K34_C9" ="CHR_C9",
"CHR_18K34_C14" = "CHR_C14",
"CHR_18K39_C-1" = "CHR_C-1",
"CHR_18K39_C9"= "CHR_C9",
"CHR_18K39_C14"= "CHR_C14",
"FEB_18K14_C-1" = "FEB_C-1",
"FEB_18K14_C9" = "FEB_C9",
"FEB_18K14_C14"= "FEB_C14",
"FEB_18K15_C-1"= "FEB_C-1",
"FEB_18K15_C9"= "FEB_C9",
"FEB_18K15_C14"= "FEB_C14",
"FEB_18K21_C-1"= "FEB_C-1",
"FEB_18K21_C9"= "FEB_C9",
"FEB_18K21_C14"= "FEB_C14")

filt <- filt %>%
  AddMetaData(
    metadata = Idents(filt),
    col.name = "timepoint")
```

After adding the labels, I proceeded to normalise the RNAseq and HTO data as well as identify the different cell clusters in the data using the following code which generated the **Figure 5-4**,

```
# Initialize the Seurat object with the raw (non-normalized data).
filt <- NormalizeData(filt)
filt <- FindVariableFeatures(filt)
filt <- ScaleData(filt)
filt <- RunPCA(filt, verbose = FALSE)
filt <- FindNeighbors(filt, dims = 1:30)
filt <- FindClusters(filt, resolution = 0.7, verbose = FALSE)
filt <- RunUMAP(filt, dims = 1:30)
DimPlot(filt, reduction = "umap",label = T)
DimPlot(filt, reduction = "umap", label = F, split.by ="timepoint",ncol=3
)
DimPlot(filt, reduction = "umap", label = F, split.by ="sample",ncol=3 )

# Normalize CITE-seq data
DefaultAssay(filt)<- "ADT"
DefaultAssay(filt)
filt <- NormalizeData(filt,normalization.method = "CLR")
ScaleData(filt,assay = "ADT", verbose = FALSE)

# Overlay antibody signal on gene expression UMAP
filt %>%
  FeaturePlot(
    reduction = "umap",
    features      =   c("adt_CD4-GCGATCCCTTGAGAT",   "CD4",   "adt_CD1c-
GAGCTACTTCACTCG", "CD1C")
  )
```

The identity of the different cell clusters was determined by running the code:

```
cluster0.markers <- FindMarkers(filt, ident.1 = 0, min.pct = 0.25,only.pos
= T)
head(cluster0.markers, n = 5)
```

The cell populations which I was interested in analysing were, monocytes (CD14 and CD16), CD4 T cells (memory, naïve and T regs), dendritic cells and plasmacytoid dendritic cells (pDCs). I proceeded to subset these cells from the entire dataset and repeating the data normalisation by running the following code, (which generated the **Figure 5-5**)

```
Combined.filt <- subset(filt, idents = c("0","1","2","4", "7","12", "16",
"15", "17", "21"), invert = FALSE)
DimPlot(Combined.filt, reduction = "umap", label = F)
DimPlot(Combined.filt, reduction = "umap", split.by = "timepoint", ncol =
3) + NoLegend()
```

```
DimPlot(Combined.filt, reduction = "umap", split.by = "sample", ncol = 3)
+ NoLegend()
DefaultAssay(object = Combined.filt) <- "RNA"

# Run the standard workflow for visualization and clustering
Combined.filt <- ScaleData(object = Combined.filt, verbose = FALSE)
Combined.filt <- RunPCA(object = Combined.filt, verbose = FALSE)
# Umap and Clustering
ElbowPlot(object = Combined.filt, ndims = 50)
Combined.filt <- RunUMAP(object = Combined.filt, reduction = "pca", dims
= 1:30)
Combined.filt <- FindNeighbors(object = Combined.filt, reduction = "pca",
dims = 1:30)
Combined.filt <- FindClusters(Combined.filt, resolution = 0.27)
DimPlot(Combined.filt, reduction = "umap", label = F) + NoLegend()
DimPlot(Combined.filt, reduction = "umap", split.by ="sample",ncol=3)
```

Once the subset data was normalised and clustered, I identified the key molecular markers that were unique to each cluster and proceeded to rename the clusters and check the cell frequencies in each cluster using the following code (which was used to generate the **Figure 5-6**)

```
Combined.filt <- RenameIdents(Combined.filt, `0` = "CD4 Memory", `1` =
"CD4 Naive", `2` = "CD14 Mono", `3` = "CD14 Mono_1", `4` = "CD16 Mono",
`5` = "CD16 CTL", `6` = "T Regs", `7` = "pDCs", `8` = "DCs", `9` = "CD8
CTL", `10` = "B Cells", `11` = "tol moDCs")

DimPlot(Combined.filt, reduction = "umap", label = T)
DimPlot(Combined.filt, reduction = "umap", split.by ="sample",ncol=3 )

Idents(Combined.filt) <- factor(Idents(Combined.filt), levels = c("CD4
Memory", "CD4 Naive", "CD14 Mono", "CD14 Mono_1", "CD16 Mono", "CD16
CTL","T Regs","pDCs", "DCs","CD8 CTL", "B Cells","tol moDCs"))

Idents(Combined.filt) <- factor(Idents(Combined.filt), levels = c("CD4
Memory", "CD4 Naive", "CD14 Mono", "CD14 Mono_1", "CD16 Mono", "CD16
CTL","T Regs","pDCs", "DCs","CD8 CTL", "B Cells","tol moDCs"))

Idents(Combined.filt)
Combined.filt$group           <-           paste(Idents(Combined.filt),
Combined.filt$timepoint, sep = "_")
Combined.filt$celltype <- Idents(Combined.filt)


Combined.filt <-SetIdent(Combined.filt, value = "celltype")

p1 <- VlnPlot(Combined.filt, features = c("CCR7", "SELL", "IL7R",
"CD14","FCGR3A", "NKG7"),pt.size = 0, combine = T, ncol = 3) +
ggplot2::theme(legend.position = "none")
```

```
p2<-    VlnPlot(Combined.filt,    features    =    c("GZMB",    "FOXP3",
"CLEC4C","NRP1","CD1C","CD8A", "MS4A1","SOCS1"),
              pt.size    =    0,    combine    =    T,    ncol    =    4)    +
ggplot2::theme(legend.position = "none")

p1
p2

Combined.filt <-SetIdent(Combined.filt, value = "timepoint")
freq_table_sample        <-        as.data.frame(prop.table(x        =
table(Idents(Ctype),margin=2))

ggplot(data=freq_table_sample,  aes(x=Var2,  y=Freq,  fill  =  Var1))  +
geom_bar(stat="identity",        color="black",        position        =
position_dodge2(reverse  =  FALSE))  +  labs(x="Cluster",  y="Percentage  of
Total Cells", fill="Sample", title = "Cell Frequencies")  +
  scale_x_discrete(limits = levels(freq_table_sample$Var2)) +
  scale_y_continuous(labels =  scales::percent)+
  theme(plot.title = element_text(hjust = 0.5))

Combined.filt <-SetIdent(Combined.filt, value = "celltype")
n_cells <- FetchData(Combined.filt,
                     vars = c("ident", "timepoint")) %>%
  dplyr::count(ident, timepoint) %>%
  tidyr::spread(ident, n)

View(n_cells)
write.xlsx   (n_cells,   file   =   "n_cells.xlsx",   append   =   FALSE,
row.names=TRUE, col.names=TRUE)
```

I then proceeded to check the differential expressed genes in the cells from febrile participants by comparing each timepoint, that is day C14 and day C9, to day C-1 (baseline). This comparison was also done in the cells from chronic patients. Differential expressed genes analysis was also done between the febrile and chronic participants but between corresponding timepoints. The following code was used for generating differentially expressed genes,

```
Idents(Combined.filt) <- "group"
c_9vsc_1_CD14<- FindMarkers(Combined.filt, ident.1 = "CD14 Mono_CHR_C9",
ident.2 = "CD14 Mono_FEB_C-1", test.use = "MAST", verbose = FALSE, min.pct
= 0.25)
c_9vsc_1_CD14$genes <- row.names(c_9vsc_1_CD14)
head(c_9vsc_1_CD14, n = 25)
```

For visualisation of the differentially expressed genes using volcano plots, I set the fold change (FC) limit to 0.3 and the adjusted p value to 10e-3 (0.01) and used the code below to generate **Figure 5-8 to Figure 5-20**,

```
FC <- 0.3
p <- 10e-3
res2<- c_9vsc_1_CD14
keyvals <- rep('grey75', nrow(res2))
names(keyvals) <- rep('NS', nrow(res2))

keyvals[which(abs(res2$avg_log2FC) > FC & res2$p_val_adj > p)] <- 'grey50'
names(keyvals)[which(abs(res2$avg_log2FC) > FC & res2$p_val_adj > p)] <-
'log2FoldChange'

keyvals[which(abs(res2$avg_log2FC) < FC & res2$p_val_adj < p)] <- 'grey25'
names(keyvals)[which(abs(res2$avg_log2FC)  < FC & res2$p_val_adj < p)] <-
'-Log10Q'

keyvals[which(res2$avg_log2FC < -FC & res2$p_val_adj< p)] <- 'blue2'
names(keyvals)[which(res2$avg_log2FC   < -FC & res2$p_val_adj < p)] <-
'Signif.down-regulated'

keyvals[which(res2$avg_log2FC > FC & res2$p_val_adj < p)] <- 'red2'
names(keyvals)[which(res2$avg_log2FC  >  FC  &  res2$p_val_adj  <  p)]  <-
'Signif.up-regulated'

unique(keyvals)
unique(names(keyvals))


EnhancedVolcano(c_9vsc_1_CD14,
                lab = c_9vsc_1_CD14[,'genes'],
                x = 'avg_log2FC',
                y = 'p_val_adj',
                xlim = c(-6.5,6.5),
                xlab = bquote(~Log[2]~ 'fold change'),
                ylab = bquote(~-Log[10] ~ italic(P)),
                title = 'Differentially Expressed Genes moDC_2',
                subtitle = NULL,
                pCutoff = 10e-3,
                FCcutoff = 0.3,
                pointSize = 2.5,
                labSize = 4,
                colCustom = keyvals,
                colAlpha = 0.75,
                legendPosition = 'right',
                legendLabSize = 15,
                legendIconSize = 5.0,
                max.overlap = 20,
                drawConnectors = T,
                widthConnectors = 0.5,
                colConnectors = 'grey50',
                gridlines.major = TRUE,
                gridlines.minor = FALSE,
                border = 'partial',
                borderWidth = 1.5,
                borderColour = 'black')
```

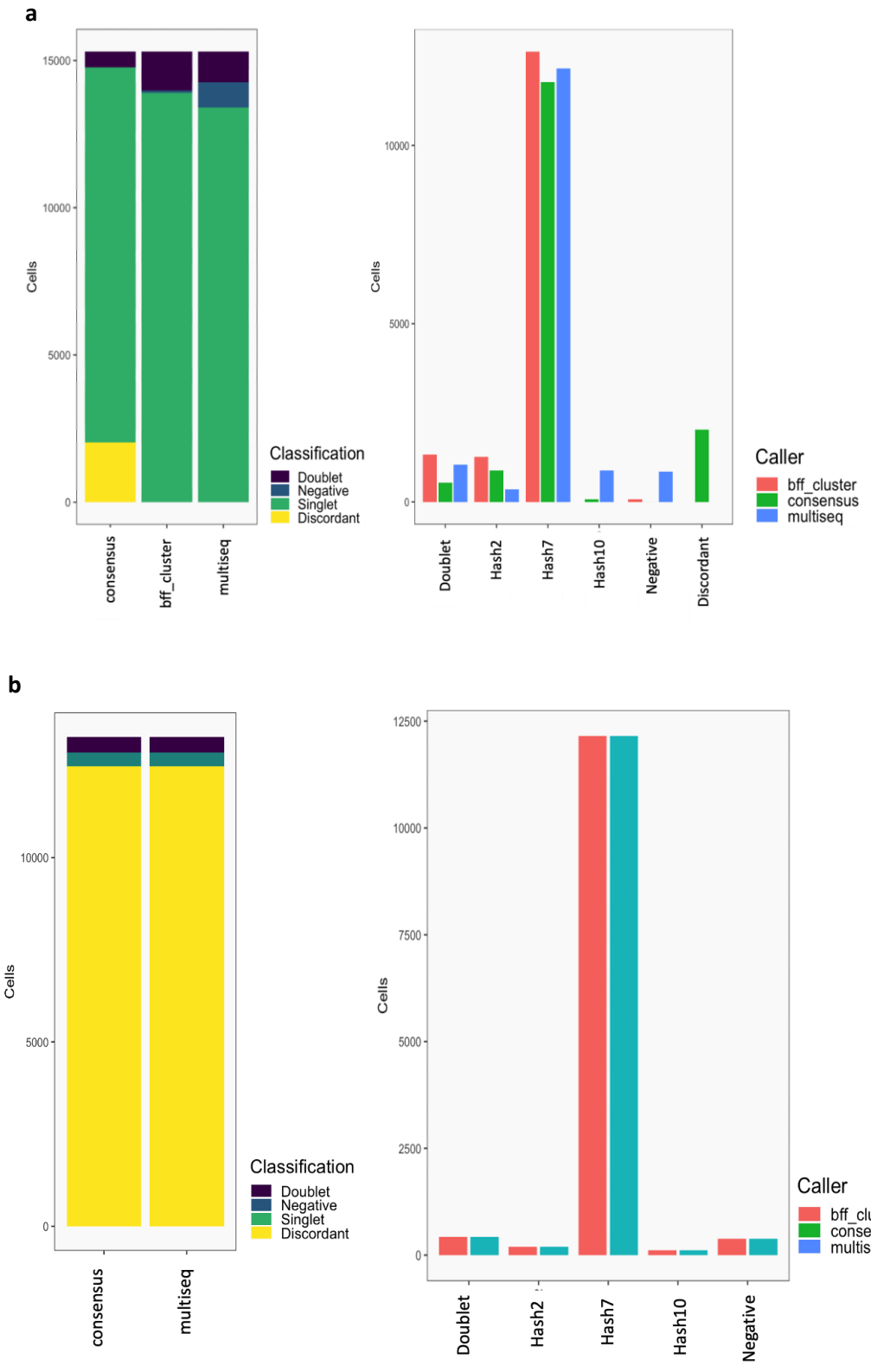
A gene list of the upregulated and downregulated genes was also generated for each comparison using the code:

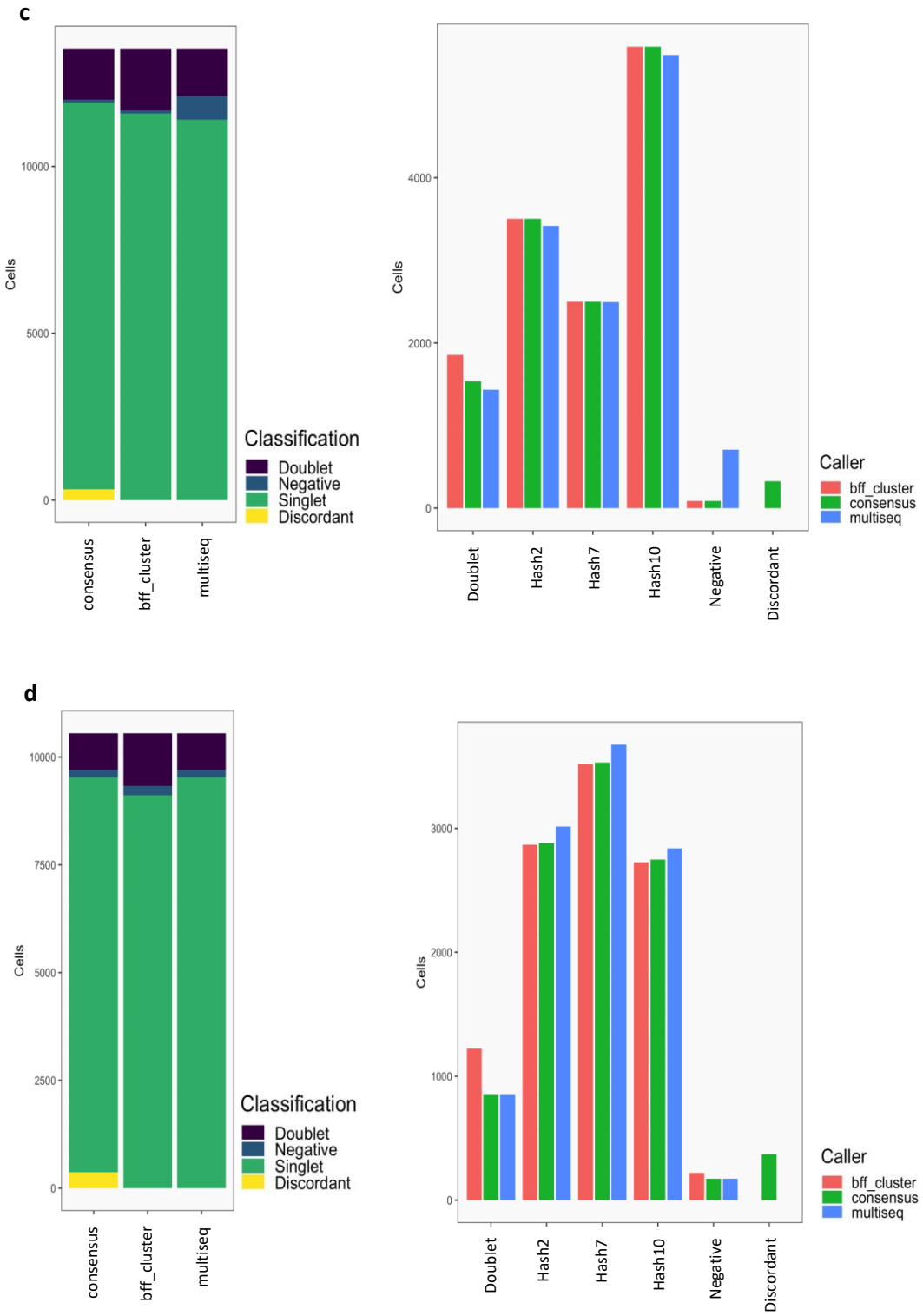
```
CD14Mono_D9DownReg<-subset(res2,res2$avg_log2FC < -0.3 & res2$p_val_adj < 0.01)
CD14Mono_D9Upregulated<-subset(res2,res2$avg_log2FC> 0.3 & res2$p_val_adj < 0.01)
```

The generated lists of both upregulated and downregulated genes were uploaded on the STRING website (https://string-db.org/) to construct a protein-protein interaction network that was used for gene ontology and pathway enrichment analysis. Each differentially expressed gene that was upregulated for the different cell types were subjected to the STRING protein-protein interaction network, gene ontology and pathway enrichment analysis. For example, CD14 monocytes from febrile participants, the enriched gene ontology (biological processes) terms for upregulated genes at day 9 post infection included mitochondria ATP synthesis (MT-CO1, MT-CO2, MT-CYB, MT-ND4L, MT-ND5), positive regulation of transcription (JUN, FOSB, KLF2, KLF4, KLF6, ETV6, CIITA, NR4A2), positive and negative regulation of NFKB signalling. CD16 monocytes from febrile participants, the enriched gene ontology terms for upregulated genes at day 9 post included negative regulation of ERK1/2 and MAPK signalling pathways (DUSP1, DUSP6), and initiation of transcription (FOS, FOSB, IER2, JUN, and KLF4). For CD4 naïve and memory T cells from febrile participants, the enriched gene ontology terms for upregulated genes at day 14 post infection were interferon-gamma-mediated signalling pathway (IRF1, STAT1 GBP1, GBP2, IFITM2, PSMB8, PSMB9, PSMB10, BST2, XAF1, SOCS3), antigen processing and presentation receptor (PSMB8, PSMB9, PSMB10, PSME1, PSME2, HLA-B, TAPBP, B2M) and signalling pathway via jak-stat. These enriched pathways were then used as a guide to develop the various biorender images.

5.3.4 HTO Data Quality Check

The HTO sequence data was checked and aligned to their respective cells using the cellhashR package in R software (as described in **section 5.3.1.1**). Each HTO was aligned with its respective cells using a consensus of two algorithms (bff_cluster and multiseq). **Figure 5-2** shows the overall alignment for Run 2 and Run 3 and **Figure 5-3** shows the total number of cells per pool that were aligned to at least one hashtag (singlets).


a
15000
10000
5000
0
Cells
consensus
bff_cluster
multiseq
Classification
Doublet
Negative
Singlet
Discordant
10000
5000
0
Cells
Doublet
Hash2
Hash7
Hash10
Negative
Discordant
Caller
bff_cluster
consensus
multiseq
b
10000
5000
0
Cells
consensus
multiseq
Classification
Doublet
Negative
Singlet
Discordant
12500
10000
7500
5000
2500
0
Cells
Doublet
Hash2
Hash7
Hash10
Negative
Caller
bff_cluster
consensus
multiseq


c
Cells
10000
5000
0
consensus
bff_cluster
multiseq
Classification
Doublet
Negative
Singlet
Discordant
Cells
4000
2000
0
Doublet
Hash2
Hash7
Hash10
Negative
Discordant
Caller
bff_cluster
consensus
multiseq
d
Cells
10000
7500
5000
2500
0
consensus
bff_cluster
multiseq
Classification
Doublet
Negative
Singlet
Discordant
Cells
3000
2000
1000
0
Doublet
Hash2
Hash7
Hash10
Negative
Discordant
Caller
bff_cluster
consensus
multiseq

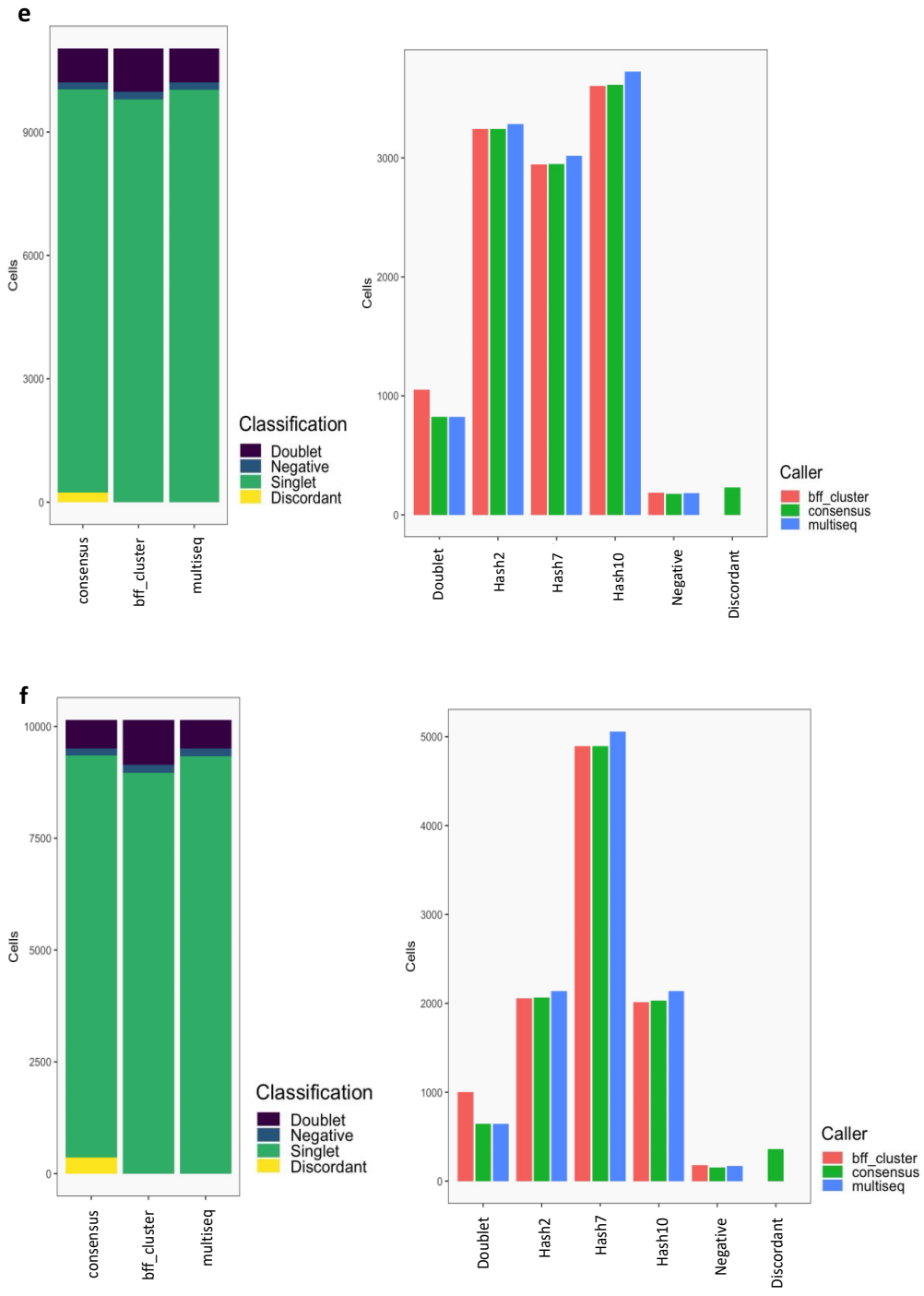
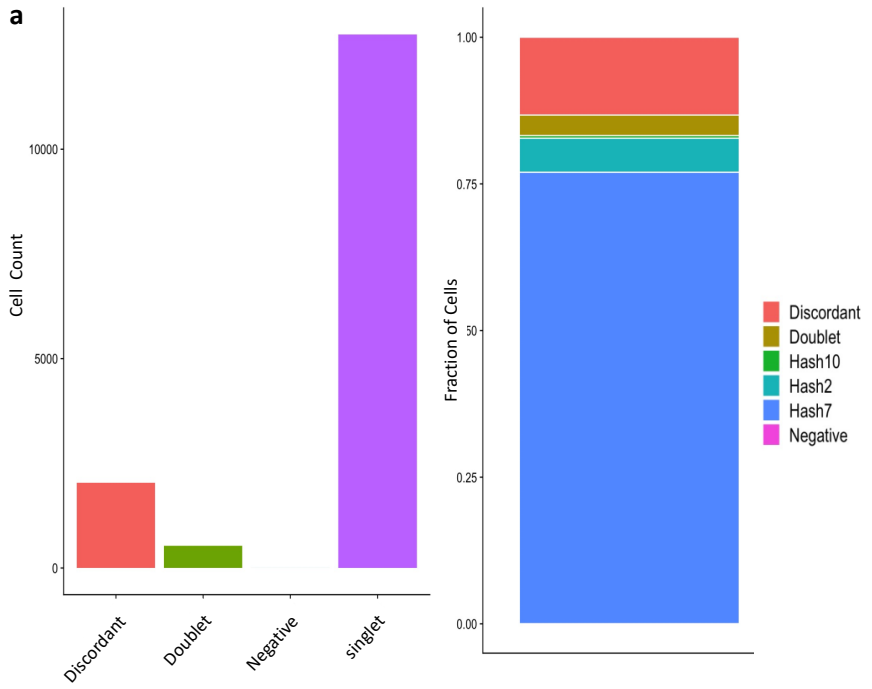
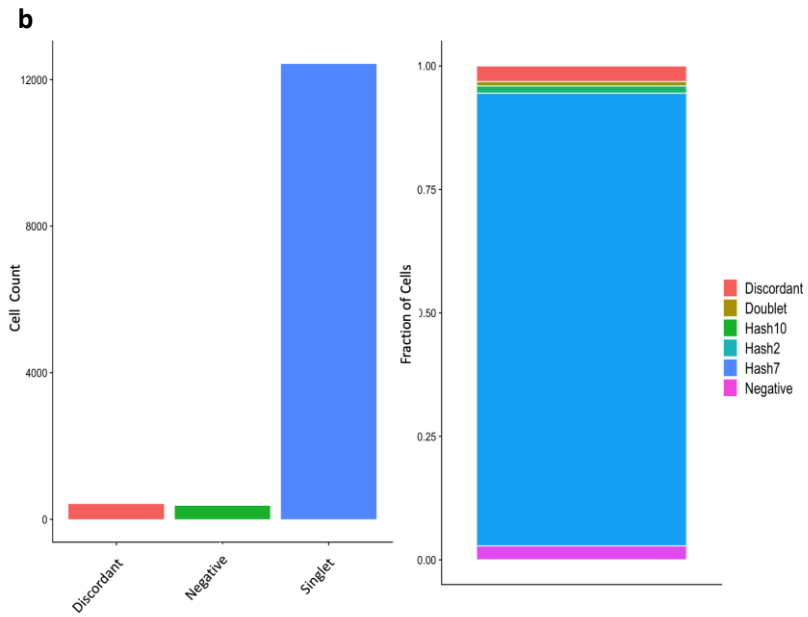


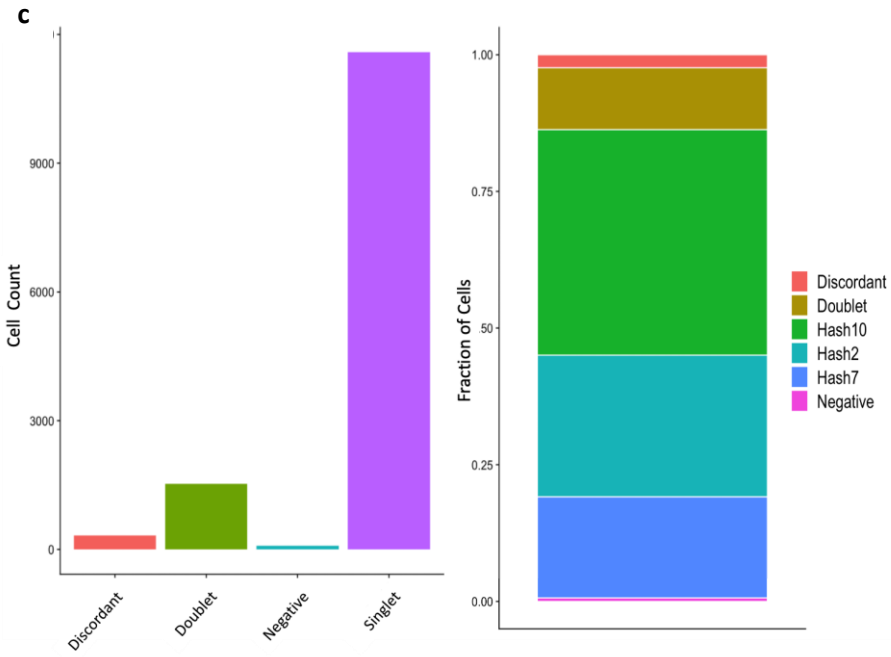
Figure 5-2 Alignment of HTO tags to cells.

Cells were aligned using a consensus of two algorithms bff_cluster and multiseq. The graphs show alignment of each hashtag for each algorithm. The cells were classified as singlets i.e., cells that contained only one of the hashtags (Hash2, Hash7 or Hash10), doublets i.e., cells that contained two or more hashtags, negative i.e. cells that lacked a hashtag and discordant i.e. cells that were matched to different hashtags by the two algorithms. **Figures a-c** shows the alignment for Run 2 pools 1, 2 and 3 respective while **figures d-f** shows the alignment for Run 3 pool 1, 2 and 3 respectively

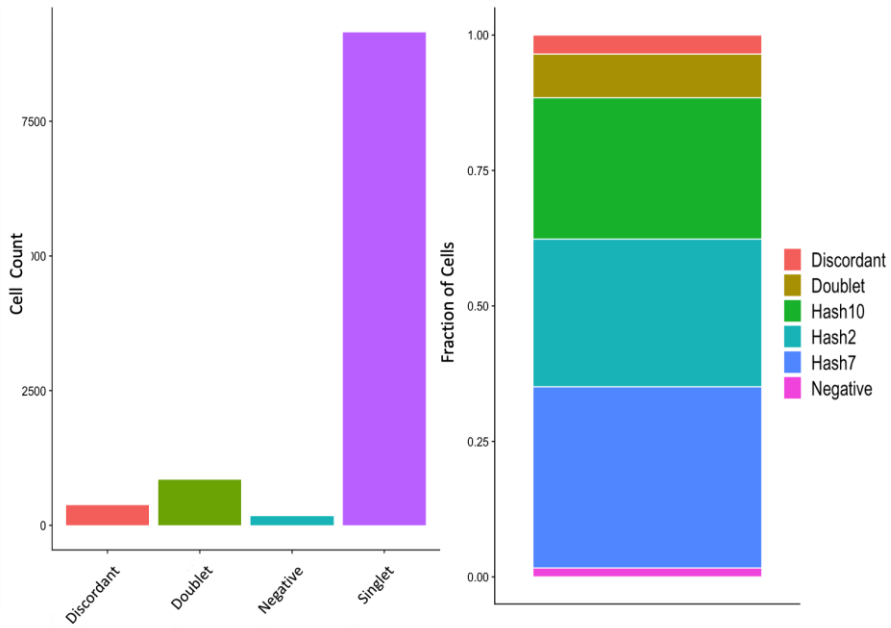

a
Cell Count
10000
5000
0
Discordant
Doublet
Negative
singlet
Fraction of Cells
1.00
0.75
50
0.25
0.00
Discordant
Doublet
Hash10
Hash2
Hash7
Negative


b
Cell Count
12000
8000
4000
0
Discordant
Negative
Singlet
Fraction of Cells
1.00
0.75
.50
0.25
0.00
Discordant
Doublet
Hash10
Hash2
Hash7
Negative

c


Cell Count
9000
6000
3000
0
Discordant
Doublet
Negative
Singlet
Fraction of Cells
1.00
0.75
.50
0.25
0.00
Discordant
Doublet
Hash10
Hash2
Hash7
Negative

d


Cell Count
7500
2500
0
Discordant
Doublet
Negative
Singlet
Fraction of Cells
1.00
0.75
0.50
0.25
0.00
Discordant
Doublet
Hash10
Hash2
Hash7
Negative

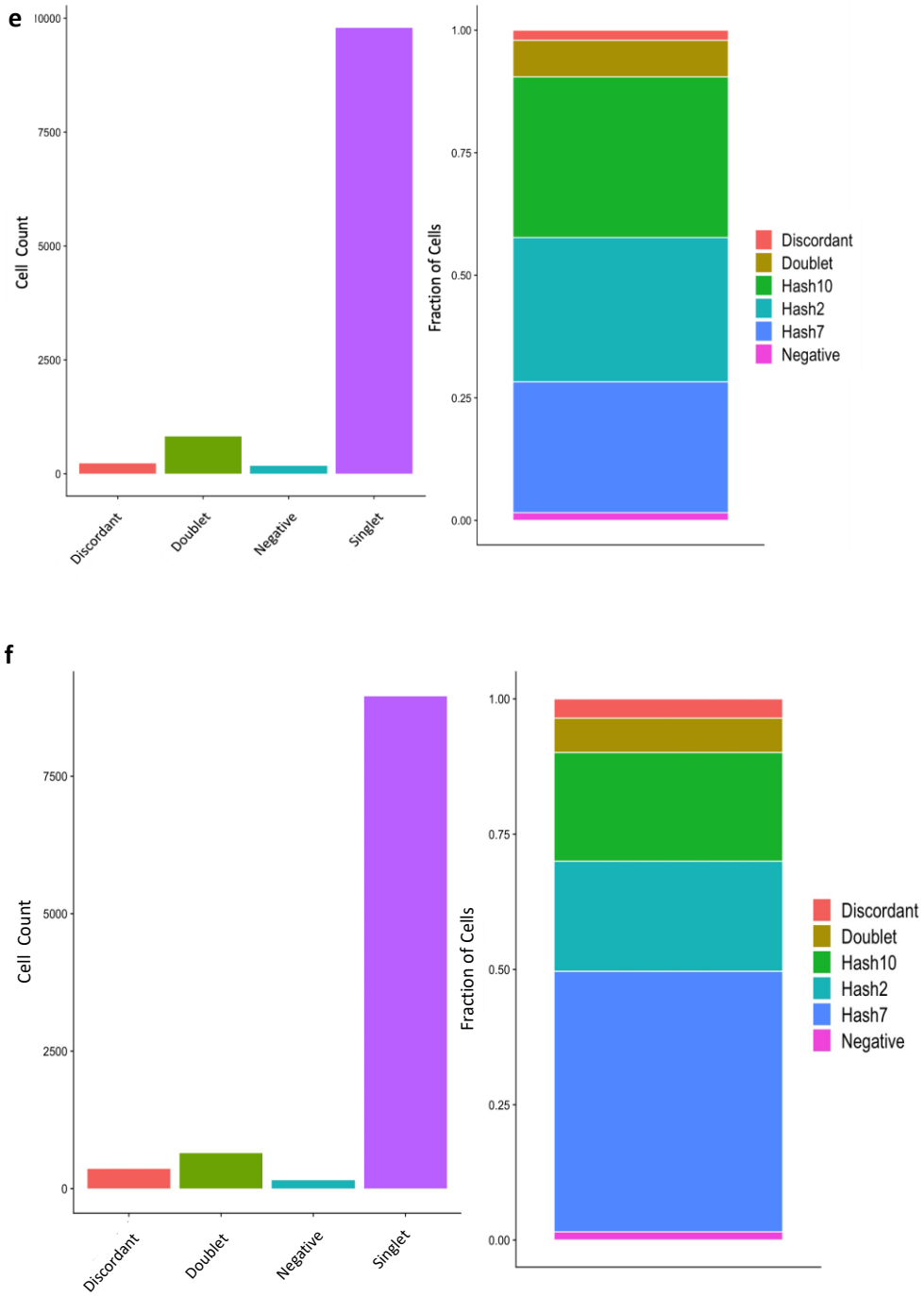


Figure 5-3 Distribution of singlets per pool

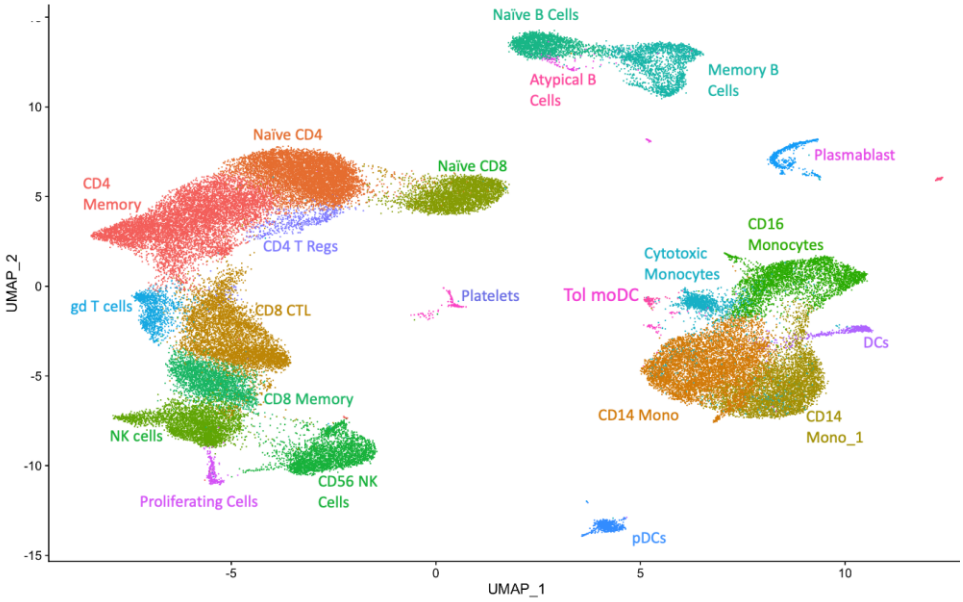
Cells with only a single hashtag were selected for further downstream analysis of the sequenced CHMI samples. The graphs show the cell number of singlets (cells that had only one hashtag) and the distribution of the various hashtags within each pool**. Figure a-c** represent Run 2 pools 1, 2, and 3 respectively while **figures d-f** represent Run 3 pools 1, 2 and 3 respectively.

5.4 Results

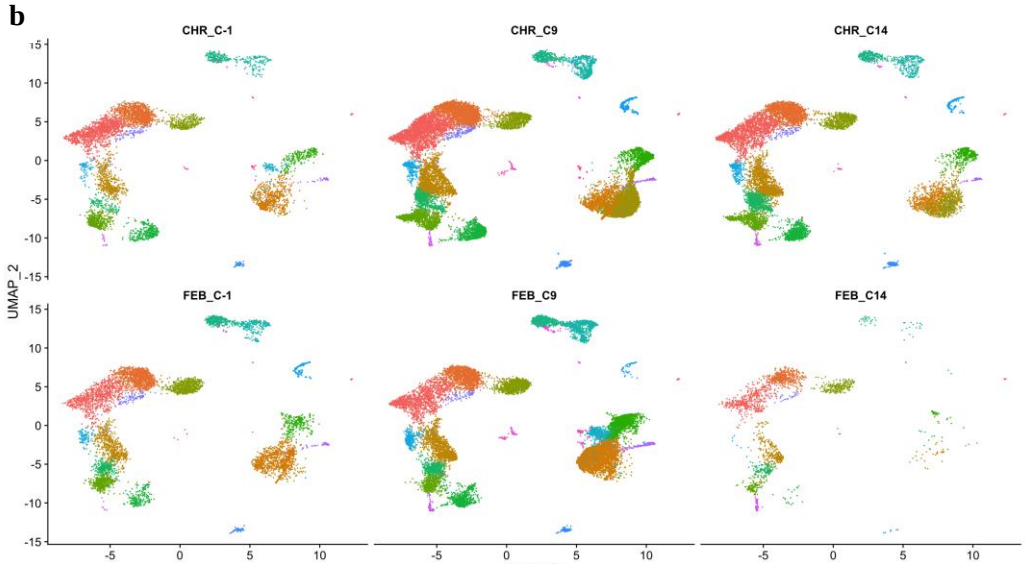
5.4.1 Identification of Cell Clusters and Cell Distribution Between Samples

Once I had aligned the HTO data with the corresponding samples and the quality of the data was confirmed, the cells with a single HTO were selected for further downstream analysis as described in **section 5.3.1.2**. **Figure 5-4** below shows the different cell populations that could be identified from the sequenced data and how they were distributed per phenotype, timepoint and sample ID.

a



b



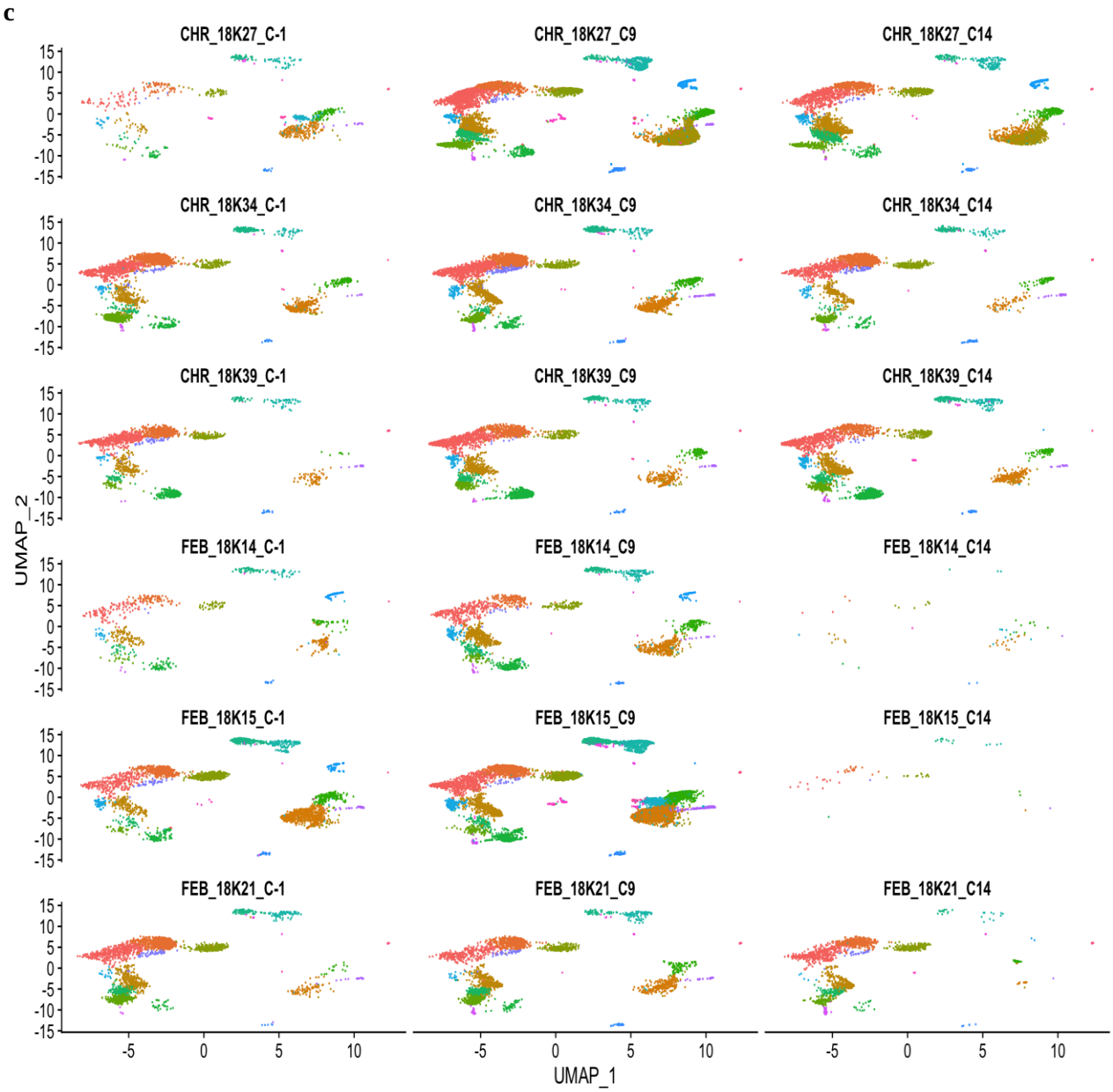


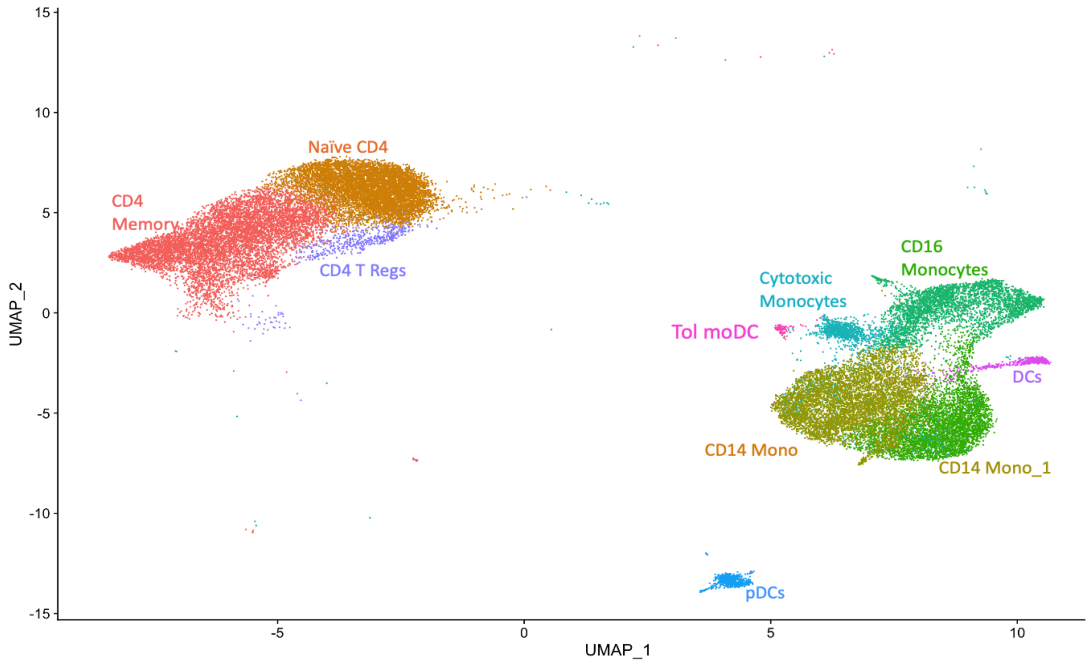
Figure 5-4 Identification of cell cluster and distribution of cells across the different samples in scRNA sequenced data.

The entire sequenced data was processed in R. **Figure a** shows the cell populations identified in the data while **figure b** shows the distribution of the samples according to phenotype and timepoint and **figure c** shows the distribution of the cells according to the phenotype, patient sample IDs, and timepoint. FEB represents the febrile participants, CHR represents the chronic participants.

5.4.2 Sub-setting of the Data Set to Focus on the Population of Interest

The sequenced data generated from the CHMI samples showed the different populations identified in the PBMC samples and so I decided to focus on four key populations that play a role in determining the immune response during a CHMI infection (Sun et al., 2019). These populations were CD4 T cells (Naïve CD4, Memory CD4 and CD4 T regs), monocytes (CD14 and CD16 monocytes), classical dendritic cells (DCs) and plasmacytoid dendritic cells (pDCs). The cells from the population of interest were selected and split from original data, shown by **Figure 5-5 a** (described in **section 5.3.1.2**). The data was then reanalysed to generate **Figure 5-5 b.**

a



b

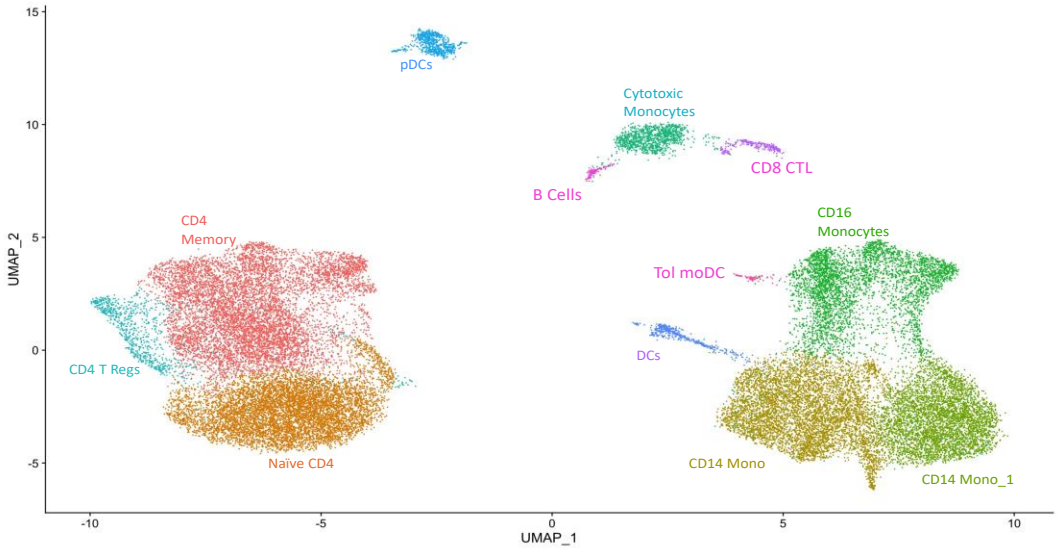


Figure 5-5 Sub-setting data from original data set.

The cells from the populations of interest, which were CD4 T cells, Monocytes, Plasmacytoid Dendritic cells, Dendritic cells were selected and separated from the original data set in **Figure 5-4 a**. and was reanalysed to generate the **Figure 5-5 b**

5.4.3 Markers Used to Identify Cell Types

Once I had identified the populations of interest, I then checked for the unique markers that were associated with each population as shown in **Figure 5-6**

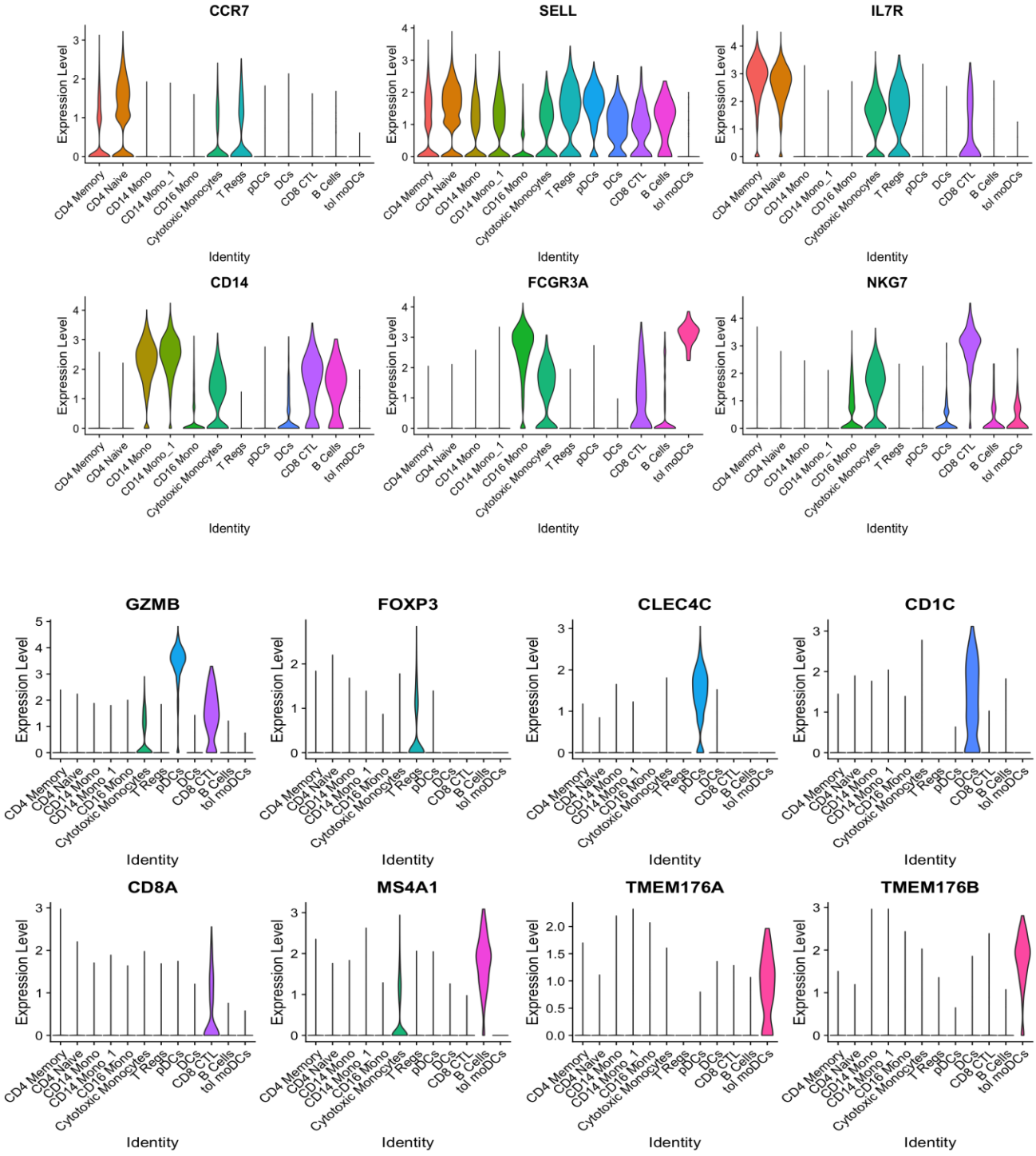


Figure 5-6 Markers used for identifying each population

Markers unique to each cell population were checked to ensure that the cell populations were the correctly labelled.

5.4.4 Cell Frequencies for each Cell Population

Once I had ensured that the cells were correctly labelled, I proceeded to check on the distribution of the cells in each population for the chronic and febrile participants at the different timepoints. It was clear that at day C9, there was an increase in the cell numbers for both patient phenotypes apart from tolerised monocyte derived dendritic cells (tol moDCs), which were unique to the febrile group with cell numbers particularly high at day C14.

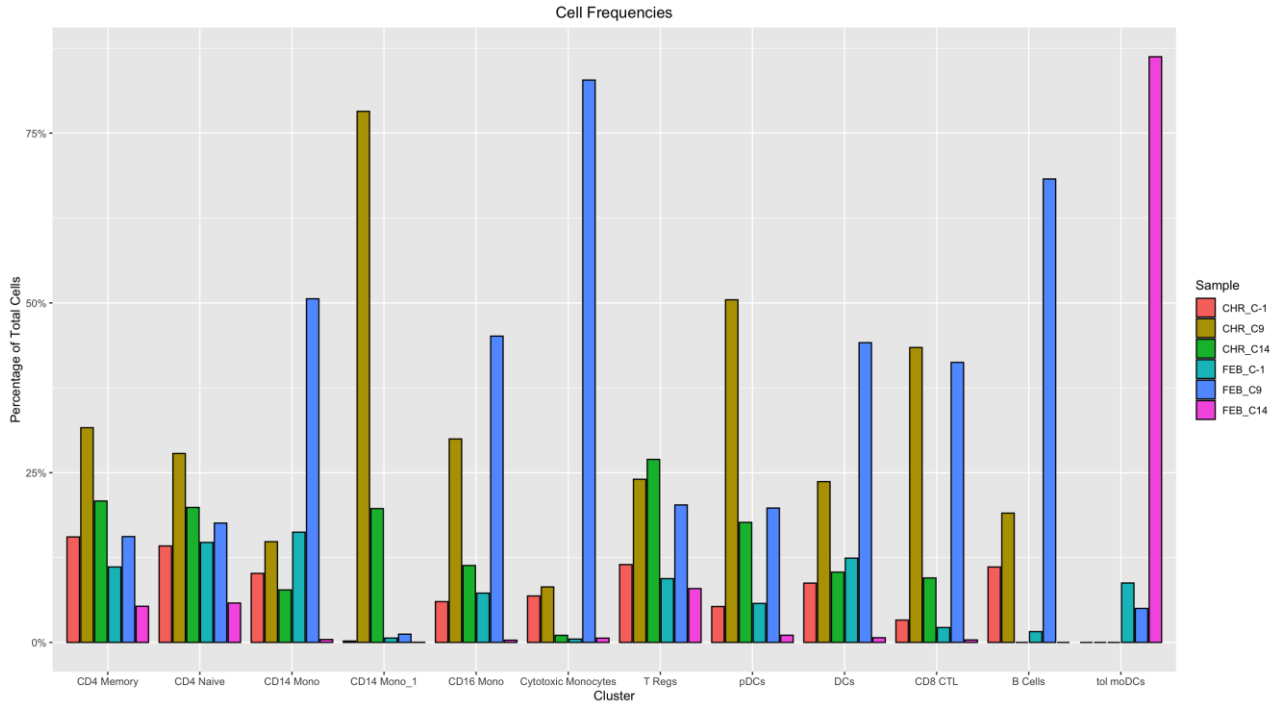


Figure 5-7 Cell numbers in the different cell populations

The figure shows the percentage of cells in each cell population for each phenotype at the different timepoints. FEB represents the febrile participants, CHR represents the chronic participants.

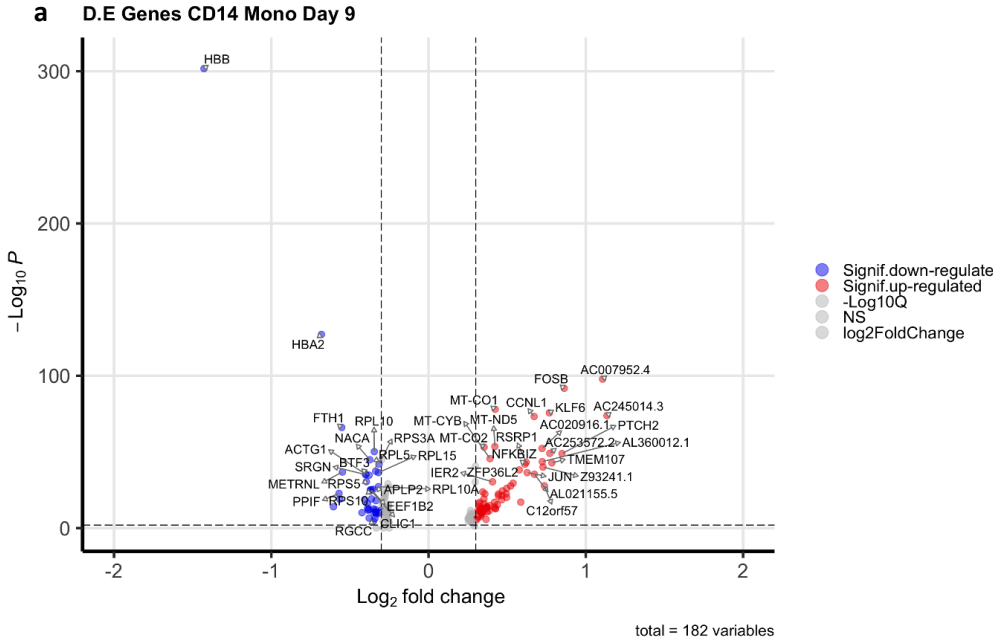
5.4.5 Differentially Expressed Genes

To understand the cellular changes that occur over the three timepoints in my cell populations of interest, I checked the differential expressed genes in each cell population for each phenotype compared with the pre-infection baseline (day C-1). I also checked the differentially expressed genes between the phenotypes at each timepoint to understand how the cells from the different phenotypes were responding to malaria infection.

5.4.6 Differential Expressed Genes in Febrile Participants

5.4.6.1 CD14 Monocytes

In CD14 monocytes the genes that were upregulated at day C9 compared to baseline (day C-1) in febrile participants were AP-1 gene, which is a dimer of FOS and JUN gene, a number of inhibitors of NF-kappa-beta signalling and transcription activity such as NFKBIZ (NF-kappa-B inhibitor zeta), KLF2 (Kruppel-like factor 2), and TNFAIP3 (Tumor necrosis factor alpha-induced protein 3). TSC22D3 (Glucocorticoid-Induced Leucine Zipper) was also upregulated, and this inhibits both the dimeric transcription factor AP-1 (a dimer of FOS and JUN genes) and NF-kappa-Beta signalling. Also, a number of mitochondrial genes and genes involved in RNA splicing were upregulated, **Figure 5-8a**.



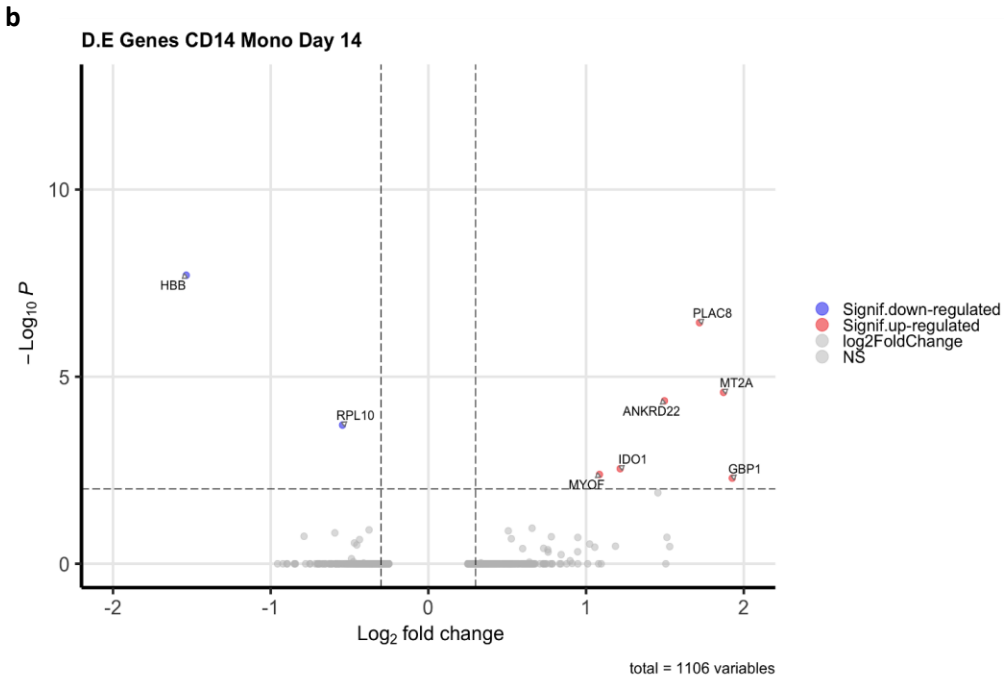


Figure 5-8 Differentially expressed genes in CD14 monocytes from febrile participants in response to challenge infection.

The differentially expressed genes in monocytes at day C9 (**a**) and day C14 (**b**) were compared to the baseline, day C-1.

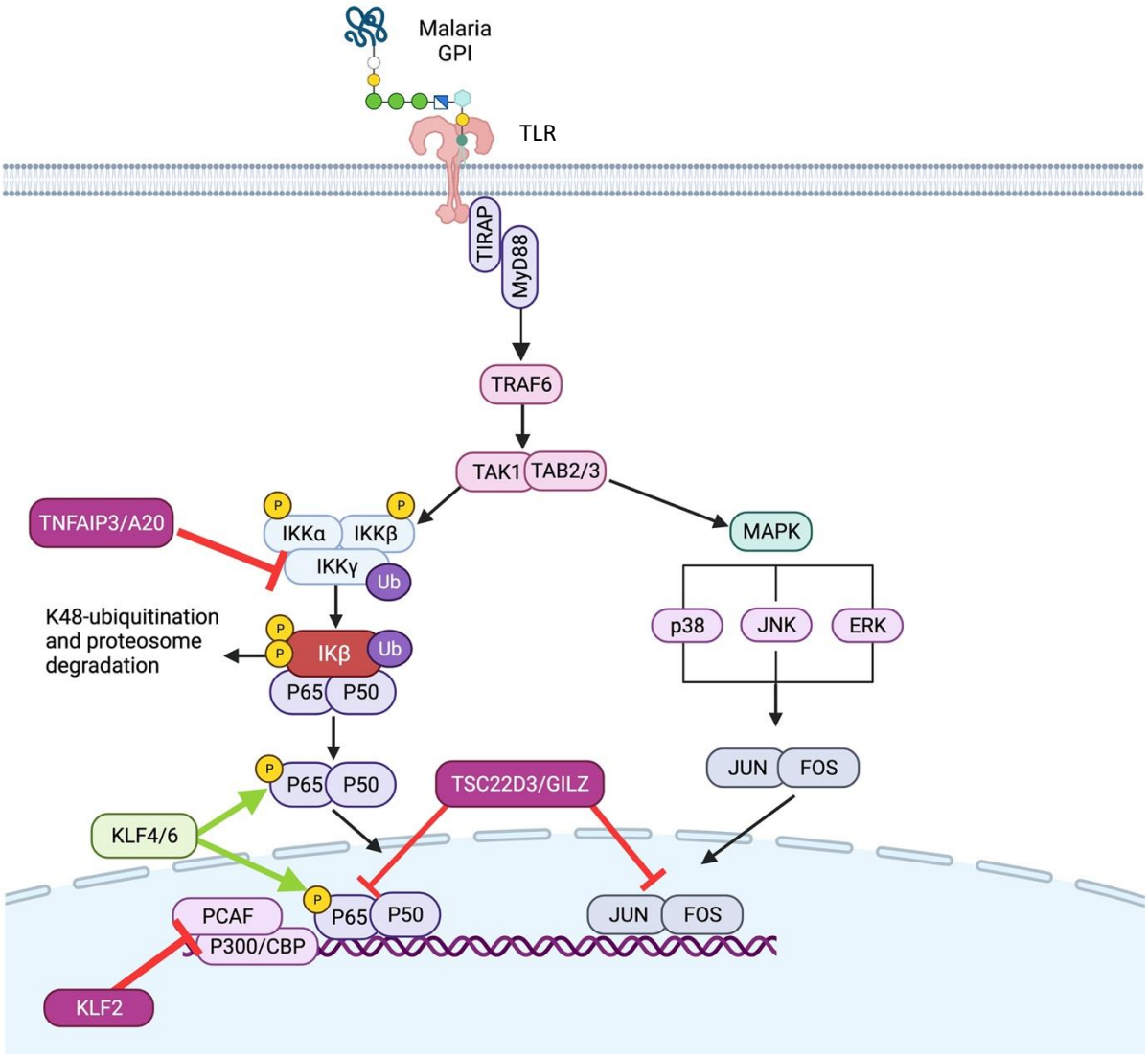


Figure 5-9 Expression of genes associated with TLR signalling in CD14 monocytes at day 9 after infection.

Malaria glycosylphosphatidylinositol (GPI) and other parasite PAMPs could possibly be recognized via Toll like receptor 2 or 4 (TLR2/4), which initiated signalling through myeloid differentiation primary response 88 (MyD88), tumor necrosis factor receptor (TNFR) associated factor 6 (TRAF6), transforming growth factor-β-activated kinase 1 (TAK1) and transforming growth factor-β-activated kinase-binding proteins (TAB2/3). This signalling cascade could lead to the dimeration and activation of JUN and FOS genes and possibly activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ). NF-κβ signaling was enhanced by Kruppel-like factor 4 and 6 (KLF4/6) but was inhibited by KLF2, tumor necrosis factor alpha-induced protein 3 (TNFAIP3) and glucocorticoid-Induced Leucine Zipper (TSC22D3). Figure created with Biorender.com

At the same timepoint, most of the downregulated genes were ribosomal genes that are involved in the initiation of translation and signalling pathways that are key in cell survival and differentiation of CD14 monocytes such as the CD63-TIMP1 signalling axis (Li et al., 1999, Liu et al., 2003, Liu et al., 2005, Jung et al., 2006). and the transcription factors MAFB, CEBPB (Huber et al., 2012, Tamura et al., 2017, Kelly et al., 2000) (**Figure 5-8a)**.

At day C14 compared to the baseline, day C-1, monocytes upregulated IDO1 (Stone and Darlington, 2002) and GBP1 (Capaldo et al., 2012, Guenzi et al., 2001, Bleiziffer et al., 2012) which induce tolerance and impedes the activation of CD4 T cells (**Figure 5-8b)**.

5.4.6.2 CD16 Monocytes

CD16 monocytes at day C9 compared to baseline (day C-1) upregulated AP-1 gene, a dimer of FOS and JUN genes, as well as genes that are known to inhibit MAPK signalling namely, Dual-specificity phosphatases (DUSP1 and DUSP6) (Grumont et al., 1996). The inflammatory chemokine receptor CX3CR1 was also upregulated (**Figure 5-10)**.

The downregulated genes at day C9 included genes involved in mitochondrial energy production, NFKBIA (NF-kappa-B inhibitor alpha), CD83 and genes involved in cell proliferation namely EMP3 and TIMP1 (**Figure 5-10)**.

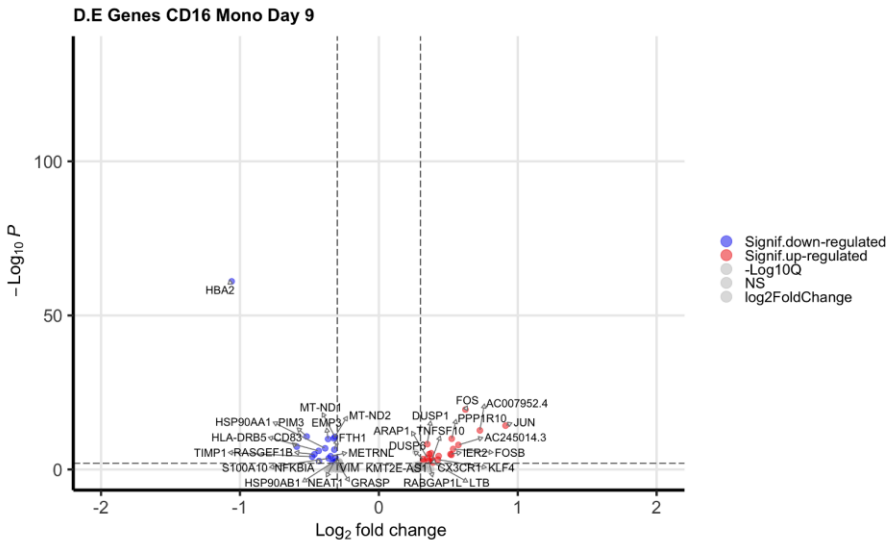


Figure 5-10 Differentially expressed genes in CD16 monocytes.

The differentially expressed gene in monocytes at day C9 compared to the baseline day C-1

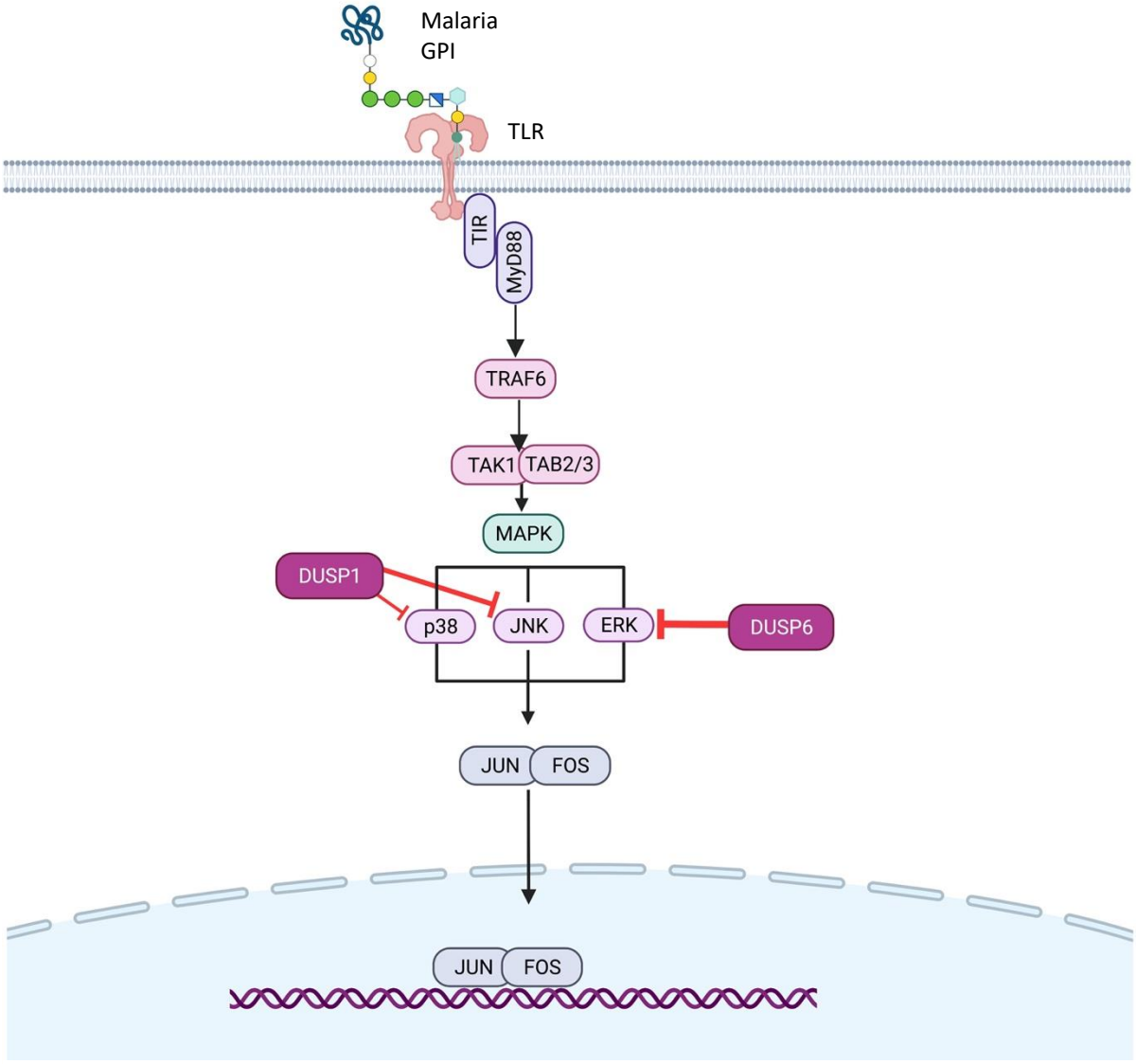


Figure 5-11 Expression of genes involved with TLR signalling in CD16 monocyte at day 9 post infection. Malaria glycosylphosphatidylinositol (GPI) and other parasite PAMPs could possibly be recognized via Toll like receptor 2 or 4 (TLR2/4), and initiate signalling through myeloid differentiation primary response 88 (MyD88), tumor necrosis factor receptor (TNFR)associated factor 6 (TRAF6) ,transforming growth factor-β-activated kinase 1 (TAK1) and transforming growth factor-β-activated kinase-binding proteins (TAB2/3). This signalling cascade could lead to the dimeration and activation of JUN and FOS genes (AP-1). Dual-specificity phosphatases 1 and 6 (DUSP1 and DUSP6) upregulation . Figure created with Biorender.com

5.4.6.3 Naïve CD4 T Cells

In naïve CD4 T cells the upregulated genes at day C9 compared to baseline (day C-1) included IFITM1 (an IFN response gene) and the transcription factor STAT1, while the chemokine receptor CXCR4 was downregulated (**Figure 5-12a)**.

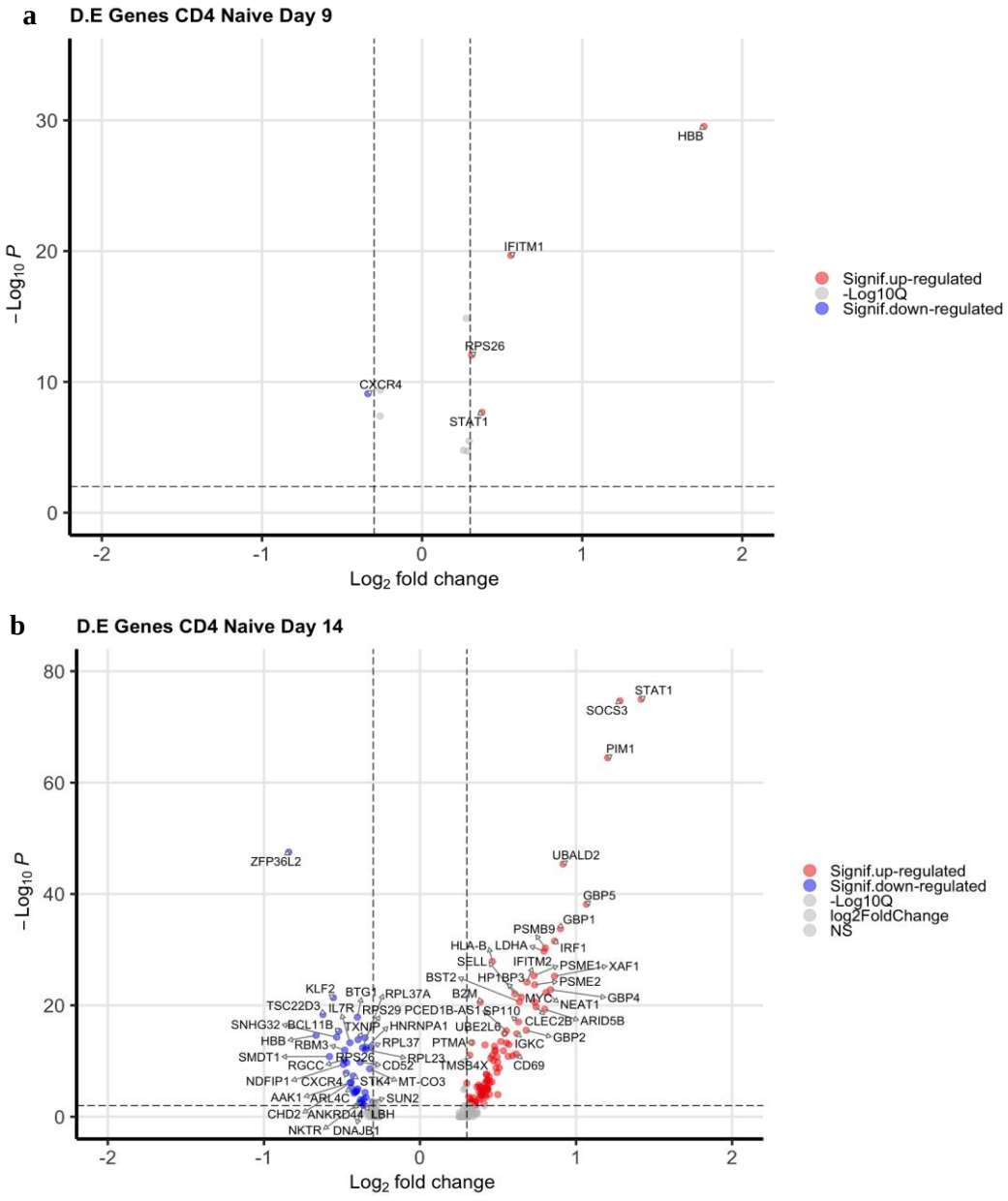


Figure 5-12 Differentially expressed genes in naïve CD4 T cells.

The differentially expressed genes in naïve CD4 T cells at day C9 (a) and day C14 (b) compared to the baseline, day C-1

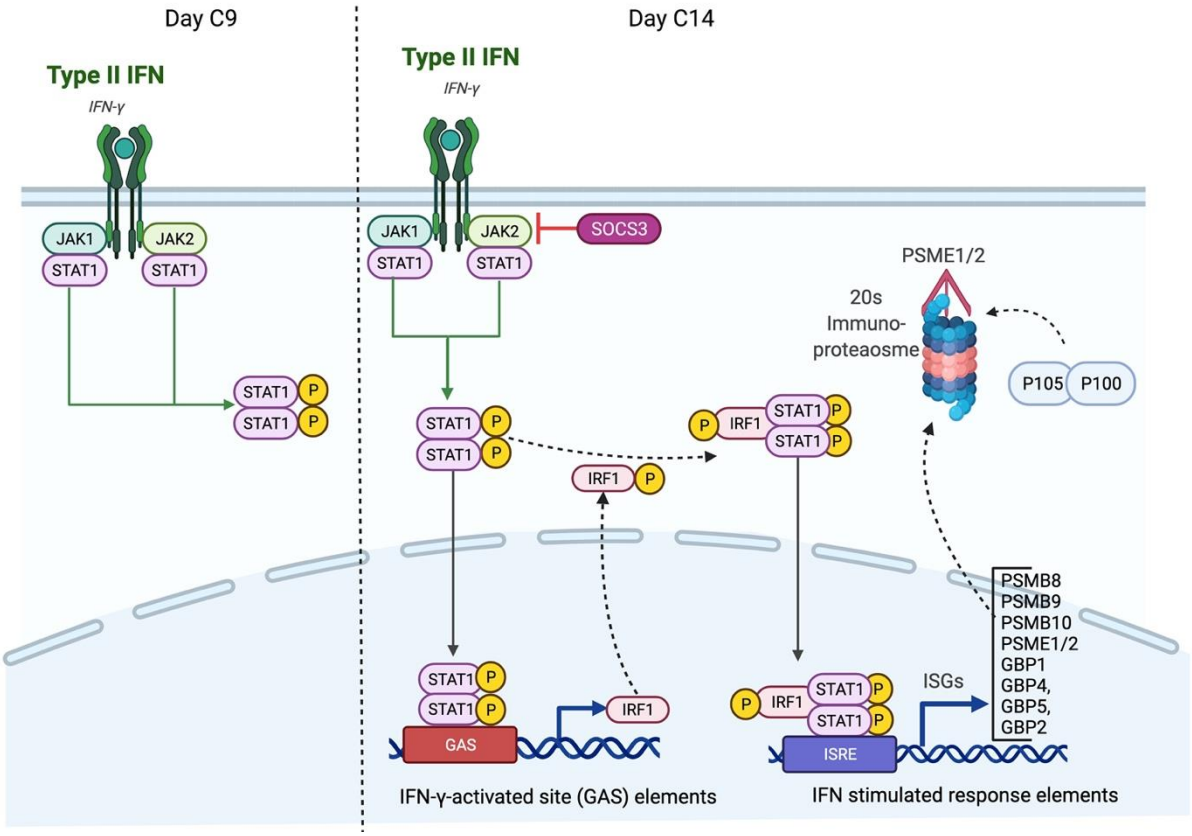


Figure 5-13 Activated signalling pathways and genes in naïve CD4 T cells at day 9 and day 14 post infection.

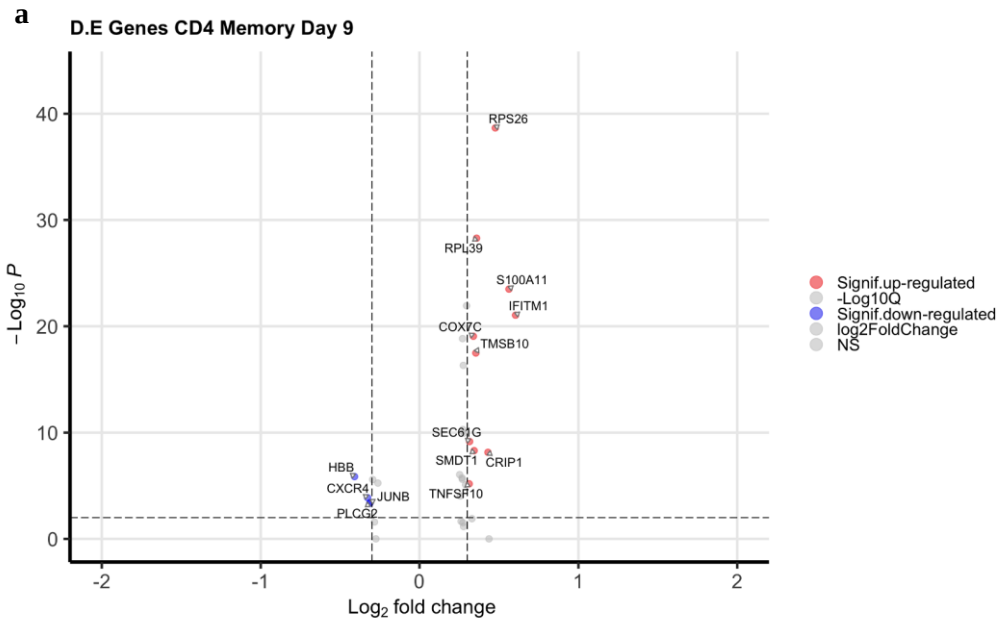
At day C9, STAT1 gene had a low log fold change a posssible indication that there was a weak type II IFN signaling at this timepoint, but at day C14 there is an upregulation of STAT1, IRF1 and a host of IFN stimulated response genes suggesting that type II IFN signalling is upregulated. Upregulation of SOCS3 may inhibit JAK2 activity thereby regulating the activity of the STAT1 gene. Figure created with Biorender.com

At day C14 compared to baseline (day C-1), naïve CD4 T cells sustained expression of STAT1, together with IRF1 and SOCS3 (Darnell et al., 1994). IRF1 and STAT1 also induced the upregulation of a number of genes such as those involved in the assembly of the 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994) and a number of Guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) (Cheng et al., 1983, Decker et al., 1989). The transcription factor STAT3 was upregulated, together with CCR7, SELL, IL2RG and CD69 (**Figure 5-12b)**.

5.4.6.4 CD4 Memory T Cells

CD4 Memory T cells at day C9 compared to baseline (day C-1) upregulated COX7C (which is involved in metabolic reprograming during T cell activation), TNFSF10, and IFITM1. Downregulated genes at the same timepoint included CXCR4, PLCG2 and JUNB. At day C14 compared to baseline (day C-1), the memory T cells upregulated STAT1, together with IRF1 and SOCS1. IRF1 and STAT1 also induced the upregulation of a number of gene such as core genes involved in the assembly of the 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2, PSMA5, PSMA7) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994) and a number of Guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) Cheng et al., 1983; Decker et al., 1989). The transcription factor STAT3 together with SELL and ICOS were also upregulated (Dong et al., 2001, Xu et al., 1996) (**Figure 5-14a**).

The downregulated genes at day C14 compared to baseline (day C-1) were mainly related to proliferation such as FOXP1, TXNIP, and BCLL11B. Negative regulators of NF-κβ signalling KLF2, TSC22D3, and ZFP36L2 were also downregulated. Ribosomal genes involved in translation initiation were also downregulated (**Figure 5-14b)**.



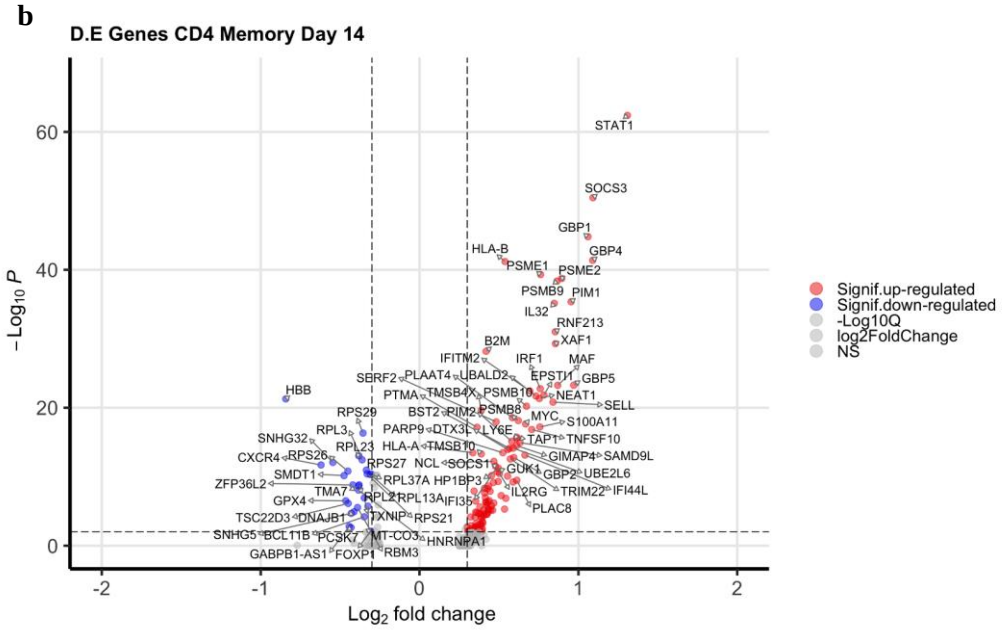


Figure 5-14 Differentially expressed genes in CD4 memory T cells.

The differentially expressed gene in CD4 memory T cells at day C9 and day C14 compared to the baseline day C-1

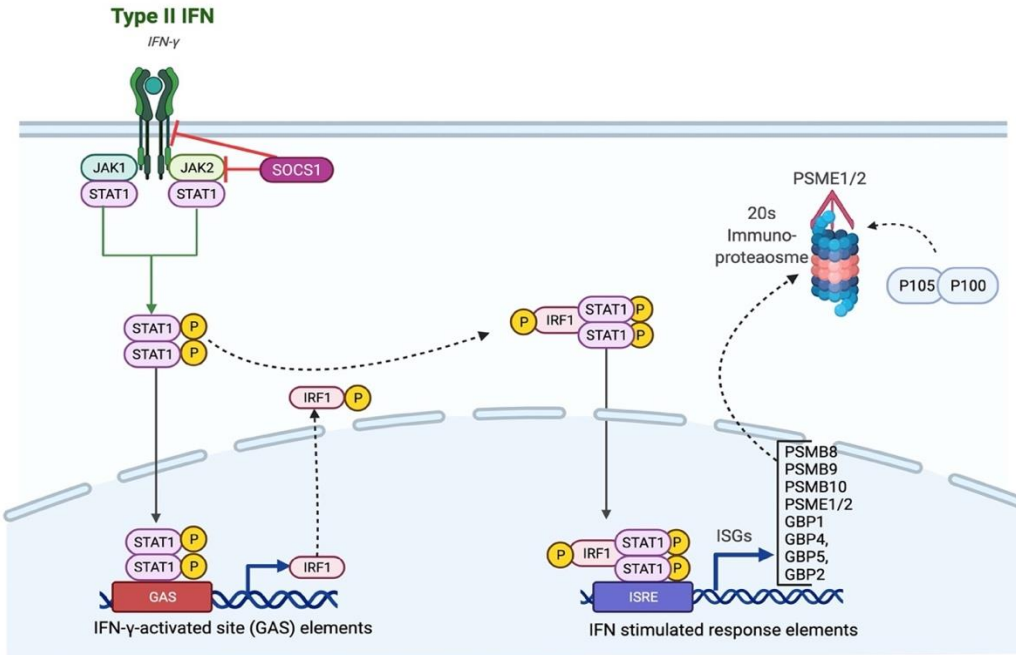


Figure 5-15 Expression of genes associated with type II IFN signalling in memory CD4 T cells at day 14 post infection

At day C14, there was an upregulation of STAT1, IRF1 and a host of IFN stimulated response genes suggesting that IFNγ signalling is upregulated. Upregulation of SOCS1 may inhibit JAK2 and IFNγ-Rα chain activity, possibly resulting in regulation of STAT1 activity. Figure created with Biorender.com

5.4.7 Differential Expressed Genes in Chronic participants

5.4.7.1 CD14 Monocytes

There were two populations of monocytes in the chronic participants. The two monocyte populations had different transcription profile. The first population of monocytes were mostly geared towards differentiation to moDCs, while the second population had an inflammatory transcription profile. The first monocyte population, CD14 Mono_1 (**Figure 5-4**), had few cells at baseline day C-1 and thus the differentially expressed genes were compared between day C14 and day C9 (**Figure 5-16**).

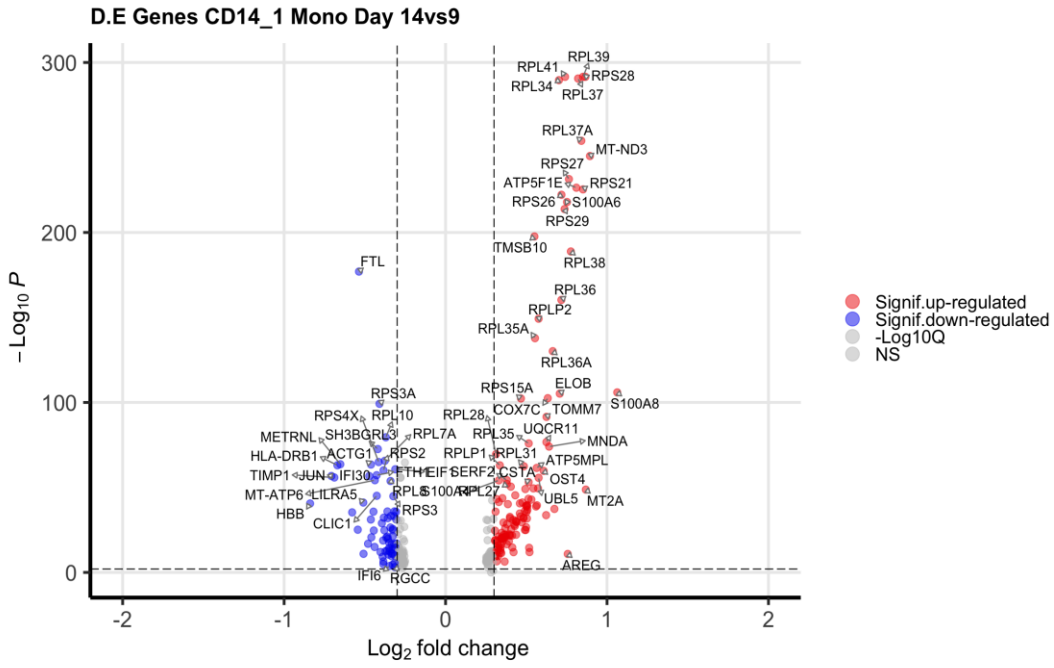


Figure 5-16 Differentially expressed genes in CD14 monocytes.

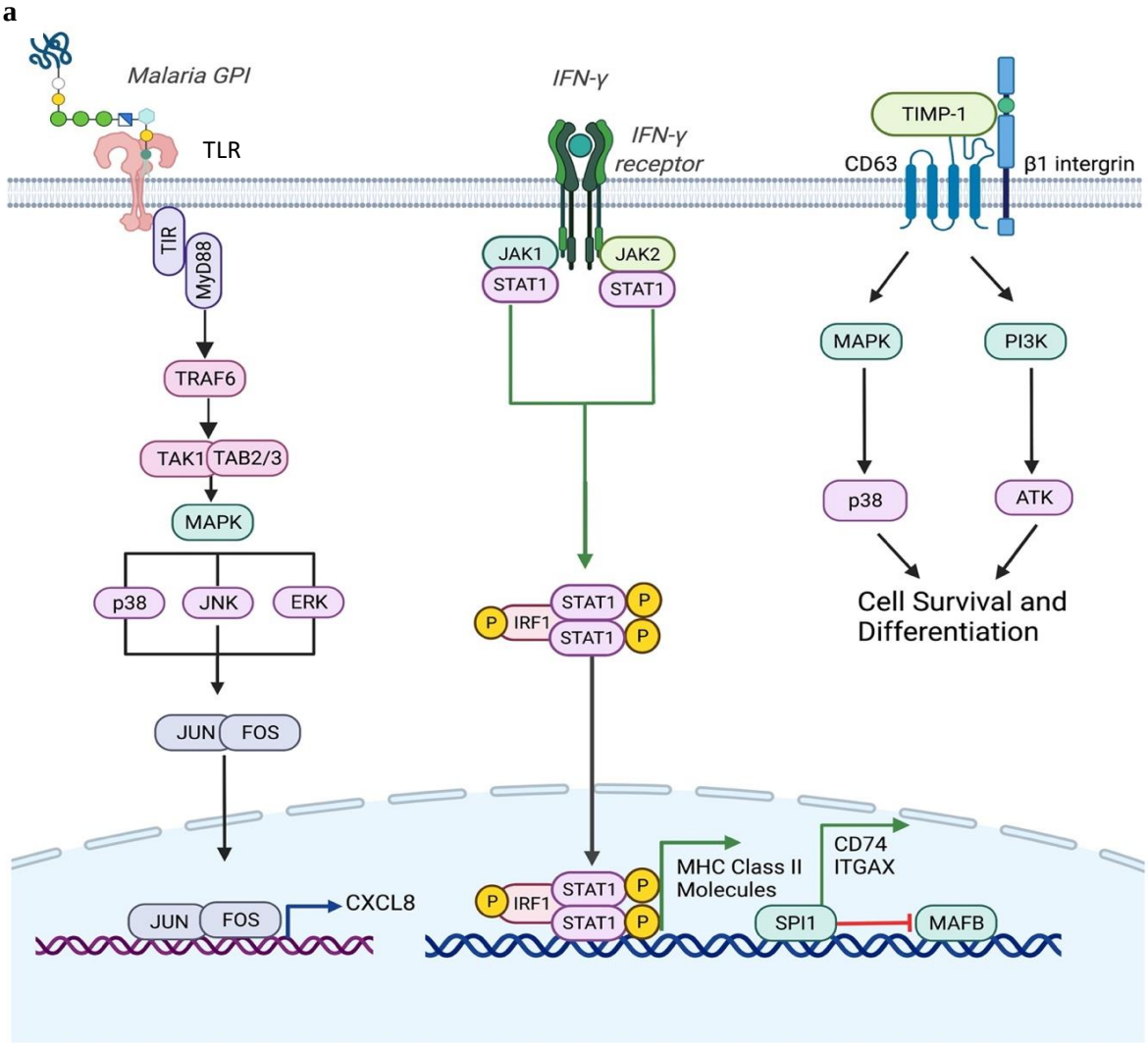
The differentially expressed gene in monocytes at day C14 compared to day C9

At day C9 compared to day C14, gene expression analysis suggest that this population of monocytes were differentiating into monocyte derived dendritic cells (moDCs), as seen by the upregulation of the transcription factors MAFB, and SPI1(PU.1) (McKercher et al., 1996, Anderson et al., 1998, Anderson et al., 2000, Bakri et al., 2005). CD63 and TIMP1 (Jung et al.,

2006) were also upregulated. Monocytes at this timepoint appear engaged in antigen uptake and processing as seen by the upregulation of a number of HLA molecules (HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB1) involved in antigen processing and presentation on MHC class II. The monocytes also upregulated CXCL8 (IL8), AP-1 transcription factor, and CXCL16 (**Figure 5-16**)

At day C14 compared with day C9, CD14 monocytes have an active JAK-STAT signalling pathway (seen by the upregulation of JAK2) that lead to the upregulation of IRF1. Other upregulated gene included, the inflammatory prostaglandins PTGS2, TNFAIP3, PTPRC IRS2 and SOCS3. Genes involved in mitochondrial oxidative phosphorylation and ribosomal assembly and initiation of translation were also upregulated.

a



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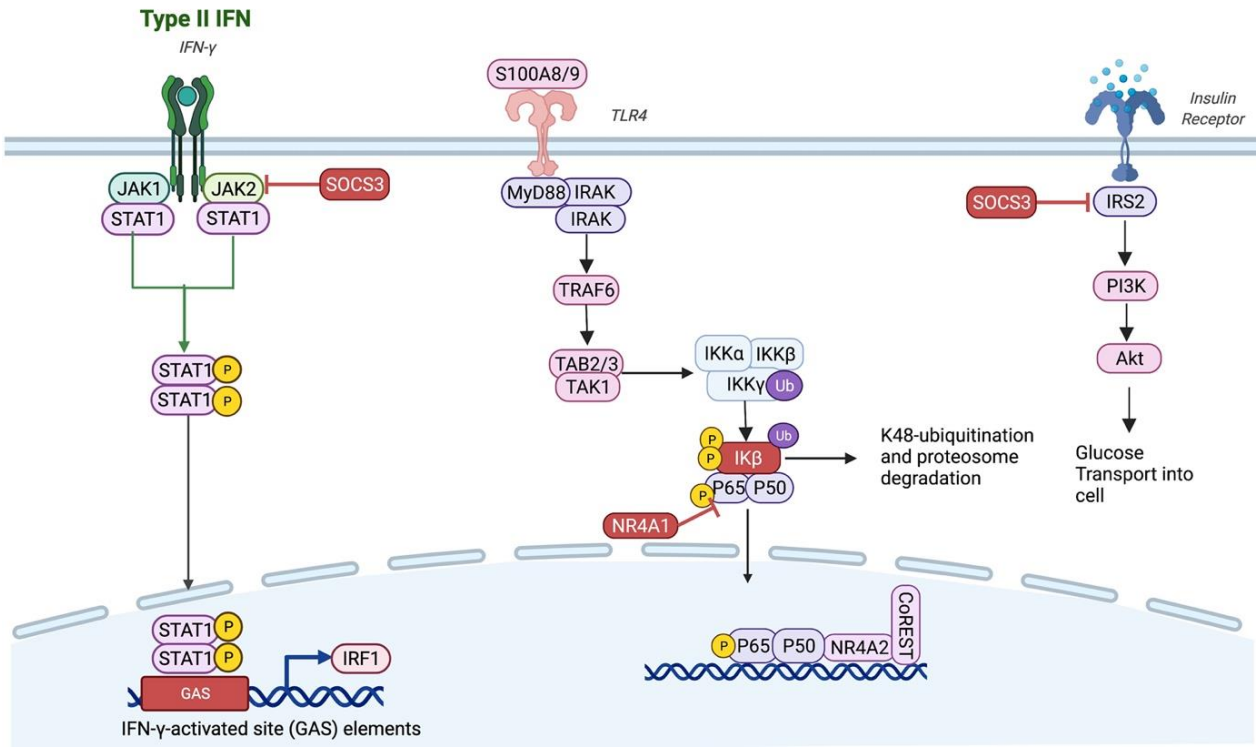


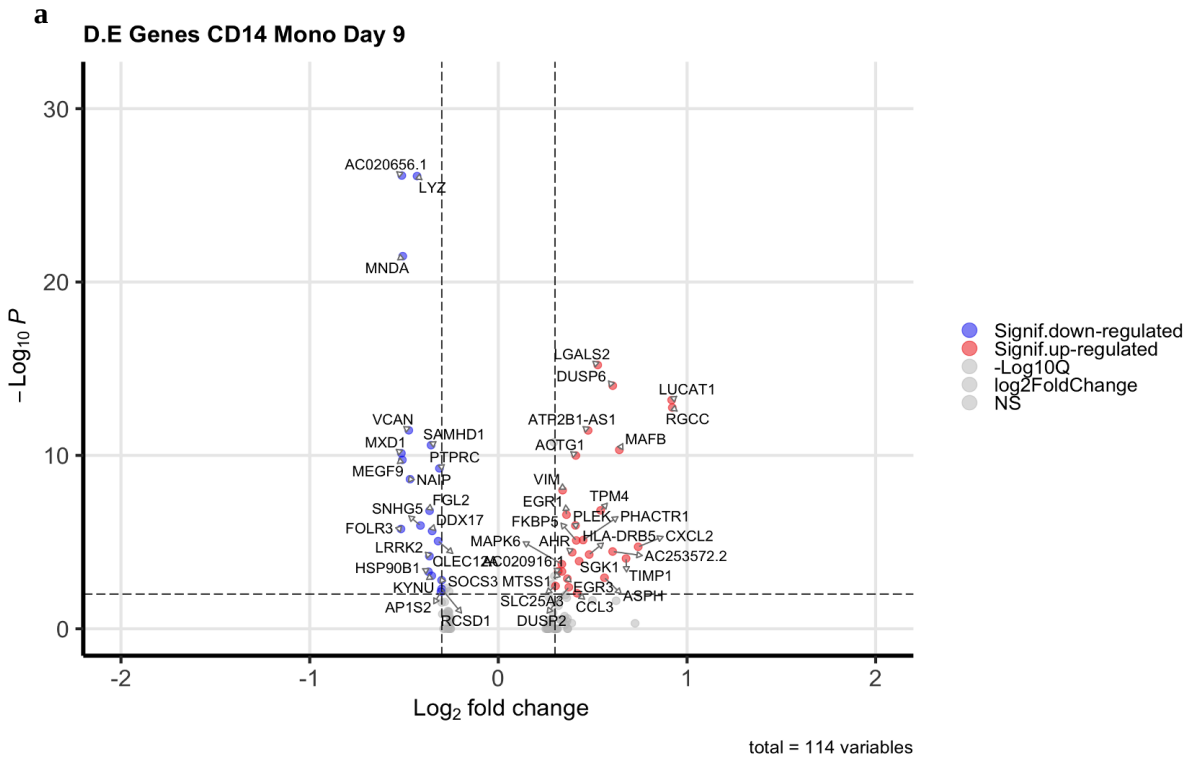
Figure 5-17 Signalling events driving the expression of genes in CD14 monocytes at day 9 post infection (a) and day 14 post infection (b).

a. At day C9, it is likely that the activation of TLR4-MyD88-TAK1signalling axis by malaria GPI could lead to the activation and dimerization of JUN and FOS gene (AP-1) and possibly upregulate transcription of CXCL8(IL-8). Upregulation of STAT1 also suggests that IFNγ signalling maybe active at this timepoint and could likely result in the upregulation of MHC Class II genes. There was also upregulation of signalling via TIMP-1 and CD63 which probably resulted in increased cell survival and enhancement of monocyte differentiation to moDCs. Transcription factors that may be involved in differentiation of monocytes to moDCs that is MAFB, SPI1(PU.1), and EST2 were upregulated at this timepoint.

b. At day C14, the signals changed from monocyte differentiation to a more inflammatory response. This is seen by the upregulation of STAT1 which suggest that IFNγ signalling was active at day C14. SOCS3 was also upregulated, this may imply that the activity of STAT1 was negative regulated. The upregulation of S100A alarmins could likely induce signalling via TLR4, which could in turn lead to activation of NF-κβ. While the upregulation of NR4A1 and NR4A2 genes could inhibit the transcriptional activity of NF-κβ. Insulin signalling via IRS2 could also have been inhibited by SOCS3, thereby inhibiting glucose uptake and metabolism in these cells. Figures were created with Biorender.com

For the second population of monocytes, CD14 Mono, at day C9 compared to baseline (day C-1), the monocytes upregulated the inflammatory chemokines CCL3 and CXCL2, the MAPK signalling inhibitors DUSP2 and DUSP6, MAPK6 and regulators of proinflammatory signalling ERG1 and ERG2. The downregulated genes at this timepoint included PTPRC, SOCS3, LYZ, and CLEC12A (**Figure 5-18a)**.

At day C14 compared to baseline (day C-1), the monocytes upregulated the chemokines IL1B and CXCR4, ZFP36L2, TNFAIP3, NR4A1, IRS2 and JUNB. At this timepoint the monocytes downregulated, LYZ and CD14 (**Figure 5-18b)**.



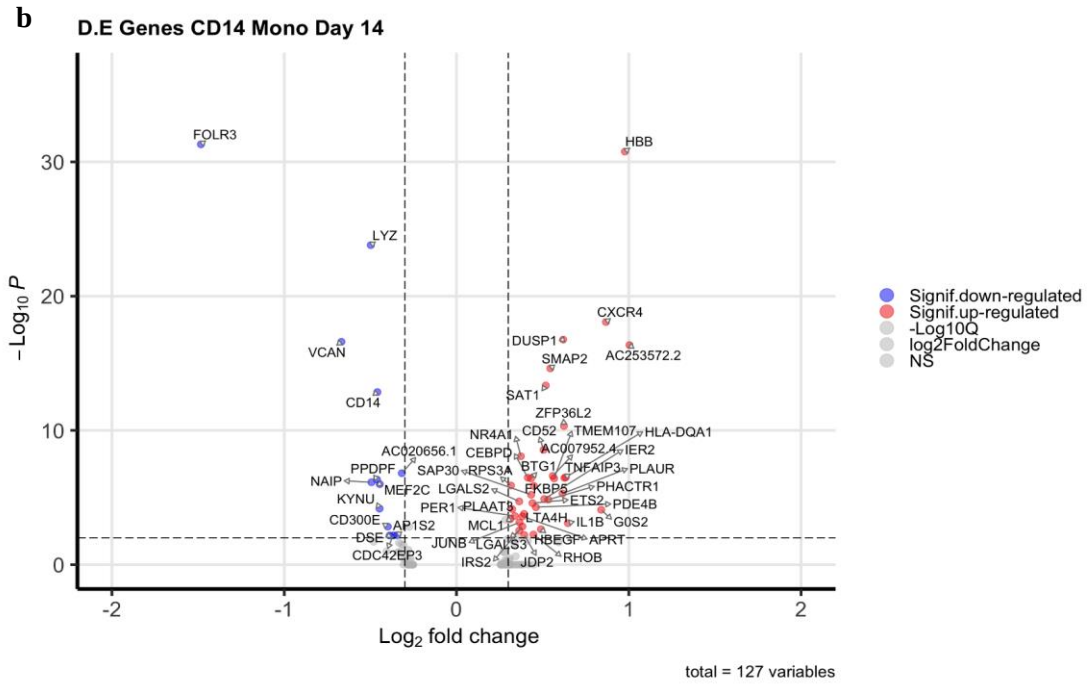


Figure 5-18 Differentially expressed genes in CD14 monocytes.

The differentially expressed gene in monocytes at day C9 (a) and day C14 (b) compared to the baseline day C-1

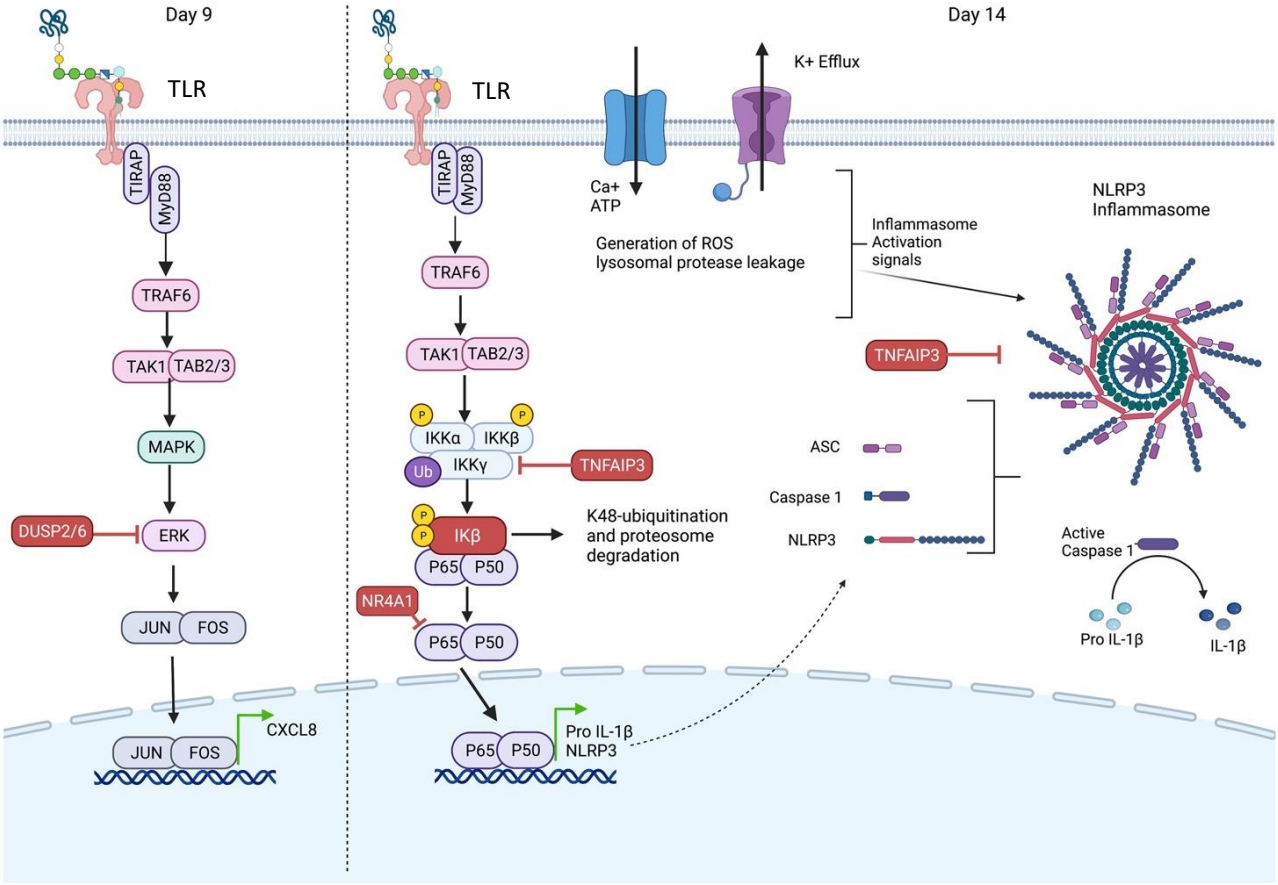


Figure 5-19 Signalling events resulting in the expression of genes in CD14 monocytes at day 9 and day 14 post infection.

At day C9, malaria GPIs could initiate signalling via TLR 2/4-MyD88-TAK1, this may result in the dimerization and activation of JUN and FOS transcription factors (AP-1), which could upregulate the transcription of CXCL8(IL-8). The upregulation of DUSP2 and DUSP6, suggests that they may inhibit the activity of ERK, thereby regulating the transcriptional activity of JUN and FOS dimers. Similarly at day C14, malaria GPIs might be responsible for initiating signalling through TLR2/4-MyD88-TAK1 leading to upregulation and activation of NF-κβ, which in turn may initiate the transcription of NLRP3 and pro-IL-1β. NLRP3 oligomerizes and may recruit ASC to the oligomer. ASC in turn recruit's caspase 1, which auto cleaves itself to an active caspase 1. The activated caspase 1 then cleaves pro-IL-1β to IL-1β. Figure created with Biorender.com

5.4.7.2 CD16 Monocytes

CD16 monocytes in the chronic participants at day C9 compared to baseline (day C-1), upregulated the transcription factors MAFB, SPI1(PU.1), and EST2 that initiate differentiation

into monocyte derived dendritic cells (moDCs). The chemokines CXCL8, CXCR4, and CXCL16 were also upregulated. MAPK signalling molecule MAPK38 was upregulated together with KLF6 a negative regulator of MAPK signalling molecule. Genes that repress oxidative species and proinflammatory response, SOD1 and SOD2 were also upregulated. CD83, ICAM3, and IFNGR1 were also upregulated in these CD16 monocytes (**Figure 5-20a)**.

The downregulated genes at this timepoint included JAK2, a key molecule in the JAK-STAT signalling pathway. IRF2 was also downregulated together with interferon stimulated genes such as IFI16, OAS1, OAS2 and IFI27L2. Ribosomal genes involved in translation initiation were downregulated and mitochondrial genes involved in ATP synthesis were also downregulated (**Figure 5-20a)**.

At day C14 compared to the baseline (C-1), the chemokine CXCR4 was still upregulated together with IFN stimulated genes IFITM1, IFITM2, and HLA-B. The transcription factor MAFB was also upregulated. The downregulated genes at this timepoint included, LZY (lysosome C), MNDA, TNFSF10 and S100A10 (**Figure 5-20b)**.

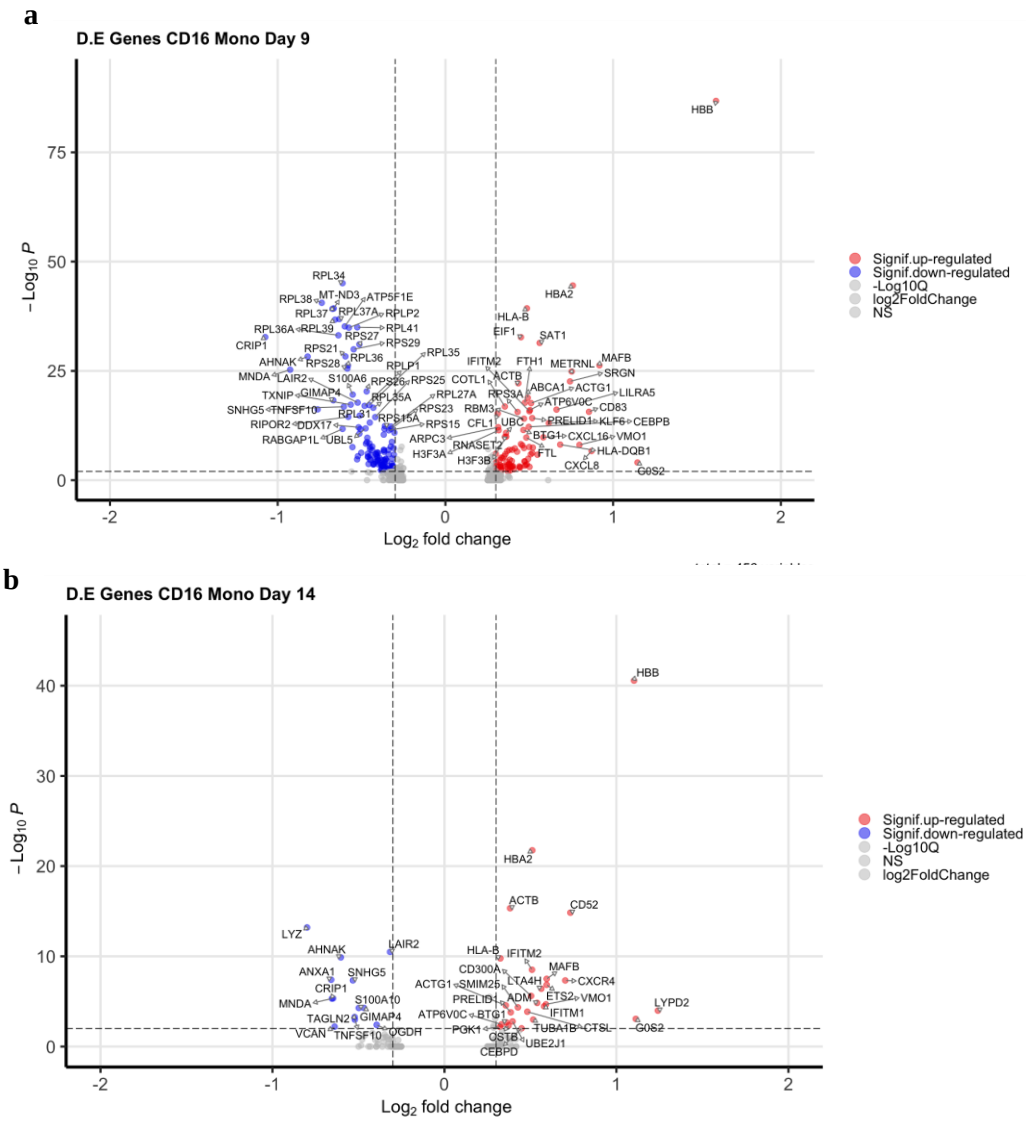


Figure 5-20 Differentially expressed genes in CD16 monocytes.

The differentially expressed gene in monocytes at day C9 (a) and day C14 (b) compared to the baseline day C-1

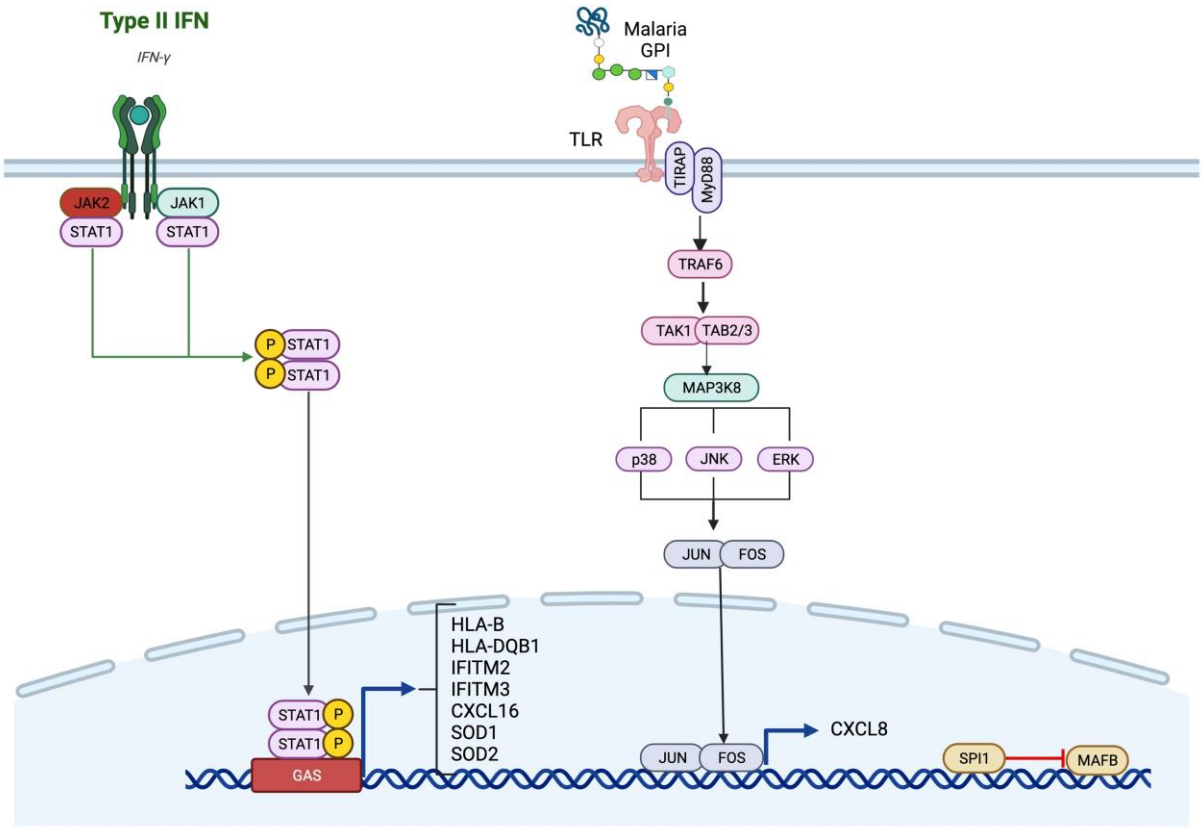


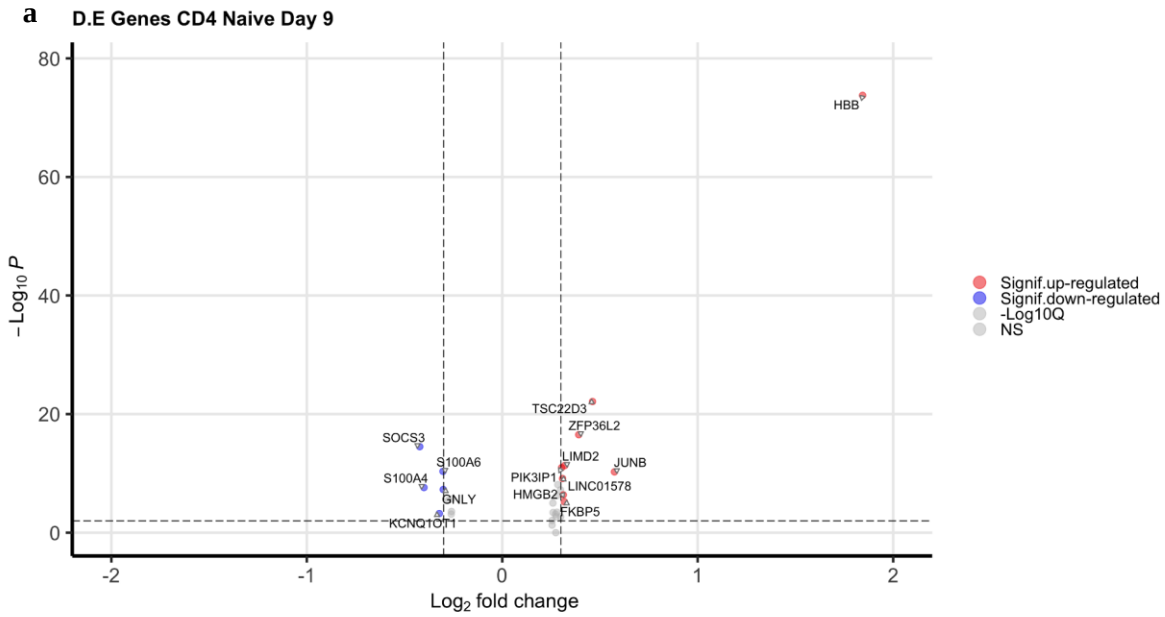
Figure 5-21 Signalling through TLR and IFNγ in CD16 monocytes leading to activation of genes at day 9 post infection.

Malaria GPIs could initiate signalling via TLR 2/4-MyD88-TAK1, this may result in the dimerization and activation of JUN and FOS transcription factors (AP-1). The active AP-1 dimer may induce the expression and upregulation of the chemokine CXCL8. The upregulation of the IFN gamma receptor 1 (IFNGR1) suggest that type II IFN signalling was active in these cells, but the adaptor molecule JAK2 was downregulated an indication that type II IFN signalling may be partially activated. The transcription factor SPI1 was upregulated in these cells, suggesting that the cells may be differentiating to dendritic cells. Figure created with Biorender.com

5.4.7.3 Naïve CD4 T Cells

Naïve CD4 T cells at day C9 compared to baseline (day C-1), upregulated negative regulators of NF-κβ signalling i.e., TSC22D3 and ZFP36L2. The transcription factor JUNB was upregulated as well as PI3KIP1, an inhibitor of the PI3K signalling pathway which is involved in cell proliferation and growth (Zhu et al., 2007, He et al., 2008). SOCS3 was downregulated at this timepoint together with GNLY, S100A6 and S100A4 (**Figure 5-22a)**.

At day C14 compared to baseline (day C-1), the T cells continued to downregulate SOCS3. While upregulating the transcription factors AP-1 (JUN and JUNB), and STAT1. The interferon response gene IFITM3 was also upregulated (**Figure 5-22b)**.



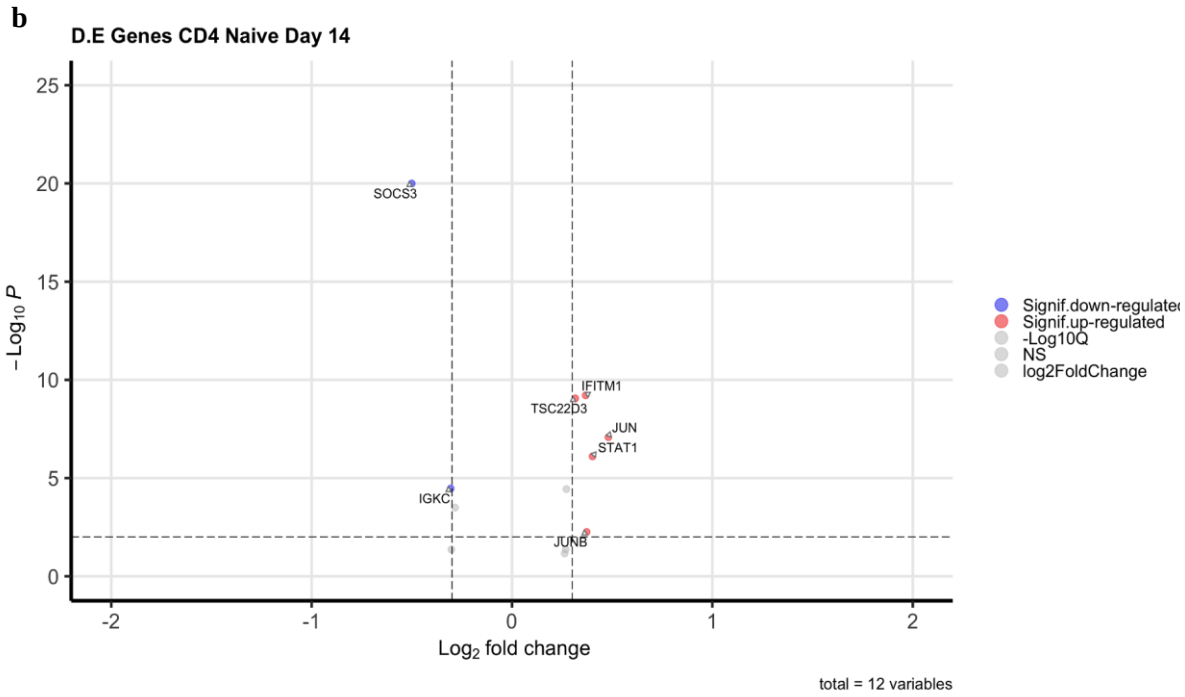


Figure 5-22 Differentially expressed genes in CD4 naïve T cells.

The differentially expressed gene in CD4 naïve t cells at day C9 and day C14 compared to the baseline day C-1

5.4.7.4 CD4 Memory T Cells

At day C9 compared to baseline (day C-1), memory CD4 T cells upregulated the transcription factor AP-1 (FOS, JUN, JUNB, FOSB), and a number of NF-κβ signalling inhibitors such as DUSP1 and DUSP2, KLF2, TNFAIP3, NFKBIA and TSC22D3. PI3KIP1 an inhibitor of the PI3K signalling pathway was also upregulated (Zhu et al., 2007, He et al., 2008). Other upregulated genes included UBC, ZFP36L2 and CXCR4 (**Figure 5-23a)**.

At day C14 compared to baseline (day C-1), there was continued upregulation of the transcription factor AP-1 (FOS, FOSB and JUN) and a number of NF-κβ signalling inhibitors such as DUSP1 and DUSP2, NFKBIA and TSC22D3. Other upregulated genes included ID2 and IFITM1(**Figure 5-23b)**.

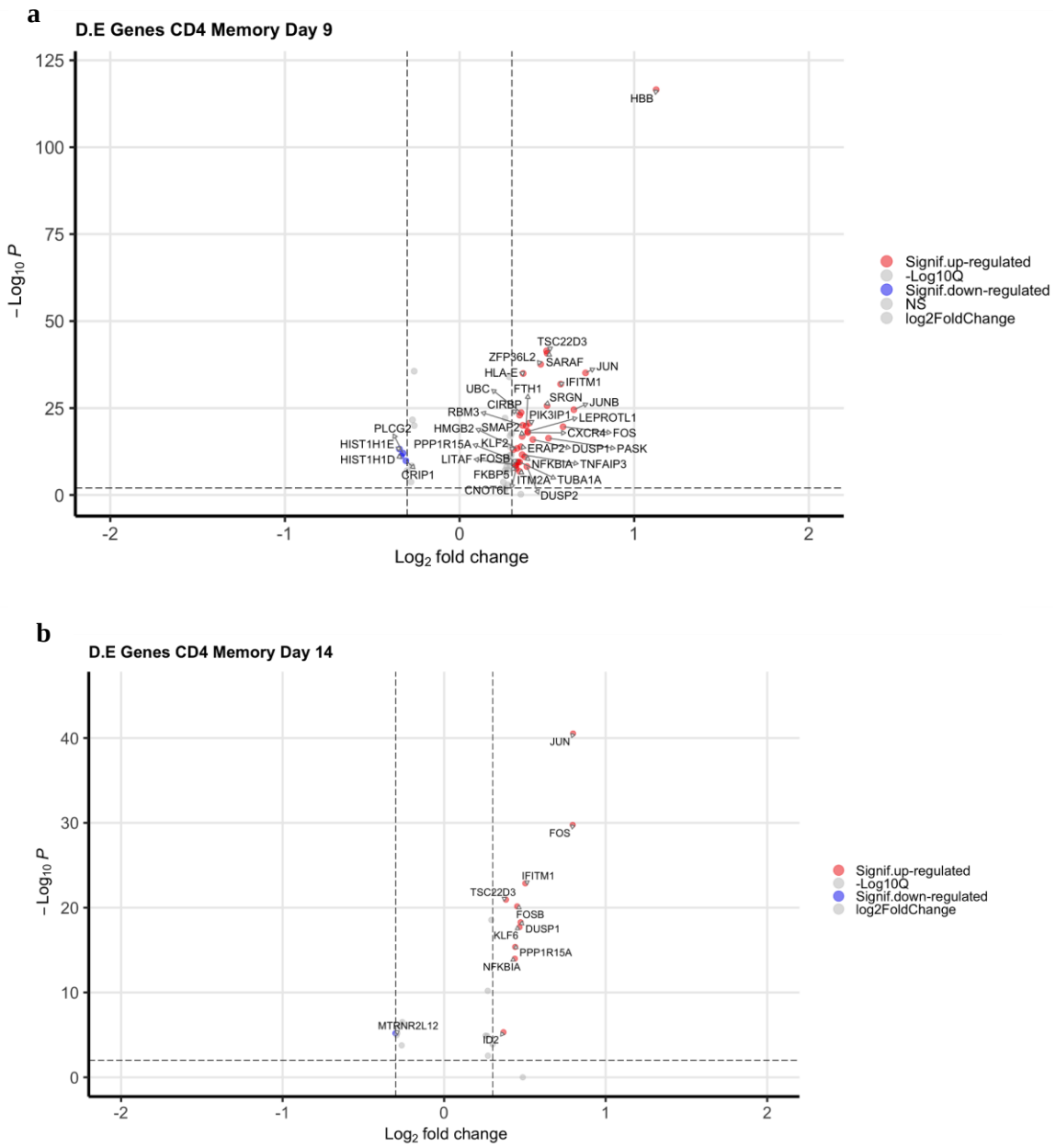


Figure 5-23 Differentially expressed genes in CD4 memory T cells.

The differentially expressed gene in CD4 Memory T Cells at day C9 and day C14 compared to the baseline day C-1

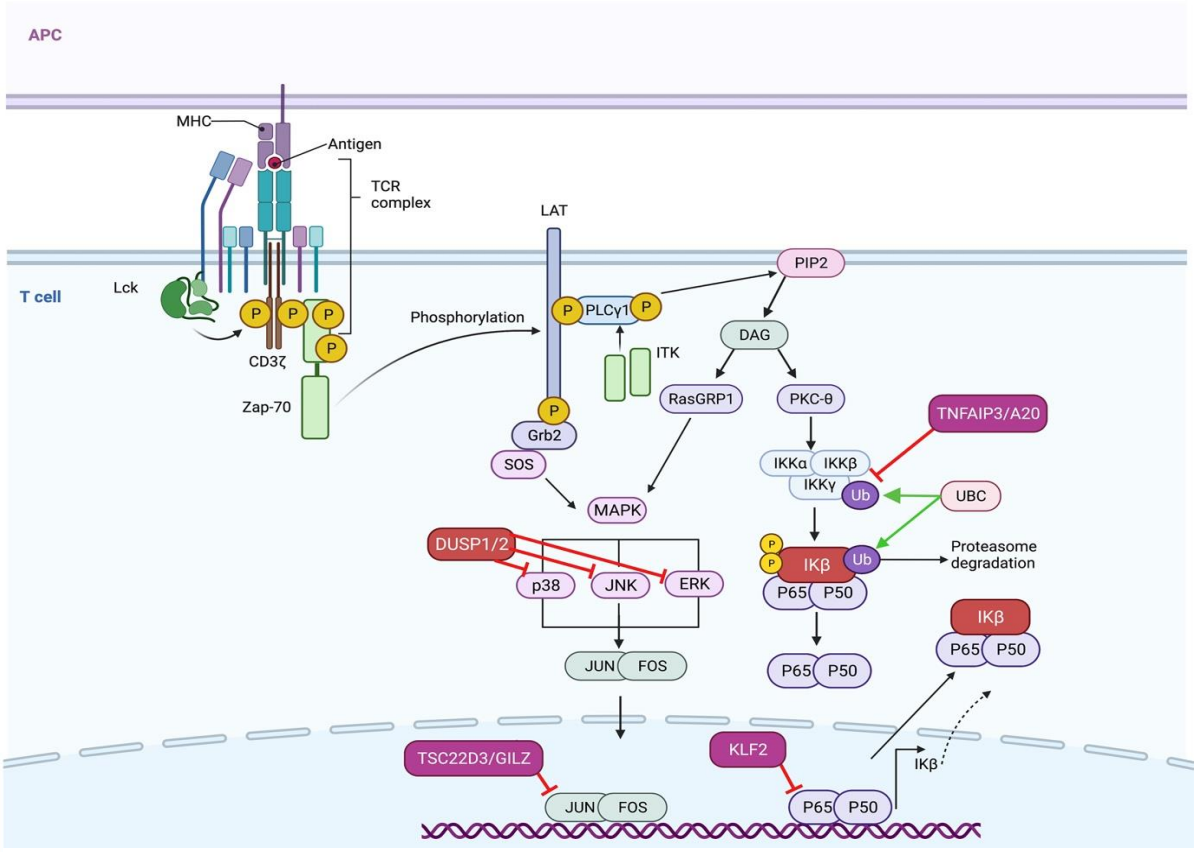


Figure 5-24 T cell receptor signalling and activation of genes in CD4 memory at day 9 and day 14 post infection.

Signals in memory CD4 T cells could be initiated via the T cell receptor complex which could lead to activation of both the MAPK-AP-1 and NF-κβ signalling pathways. Upregulation of DUPS1/2 might inhibit MAPK signalling while the upregulation of TSCD22D3 could negatively regulate AP-1 activity. Upregulation of TNFAIP3, Iκβ and KLF2 may have inhibited NF-κβ signalling. Figure created with Biorender.com

5.4.8 Differential Expressed Genes between Febrile and Chronic participants

5.4.8.1 CD14 Monocytes

Comparison of differentially expressed genes in CD14 monocytes at day C9 showed that the febrile participants upregulated genes involved in type I interferon signalling (STAT1, STAT2, IRF1, XAF1, MX1, IFI6, IFITM3, ISG15, ADAR and SAMHD1) when compared with the chronic participants. Inhibitors of NF-κβ signalling were also upregulated such as KLF2 and

NR4A1. The zinc finger proteins ZFP36L1 and ZFP36L2, which target proinflammatory cytokine mRNA were also upregulated. The receptors CLEC7A (Dectin-1) and CLEC12A were upregulated at this timepoint in the febrile participants when compared with the chronic participants. Other genes expressed at this timepoint in the febrile participants were related to ribosomal assembly and initiation of transcription as well as genes involved in the mitochondrial TCA cycle (**Figure 5-25a)**.

CD14 monocytes from the chronic participants upregulated immunomodulatory molecules LGALS3 (Galectin-3), and HAVCR2 (TIM-3) when compared to febrile participants. HAVCR2 (TIM-3) inhibits IL-12 production by monocytes (Zhang et al., 2011, Ma et al., 2013) and also upregulates the expression of PD-1 molecule on monocytes (Zhang et al., 2012) which hampers Th1 responses in naïve CD4 T cells (Li et al., 2017). Galectin 3 (LGALS3) modulates monocytes to secrete IL-10, reducing surface expression of MHC molecules and inhibits IL-12 production (Chen et al., 2015, Chung et al., 2013). CXCL8(IL-8), CXCL2 and CCL3 proinflammatory chemokines released by activated monocytes were also upregulated in the chronic participants at this timepoint. CD63-TIMP1 (Jung et al., 2006) signalling was active at this timepoint, together with MAPK signalling molecules MAPK6 and MAP3K8. A number of alarmins (S100A8/9/12) were also expressed and these monocytes still maintained expression of CD14 unlike the monocytes from the febrile phenotype. Genes involved in ribosomal assembly, translation initiation and mitochondria ATP synthesis were also upregulated (**Figure 5-25a)**.

At day C14, the monocytes from the febrile participants upregulated interferon response genes IFITM3, GBP5 GBP1 and MT2A when compared to chronic participants. IDO1 involved in tryptophan metabolism was also upregulated (**Figure 5-25b)**.

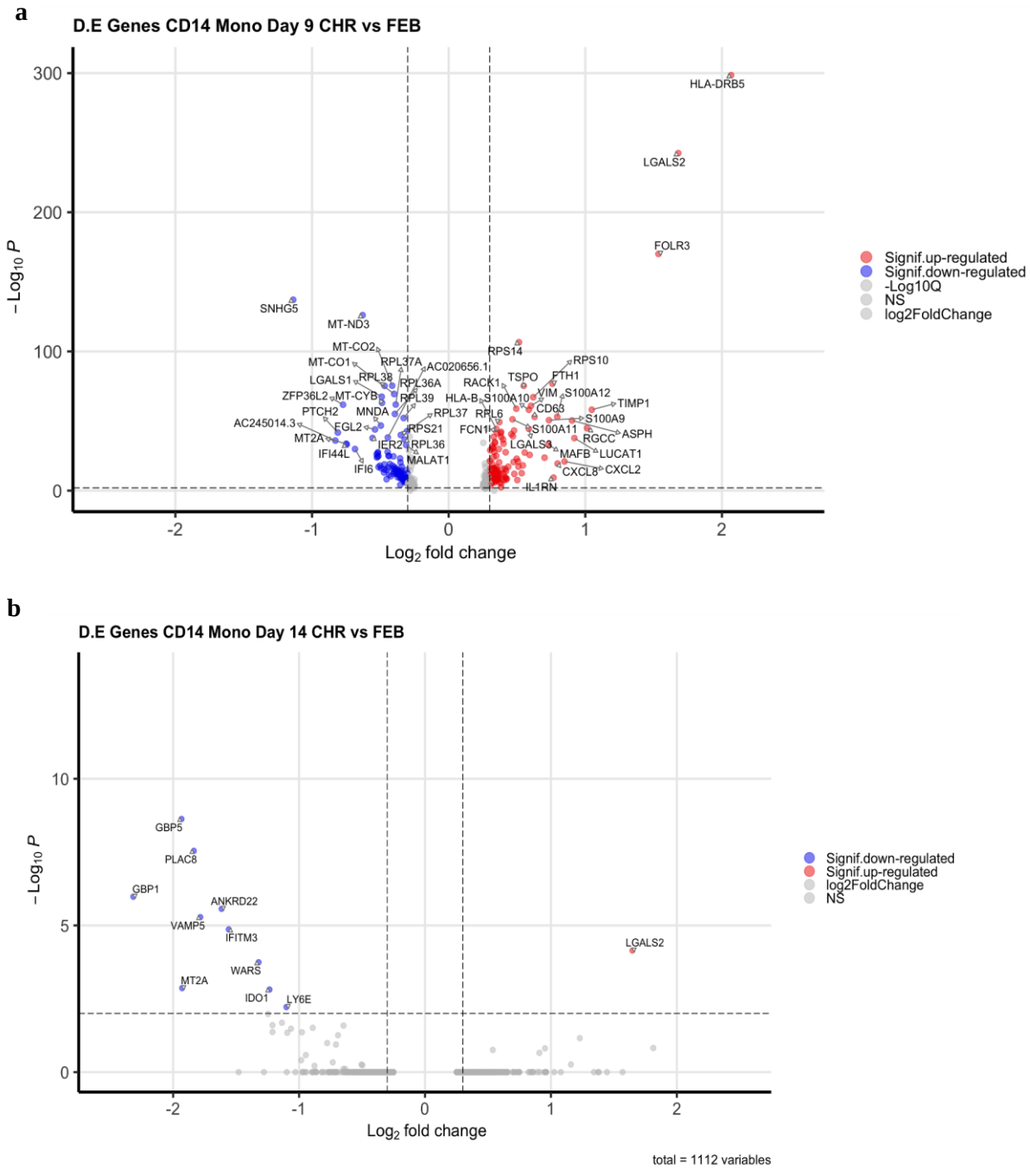


Figure 5-25 Differentially expressed genes between chronic and febrile CD14 monocytes.

The graph shows differentially expressed gene in CD14 monocytes between the chronic and febrile groups at similar timepoints

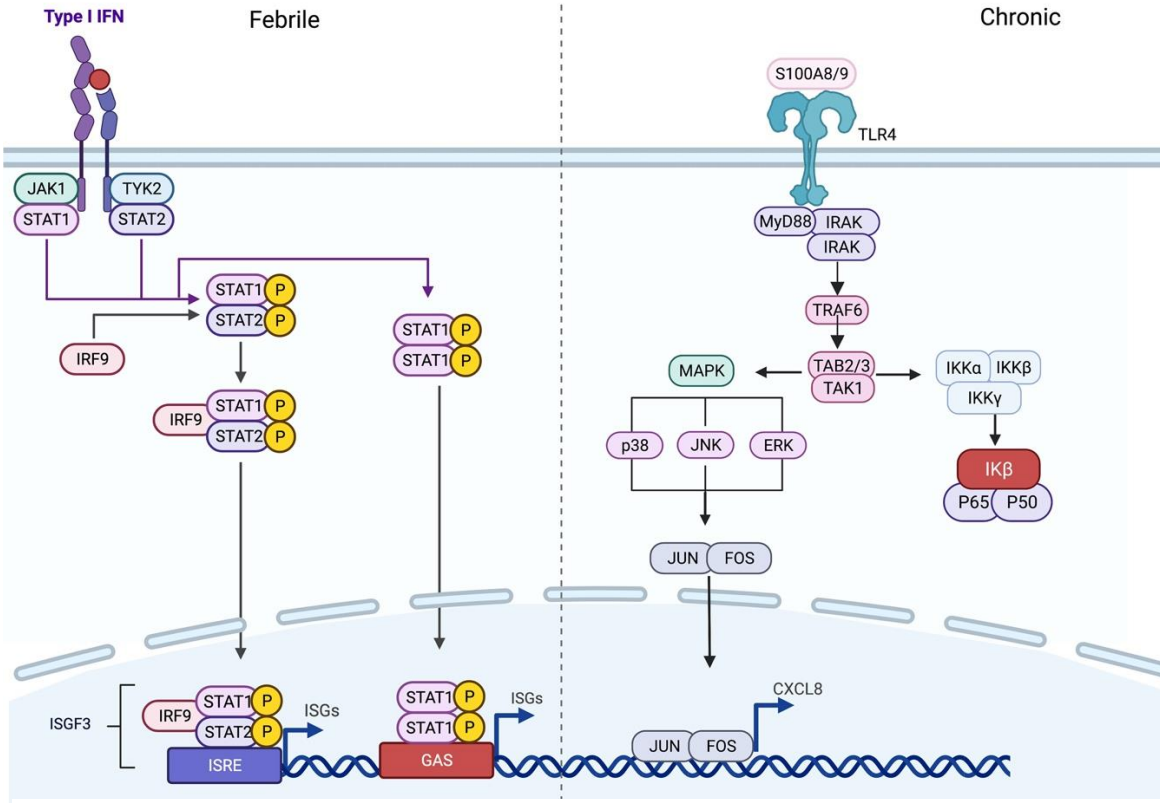


Figure 5-26 The different activated signalling pathways and genes in CD14 monocytes at day 9 post infection in febrile and chronic participants.

CD14 monocytes from the febrile participants upregulated STAT1 and STAT2, this implies that signalling via type I IFN signalling pathway was active, while CD14 monocytes from the chronic participants upregulated S100a alarmins that could activate TLR4 signalling may result in the dimerization and activation of JUN and FOS transcription factors (AP-1). Figure created with BioRender.com

5.4.8.2 CD16 Monocytes

CD16 monocytes from febrile participants when compared to chronic participants, upregulated genes involved in type I and II IFN signalling namely of JAK2, IRF2, IRF1, STAT1 and STAT2 and IFN response elements, MX1, ISG15, XAF1, IFI6, IFI16, IFIT3, OAS1. These monocytes also upregulated cytotoxic granules NKG7 and LYZ. The zinc finger proteins ZFP36L1 and ZFP36L2, which target proinflammatory cytokine mRNA for destruction. Genes involved in ribosomal assembly, translation initiation and mitochondria ATP synthesis were also upregulated.

CD16 monocytes from chronic participants when compared to febrile participants, upregulated CEPBPD, CEBPB, and SPI1 which initiate the differentiation of monocytes to moDCs. SPI1 also initiated the transcription of ITGAX (CD11c), which was upregulated in these cells. These monocytes also upregulated the costimulatory molecules CD83 and CD86. Signalling pathways involved in cell survival and differentiation of CD16 monocytes to moDCs namely the CD63-TIMP1 signalling axis (Jung et al., 2006), were also expressed. These cells also upregulated IFNGR1 and IFNγ response elements IFITM2, IFITM3, HLA-B, HLA-DQB1, HLA-DRB5, and CXCL16. Other genes upregulated in the cells were MAPK3K8, the proinflammatory cytokines CXCL8(IL-8), the chemokine CXCR4, superoxide dismutase 1 and 2 (SOD1 and SOD2).

The comparison of CD16 monocytes at day C14 yielded genes that were below the threshold of a p value of 0.01 and average log fold change of 0.3

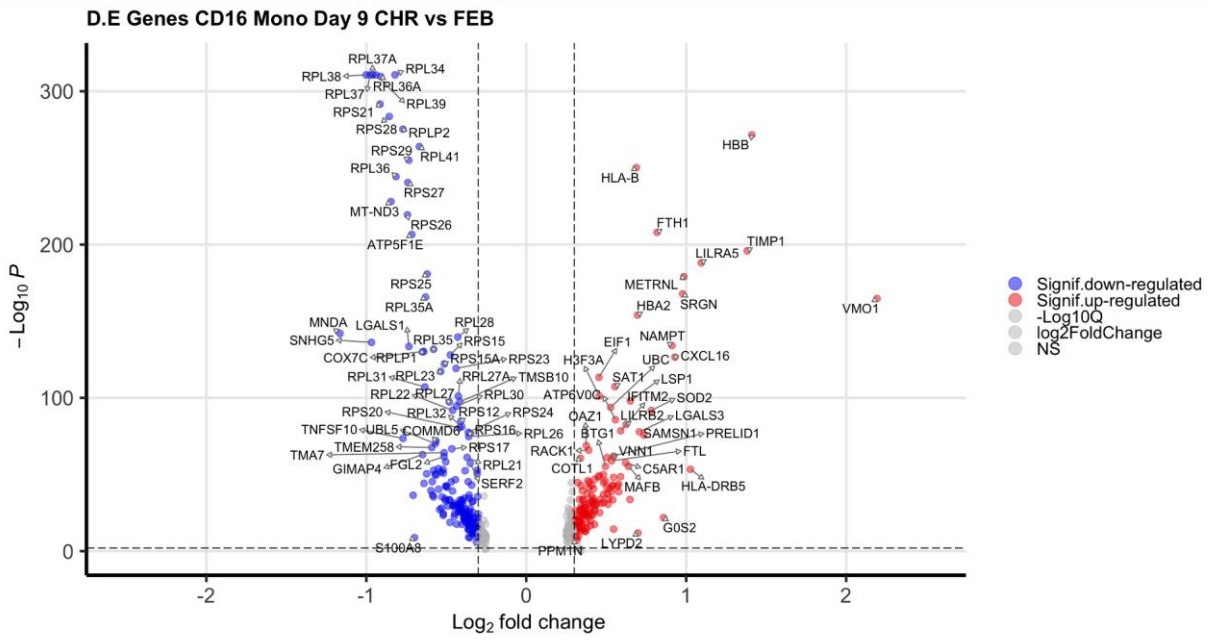


Figure 5-27 Differentially expressed genes in CD16 monocytes between chronic and febrile participants.

The graph shows differentially expressed gene in CD16 monocytes between the chronic and febrile groups at similar timepoints

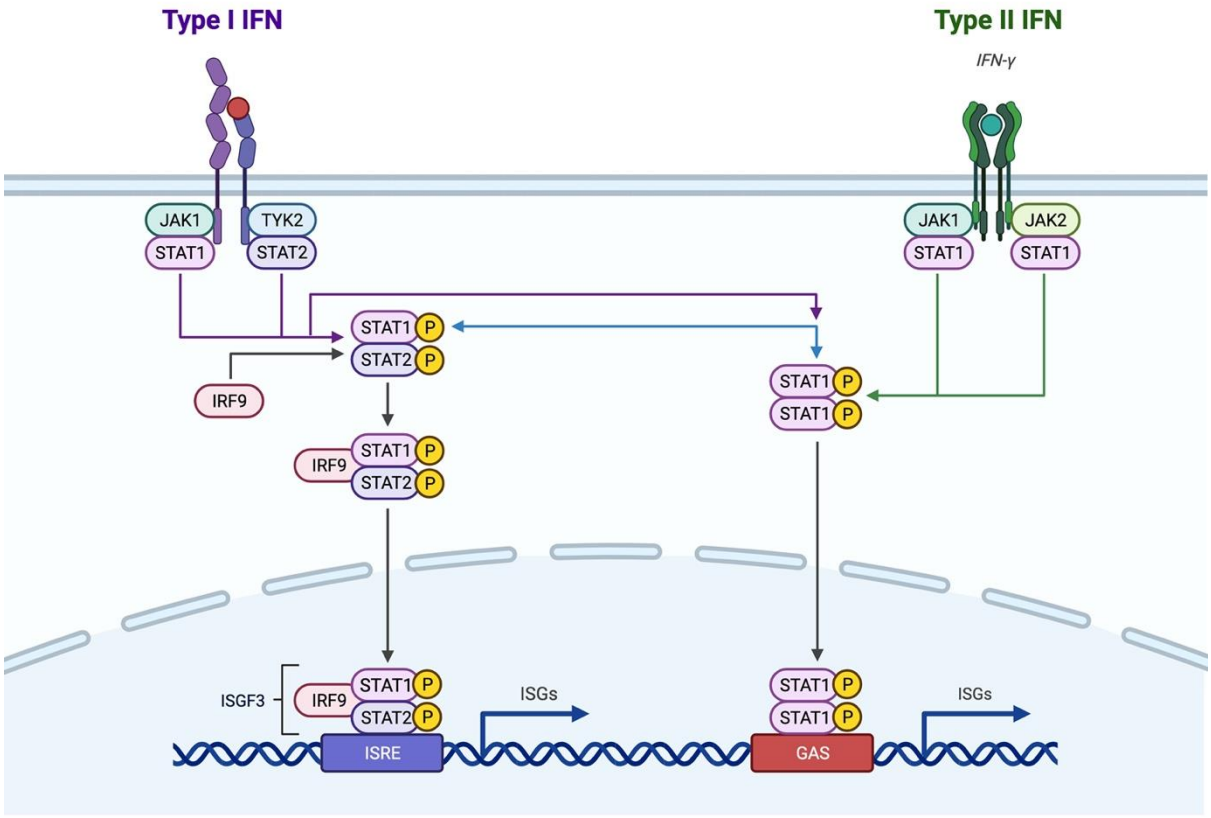


Figure 5-28 Type I and Type II IFN signalling in CD16 monocytes from febrile participants at day 9 post infection.

CD16 monocytes from the febrile participants upregulated JAK2, STAT1, STAT2 and a number of IFN response genes which suggests that IFN signalling was active in these cells. CD16 monocytes from chronic participants also upregulated IFNGR1 and several IFN response genes which could indicate that type 1I IFN was active in these cells. Figure created with Biorender.com

5.4.8.3 Naïve CD4 T Cells

At day C9 the naïve CD4 T cells from the febrile participants mostly expressed ribosomal genes involved in ribosomal assembly, and translation initiation.

The naïve CD4 T cells from the chronic participants on the other hand expressed the transcription factor AP-1 (JUN and JUNB). A number of NF-κβ signalling inhibitors such as TNFAIP3, NFKBIA, KLF2 and TSC22D3. The inhibitor of PI3K signalling pathway, PI3KIP1, the activation marker CD69 and chemokine receptor CXCR4 were also expressed at this timepoint.

At day C14, naïve CD4 T cells from the febrile participants expressed genes from type II IFN signalling pathway namely IRF1, STAT1 and SOCS3 together with IFN response elements, IFITM2, XAF1, IFITM2, and BST2. Genes involved in the assembly of the 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2, PSMA5, PSMA7) and a number of Guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2). Genes involved in T cell activation such as CCR7, SELL, PTPRC, and CD69 were also expressed by these cells.

The naïve CD4 T cells from the chronic participants at day C14 expressed the transcription factor JUND, NF-κβ signalling inhibitors such as DUSP1, KLF2, TNFAIP3, and TSC22D3. The zinc finger proteins ZFP36L2, and a number of ribosomal genes were also expressed at this time point.

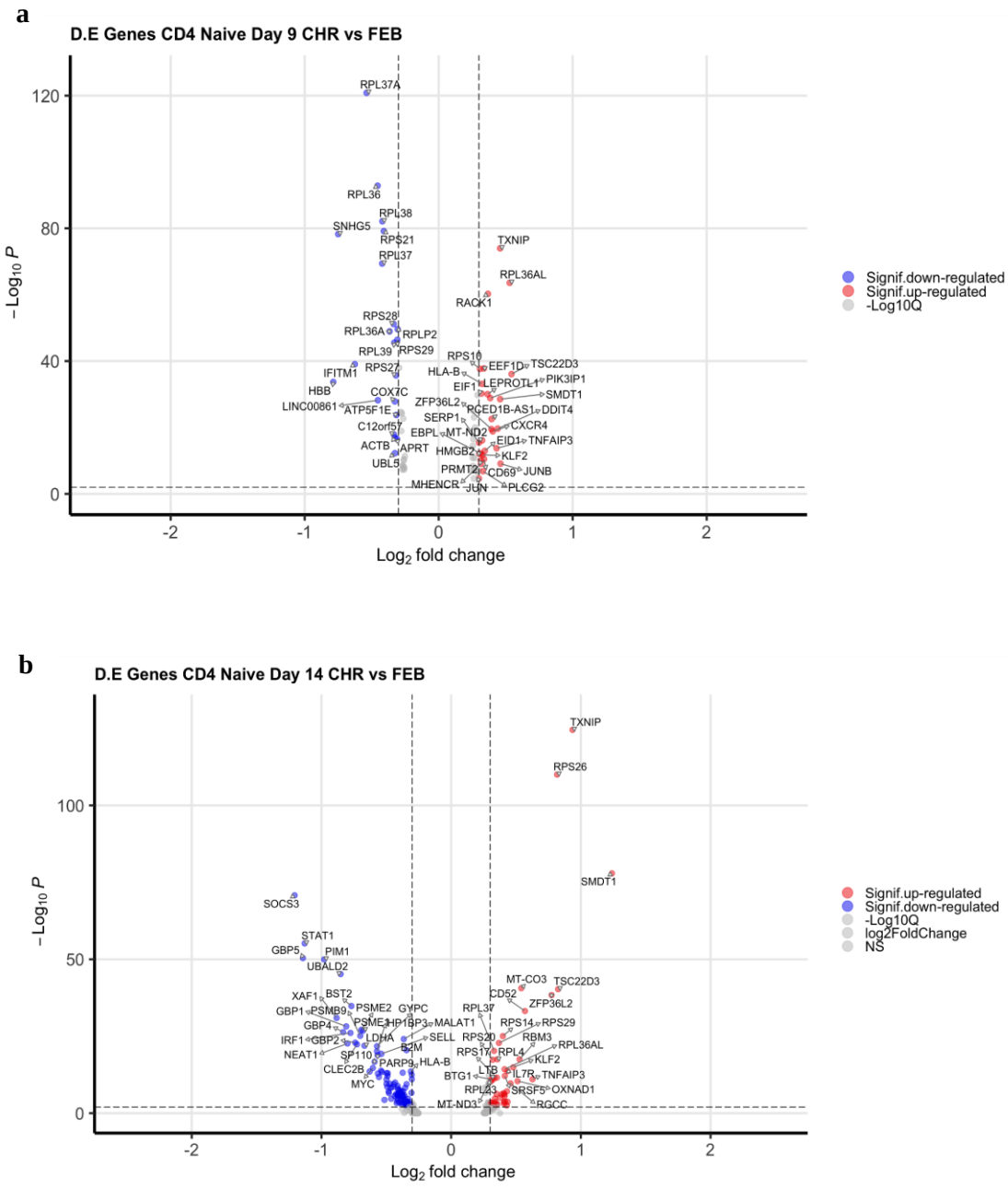
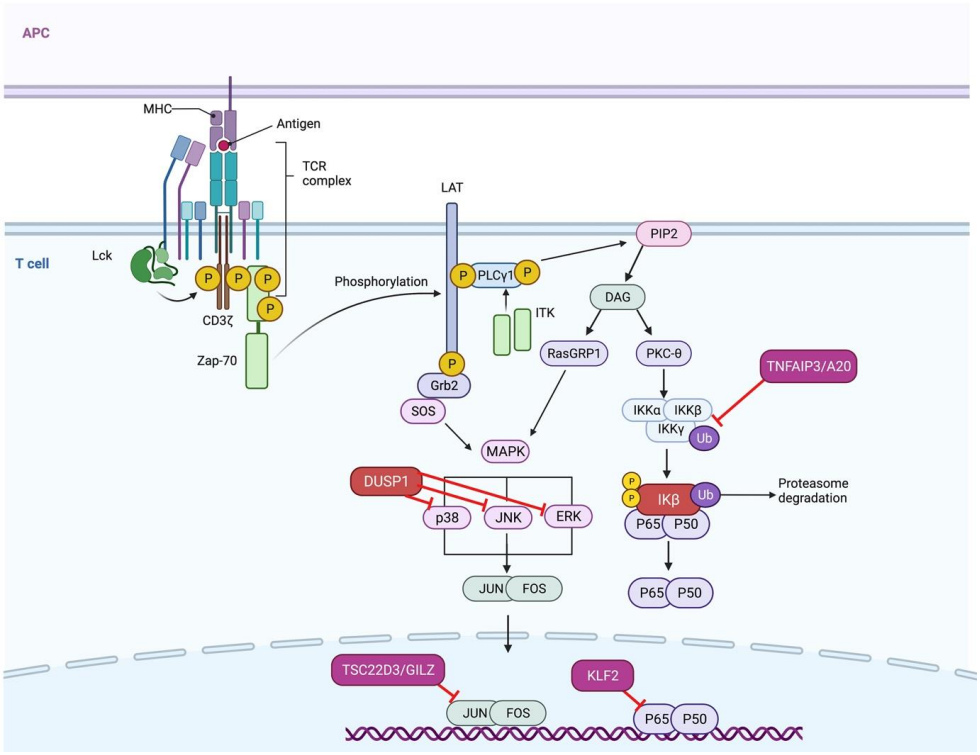


Figure 5-29 Differentially expressed genes in naïve CD4 T cells from chronic and febrile participants.

The graph shows differentially expressed gene in CD14 monocytes between the chronic and febrile participants at day 9 (a) and day 14 (b) post infection

a



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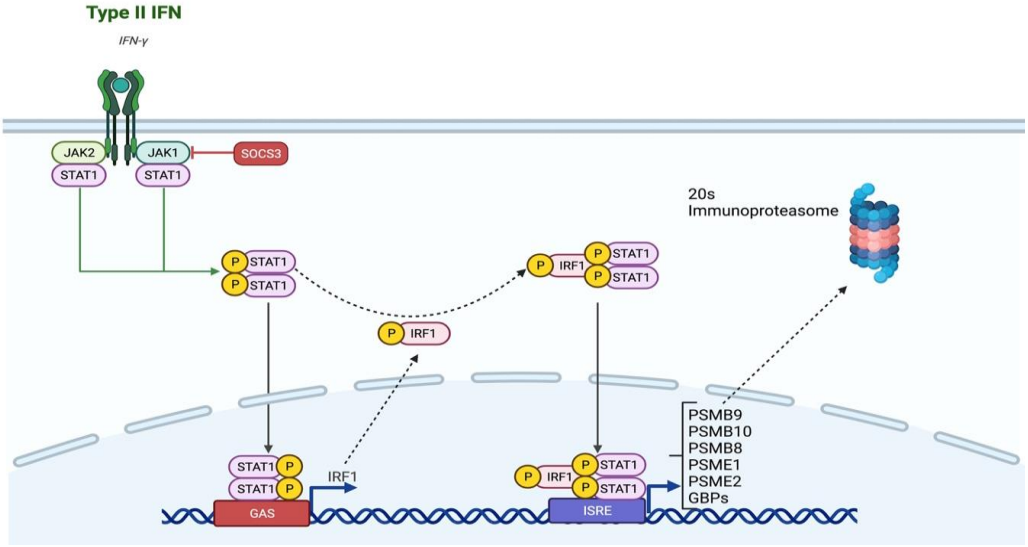


Figure 5-30 T cell receptor signalling and Type II IFN signalling in CD4 naïve T cell from chronic (a) and febrile (b) participants respectively.

a. CD4 naïve T cells from the chronic participants could be initiate signalling via the T cell receptor complex which could lead to activation of both the MAPK-AP-1 and NF-κβ signalling pathways. Upregulation of DUPS1/2 might inhibit MAPK signalling, while the upregulation of TSCD22D3 could negatively regulate AP-1 activity. Upregulation of TNFAIP3, Iκβ and KLF2 may have inhibited NF-κβ signalling.

b. In the febrile participants at day C14, there was an upregulation of STAT1, IRF1 and a host of IFN stimulated response genes suggesting that IFNγ signalling is upregulated. Upregulation of SOCS1 may inhibit JAK2 and IFNγ-Rα chain activity, possibly resulting in regulation of STAT1. Figures were created with Biorender.com

5.4.8.4 CD4 Memory T Cells

At day C9, CD4 memory T cells from the febrile participants upregulated genes from type I IFN signalling pathway namely of IRF1 and STAT1 when compared to chronic participants. IFN response elements, IFITM1, CCL5 AND ACTR3 were also upregulated. Genes involved in ribosomal assembly and translation initiation were also expressed.

CD4 memory T cells from chronic participants at day C9 expressed NF-κβ signalling inhibitors such as KLF2, TNFAIP3, and TSC22D3 when compared to febrile participants. The inhibitor of PI3K signalling pathway, PI3KIP1 was also upregulated at this timepoint.

At day C14 CD4 memory T cells from febrile participants, upregulated genes associated with type II IFN signalling pathway namely IRF1, STAT1 and SOCS3 together with IFN response elements, IFITM2, MX1, MX2, IFI35 XAF1, IFI6, ISG20, and BST2 when compared to CD4 memory T cells from chronic participants. Genes involved in the assembly of the 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2, PSMA5, PSMA7) and a number of Guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) were also upregulated. Genes involved in T cell activation such as CCR7, ICOS, SELL, and PTPRC were also upregulated in these cells.

CD4 memory cells from the chronic participants at day C14, expressed the transcription factor AP-1 (JUN and JUNB) and several NF-κβ signalling inhibitors such as DUSP1, TNFAIP3, KLF2 and TSC22D3 when compared to CD4 memory T cells from febrile participants. The chemokine receptor CXCR4 and CD48 signalling molecule were also expressed at this timepoint.

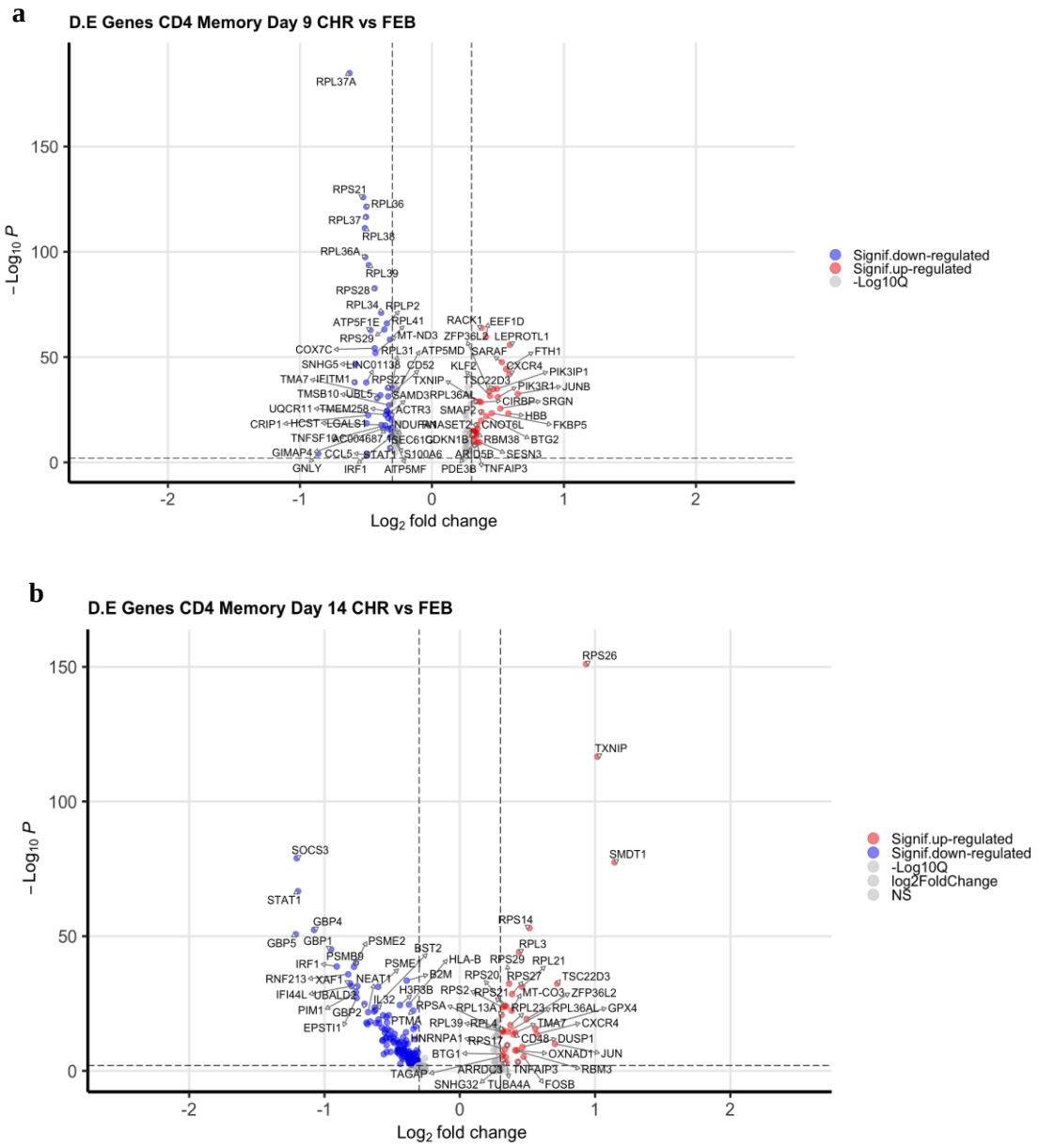


Figure 5-31 Differentially expressed genes between chronic and febrile CD4 memory T cells.

The graph shows differentially expressed gene in CD4 memory T cells between the chronic and febrile participants at day 9 (a) and day 14 (b) post infection

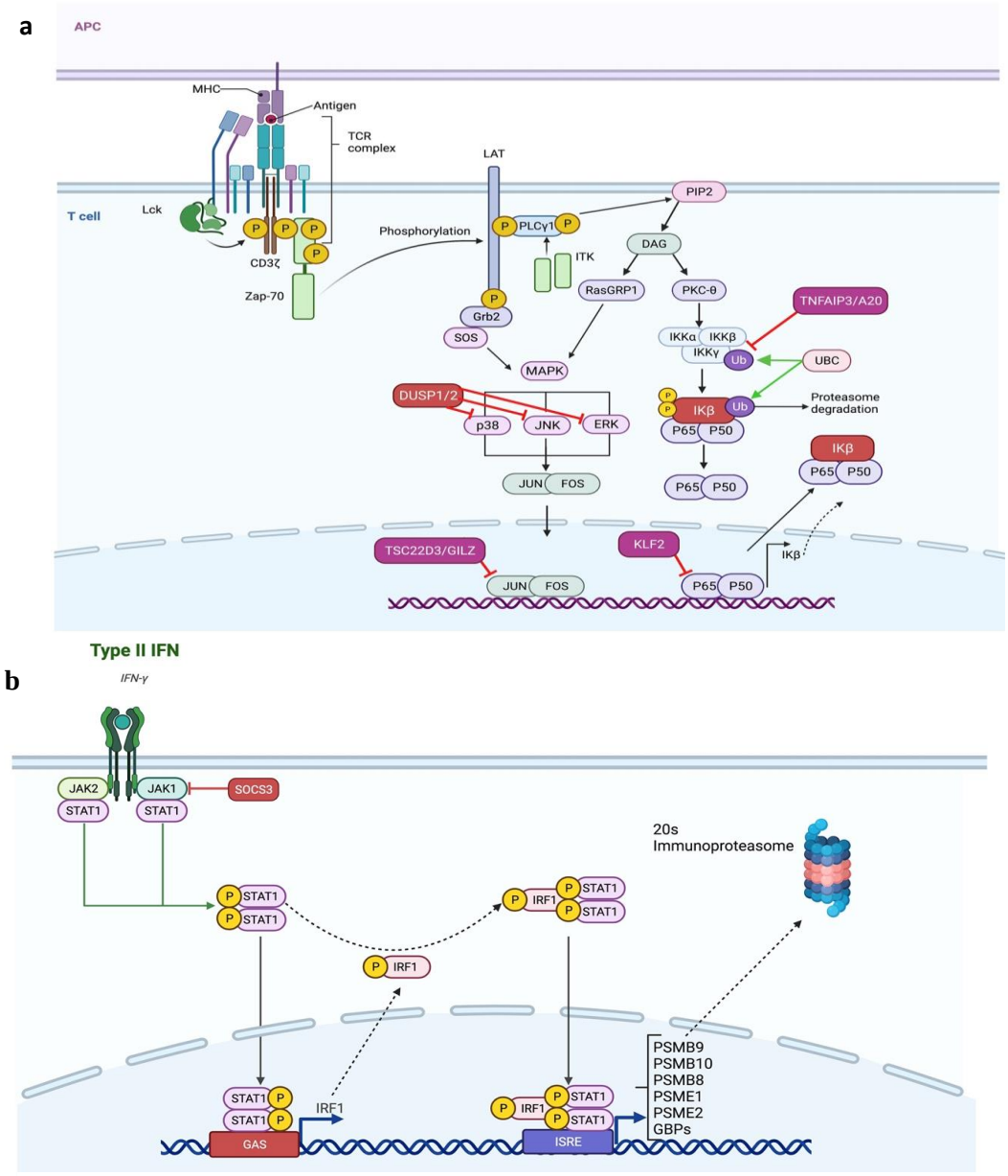


Figure 5-32 T cell receptor signalling and Type II IFN signalling in CD4 naïve T cell from chronic (a) and febrile (b) participants respectively.

a. CD4 naïve T cells from the chronic participants could be initiate signalling via the T cell receptor complex which could lead to activation of both the MAPK-AP-1 and NF-κβ signalling pathways. Upregulation of DUPS1/2 might inhibit MAPK signalling, while the upregulation of TSCD22D3 could negatively regulate AP-1 activity. Upregulation of TNFAIP3, Iκβ and KLF2 may have inhibited NF-κβ signalling.

b. In the febrile participants at day C14, there was an upregulation of STAT1, IRF1 and a host of IFN stimulated response genes suggesting that IFNγ signalling is upregulated. Upregulation of SOCS1 may inhibit JAK2 and IFNγ-Rα chain activity, possibly resulting in regulation of STAT1. Figures were created with Biorender.com

Table 5-2 Gene expression profile of the different cells from febrile participants at day 9 and day 14 post infection compared to baseline

	CD14 Monocytes Day C9		CD14 Monocytes Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	FOS, JUN	CEBPB, MAFB		
Negative Regulators of NF-κβ signalling	KLF2, TSC22D3, TNFAIP3, NR4A2, NFKBIZ			
Positive Regulators of NF-κβ signalling	KLF6, KLF4			
Mitochondrial Oxidative Phosphorylation/Electron Transport	MT-CO1, MT-ND5, MT-CO2, MT-CYB, MT-ND4L			
Ribosomal Assembly and Initiation of Translation		RPS3A, RPL10A, RPS10, RPS5, RPL5, RPL15, EEF1B2, BTF3, NASA		RPL10
Haemoglobin genes		HBB, HBA2		HBB
Cell Proliferation and Survival		CD63, TIMP1		
Negative regulator of MAPK signalling		DUSP2		
Tryptophan Metabolism			IDO2	

	CD16 Monocytes Day C9			
	Upregulated	Downregulated		
Transcription Factor	FOSB, FOS, JUN, IER2			
Negative Regulator of MAPK signalling	DUSP2, DUSP6			
Negative Regulators of NF-κβ signalling		NFKBIA		
Positive Regulators of NF-κβ signalling	KLF4			
Mitochondrial Oxidative Phosphorylation/Electron Transport		MT-ND1, MT-ND2		
Haemoglobin genes		HBB, HBA2		
Cell Proliferation and Survival		CD63, TIMP1, PIM3		
Chemokine/Cytokine	CSF1			
Cell Surface Receptor		CD83		

	Naïve CD4 T Cells Day C9		Naïve CD4 T Cells Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
IFN Signalling Molecules	STAT1			
Negative Regulators of NF-κβ signalling			NFKBIZ	KLF2, TSC22D3
Type II IFN Gamma Signalling	IFITM1		STAT1, IRF1	
Type II IFN Gamma Induced Gene			XAF1, IFITM2, IFI6, PSMB9, PSMB10, PSMB8, PSME1, PSME2, GBP1, GBP4, GBP5, GBP2	
T Cell Activation			CD69, SELL, CCR7	
Negative regulators of IFN Gamma Production			SOSC3	
Ribosomal Assembly and Initiation of Translation				RPL23, RPL37, RPL37A, RPS26,
Haemoglobin Genes	HBB			
Chemokine Receptor		CXCR4		CXCR4, IL7R

	Memory CD4 T Cells Day C9		Memory CD4 T Cells Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor		JUNB		
Negative Regulators of NF-κβ signalling			NFKBIA	TSC22D3
IFN Induced Gene	IFITM1			
Type II IFN Gamma Signalling			STAT1, IRF1	
Type II IFN Gamma Induced Gene			XAF1, IFITM2, IFI6, IFI35, ISG20, PSMB9, PSMB10, PSMB8, PSME1, PSME2, GBP1, GBP4, GBP5, GBP2	
T Cell Activation			SELL, ICOS	
Negative regulators of IFN Gamma Production			SOSC3, SOCS1	
Ribosomal Assembly and Initiation of Translation				RPL13A, RPL21, RPL23, RPL3, RPL37A, RPS21, RPS26, RPS27, RPS29
Haemoglobin Genes		HBB		
Cell Proliferation and Survival				
Negative regulator of MAPK signalling				TSC22D3
Chemokine Receptor		CXCR4		CXCR4

Table 5-3 Gene expression profile of the different cells from chronic participants at day 9 and day 14 post infection compared to baseline

	CD14 Monocytes Day C9		CD14 Monocytes Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	MAFB, SPI1(PU.1), JUN, FOS			
Negative Regulators of NF-κβ signalling			KLF2, TSC22D3, TNFAIP3, NR4A2, NFKBIZ	
Negative Regulators of IFN Gamma Production			SOCS3	
Mitochondrial Oxidative Phosphorylation/Electron Transport	MT-CO1, MT-ND5, MT-CO2, MT-CYB, MT-ND4L		NDUFA1, NDUFA3, NDUFB1, NDUFB2, NDUFB4, NDUFC1	
Ribosomal Assembly and Initiation of Translation	RPL10, RPL3, RPL7A, RPL8, RPLP0, RPS2, RPS3, RPS3A, RPS4X, RPS4Y1, RPS5		RPL10, RPL3, RPL7A, RPL8, RPLP0, RPS2, RPS3, RPS3A, RPS4X, RPS4Y1, RPS5	
Haemoglobin genes	HBB, HBA2			
MHC Molecules	HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB1			
Type II IFN Signalling	IFNGR2		JAK2, IRF1	
Type II IFN Induced Gene	IFI30, IFI6, IFITM2			
Chemokines	CXCL16, CXCL8			

Integrins			ITGA4,ITGAL, ITGB1, ITGA4, ITGB1	
S100 alarmins			S100A6, S100A4, S100A8 S100A9, S100A12	

	CD14 Monocytes Day C9		CD14 Monocytes Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	MAFB	CEBPB, MAFB	CEBPD, JDP2, JUNB	
Transcriptional Regulators	ERG1, ERG3			
Negative Regulators of NF-κβ signalling			TNFAIP3, NR4A2	
MAPK Signalling	MAPK6			
Type II IFN Induced Gene			IRF2	
Negative regulator of MAPK signalling	DUSP6, DUSP2		DUSP1	
Haemoglobin genes			HBB, HBEGF	
Negative Regulators of IFN Gamma Production		SOCS3		
Chemokines/Cytokine	CXCL2, CCL3		IL-1B	
Chemokine Receptor			CXCR4	
Cell Surface Receptor		CLEC12A, PTPRC		CD14, CD300E

	CD16 Monocytes Day C9		CD16 Monocytes Day C9	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	MAFB, SPI1(PU.1), CEBPB, CEBPD, EST2		MAFB, CEBPD	
Negative Regulator of MAPK signalling	DUSP2, DUSP6			
Negative Regulators of NF-κβ signalling			TSC22D3	
Positive Regulators of NF-κβ signalling	KLF6			
Type II IFN Signalling	IFNGR1	JAK2		
Type II IFN Induced Gene	IFITM2, IFITM2	IFI16, IGSF6, IRF2, OAS1, OAS2	IFITM2, IFITM2	
MAPK Signalling	MAP3K8			
MHC Molecules	HLA-B, HLA-DQB1			
Ribosomal Assembly and Initiation of Translation		RPL13A, RPL21, RPL23, RPL3, RPL37A, RPS21, RPS26, RPS27, RPS29		
Haemoglobin genes	HBB, HBA2		HBB, HBA2	
Cell Surface Receptors				TNFSF10, LAIR2
Chemokines/Cytokine	CXCL16, CXCL8			
Chemokine Receptor	CXCR4	CX3CR1	CXCR4	

	Naïve CD4 T Cells Day C9		Naïve CD4 T Cells Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	JUNB		JUN, JUNB	
Negative Regulator of PI3K activity	PIK3IP1			
Negative Regulators of NF-κβ signalling	TSC22D3		TSC22D3	
Type II IFN Signalling			STAT1	
Type II IFN Induced Gene			IFITM1	
Negative regulators of IFN Gamma Production		SOCS3		SOCS3
S100A alarmins		S100A6, S100A4		
Haemoglobin Genes	HBB			

	Memory CD4 T Cells Day C9		Memory CD4 T Cells Day C14	
	Upregulated	Downregulated	Upregulated	Downregulated
Transcription Factor	FOSB, FOS, JUN, JUNB		FOSB, FOS, JUN	
Negative Regulators of NF-κβ signalling	KLF2, NFKBIA, TSC22D3, TNFAIP3		NFKBIA, TSC22D3	
IFN Induced Gene	IFITM1		IFITM1	
Negative Regulators of MAPK signalling	DUSP1, DUSP2		DUSP1	
Negative Regulator of PI3K activity	PIK3IP1			
Transmembrane Signalling enzyme		PLCG2		
Positive Regulator of NF-κβ signalling			KLF6	
Chemokine Receptor	CXCR4			
Chromatin Modifiers and Remodellers		HIST1H1E, HIST1H1D		

5.5 Discussion

The single cell RNA sequencing of CHMI samples showed that different cell phenotypes (except for tolerised moDCs) increased in numbers from the baseline to day 9 post infection in both the chronic and febrile participants. The cell expansions at day 9 post infection correlates with the exposure of the immune system to blood stage parasites, which normally appear from day 7 of CHMI. The tolerised moDCs were a population unique to the febrile participants and their numbers increased at day C14, which coincided with a decrease in number of circulating monocytes (both CD14 and CD16), DC, and plasmacytoid DC. While this population of monocytes are yet to be described in adults during an acute malaria infection, in a cohort of Malian children, it was observed that recurring/continuous malaria infection in children skewed their monocytes towards a tolerised phenotype that attenuated *Plasmodium*-induced inflammation, but monocytes returned to a non-tolerised phenotype in the absence of ongoing malaria infection, which coincided with the dry season (Portugal et al., 2014). In Uganda where malaria transmission is all year round, older children dampened immune responses during acute malaria infection compared to younger children (Farrington et al., 2017). This indicates that persistent exposure may be a prerequisite to altering monocyte metabolic state, chromatin, surface receptors expression and the frequencies of each monocyte subset (Nahrendorf et al., 2021), thus contributing to maintaining monocytes in a tolerogenic state and controlling *Plasmodium*-induced inflammation. Another population of cells that was unique to the febrile participants were the cytotoxic monocytes. These cytotoxic monocytes were double positive, they expressed both CD14 and CD16 (intermediate monocytes), and expressed several cytotoxic molecules such as, lysozyme, granzyme, perforin, granulysin, and natural killer cell granule protein 7, an indication that these cells may be specialised in phagocytosis and elimination of parasite infected RBCs. Expansion of cytotoxic monocytes (that express both CD14 and CD16 (intermediate monocytes)) has been observed in children with both uncomplicated and severe malaria from western Kenya (Ogonda et al., 2010, Dobbs et al., 2017) and in both adults and children these intermediate monocytes have been shown to have a better phagocytic capacity than classical monocytes in phagocytosis of *P. falciparum* (Dobbs et al., 2017) and *P. vivax* infected RBCs (Antonelli et al., 2014). The intermediate monocytes also expressed a number of HLA molecules involved in antigen presentation. This suggests that the double positive

monocytes in the febrile participants at day C9 may play an active role in controlling parasitaemia. These double positive monocytes are unable to adequately control parasitaemia as febrile participants show a steady increase in parasitaemia over time (**Figure 5-1**) and were later treated with antimalarials. At day 14 post infection, the double positive monocytes may be replaced with tolerised moDCs that try to limit the inflammatory response against the parasite.

5.5.1 Active Signalling Pathways in Febrile Participants

5.5.1.1 CD14 Monocytes

In the samples from febrile participants, the transcription factor activator protein-1 (AP-1) which is a dimer of JUN and FOS transcription factors, and several regulators of the NF-κβ signalling pathway were the main differentially upregulated genes in CD14 monocytes at day C9, while at day C14 the main upregulated genes were mostly involved in immunosuppression and inhibition of proliferation these were IDO1 and GBP1. At day C9 compared with baseline, there was an implication that both the mitogen-activated protein kinases (MAPK) signalling which activates AP-1 (Whitmarsh and Davis, 1996) and NF-κβ signalling pathway were active. The activation of these two pathways could possibly occurs through activation of TLR signalling (Hwang et al., 1997, Kuper et al., 2012). TLRs may be able to recognise *Plasmodium* GPI and other *Plasmodium* PAMPs that activate signalling through myeloid differentiation primary response 88 (MyD88), tumour necrosis factor receptor-associated factor 6 (TRAF6) and transforming growth factor-β-activated kinase 1 (TAK1). TAK1 might activate both NF-κβ and AP-1 transcription factors which in turn may initiate transcription of proinflammatory genes (Krishnegowda et al., 2005, Zhu et al., 2005). The activated inflammatory signals must be kept in check to ensure that the inflammatory response is not harmful to the host. As seen in the sequence data, the CD14 monocytes upregulate several genes that keep inflammatory signal in check, majority of which were directed at NF-κβ signalling molecules and its transcription activity. These include, the Kruppel-like transcription factors, zinc finger proteins with the ability to bind DNA elements that could act as transcriptional repressors (KLF2) or activators (KLF4 and KLF6) (Nagai et al., 2009). KLF2, KLF4, and KLF6 were upregulated in CD14 monocytes each with a specific role that may shape the CD14 monocyte response to

Plasmodium infection. KLF2 has the potential to inhibit transcriptional co-activators of NF-κβ, possibly preventing transcription of proinflammatory genes (Das et al., 2006, Nayak et al., 2013), thus regulating inflammatory signals in the monocytes. KLF4 on the other hand could be an activator of monocyte inflammatory response through its interaction with NF-κβ subunit p65 which could potentially enhance the transcription of proinflammatory genes (Feinberg et al., 2005). KLF4 may play a role monocyte differentiation process and development of inflammatory monocytes (Feinberg et al., 2005, Alder et al., 2008). KLF6 functions similar to KLF4, in that it could promote inflammation in monocytes through binding to the NF-κβ subunit p65 and potentially enhance the transcription of proinflammatory genes (Date et al., 2014, Zhang et al., 2014). TNFAIP3, also known as A20, is a ubiquitin-editing enzyme that can has the ability to deubiquitinate and ubiquitinate its target proteins (Martens and van Loo, 2020, Lu et al., 2013). TNFAIP3 is a potential negative regulator of NF-κβ signalling, it does this through binding to NEMO (IKKγ) which forms part of the IKK complex with IKKα and IKKβ, inhibiting the phosphorylation of IKK (IKKα and IKKβ) by TAK1 (Verhelst et al., 2012, Tokunaga et al., 2012, Skaug et al., 2011). Apart from regulating NF-κβ signalling, TNFAIP3 also can negatively regulate the activation of the inflammasome, restricting the secretion of cytokines (Walle et al., 2014). TSC22D3, which encodes for the glucocorticoid-induced leucine zipper (GILZ), is a potent anti-inflammatory mediator that could inhibit the transcriptional activities of both AP-1 and NF-κβ. It could inhibit NF-κβ transcription activities through binding to the NF-κβ subunit p65, thereby preventing NF-κβ from binding to its gene promoter region (Ayroldi et al., 2001, Mittelstadt and Ashwell, 2001). TSC22D3 can also inhibit the transcriptional activities of AP-1 by binding to either JUN and FOS thereby regulating its transcription activity (Mittelstadt and Ashwell, 2001) (**Figure 5-9**).

The downregulated genes at this timepoint were genes that could be involved in cell survival and differentiation. This includes transcription factor MAFB and CEBPB, which may be involved in monocyte differentiation, proliferation, and survival (Huber et al., 2012, Tamura et al., 2017, Kelly et al., 2000). CD63 and Tissue Inhibitor of Metalloproteases 1 (TIMP1) genes were also downregulated, and these genes could be involved in cell survival and differentiation and signalling through TIMP1-CD63 could also to inhibit apoptosis (Li et al., 1999, Liu et al., 2003, Liu et al., 2005, Jung et al., 2006). TIMP1 main function is to inhibit the activity of matrix

metalloproteases, but it has been shown to also function as a cytokine through interacting with CD63, a binding partner for β1-integrin (Jung et al., 2006). TIMP1/CD63/β1-integrin complex can initiate cell survival signals via FAK and ERK, and pro-apoptotic signals via caspase-3 (Jung et al., 2006). Signalling through the TIMP1/CD63/β1-integrin complex potentially activates different signalling pathways depending on the cell type (Olafsson et al., 2019, Wilk et al., 2013, Nicaise et al., 2019). Ribosomal genes were also downregulated in these monocytes.

At day C14 compared to baseline, the monocytes upregulated the tryptophan degrading enzyme IDO1, which might initiate the tryptophan metabolic pathway (Stone and Darlington, 2002). IDO1 and other downstream enzymes in this pathway might produce several immunosuppressive by-products from tryptophan metabolism. These by-products may supress the function of antigen presenting cells and monocytes, potentially hindering their ability to activate T cells (Mellor and Munn, 2004, Munn et al., 1999). GBP1, an interferon induced gene was also upregulated, suggesting that it may act to inhibit proliferation and apoptosis in cells during inflammation, therefore increasing cell survival in inflammatory conditions (Capaldo et al., 2012, Guenzi et al., 2001, Bleiziffer et al., 2012). Monocytes at this time point have reduced effector function and are likely skewed towards a suppressive phenotype.

Overall, at day C9 CD14 monocytes show early signs of inflammatory activation, likely through the TLR2/4-MyD88 axis which may activate both NF-κβ and MAPK signalling pathway, resulting in the activation of AP-1 (JUN and FOS dimers) and NF-κβ transcription factors. NF-κβ signalling and transcriptional activities could be negatively regulated by TSC22D3, TNFAIP3, and KLF2. TSC22D3, may also inhibited the transcriptional activities of AP-1 by binding to either JUN and FOS thereby regulating its transcription activity (Mittelstadt and Ashwell, 2001). Interestingly there was competition for binding to the p65 subunit of NF-κβ, as KLF4 and KLF6 also bind to p65 and enhance NF-κβ transcriptional activity, while TSC22D3 binding to p65 subunit inhibits NF-κβ from binding to its promoter region. Generally, it seems that once NF-κβ and AP-1 are activated in these cells, negative regulators of both transcription factors can be initiated, limiting the proinflammatory signals from these pathways. Activation of these proinflammatory signals result in downregulation of genes involved in cellular proliferation and survival of the monocytes. At day C14, there is an upregulation of anti-

apoptotic and anti-proliferative genes, in this case GBP1, that ensures the cells survive in the inflammatory setting, while IDO1 drives the cells towards an immunosuppressives phenotype.

While such an immune profile is yet to be described in literature, this data shows the inflammatory pathways that are initiated early during a malaria infection in febrile participants. In Malian adults, monocytes simulated *ex-vivo* with *P. falciparum* infected RBCs resulted in downregulation of NKFB1, TLR5, TLR7, CXCL9, CXCL10, NLRP1, NLRP3, FCGR3A, PTX3 and various HLA molecules when compared to monocytes from children, who most likely have had less malaria episodes (Guha et al., 2021). The downregulation of NKFB1 can be attributed to the regulatory mechanism described above that ensures its activation is transient.

5.5.1.2 CD16 Monocytes

In CD16 monocytes at day C9 compared to baseline, the upregulated genes were mostly involved in the MAPK signalling pathway, which suggests that the transcription factor AP-1 (FOS, FOSB, and JUN dimers) were activated. The pathway involved in AP-1 activation could be the TLR2/4-MYD88-TRAF6-TAK1-MAPK pathway. The activation of TLR-MAPK signalling pathway might also lead to the activation of dual-specificity phosphatases (DUSP) 1 and DUSP6 (Grumont et al., 1996). DUSP1 transcription can be initiated by a number of inflammatory signals and in this case AP-1 and NF-κβ signalling might provide the signals (Kwak et al., 1994). Once activated, DUSP1 might inhibit p38 MAPKs and JNK signalling cascades, but it has been shown to preferentially bind to JNK with a higher affinity (Chen et al., 2002, Hammer et al., 2006, Zhao et al., 2006, Slack et al., 2001, Wu et al., 2005) thereby limiting signalling through the MAPK pathway. DUSP6 could dephosphorylates ERK1/2, thereby inhibiting the ERK1/2 signalling cascade (Arkell et al., 2008). This then leaves the p38 MAPK signalling cascade of MAPK pathway active as the JNK and ERK1/2 cascaded are inhibited by DUPS1 and DUSP6 respectively. Thus, the p38 MAPK signalling cascade might be activate in CD16 monocytes, and this may lead to continued activation of AP-1 (JUN and FOS dimers) transcription factors and upregulation of proinflammatory genes (**Figure 5-11**).

In children, a low number of CD16 positive non-classical monocytes ($CD14^{-}CD16^{+}$) have been associated with a more severe malaria and a higher risk of death (Royo et al., 2019), but these observations have not been seen in adults especially those with previous exposure to malaria. While these non-classical monocytes play a role in severe malaria in children, their role in adults is yet to be determined, however, my data shows the key pathways that are activated in these monocytes and as with CD14 monocytes, the pathways are tightly controlled with negative regulators activated to ensure inflammation is kept in check.

CD14 and CD16 monocytes both seem to have a similar role in that they both seem to initiate inflammatory signals. The only difference being the activated signalling pathway with CD14 monocytes having an active MAPK and NF-κβ signalling pathway while CD16 only had an active MAPK signalling pathway.

5.5.1.3 Naïve. CD4 T Cells

Naïve CD4 T cells at day C9 compared to baseline, upregulated STAT1, suggesting that the cells had an active IFNγ-JAK/STAT signalling pathway that could eventually lead to activation and upregulation of STAT1 and the IFN response gene IFITM1 (Darnell et al., 1994). STAT1 in turn could regulate the expression of the transcription factor T-bet, which is vital in Th1 differentiation of naïve CD4 T cells (Lighvani et al., 2001). At day C14 compared to baseline, the naïve CD4 T cells still had an active IFNγ-JAK/STAT signalling pathway, as seen by the upregulation of STAT1. Signalling through the IFNγ-JAK/STAT pathway could have led to the activation of IRF1 which was upregulated through the transcriptional activities of STAT1 homodimers. STAT1-IRF1 dimers can initiate transcription of several interferon response elements including the guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) (Cheng et al., 1983, Decker et al., 1989) and the core subunits of 20s immunoproteasome (PSMB9, PSMB10, PSMB 8, PSME1, PSME2) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994) all of which were upregulated in the naïve CD4 T cells. The role of GBPs in CD4 T cells is still unclear but GBP1 is known to negatively regulate early signalling in the T cell receptor (Forster et al., 2014). Components of the 20s immunoproteasome were upregulated possibly as to response to IFNγ signalling. The 20s immunoproteasome assembly in response

to cytokines, in this case IFNγ, is faster than the standard proteasome, this is to ensure that the cell is able quickly adapts to its changing environment. The rapid assembly is accompanied by substantially fast turnover, as it mostly relies on cytokine signals to be induced, ensuring that the cell returns to a homeostatic state once the stimuli has subsided (Heink et al., 2005). The activation of proinflammatory signals in the naïve CD4 T cells is accompanied by upregulation of protein synthesis and production of defective ribosomal products (misfolded and/or oxidized proteins), which can be degraded by the immunoproteasome to avoid the toxic effects of accumulated polyubiquitinated protein aggregation as seen in immunoproteasome deficient mice (Seifert et al., 2010). The immunoproteasome might also function an activator of NF-κβ, through the proteolytic processing of NF-κβ precursors p105 and p100, to its active transcription factor p52/p50 as mice lacking the immunoproteasome have defects in proteolysis of IκB and processing of p100/p105 (Hayashi and Faustman, 1999, Hayashi and Faustman, 2000). Naïve CD4 T cells at this timepoint also upregulated STAT3 and SOSC3, though it was not clear which pathway activates the two signals as there was no evidence that IL-6 signalling was active in these cells. However, it has been shown that IFNγ can weakly activate STAT3 (Qing and Stark, 2004), which in turn can initiates transcription of SOSC3. SOCS3 may function as an inhibitor of the JAK-STAT pathway, by binding to JAK1, JAK2, and TYK2, inhibiting them from activating downstream signalling molecules in the pathway (Babon et al., 2012). Downregulated genes in the naïve CD4 T cells were mostly related to inhibition of NF-κβ activities that is, TSC22D3 and KLF2 (**Figure 5-13**).

Overall, naïve CD4 T cells upregulated CCR7, SELL and CD69 at day C14 post infection, this suggests that the CD4 T cells were active at this timepoint. Proinflammatory signals were also enhanced at day C14 compared to day C9, due to upregulation of STAT1 and IRF1which would suggest an active IFNγ-JAK/STAT pathway, and the upregulated STAT1 could push the naïve cells towards a Th1 phenotype. A number of IFNγ induced genes are also upregulated by STAT1 and IRF1, particularly the 20s immunoproteasome which could activate the NF-κβ signalling further enhancing the proinflammatory cytokines in these cells. The IFNγ-JAK/STAT pathway is kept in check by SOCS3, which inhibits the activity of JAK2, thereby limiting the inflammatory signal.

5.5.1.4 Memory CD4 T Cells

The upregulated genes identified in memory CD4 T cells from febrile participants at day C9 compared to baseline, were metabolic related genes, COX7C which changes the metabolic activity of T cells during activation, the chemokine TNFSF10 (tumour necrosis factor ligand superfamily member 10) which induces apoptosis, and the interferon induced gene IFITM3. At day C14 compared to baseline, the upregulated pathways in the CD4 memory T cells from febrile participants were similar to those described in naïve CD4 T cells above. Upregulation of STAT1 and IRF1 could be an indication of an active IFNγ-JAK/STAT signalling pathway in the memory cells at this timepoint. STAT1 and IRF1 together could initiate the upregulation of number of interferon response elements which included the guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) (Cheng et al., 1983, Decker et al., 1989) and the core subunits of 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994). The major difference between the naïve and memory response was the upregulation of SOCS1 in the memory CD4 T cells. Upregulation of SOCS1 implies that the activation of STAT1 can be inhibited, as SOCS1 can bind to JAK2 and IFNγ-Rα chain (Yasukawa et al., 1999, Alexander et al., 1999, Starr et al., 1998) thus disrupting the activation of STAT1. This effectively disrupts downstream signals involved in Th1-like skewing of the memory cells. There was also upregulation the ubiquitin E2 enzyme UBE2L6, which could tag proteins with ubiquitin for degradation and in this case, it could tag NFKBIA for degradation freeing up NF-κβ to translocate to the nucleus and initiate transcription of proinflammatory genes (Hinz et al., 2012). Memory CD4 T cells at this timepoint are activated as seen by the upregulation of ICOS and SELL suggesting that the CD4 T cells were in an active state (Dong et al., 2001, Xu et al., 1996) (**Figure 5-15**).

In summary, the memory CD4 T cells could be activated at day C14 as seen by the upregulation of ICOS and SELL. The upregulation of SAT1 and IRF1 also suggests that the IFNγ-JAK/STAT signalling pathway was activated in these cells and could possibly push the cells towards a Th1-like phenotype. A number of IFNγ induced genes are also upregulated by the transcriptional activities of STAT1 and IRF1 dimers, particularly the 20s immunoproteasome which could activate NF-κβ further enhancing the proinflammatory signals in these cells. The

proinflammatory signals that could be initiated by the IFNγ-JAK/STAT signalling pathway were kept in check by SOCS3 and SOCS1 which could inhibit JAK2 activity. SOCS1 suppression further extends to the IFNγ-Rα chain further inhibiting IFNγ signals and limiting the development of Th1 like memory cells.

5.5.2 Active Signalling Pathways in Chronic Participants

5.5.2.1 CD14 Monocytes

Looking at cells from the chronic participants, there were two groups of CD14 monocytes, the first group of CD14 monocytes (CD14 Mono_1, **Figure 5-5 b**), the differentially expressed gene (which was a comparison of day C9 and C14 due to low number of cells at baseline), showed that at day C9 compared to day C14, there was an upregulation and dimerization of JUN and FOS genes (AP-1), suggesting that the TLR-MYD88-TRAF6-TAK1-MAPK signalling pathway was active in these cells (Krishnegowda et al., 2005, Zhu et al., 2005). Activation of AP-1(JUN and FOS dimers) could lead to transcription and upregulation of the chemokine CXCL8 (IL-8), which can attract other immune cells to the site of inflammation (Hoffmann et al., 2002). The monocytes at this timepoint also upregulated interferon gamma receptor 2, (IFNGAR2) an indication that IFNγ signalling was active in these cells. IFNγ signalling enhanced monocyte activation through upregulation of TLR expression on the surface of monocytes as well as upregulation of TLR signalling molecules MyD88, MD-2 (Kang et al., 2019, Bosisio et al., 2002) and upregulation of MHC class II molecules (Steimle et al., 1994). Monocytes at this timepoint also upregulated the transcription factors SPI1(PU.1) and MAFB. SPI1 is critical in initiating the differentiation of monocytes to monocyte derived dendritic cell (moDCs) (McKercher et al., 1996, Anderson et al., 1998, Anderson et al., 2000, Bakri et al., 2005), while MAFB drives differentiation of monocytes to macrophages (Kelly et al., 2000, Bakri et al., 2005), but MAFB activity can be inhibited by SPI1 (Bakri et al., 2005). SPI1 can also initiate the transcription of ITGAX (CD11c) , CD74, and a number of MHC class II molecules namely HLA-DMA, HLA-DMB, HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1 all of which were upregulated in these monocytes (Kitamura et al., 2012, Zhu et al., 2012, Miura et al., 2016, Yashiro et al., 2017). DNA-binding

protein inhibitor 2 (ID2) is also another transcription factor that was upregulated and may be important in differentiation of monocytes to moDCs (Jackson et al., 2011, Chopin et al., 2013). Upregulation of TIMP1 and CD63 was an indication that signalling via TIMP1-CD63 was active at this time and this could promote monocyte differentiation, proliferation, and growth (Hayakawa et al., 1992, Murate et al., 1993) (**Figure 5-17 a**).

At day C14 the monocytes, had active IFNγ signalling, evident by the upregulation of JAK2 adaptor protein that activates STAT1, and subsequently STAT1 can induces the transcription of IRF1. IFNγ signalling was kept in check by SOCS3 which inhibited JAK2 activity. The monocytes also upregulated the S100A alarmins, an indication that the monocytes were still in an inflammatory state. The alarmins S100A8/9 and S100A12 also interact with TLR4 to initiate signalling via the NF-κβ pathway, leading to upregulation of proinflammatory cytokines (Sunahori et al., 2006, Vogl et al., 2007). The activated NF-κβ pathway was negatively regulated by nuclear receptor sub-family 4 group A (NR4A) 1 and NR4A2. NR4A1 directly binds to the p65 subunit of NF-κβ blocking the transcriptional activity of NF-κβ (Li et al., 2015), while NR4A2 recruits the CoREST transrepressor to the promoters regions of NF-κβ target genes, impeding transcription (Saijo et al., 2009). Integrins were upregulated at this timepoint, namely ITGA4, ITGAL, ITGB1. ITGA4 and ITGB1 encode for proteins that form the integrin heterodimer very late activation antigen 4 (VLA-4) that is responsible for transmigration of monocytes to sites of inflammation (Gerhardt and Ley, 2015) and are also involved in the formation of immune synapse with T cells initiating their activation (Mittelbrunn et al., 2004). The insulin receptor substrate 2 (IRS2) was upregulated in the monocyte, it mainly regulates glucose metabolism (Sun et al., 1995) and during an inflammatory response its activity inhibited by SOCS3. SOCS3 binds to IRS2 promoting its ubiquitination, and eventual degradation by proteasomes (Rui et al., 2002). Degradation of IRS2 results in a decrease in glucose uptake and metabolism in the cells (**Figure 5-17 b**).

Overall, this group of monocytes at day C9 are actively differentiating into moDCs, as seen by upregulation of key transcription factors required for moDC differentiation as well as upregulation of MHC- class II molecules. The monocytes at this timepoint are likely adept at antigen capture, processing and presentation and release the chemokine CXCL8 (IL-8) to attract

other cells to the site of inflammation. By day C14 the monocytes no longer express the transcription factors required for moDC differentiation, instead there is an upregulation of inflammatory signals. These inflammatory signals initiated by S100A alarmins and IFNγ are transient as negative regulators of IFNγ and NF-κβ signalling are in play to control inflammation.

In the second group of monocytes (CD14 Mono, **Figure 5-5 b**), from chronic participants, at day C9 compared to baseline, there was an upregulation of MAPK6 (ERK3), which is part of the unconventional MAPKs that lack the typical kinase domain of classical MAPK (Cargnello and Roux, 2011). This indicates that the cells had an active MAPK signalling pathway. Activated MAPK6 can interact with c-Jun, thus positively regulating the activity of AP-1(JUN and FOS dimers), which is key in inducing cells to secrete CXCL8 (IL-8) a chemokine that may recruit neutrophils to sites of inflammation (Bogucka et al., 2020). The dual-specificity phosphatases (DUSP) 2 and DUSP6 were also upregulated at this timepoint. DUSP2 can dephosphorylates ERK1/2 and p38 MAPKs (Ward et al., 1994), and may also able to bind to and inactivate the "atypical" MAPK kinases MAPK6 (ERK3) and ERK4 (Perander et al., 2017), while DUSP6 can dephosphorylates ERK1/2, thereby inhibiting the ERK1/2 signalling cascade. Thus, MAPK6 (ERK3) activity can be regulated by DUSP2, ensuring that cytokines induced by the ERK3/4 cascade were only transiently expressed. These monocytes also upregulated the transcription factor, early growth response (ERG)-1 and ERG3. ERG1 transcription factors might inhibit inflammatory responses by recruiting the nucleosome remodelling and deacetylase (NuRD) complex, to promoter sites of inflammatory genes, thus inhibiting their transcription (Trizzino et al., 2021). SOSC3 was downregulated in these monocytes, an indication that the IFNγ signalling pathway was not being regulated in these cells (**Figure 5-19**).

At day C14 compared with baseline, the monocytes upregulated the transcription factors JUNB, Jun dimerization protein 2 (JDP2), and CEBPD. JDP2 can inhibit the transcriptional activity of CEBPD by binding to the CEBPD promoter (Nakade et al., 2007). It can also inhibit the activity of histone acetyltransferase (HAT) such as p300 and ATF-2, therefore limiting access of transcription factors to genes (Kawasaki et al., 2000, Jin et al., 2006). JDP2 may be able to form dimers with members of the AP-1 (JUN and FOS) family, in this case JUNB, and this can inhibit

AP-1(JUN and FOS dimer) dependent transcription and cellular transformation (Aronheim et al., 1997). The proinflammatory cytokine IL-1B was upregulated at this timepoint an indication of an active inflammasome signalling. IL-1B activation is a two-step signalling process that is can be initiated by a priming signal (signal 1) and an activation signal (signal 2) that results in activation of the NLRP inflammasome (Bauernfeind et al., 2009). Signal 1 can be initiated through the TLR4-MYD88-TRAF6-TAK1-NF-κβ signalling pathway, which could be activate in the monocytes at timepoint C14, resulting in the transcription and expression of pro IL-1B, which is then cleaved and activated by NLRP3 (Sun et al., 1995, Sutterwala et al., 2014). Signal 2 mostly involves activation of the NLRP3 inflammasome which could be induced through various pathways and stimuli such as generation of reactive oxygen species (ROS), lysosomal protease leakage, and the potassium efflux (He et al., 2016a). The activated inflammasome consist of the NLRP3 (that contains a Pyrin domain), binding to the Pyrin domain of apoptosis speck protein (ASC). The ASC recruits procaspase-1 through CARD–CARD homotypic interaction, and procaspase-1 auto cleaves itself to the active caspase-1 which cleaves pro-IL-1β to the active IL-1β cytokine (Schroder and Tschopp, 2010). IL-1β induces the transcription and upregulation of serglycin (SRGN), a proteoglycan peptide activated by inflammatory signals (in this case IL-1β) and localises in secretory granules and vesicles participating in intracellular storage and secretion of bioactive molecules (Grujic et al., 2005, Åbrink et al., 2004, Niemann et al., 2007). IL-1β can also induce monocytes to upregulate CXCR4 expression (Sun et al., 2015, Nie et al., 2020) a chemokine that is a crucial regulator of monocyte trafficking. Galectin 3 (LGALS3) was upregulated in these monocytes, and it may play a role in enhancing inflammasome activation (Lo et al., 2021, Arsenijevic et al., 2020) and is also a chemoattractant for monocytes recruiting more monocytes to the site of inflammation (Sano et al., 2000). Galectin 3 (LGALS3) might also modulate monocytes to secrete IL-10, which causes them to reduce MHC expression and inhibit IL-12 production (Chen et al., 2015, Chung et al., 2013). Additionally, galectin 3 may inhibit monocyte differentiation to moDCs (Chung et al., 2013). The activated NF-κβ pathway was negatively regulated by nuclear receptor sub-family 4 group A (NR4A) 1. NR4A1 directly could binds to the p65 subunit of NF-κβ blocking the transcriptional activity of (Li et al., 2015). TNFAIP3 a negative regulator of NF-κβ signalling was upregulated and can bind to NEMO (IKKγ) which forms part of the IKK complex with IKKα and IKKβ, inhibiting the phosphorylation of IKK (IKKα and IKKβ) by TAK1 (Verhelst

et al., 2012, Tokunaga et al., 2012, Skaug et al., 2011) TNFAIP3 also negatively regulates the activation of the inflammasome, restricting the secretion of cytokines (Walle et al., 2014, Zhai et al., 2018) (**Figure 5-19**).

In summary this group of monocytes transiently express proinflammatory cytokines that are then regulated by various negative feedback mechanisms. There is also a shift in the inflammatory signals from AP-1 signalling and monocyte recruitment through CXCL8 at day C9 to NF-κβ and inflammasome signalling at day 14. At day C9, the MAPK signalling pathway is activated through the atypical ERK3 signalling cascade, eventually leading to secretion of CXCL8 (IL-8) by the monocytes. ERK3 signalling is negatively regulated through the inhibitory activities of DUSP2 and DUSP6, but DUSP2 plays the major role in inhibiting the ERK3 signalling. ERG1 also provides a negative signal that inhibits AP-1 transcriptional activities through the NuRD complex, thereby interfering with further transcription of IL-8 cytokine. At day C14, TLR- NF-κβ signalling was activated, together with the inflammasome, leading to the activation of IL-1 β. These signals were negatively regulated by NR4A1 which inhibits NF-κβ transcriptional activity, and TNFAIP3 which inhibits both inflammasome activation and NF-κβ activities. At day C14 the monocytes transcription activities through JUNB and CEBPD were all inhibited by JDP2.

5.5.2.2 CD16 Monocytes

At day C9 compared to baseline, CD16 monocytes from chronic participants upregulated interferon gamma receptor 1, (IFNGR1) an indication that IFNγ signalling was active in these monocytes.

The CD16 monocytes at this timepoint also expressed the transcription factors SPI1(PU.1) and MAFB. SPI1 is a gene that is critical in initiating the differentiation of monocytes to monocyte derived dendritic cell (moDCs) (McKercher et al., 1996, Anderson et al., 1998, Anderson et al., 2000, Bakri et al., 2005), while MAFB drives differentiation of monocytes to macrophages (Kelly et al., 2000, Bakri et al., 2005). MAFB expression was inhibited by SPI1 in the CD16 monocytes (Bakri et al., 2005). The transcription factors CEBPD and CEBPD were upregulated

by inflammatory signals from the MAPK and IFNγ pathway (Salmenpera et al., 2003). These transcription factors bind to SPI1, initiating the transcription of a number of myeloid genes that drive the CD16 monocytes to become moDCs (Lekstrom-Himes and Xanthopoulos, 1998, Iwama et al., 2002). MAP3K8 was also upregulated in theses monocytes an indication of an active MAPK signalling pathway. CD83 costimulatory molecule was upregulated in as well, together with the adhesion molecule ICAM3. Superoxide dismutase 1 (SOD1) was highly expressed at this timepoint and responsible for destroying free superoxide radicals in the protecting the cells and supressing proinflammatory responses (Hwang et al., 2020) (**Figure 5-21**).

The downregulated genes at day C9 included JAK2, a crucial signalling molecule in the IFN-JAK-STAT pathway, an indication that signalling via IFNGAR2 -JAK2 was inhibited, thus IFNγ signalling was not as effective. The downregulation of JAK-STAT pathway led to a decrease in IFN stimulated genes, IRF2, OAS1, OAS2 and MT2A. Genes involved in mitochondrial ATP synthesis, ribosomal assembly and translation initiation were also downregulated.

At day C14 compared to baseline, the CD16 monocytes from chronic participants upregulated the transcription factor MAFB, which initiates differentiation of monocytes to macrophages (Kelly et al., 2000, Bakri et al., 2005). A number of IFN response genes namely IFITM2 and IFITM1 were upregulated together with the chemokine CXCR4, and genes involved with cell motility and cytoskeleton remodelling (ACTG1, ACTB and TUBA1B).

Overall CD16 monocytes at day C9 are geared towards moDCs differentiation as seen by the upregulation of SPI1, CEBPB and CEBPD, which drive the differentiation programme. The monocytes also upregulated the costimulatory marker CD83. While IFGNR1 was upregulated, signalling through its partner receptor IFGNR2 was inhibited as a key signalling molecule, JAK2, was downregulated in these monocytes indicating that the JAK-STAT pathway was partially active.

5.5.2.3 Naïve CD4 T Cells

Naïve CD4 T cells from chronic participants at day C9 compared to baseline upregulated the transcription factor JUNB, which can play a role in maintaining cell homeostasis and driving the naive cells to differentiate to T regs (Koizumi et al., 2018, Katagiri et al., 2019). TSC22D3 was also upregulated in these cells, an indication that the proinflammatory activity of the NF-κβ transcription factor may have been inhibited by TSC22D3, which encodes for the glucocorticoid-induced leucine zipper (GILZ), a potent anti-inflammatory mediator that can also inhibit the transcriptional activities of both AP-1 and NF-κβ. TSC22D3 may also inhibit the differentiation of naïve CD4 T cells to a Th1 or a Th17 phenotype but can favour the development of a Th2 or Treg (by activating transforming growth factor-β (TGF-β) signalling) (Jones et al., 2015, Bereshchenko et al., 2014, Cannarile et al., 2006). TSC22D3, might also inhibit the transcriptional activities of AP-1 (JUN and FOS dimers) by binding to either JUN and FOS thereby regulating its transcription activity (Mittelstadt and Ashwell, 2001). The zinc finger proteins ZFP36L2 was also upregulated and might be involved in maintaining CD4+ T cells in a naïve state (Makita et al., 2020). Phosphatidylinositol-3 kinases interacting protein 1 (PIK3IP1) was also upregulated and it may be able to inhibited the activity of PI3K signalling pathway which is involved in cell proliferation and growth (He et al., 2008), and can also negatively regulate T cell activation (Uche et al., 2018). SOCS3 was downregulated at this timepoint, indicating that inflammatory signals were not being suppressed.

At day C14, the naïve CD4 T cells upregulated STAT1, indicating that the naive cells had initiated signalling via IFNγ-JAK/STAT pathway that eventually led to activation and upregulation of STAT1 and the IFN response gene IFITM1 (Darnell et al., 1994). STAT1 in turn can upregulate the expression of the transcription factor T-bet, which is vital in Th1 differentiation of naïve CD4 T cells (Lighvani et al., 2001). TSC22D3 was upregulated at this timepoint, and it might inhibit the transcriptional activities of both AP-1 and NF-κβ while favouring the development of Th2 or Treg, depending on the cytokine milieu in the environment (Jones et al., 2015, Bereshchenko et al., 2014, Cannarile et al., 2006). SOCS3 was downregulated at this timepoint, thus it was unable to inhibit the activity of the JAK-STAT pathway.

In the naïve CD4 T cells at day C9, there is a host of signals pushing the naïve CD4 T towards differentiation into CD4 T regs, while at day C14 the push is for differentiation of naïve T cells to a Th1 phenotype.

5.5.2.4 Memory CD4 T Cells

Memory CD4 T cells from chronic participants at day C9 compared to baseline upregulated the transcription AP-1 (which was a dimer of JUN, FOS and JUNB, FOSB), which potentially could be activated via mitogen-activated protein kinase (MAPK) signalling. NF-κβ signalling was active at this timepoint as well, as seen by the upregulation of NF-κβ inhibitor alpha (NFKBIA) and ubiquitin-c (UBC). NFKBIA can bind to NF-κβ transcription factors, and this masks their nuclear localization signals, retaining them in the cytosol (Huxford et al., 1998, Jacobs and Harrison, 1998). To release NF-κβ from the inhibitory action of NFKBIA requires the phosphorylation by the Iκβ kinase complex which is made up of three subunits, IKKα, IKKβ and NEMO (IKKγ) (Mercurio et al., 1997). The phosphorylated NFKBIA is then polyubiquitinated at Lys-48 by UBC, possibly tagging it for degradation by the proteasome and releasing NF-κβ dimers to translocate to the nucleus driving transcription of target genes (Wertz and Dixit, 2010, Hoffmann et al., 2006). The activated NF-κβ can be negatively regulated by TNFAIP3 (which was upregulated in these cells) which binds to NEMO (IKKγ) subunit of the IKK, inhibiting the phosphorylation of IKK subunits IKKα and IKKβ by TAK1 (Verhelst et al., 2012, Tokunaga et al., 2012, Skaug et al., 2011). TSC22D3 can also regulate NF-κβ signalling through possibly binding to the NF-κβ subunit p65, thereby preventing NF-κβ from binding to its gene promoter region (Ayroldi et al., 2001, Mittelstadt and Ashwell, 2001). TSC22D3, can also inhibit the transcriptional activities of AP-1 by binding to either JUN and FOS thereby regulating its transcription activity (Mittelstadt and Ashwell, 2001). The Kruppel-like transcription factors 2 (KLF2) a zinc finger protein that has the ability to bind DNA elements was upregulated, it can bind to the transcriptional co-activators of NF-κβ thereby preventing it from initiating transcription of proinflammatory genes (Das et al., 2006, Nayak et al., 2013).The dual-specificity phosphatases (DUSP) 1 and DUSP2 were also upregulated at this timepoint. DUSP1 transcription is initiated by a number of inflammatory signals and in this case AP-1 and NF-κβ signalling provided the inflammatory signals (Kwak et al., 1994). Once activated DUSP1

could inhibit the activity of ERKs, p38 MAPKs and JNK signalling molecules, but it can preferentially bind to JNK with a higher affinity (Chen et al., 2002, Hammer et al., 2006, Zhao et al., 2006, Slack et al., 2001, Wu et al., 2005) thereby limiting signalling through the MAPK pathway. DUSP2 on the other hand could dephosphorylates ERK1/2 and p38 MAPKs (Ward et al., 1994), and can also bind to and inactivate the "atypical" MAPK kinases ERK3 and ERK4 (Perander et al., 2017) (**Figure 5-24**).

At day C14 compared to baseline, the cells showed sustained expression of the transcription factor AP-1 (JUN, FOS/FOSB dimers), whose activity was negatively regulated by TSC22D3. The MAPK pathway that activates AP-1 was also negatively regulated by DUSP1.

In memory CD4 T cells at day C9, active MAPK and NF-κβ signalling was seen by upregulation of the transcription factor AP-1 and a host of negative regulators for NF-κβ signalling such as TSC22D3, TNFAIP3, KLF2 and NFKBIA. The negative regulators of MAPK signalling, DUSP1, and DUSP2 were also upregulated. This shows that the inflammatory pathways are tightly regulated, and most inflammatory signals are transient as negative regulators of each pathway are in play to limit the negative effect of the inflammation. At day C14 there was sustained expression of AP-1 indicating that the cell had initiated inflammatory signals, but these signals were transient and tightly regulated by TSC22D3 and DUSP1. NFKBIA was also upregulated indicating that NF-κβ was bound to its inhibitor and restricted to the cytoplasm, thus NF-κβ signalling and transcriptional activities at this timepoint were inhibited. Memory CD4 T cells at day 14 were in a less inflammatory state than those at day C9.

5.5.3 Comparison of Genes Expressed in Chronic and Febrile Participants

5.5.3.1 CD14 Monocytes

CD14 monocytes from febrile participants at day C9 compared to CD14 monocytes from chronic participants at the same timepoint upregulated IRF1, STAT1, and STAT2 an indication that the cells had an active type I IFN signalling. Type I IFNs can initiate signalling through IFN alpha receptor (IFNAR), composed of IFNAR1 and IFNAR2 subunits. Receptor binding

leads to the activation of the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (Ihle, 1995, Chen et al., 2004), which in turn phosphorylates the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2. STAT1 and STAT2 form dimers which can associate with IRF9, to form the IFN-stimulated gene factor 3 (ISGF3) (Fu et al., 1990). ISGF3 translocate to the nucleus, binding to DNA sequences known as IFN-stimulated response elements (ISREs) leading to transcription of IFN-stimulated gene such as ISG15, XAF1, IFI6, IFITM3, MX1, ADAR and SAMHD1 all of which were upregulated in these cells (Levy and Darnell, 2002, Wesoly et al., 2007). STAT1 can also form homodimers which translocate to the nucleus and initiate the transcription of gamma interferon activation site (GAS) DNA elements, which includes IRF1 (Levy and Darnell, 2002, Wesoly et al., 2007, Li et al., 1996). STAT1 can form dimers with IRF1 to induce transcription of a number of IFN response genes (Abou El Hassan et al., 2017) that drive inflammatory response in these cells (**Figure 5-26**).

CD14 monocytes from chronic participants when compared to febrile participants, upregulated the S100A alarmins (S100A10, S100A11, S100A12, S100A6, S100A8, S100A9), an indication that the monocytes were in an inflammatory state. The alarmins S100A8/9 and S100A12 can interact with TLR4 to initiate signalling via the NF-κβ pathway, leading to upregulation of proinflammatory cytokines (Sunahori et al., 2006, Vogl et al., 2007). NF-κβ activation can increases the expression levels of S100A8 and S100A9, initiating a positive feedback loop which amplifies the inflammatory signal (Ehrchen et al., 2009). S100A9 can rapidly induces NF-κβ activation when compared to LPS stimulation in THP-1 cells (Riva et al., 2012) and the activated NF-κβ may induce the transcription of a number of pro-inflammatory cytokines. Thus, alarmin signalling seems to be more potent in initiating pro inflammatory cytokine secretion via TLR4- NF-κβ pathway. NF-κβ signalling is negatively regulated by NFKBIA, which binds to NF-κβ transcription factors and masks their nuclear localization signals, retaining them in the cytosol (Huxford et al., 1998, Jacobs and Harrison, 1998). Apart from alarmins, these cells also upregulated MAPK signalling molecules, namely MAP3K8 (TPL2) and MAPK6 (ERK3). MAP3K8 can be activated through a number of receptors such as TLRs, IL-1R and TNFR which are key in activating downstream signals in the MAPK signalling by possibly phosphorylating a number of MAP2K proteins which eventually lead to activation of proinflammatory signals.

MAPK6 belongs to the unconventional MAPKs that lack the typical activation motif found in most MAPKs (Cargnello and Roux, 2011) and can potentially initiate the secretion of CXCL8 (IL-8) a chemokine that recruits neutrophils to sites of inflammation (Bogucka et al., 2020). The inhibitory costimulatory molecule, hepatitis A virus cellular receptor 2 (HAVCR2/TIM3) was upregulated, and is known to inhibit IL-12 production by monocytes (Zhang et al., 2011) and also hampers Th1 responses in naïve CD4 T cells (Li et al., 2017). Galectin 3 (LGALS3) modulates monocytes to secrete IL-10, which causes them to reduce surface expression of MHC and also inhibits IL-12 production (Chen et al., 2015, Chung et al., 2013). (**Figure 5-26**).

On day C14, monocytes from the febrile participants when compared to chronic participants, upregulated the tryptophan degrading enzyme IDO1, which can initiate the tryptophan metabolic pathway (Stone and Darlington, 2002). IDO1 and other downstream enzymes in this pathway can produce a number of immunosuppressive by-products from the tryptophan metabolism that supress the function of antigen presenting cells and monocytes, hindering their ability to activate T cells (Mellor and Munn, 2004, Munn et al., 1999). GBP1, an interferon induced gene was also upregulated. GBP1 may inhibit proliferation and cell sensitivity to apoptosis during inflammation (Capaldo et al., 2012, Guenzi et al., 2001, Bleiziffer et al., 2012). Therefore, monocytes at this time point have reduced effector function and are likely skewed towards a suppressive phenotype.

CD14 monocytes from febrile participants at day C9 were responding to type I IFN, triggering the activation of the JAK-STAT pathway, resulting in induction of a number of proinflammatory genes that drive immune response. Interestingly there were no negative feedback mechanisms that were active at this timepoint to limit the type I IFN signalling, leading to uncontrolled inflammation. In contrast, CD14 monocytes from the chronic participants at the same point (day C9) showed that the inflammatory signals could be initiated by S100A alarmins and not type I IFN. S100A8/9 alarmins are constitutively expressed at a high level in neutrophils while their expression is low in monocytes (Foell et al., 2004). These alarmins can be released into the extracellular milieu during infection induced inflammation similar to how infections such as *Mycobacterium tuberculosis* (Pechkovsky et al., 2000) and HIV (Ryckman et al., 2002) result in upregulation of S100A extracellular alarmins. These alarmins can also recruit monocytes and

neutrophils to sites of inflammation (Ryckman et al., 2003). While their role in malaria is not known it is clear that alarmins amplify inflammatory response in CD14 monocytes but not CD16 monocytes as CD14 acts as a co-receptor for alarmins (He et al., 2016b).

On day C14, monocytes from the febrile participants were skewed towards a suppressive phenotype as seen by the upregulation of IDO1 and GBP1 when compared to chronic participants. While CD14 monocytes from the chronic participants, upregulated immunomodulatory molecules HAVCR2 and LAGLS3 on day C9 rather than on day C14. These immunomodulatory molecules inhibits IL-12 production by monocytes (Zhang et al., 2011, Ma et al., 2013) while promoting the production of IL-10, which reduces the surface expression of MHC molecules (Chen et al., 2015, Chung et al., 2013). These molecules also hinder Th1 responses in CD4 T cells and may limit any inflammatory signals that are activate by the MAPK and NF-κβ signalling pathways.

5.5.3.2 CD16 Monocytes

On day C9, CD16 monocytes from the febrile participants had upregulated STAT1 and STAT2 an indication that the cells had an active IFN signalling response. JAK2, a signalling molecule downstream from IFN gamma receptor, was upregulated and is key in activation of STAT1 (Keil et al., 2014). The cells also upregulated STAT2, which can be activated via the type I IFN signalling pathway (Darnell et al., 1994). Activated STAT1 and STAT2 can form dimers and associate with IRF9, to form the IFN-stimulated gene factor 3 (ISGF3) (Fu et al., 1990). ISGF3 translocates to the nucleus, binding to DNA sequences known as IFN-stimulated response elements (ISREs) leading to transcription of IFN-stimulated gene such as ISG15, XAF1, IFI6, IFIT3, MX1, OAS1 and SAMHD1 all of which were upregulated in these cells (Levy and Darnell, 2002, Wesoly et al., 2007). STAT1 can also form homodimers which translocate to the nucleus and initiate the transcription of Gamma interferon activation site (GAS) DNA elements, which includes IRF1 (Levy and Darnell, 2002, Wesoly et al., 2007, Li et al., 1996). STAT1 can also form dimers with IRF1 to induce transcription of a number of IFN response genes such as MT2A, TRIM22, AOS1, CIITA, GBP1 and GBP4, which were all upregulated in these cells, (Abou El Hassan et al., 2017) and drove inflammatory response. IRF2 was also upregulated at

this timepoint and is a negative regulator of IRF1 transcription activities (Harada et al., 1993). IRF1 and IRF2 binds to the same DNA region making them a competitive inhibitor of each other, but IRF2 has a longer half-life than IRF1 (Taniguchi et al., 1995), thus can effectively inhibit IRF1 transcription activities. The GTPase of the immunity-associated protein family (GIMAP) proteins were also upregulated in the cells (**Figure 5-29**).

On day C9, CD16 monocytes from the chronic participants expressed the transcription factors CEBPB, CEBPD, SPI1(PU.1) and MAFB. SPI1 is a transcription factor that has been implicated in initiating the differentiation of monocytes to monocyte derived dendritic cell (moDCs) (McKercher et al., 1996, Anderson et al., 1998, Anderson et al., 2000, Bakri et al., 2005), while MAFB may initiate the differentiation of monocytes to macrophages (Kelly et al., 2000, Bakri et al., 2005). MAFB expression can be inhibited by SPI1 inhibiting macrophage differentiation of the CD16 monocytes (Bakri et al., 2005). SPI1 could also lead to the transcription of ITGAX (CD11c). The transcription factors CEBPB and CEBPD can bind to SPI1, further driving the differentiation of CD16 monocytes to moDCs (Lekstrom-Himes and Xanthopoulos, 1998, Iwama et al., 2002).The monocytes also expressed the costimulatory molecules CD86 and CD83, and upregulated TIMP1 and CD63 which could potentially enhance monocyte differentiation to moDCs (Westbrook et al., 1984, Hayakawa et al., 1992).

CD16 monocytes from the febrile participants had an active IFN signalling pathway, that could result in induction of a number of proinflammatory genes. Interestingly there were no negative feedback mechanisms that were active at this timepoint to regulate the IFN signalling, but IRF2 could possibly regulated the transcription activates of IRF1 limiting the inflammatory response. While CD16 monocytes from the chronic participants did not show signs of IFN signalling but had initiated differentiation into moDCs, as seen by the upregulation of transcription factors involved in moDCs differentiation (SPI1, MAFB, CEBPD, CEBPB, TIM1 and CD69) and the upregulation of CD86, CD83 and ITGAX (CD11c).

Both CD14 and CD16 monocytes from the febrile participants have initiated signalling by type I IFN, with the only difference being that CD16 monocytes also had an active type II IFN

signalling response. Overall, it seems that the two monocyte populations had similar function that is to initiate inflammation during the course of the infection.

5.5.3.3 CD4 Naïve T Cells

On day C9, naïve CD4 T cells from febrile participants when compared to chronic participants, upregulated genes relating to ribosomal assembly and initiation of translation. Naïve CD4 T cells from chronic participants upregulated the AP-1 transcription factor, a dimer of JUN and JUNB, which can activate a number of proinflammatory genes. However, TSC22D3 was upregulated, which inhibits the transcriptional activities of AP-1 by binding to either JUN and FOS thereby regulating its transcription activity (Mittelstadt and Ashwell, 2001). Negative regulators of NF-κβ signalling were also upregulated at this timepoint, these were, the zinc finger protein KLF2 which bound to NF-κβ transcription coactivator, limiting the transcriptional activity of NF-κβ, and TNFAIP3 inhibiting the phosphorylation of IKK subunits IKKα and IKKβ by TAK1, thereby attenuating the inflammatory signal (Wertz et al., 2004, Shembade et al., 2010). These cells also expressed the early activation marker CD69, an indication that the cells were being activated (**Figure 5-30**).

On day C14, the naïve CD4 T cells from the febrile participants upregulated STAT1 and IRF1, indicating that the naive cells had initiated signalling via IFN-JAK/STAT pathway that eventually led to activation of STAT1 (Darnell et al., 1994). STAT1 in turn regulates the expression of the transcription factor T-bet, which is vital in Th1 differentiation of naïve CD4 T cells (Lighvani et al., 2001) and can activate IRF1. STAT1-IRF1 dimers can initiate transcription of several interferon response elements including the guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) (Cheng et al., 1983, Decker et al., 1989) and the core subunits of 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994) . The 20S immunoproteasome can rapidly assembled in response to IFN stimulation, and its assembly is faster than that of the standard proteasome, to ensure that the cell quickly adapts to it changing environment. The rapid assembly is accompanied by substantially fast turnover/degradation, when the cytokine signals in this case to IFN subsides, this ensures that the cell returns to a homeostatic state once stimuli

has subsided (Heink et al., 2005). The naïve T cells also upregulated SOCS3, which can bind to JAK1, JAK2, and TYK2, inhibiting them from activating downstream signalling molecules the JAK-STAT pathway (Babon et al., 2012). These cells also upregulated the early activation marker CD69, and the signalling molecule PTPRC (CD45), a transmembrane protein tyrosine phosphatase that is critical in regulating T-lymphocyte activation (Hermiston et al., 2003). As for the naïve CD4 T cells from the chronic participants, they upregulated the transcription factor JUND and a host of negative regulators for NF-κβ signalling such as TSC22D3, TNFAIP3, and KLF2 together with the negative regulator of MAPK signalling, DUSP1 (**Figure 5-30**).

Overall, naïve CD4 T cells from the chronic participants were activated at day C9, evident by the upregulation of the transcription factor AP-1 and early activation marker CD69. A number of regulators for the NF-κβ and MAPK signalling pathway were also expressed and by day C14 these naïve CD4 T cells still expressed the same genes. This indicates that naïve CD4 T cells from the chronic participants tightly regulate the inflammatory response to ensure that it is transient and controlled to ensure homeostasis is resorted once the signals had subsided.

In contrast, naïve CD4 T cells from febrile participants were mostly responding to IFN at day C14, resulting in activation of the IFN-JAK/STAT signalling pathway which was driving inflammatory responses in these cells. IFN production was regulated by SOCS3 protecting the cells from harmful effects of a prolonged inflammatory response. The late activation of CD4 T cells in the febrile participants was ineffective at controlling malaria infection as at this timepoint (day C14) parasitaemia was high.

5.5.3.4 Memory CD4 T Cells

On day C9, memory CD4 T cells from febrile participants when compared to chronic participants upregulated STAT1, IRF1 and the IFN response gene IFITM1 an indication that the cells had an active IFNγ-JAK/STAT signalling pathway (Darnell et al., 1994). On the other hand, memory CD4 T cells from the chronic participants upregulated several negative regulatory genes which are associated with inhibiting NF-κβ signalling such as TSC22D3, TNFAIP3, and KLF2. They cells also upregulated PIK3IP1, an inhibitor of PI3K signalling pathway (He et al.,

2008) and the zinc finger proteins ZFP36L2, which targets proinflammatory cytokine mRNA for destruction (Lai et al., 1999, Brooks and Blackshear, 2013). The transcription factor JUNB was also upregulated. **(Figure 5-32**)

On day C14, memory CD4 T cells from febrile participants upregulated STAT1, IRF1 and the IFN response gene IFITM1 an indication that the cells had an active IFNγ-JAK/STAT signalling pathway (Darnell et al., 1994). STAT1can form dimers with IRF1, and STAT1-IRF1 dimers can initiate transcription and upregulation of several interferon response genes including IFITM2, MX1, MX2, IFI35 XAF1, IFI6, ISG20, BST2, the guanylate-binding proteins (GBP1, GBP4, GBP5, and GBP2) (Cheng et al., 1983, Decker et al., 1989), the core subunits of 20s immunoproteasome (PSMB9, PSMB10, PSMB8, PSME1, PSME2) (Chatterjee-Kishore et al., 2000, Barton et al., 2002, Aki et al., 1994). STAT1 can also initiate the transcription of SOCS1. SOCS1 can inhibit STAT1 activation by binding to JAK2 and IFNγ-Rα chain (Yasukawa et al., 1999, Alexander et al., 1999, Starr et al., 1998). JAK3 and IL-2RG were upregulated by the cells, potentially indicating that signalling via the IL-2RG-JAK3-STAT3 was active. A number of cytokines can activate this signalling pathway this include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which bind to the IL-2RG activating JAK3 which in turn phosphorylates STAT3 (Leonard et al., 2019, Ghoreschi et al., 2009) and STAT3 may initiate the transcription of SOSC3. SOCS3 is an inhibitor of the JAK-STAT pathway, it binds to JAK1, JAK2, and TYK2, inhibiting them from activating downstream signalling molecules in the pathway (Babon et al., 2012). Memory CD4 T cells at this timepoint expressed the activated as seen by the upregulation of ICOS, PTPRC and SELL (Dong et al., 2001, Xu et al., 1996) (**Figure 5-32**).

Memory CD4 T cells from the chronic participants had upregulated the transcription factor AP-1 (a dimer of JUNB and FOS), and TSC22D3 genes which regulates the activity of NF-κβ. TSC22D3, can also inhibit the transcriptional activities of AP-1 by binding to either JUN and FOS (Mittelstadt and Ashwell, 2001). The negative regulator of MAPK signalling DUSP1 and the zinc finger proteins ZFP36L2, which targets proinflammatory cytokine mRNA for destruction were both upregulated at this time point (Lai et al., 1999, Brooks and Blackshear, 2013) (**Figure 5-32**).

In the chronic participants, most of the inflammatory signals were initiated by the MAPK and NF-κβ signalling pathways as several regulators for the NF-κβ and MAPK signalling pathway were expressed. And as seen in the naïve CD4 T cells, the transcription factor AP-1 is central to initiating the inflammatory and activation signals. For memory CD4 T cells from the febrile participants, the IFN-JAK/STAT signalling pathway is key in initiating inflammatory signals in these cells, with SOCS3 playing a negative regulatory role in IFN signalling.

5.5.4 Summary

In summary, the data here shows the different immune pathways that are present in response between the two groups of individuals with different clinical responses to infection. In the febrile participants, monocytes (both CD14 and CD16) can be seen to have an active TLR-MYD88-MAPK/NF-κβ signalling pathway. These inflammatory signals can potentially be negatively regulated by several genes such as KLF2, TSDC22D3, TNFAIP3, DUSP1 and DUSP6. Activation of these proinflammatory signals may result in downregulation of genes involved in cellular proliferation and survival of the monocytes. At day C14, CD14 monocytes expressed the anti-apoptotic and anti-proliferative genes, GBP1 which might ensure that the cells were able survive in an inflammatory setting, and IDO1 drives the cells towards an immunosuppressives phenotype.

Naïve CD4 T cells from the febrile participants could be activate at day C14, as upregulation of STAT1 and IRF1 genes suggest signalling could be occurring through the IFNγ-JAK/STAT pathway. This pathway may be negatively regulated by SOCS3, which inhibits the activity of JAK2, thereby limiting the inflammatory signal. Memory CD4 T cells from the same participants at day C9, upregulation of the transcription factors JUN and FOS (AP-1) an indication that MAPK and NF-κβ signalling were active in these cells. A host of negative regulators for NF-κβ signalling such as TSC22D3, TNFAIP3, KLF2 and NFKBIA were also upregulated to keep inflammatory signals in check. The negative regulators of MAPK signalling, DUSP1, and DUSP2 were also upregulated. This implies that the inflammatory pathways are tightly regulated, and most inflammatory signals are transient and negative regulators of each pathway are in play to limit uncontrolled inflammation. At day C14 there was

upregulation of JUN and FOS genes (AP-1) together with its negative regulator TSC22D3. NFKBIA was also upregulated indicating that NF-κβ was bound to it inhibitor and restricted to the cytoplasm.

In the chronic participants, both monocyte populations (CD14 and CD16) at day C9 had upregulated the transcription factors SPI1, CEBPB and CEBPD which suggests that the monocytes were the differentiation to moDCs. This differentiation could be further enhanced by signals from TIMP-1 and CD63. SPI1 expression might also leads to upregulation of ITGAX (CD11c), CD74 and several MHC class II molecules, suggesting that the cells could actively be involved in antigen presentation and T cell activation. At day C14, the CD14 monocytes had an active inflammatory signal that replaced the moDC differentiation signals. These signals could be driven by IFNγ and NF-κβ signalling, while CD16 monocytes at day C14 upregulated the transcription factor MAFB, which could drive differentiation of monocytes to macrophages.

Transcriptional analysis of monocytes has been conducted in adults and children from Papua during an acute malaria episode and convalesce (Loughland et al., 2020). In the study they found that adults upregulated MAP3K1 and NF-κβ signalling molecules together with IL-4 and IL-6. While children on the other hand expressed IFNG, STAT1, IL-8 and CXCR4 (Loughland et al., 2020). My data shows that MAPK and NF-κβ signalling were active in the CD14 monocytes from the febrile participants, but they did not produce any IL-4 and IL-6. While the signals that were upregulated in the children corresponded with those seen in CD14 monocytes from chronic participants. Another study also investigated the transcription profile of monocytes in malaria naïve individuals enrolled in CHMI (Walk et al., 2020). They found that 9 days post infection the participants downregulated the cytokines IL-1β, CXCL8 (IL8) and CCL3, which was contrary to my data as IL-1β, and CXCL8 (IL8) were upregulated in CD14 monocytes from chronic participants 9 days post challenge. While at 36 days post infection the participants downregulated several inhibitors of the NF-kB and MAPK signalling pathways namely NFKBID, NFKBIZ, DUSP-1, DUSP-2 and NR4A2. These negative inhibitors were upregulated in my data at 9 days and 14 days post infection as they form part of the negative feedback mechanism that limits inflammation. Their downregulation 36 days post infection could be attributed to the lack of inflammatory signals at this timepoint as parasites had been cleared.

Overall majority of studies have looked at the function of monocytes in children during an acute infection and very few have examined monocytes in adults especially from an endemic region and are able to control parasitaemia during a CHMI study, thus data is limited.

For the CD4 T cells from the chronic participants at day C9 naïve CD4 T cells had a host of signals pushing their differentiation to T regs but at day C14 the push is for differentiation of naïve T cells to a Th1 phenotype. In memory CD4 T cells at day C9, the transcription factor JUN and FOS dimers (AP-1) was active, but its activity was regulated by TSC22D3. A host of negative regulators for NF-κβ signalling such as TSC22D3, TNFAIP3, KLF2 and NFKBIA were upregulated at this time point, together with negative regulators of MAPK signalling, DUSP1, and DUSP2. This shows that the inflammatory pathways are tightly regulated, and most inflammatory signals are transient as negative regulators of each pathway are in play to limit uncontrolled inflammation. At day C14 there was sustained expression of AP-1 together with its negative regulator TSC22D3. NFKBIA was also upregulated indicating that NF-κβ was bound to its inhibitor and restricted to the cytoplasm.

Naïve and memory CD4 T cells from febrile participants were activated on day C14, unlike CD4 T cells from chronic participants which were activated at day C9. This delay could be attributed to early type I IFN production by febrile participants that dampens the immune response. This is immunoregulatory signal by type I IFN is replaced by IFNγ later at day C14. Type I IFN can be immunoregulatory as it been shown to impair DC function and development of IFN-γ producing CD4+ T cells for parasite control during *P. berghei* ANKA and *P. chabaudi chabaudi* AS infections (Haque et al., 2011, Haque et al., 2014). Type I IFN and IFN-γ have been shown to induce DC death in mice infected with *P. berghei* ANKA (Tamura et al., 2015). Type I IFN also has the ability to expand the population of T regulatory 1 (Tr1) cells in mice infected with non-lethal *P. yoelii* 17XNL (Zander et al., 2016), and suppress innate immune responses. In humans, type I IFN signalling has also been associated with immune suppression and increase in severity of disease. While polymorphism in IFN-α/β receptor (IFNAR) have been linked to progression of cerebral malaria (Ball et al., 2013, Aucan et al., 2003).

IFNγ has been shown to have antiparasitic effect as mice treated with IFNγ neutralising antibodies failed to control the parasitaemia of both lethal (Yoneto et al., 1999) and non-lethal (Stevenson et al., 1990) forms of *Plasmodium.* IFNγ deficient mice are unable to control the parasitaemia when infected with non-lethal *Plasmodium* species and these mice also produced less parasite-specific IgM, IgG2a, and IgG3 when compared to wild type control mice (Su and Stevenson, 2000). Human volunteers repeatedly injected with ultra-low doses of blood stage parasites, and subsequently treated with anti-malarial drugs before they got ill, developed strong IFN-γ-producing CD4 T cell mediated immune response, which offered them protection against consequent blood stage challenge (Pombo et al., 2002). Several other field studies have shown that IFN-γ responses correlate with protection from clinical symptoms of malaria (D'Ombrain et al., 2008, Robinson et al., 2009, McCall et al., 2010a).

The shift from immunosuppression by type I IFN to antiparasitic IFNγ in the febrile participants warrants further investigation to determine the cellular cues that cause the shift in cytokine production.

When comparing chronic and febrile participants, it was evident that most febrile participants had an active IFN-JAK/STAT signalling pathway that was not regulated by any negative feedback mechanism to control the inflammatory response this was mostly evident in both CD14 and CD16 monocytes. While in the chronic participants, both CD14 and CD16 monocytes, most of the active signals were skewed towards monocyte differentiation to moDCs which was initiated at day C9. Apart from one group of CD14 monocytes that expressed several immunomodulatory signals that is HAVCR2 and LAGLS3 that promote IL-10 production, and hinder moDC differentiation.

The signalling pathways that were activated in the chronic participants varied from those in the febrile participants. S100A alarmins seemed to be the preferred signalling molecule in CD14 monocytes from chronic participants. These alarmins also act as chemokines, recruiting more neutrophils and monocytes to sites of inflammation and during an infection, where they are seen to amplify the inflammatory response. CD14 monocytes from the febrile participants relied on type I IFN to initiate immune response but type I IFN signalling is associated with immune

suppression and increase in severity of disease (Ball et al., 2013, Aucan et al., 2003). Type I IFN, also directs APCs to suppress CD4 T cell activation (Haque et al., 2011) and this could account for the delay in activation of both naïve and memory CD4 T cells from febrile participants.

As for CD4 T cells, naïve cells were mostly activated by day C9 in the chronic participants, evident by the upregulation of the transcription factor AP-1 and early activation marker CD69 and these signals were sustained up to day C14. This early activation can be attributed to the S100A alarmins which activated the monocytes in a similar manner to LPS stimulated monocytes (Fassl et al., 2015) thereby ensuring an adequate immune response was initiated. While naïve and memory CD4 T cells from febrile participants, were activated on day C14, through the IFNγ-JAK/STAT signalling pathway. This delay is attributed to early type I IFN production by CD14 monocytes from febrile participants that dampens the immune response.

It was interesting to see genes relating to haemoglobin that is HBB and HBA in the data set. These genes were upregulated in some cells populations and downregulated in others. This could probably be due to contamination with RBC during the PBMC isolation. Thus when the PBMC were being sequenced the RBCs were subsequently sequenced.

Chapter 6 General Discussion

Malaria remains a significant burden in sub-Saharan Africa, especially in children under five years, where most of the morbidity and mortality is experienced. Current malaria control measures are insufficient to eradicate malaria and continue to be thwarted by the emergence of parasite resistance strains to antimalarial drugs, inadequate funding, and poor uptake of insecticide-treated bed nets (Dhiman, 2019, Nkumama et al., 2017). While some individuals can develop some form of immunity against disease after repeated infections, this immunity is nonsterile (Langhorne et al., 2008). The inability to achieve sterile immunity leaves individuals still at risk of being infected, partly due to the parasite's ability to constantly change immunogenic antigens (antigenic variation) (Roberts et al., 1992, Doolan et al., 2009) as an immune evasive strategy. The exact mechanism of how disease immunity develops remains poorly understood. Still, the observation of naturally acquired immunity has driven research into developing malaria vaccines that target the parasite at various stages of its life cycle (Arama and Troye-Blomberg, 2014, Ouattara and Laurens, 2015, Duffy and Patrick Gorres, 2020). Despite, clinical trials of over 100 candidate malaria vaccines so far, only RTS,S/AS01 has been approved for use in children and infants despite having a modest efficacy (Rts, 2015). The inability of individuals to achieve sterile immunity and the reduced effectiveness observed in the vaccine administered in malaria endemic areas could be attributed to the parasite's ability to impede dendritic cell function and disrupt CD4 T cell-dendritic cell interaction. The disruption of these interactions results in an impairment of T cell function and limited protective antibody responses.

6.1 Summary of Key Findings

The first aim of this study was to investigate how dendritic cell phenotype and function is affected by *Plasmodium falciparum.* To achieve this, I generated monocyte-derived dendritic cells (moDCs) and exposed them to *P. falciparum*-infected RBC after which they were stimulated with LPS. The parasite exposed moDCs responded to LPS with lower expression levels of costimulatory molecules when compared to LPS stimulated moDCs pre-exposed to uninfected red cells. This effect was seen when using moDCs from both malaria naive

(individuals who have not been exposed to malaria infection) and individuals who reside in a malaria-endemic area. However, a few individuals from the malaria endemic area had moDC that were able to upregulate costimulatory molecules after exposure to the parasite, followed by stimulation with LPS.

I then assessed if the exposure of moDCs to *P. falciparum* affected their ability to interact with and activate CD4 T cells. CD4 T cells could be activated by moDCs pre-exposed to *P. falciparum* as seen by expression of CD69 (**Figure 3-14** and **Figure 3-15**), but other early markers of activation were not upregulated such as PD-1 and CD25. To have a better understanding of the molecular changes that occur when moDCs (pre-exposed to *P. falciparum* iRBC) interact with CD4 T cells, a co-culture of moDC (pre-exposed to either *P. falciparum* Infected RBCs or uninfected RBCs) and CD4 T cells was sent to Glasgow Polyomics for single cell sequencing. The sequenced data showed that TGFB1, and HMGB2 were the major ligands expressed by moDCs that influenced their function, and interaction with CD4 T cells. Binding of HMGB2 to *P. falciparum* DNA is required for recognition by nucleic-acid-sensing cytosolic receptors, initiating signalling via cyclic GMP-AMP synthase (cGAS) (Gallego-Marin et al., 2018). TGFB1, is a potent immunoregulatory cytokine that impairs moDC function by downregulating costimulatory molecules CD80, CD86, CD83 (Geissmann et al., 1999, Ohtani et al., 2009, Mou et al., 2011), and impeded their ability to process and present antigens, leading to a downregulation of MHC-antigen complex on the cells surface (Strobl and Knapp, 1999). TGFB1 also inhibited the production of pro-inflammatory cytokines (Fainaru et al., 2007, Bonnefoy et al., 2011) and induced the expression of human leucocyte antigen-G (HLA-G) which interferes with CD4 T cell interaction and activation (Abediankenari et al., 2011). Overall TGFB1 affected moDC maturation status and its interaction with CD4 T cell. In CD4 T cells, TGFB1 inhibited the differentiation of naïve CD4 T cells to Th1 cells by inhibiting the production of Tbet and GATA-3 (Gorelik et al., 2002). TGFB1 could also downregulate the expression of IL-12Rβ2 on T cells (Gorham et al., 1998) and inhibit Th2 differentiation by modulating GATA3 expression (Kuwahara et al., 2012), while promoting the expression of FOXP3 resulting in generation of CD4 T regs (Lu et al., 2010, Martinez et al., 2010, Zheng et al., 2010, Tone et al., 2008). TGFB1 can inhibit IL-27 activated STAT1 signalling via its SMAD2/3, thereby limiting the activity of IL-27 (Sugiyama et al., 2012). This strongly suggests

that TGFB1 is key in shaping T cell immune responses in malaria naïve individuals, as it acts to counter the effect of proinflammatory cytokines that are released during the initial stages of malaria infection. In field studies, it has been shown that low levels of TGFB correlated with increase in disease severity (Walther et al., 2006, de Jong et al., 2020, Wenisch et al., 1995) and a similar observation has been seen in mouse models of malaria (Omer and Riley, 1998a).

The sequenced data from the CHMI PBMCs suggested that TLR signalling played a key role in initiating immune responses in both CHMI groups. At day 9 post infection which coincides with the onset of blood stage of infection, both groups showed an increase in cell numbers (except for tolerised moDCs whose cells numbers increased at day 14 post challenge), this may be in an attempt to control parasitaemia. By day 9 post infection, distinct signalling pathways were associated with febrile and chronic outcome of the infection. At the later timepoint, day 14 post challenge, the cell number in peripheral blood had reduced especially that of dendritic cells and monocytes. I was unable to find any differential expressed gene in the myeloid dendritic cell population most likely because activated dendritic cells would home to lymphoid organs or encounter malaria parasites in lymphoid tissues.

Sequenced data from the CHMI samples showed that the CD14 and CD16 monocytes from febrile participants were activated through two pathways. The first pathway was the TLR-MYD88-TRAF6-NF-κβ/MAPK signalling pathway and the second was the IFN signalling pathway. TLRs are pattern recognition receptors (PRRs) that are able to sense malaria PAMPs, and it has been shown that TLR2-TLR6 or TLR1-TLR2 heterodimers and TLR4 are the major TLRs involved in malaria sensing (Durai et al., 2013, Krishnegowda et al., 2005, Nebl et al., 2005). TLR activation led to signalling via MYD88 and TRAF6, as seen by the activation of both NF-κβ and AP-1 transcription factors. Downstream signalling through TLRs not only activated proinflammatory cytokines, but also initiated negative feedback mechanisms to limit the duration and extent of the inflammatory signal. The negative feedback mechanisms activated inhibitory signalling molecules upstream of AP-1 and NF-κβ and also activated transcription repressors that interfered with the transcriptional activities of both AP-1 and NF-κβ. IFN signalling varied between CD16 and CD14 monocytes as type I IFN signalling pathway was active in CD14 monocytes from day 9 post challenge, while in CD16 at the same timepoint

had an active type I and type II IFN signalling pathway. Type I IFN is vital for antiviral defences but it has been shown to promote pathogen survival by suppressing immunity during infection caused by *Mycobacteria tuberculosis* (Teles et al., 2013, Desvignes et al., 2012) , *Leishmania* (Xin et al., 2010), and *Plasmodium falciparum* (Haque et al., 2011, Sharma et al., 2011). In humans, pDCs are the major source of type I IFNs during a malaria infection and can be stimulated by infected RBC via TLR9 dependant pathway (Pichyangkul et al., 2004a). Type I IFNs have been shown to inhibit the activity of monocytes by suppressing their ability to produce IL-6 (de Oca et al., 2016). Type I IFNs also inhibit production of IFNγ in CD4 T cells (de Oca et al., 2016) and supress the antiparasitic CD4 T cell responses (Haque et al., 2011, Haque et al., 2014). Type II IFN on the other hand has been shown to be protective against *P. falciparum* infection, as early production of IFNγ been shown to be protective against cerebral malaria in both humans (Prakash et al., 2006) and mice (Mitchell et al., 2005), while in children it has been shown to be protective against clinical symptoms of malaria (D'Ombrain et al., 2008). However, production of high levels of type II IFN during the blood stage of malaria has been linked to experimental cerebral malaria in mice (Rudin et al., 1997, Amani et al., 2000). This indicates that the timing of type II IFN production dictates whether it is protective or harmful. The weak type II IFN signalling seen in CD16 monocytes at day 9 post infection could be attributed to the effect of type I IFN being able to downregulate type II IFN receptors on CD16 monocytes making them less sensitive to IFNγ stimulation, similar to how some bacterial infection reduce IFNγ sensitivity in macrophages (Eshleman et al., 2017).

In both memory and naïve CD4 T cells from febrile participants, the IFNγ-JAK-STAT pathway was driving majority of the cellular processes, and its effect was clearly seen at the later timepoint, that is at day 14 post challenge. There was a delay in activating both compartments of CD4 T cells, and this could be because cells at day 9 post challenge, monocytes are geared towards limiting inflammation through the upregulation of type I IFNs, which results in the immunosuppression of antigen-presenting cells and other immune cells (de Oca et al., 2016, Haque et al., 2011, Haque et al., 2014). Type I IFN, therefore, directs APCs to suppress CD4 T cell activation (Haque et al., 2011) which is seen at day 9 post challenge. Type I IFN induced suppression that is active at day 9 post infection is replaced by IFNγ on day 14 post challenge, indicating a shift from immunoregulation to immunocompetent cells.

Overall, in febrile participants we see type I IFN as the major cytokine driving cellular responses. As seen from the data, type I IFN reduced CD16 sensitivity to IFNγ, and inhibits the differentiation of CD4 T cell to Th1 or Tfh phenotypes (Ryg-Cornejo et al., 2016) at day 9 post infection. Type I IFN also inhibits production of interleukin-6 (IL-6) by monocytes (de Oca et al., 2016). and induces dendritic cells to produce interleukin-10 (IL-10) (Haque et al., 2014). The combination of IL-10 and type I IFN also drives naïve CD4 T cells to a T regulatory type 1 cell (Tr1), that produces IL-10 (Zander et al., 2016) further supressing immunity during blood-stage *Plasmodium* infection. Thus, early production of type I IFN in the febrile participants is detrimental in initiating immune responses against the parasite. But this type I IFN mediate immune suppression is diminished at day 14 post infection as CD4 T cells begin to initiate type II IFN signalling.

The sequenced data of PBMCs of chronic participants identified two distinct clusters of CD14 monocytes. The first cluster of CD14 monocytes were actively differentiating into moDCs and had an active type II IFN and TLR signalling pathway at day 9 post infection. At day 14 post challenge, these CD14 monocytes switched from moDC differentiation to inflammatory monocytes that still had an active type II IFN signalling, and TLR4 signalling was stimulated through S100A8/9 alarmins. The second cluster of CD14 monocytes from the chronic participant had an active MAPK signalling pathway which initiated secretion of CXCL8 (IL-8) at day 9 post challenge. At day 14 post challenge, the CD14 monocytes had an active NF-κβ signalling pathway and were actively secreting IL-1β. CD16 monocytes as well had an active type II IFN signalling pathway but a key signalling molecule in this pathway that is JAK2 was significantly downregulated. Type II IFN was active at an early time point in the chronic participants, and this has been shown to be protective against clinical symptoms of malaria in children (D'Ombrain et al., 2008). In mice, exogenous administration of type II IFN resulted in control of parasitaemia in *P. chabaudi adami* 556KA-infected CBA/CaH mice, and after recovering from infection, continuous type II IFN treatment offered full protection against subsequent infection (Clark et al., 1987). Mice treated with antibodies against type II IFN during a *P. chabaudi* AS-infection were unable to control parasitaemia compared to control mice (Stevenson et al., 1990). This further showed the importance of type II IFN in parasite control. Interestingly CD16 monocytes downregulated JAK2, a signal transduction molecule that

associates with interferon gamma receptor 2 (IFNGR2) and is essential for activating STAT1 molecule. Despite its downregulation, the cells were still able to initiate several type II IFN stimulated genes (**Figure 5-21)**. Possibly indicating that a compensatory mechanism maybe active to ensure that type II IFN signalling occurs in these cells. CXCL8 (IL-8) was secreted by CD14 monocytes at both timepoints, that is day 9 and day 14 post infection. CXCL8 (IL-8) has been shown to attract neutrophils to the site of inflammation and promote their activation (Martinez et al., 2004). CXCL8 (IL-8) has been shown to increase in patients during a malaria infection (Chandrasiri et al., 2014). During a malaria infection it is most likely that endothelial cells increase the expression of several receptors that are involved in neutrophil migration and adhesion (Chakravorty et al., 2007). ICAM-1, VCAM-1 and endothelial leukocyte adhesion molecule E-selectin have been observed to increase *in vitro* when exposed to iRBCs (Chakravorty et al., 2007). In Malawian children, soluble E-selectin and soluble ICAM-1 increased in the blood of *P. falciparum* infected children when compared to healthy control (Moxon et al., 2014), further supporting the notion of neutrophils adhering to endothelial surfaces. Although malaria increases IL-8 and adhesion molecule expression, neutrophils are rarely found in sites of parasite sequestration such as the brain microvasculature (Feintuch et al., 2016) and the placenta in malaria infected pregnant women (Carmona-Fonseca et al., 2013, Souza et al., 2013). Neutrophils can phagocytose iRBC, as observed in blood films of children (Sun and Chakrabarti, 1985), and merozoites (Wickramasinghe et al., 1987). The activation of these neutrophils also results in the release of S100A alarmins are constitutively expressed at a high level in cytosol of neutrophils (Foell et al., 2004). These alarmins are seen to trigger TLR4 signalling and recruit more neutrophils and monocytes to the site of inflammation (Ryckman et al., 2003) further amplifying the inflammatory signal.

Both memory and naïve CD4 T cells from the chronic participants, were activated early on day 9 post infection and this was mainly due to the early activation of APCs and monocytes as described above.

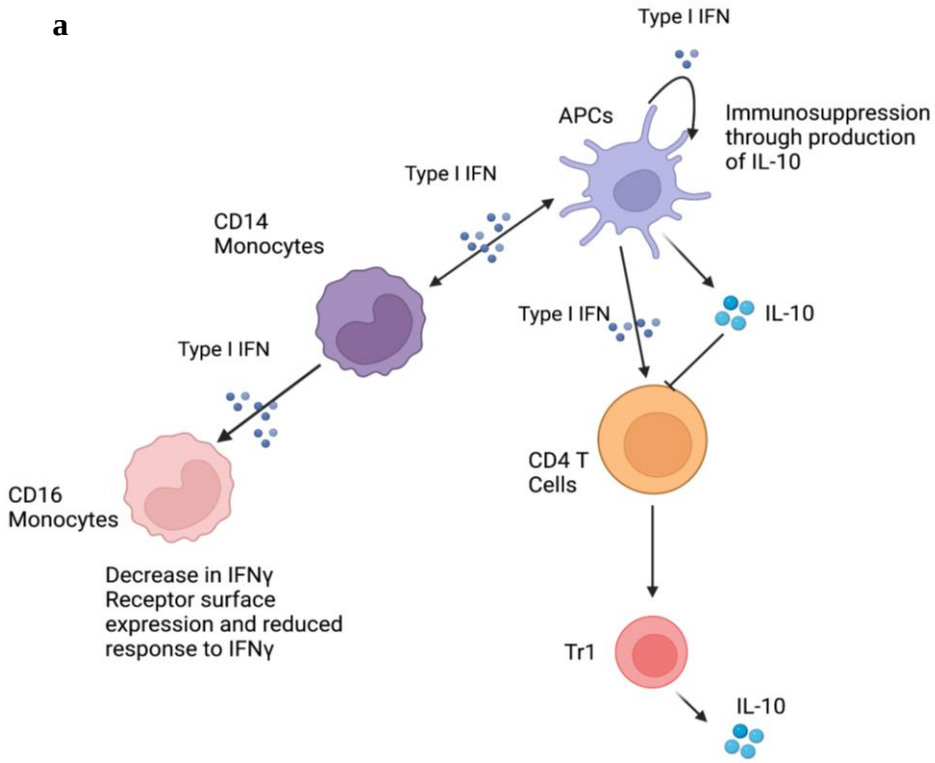
The major differences between the chronic and febrile participants were the in the type of IFN signalling initiated by each and the S100A alarmins signalling seen in the chronic participants. The chronic participants had an active type II IFN signalling at both day 9 post challenge, and

day 14 post challenge, while the febrile participants only initiated type II IFN signalling at day 14 post challenge. At day 9 post infection, type I IFN signalling in the febrile participants was active and this suppressed immune response leading to a delay in activation of CD4 T cells in these participants. The other key difference appears to be the S100A alarmins that were driving TLR4 signalling in the chronic participants, while this was not evident in the febrile participants. Apart from the signalling pathway initiated, cells from the chronic participants had tightly regulated inflammatory responses to ensure that the inflammation is transient, and cell homeostasis is restored as quickly as possible, consistent with their non-febrile status. While the febrile participants did initiate negative feedback mechanisms to some signalling pathways, others like the type I IFN pathway lacked this mechanism, thus inflammatory signals in chronic participants were more transient and tightly regulated than in the febrile participants.

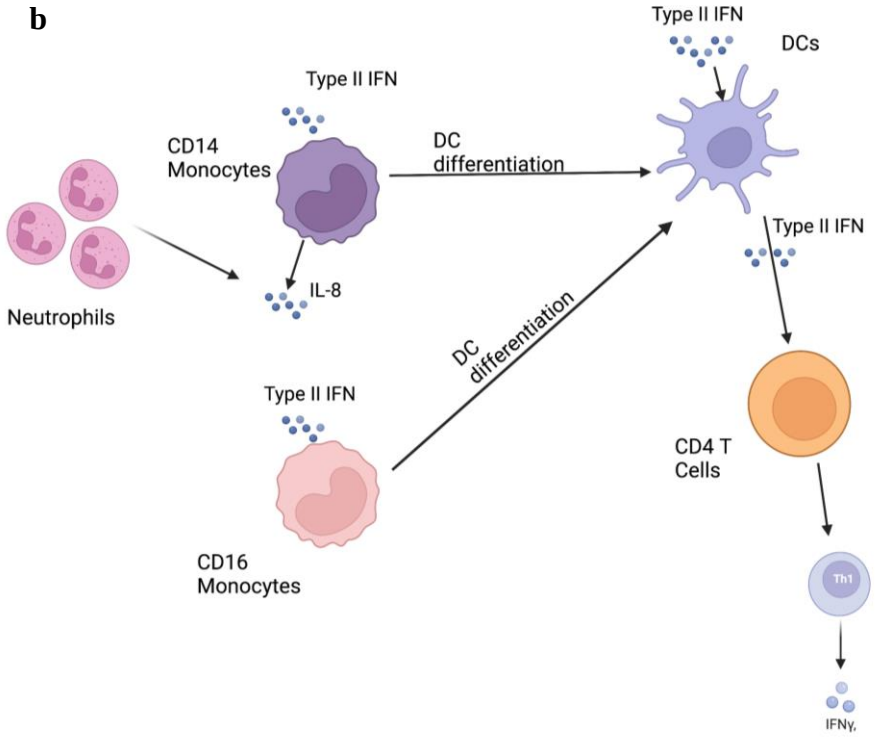
Overall, the *in vitro* data showed that malaria infected RBCs can downregulate dendritic cells costimulatory molecules CD80, CD86 CD40 and HLA-DR (**Figure 3-10, Figure 3-12**). This in turn affected dendritic cell's ability to activate CD4 T cells as shown by the downregulation of early activation markers in the T cells, which could affect their function (**Figure 3-10, Figure 3-12**). This was also seen in the sequenced data from *in vitro* co-culture where dendritic cells costimulatory molecules were downregulated and CD4 T cells were inadequately activated, and this could push the T cells towards a regulatory phenotype. Similar, the sequenced data from the CHMI samples from the study participants showed a similar trend to that seen in the *in vitro* data, with costimulatory molecules being downregulated at day 9 and 24 post infection when compared to baseline.

The *in vitro* and *in vivo* sequenced data differed in the type of cytokine that was driving response in the cells. TGFB1 was the major cytokine upregulated in the *in vitro* sequenced data and was seen to impede DC function and cause expansion of CD4 Treg cells. While in the *in vivo* data, type I IFN was driving immune responses in the febrile participants and seemed to impede DC function and inhibit differentiation of CD4 T cell to Th1 or Tfh phenotypes thus hampering development of immunity against the malaria parasite. Apart from the difference in cytokine production, sequenced data from both the febrile participants and *in vitro* co-culture showed that the initial malaria infection could impeded dendritic cell function, which may result in

inadequate immune response. Type II IFN was also seen in the *in vivo* data and was driving immune responses in the chronic participants. Early production of type II IFN seemed to play a role in aiding chronic participants in mounting an immune response that was able to control parasitaemia. The difference in cytokine secretion *in vivo,* could be attributed to the parasite being exposed to different physiological and immunological condition that require the parasite to constantly adapt to its dynamic environment. Additionally, *in vivo* different cells are constantly interacting with the parasite, parasite infected RBCs, and other parasite products that elicit different immune reactions. While the *in vitro* environment the parasites are not put under any immune pressure, and in the *in vitro* system that I set up the parasite was only exposed to DCs, unlike *in vivo* where various innate and adaptive immune cells were interacting with the parasite and parasite infected RBCs. The *in vitro* system might be a true reflection of interacting DCs and CD4 T cells in the lymphoid organs, unlike the PBMCs which are obtained from circulating blood which is an indirect measure of cellular interaction.



b


Type II IFN
DCs
Type II IFN
CD14
Monocytes
DC
differentiation
Type II IFN
IL-8
Neutrophils
DC
differentiation
Type II IFN
CD4 T
Cells
CD16
Monocytes
Th1
IFNγ,

c

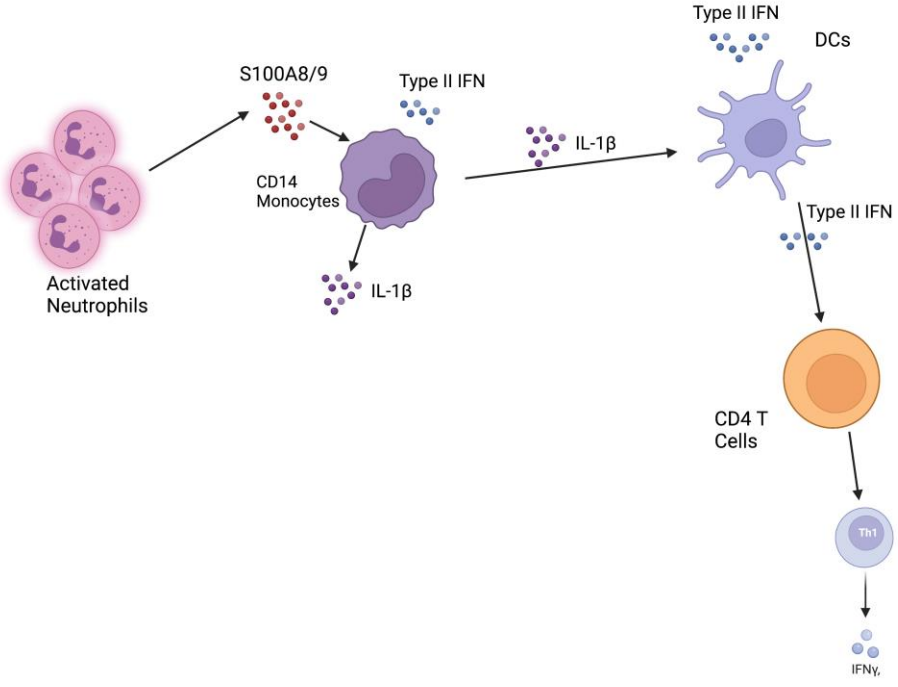

Type II IFN
DCs
S100A8/9
Type II IFN
IL-1β
CD14
Monocytes
Type II IFN
Activated
Neutrophils
IL-1β
CD4 T
Cells
Th1
IFNγ,

Figure 6-1 Signalling events in febrile (a) and chronic (b and c) participants

a. Type I IFN released at day 9 post infection in febrile participants leads to the downregulation of MHC molecules in monocytes and dendritic cells. In CD16 monocytes type I IFN reduces their ability to adequately respond to interferon gamma by reducing the surface expression of interferon gamma receptor. Type I IFN also inhibits production of interleukin-6 by monocytes and stimulates dendritic cells to produce interleukin-10 (IL-10). IL-10 impedes dendritic cell activation and function, and a combination of type I IFN and IL-10 inhibits CD4 T cells from differentiating into interferon gamma producing T helper 1 cells. Type I interferon drives the differentiation of CD4 T cells to T regulatory type 1 (Tr1) cells that inhibits the generation of malaria specific immune responses. T

b. In chronic participants at day 9 post challenge, we see both monocyte subsets responding to type II IFN and initiating differentiation into dendritic cells. Type II IFN enhances dendritic cells maturation by increasing expression of costimulatory molecules and increasing dendritic cell's ability to process and present antigens on MHC molecules. A similar effect is also seen in monocytes. Type II IFN also enhances the expression of IL-12 which is vital in inducing CD4 T cells to differentiate to interferon gamma producing T helper 1 cells that are important in malaria immunity. CD14 monocytes also produce IL-8 which recruits neutrophils and other monocytes to the site of inflammation.

c. At day 14 post infection in the chronic participants we see a sustained type II IFN response, and the recruited neutrophils become activated and assist in parasite control, resulting in secretion of S100A alarmins that drive responses in CD14 monocytes resulting in induction and production of IL-1β, which enhanced dendritic cell function and induced dendritic cells to produce IL-12. The mature DCs which were producing IL-12 and type II IFN, induced CD4 T cells to differentiate to interferon gamma producing Th1 cells

6.2 Conclusions

The research presented in this thesis aims to understand how malaria parasite affects dendritic cell function and how it influences interactions with CD4 T cells. It also provides a unique insight into the signalling pathways that are either activated or suppressed by *P. falciparum* exposure and how these pathways shape immune responses against malaria. The data presented helps us to understand how individuals with some level of naturally acquired immunity control parasitaemia and the clinical symptoms of malaria. The data analysed from the sequenced PBMCs from the CHMI study focused on monocytes and CD4 T cells and showed that early type II IFN production was associated with the ability to control parasite burden. Other cell populations in this data set also play a vital role in shaping the immune response. This includes NK cells, γδ T cells, and CD8+ T cells all of which are key producers of type II IFN during a malaria infection. NK cells and γδ T cells are known to be early producers of type II IFN (IFNγ), during the onset of malaria infection and these innate cells maintain IFNγ levels until the adaptive response is initiated, where CD4 and CD8 T cells become the major source of IFN-γ (Connelly et al., 1997). Understanding the interplay between these innate and adaptive immune cells how they contribution to the immune response during a malaria infection will help paint a clear picture of host-parasite interactions.

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