

The Transcription Factor T-bet in the Control of Germinal Centre Dynamics in Malaria

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To my grandma, Wan Sau Kwai.

Abstract

With reductions in the global malaria burden stalled, this preventable and curable infectious disease caused by the *Plasmodium* parasites, remains a public health challenge that affects the world's most vulnerable populations. Naturally acquired immunity plays an important role in protection from disease; however, there is long-standing evidence that it requires years of repeated infections to develop. The reasons for this are largely elusive, but immuno-epidemiological studies support that protective antibodies and memory B cells are short-lived and inefficiently generated to infection. Moreover, recurrent infections are associated with an expansion of atypical memory B cells that may have impaired function. Histological analyses revealed significant disorganisation of the spleen in severe malaria patients, which led to the concept that acute infection may undermine the acquisition of B cell memory. T helper 1 pro-inflammatory responses induced by blood-stage infection were subsequently shown to compromise the induction of humoral immunity by inhibiting effective T follicular helper (Tfh) cell differentiation and germinal centre (GC) reactions.

The relative contribution of the T helper 1 lineage-defining transcription factor, T-bet, in CD4⁺ T cells and B cells to GC development in malaria, was investigated using the *P. berghei* ANKA blood-stage infection model. T-bet expression in CD4⁺ T cells limited the differentiation of Tfh cells that supported GC development in the spleen. This led to an impaired generation of antibody-secreting cells and memory B cells following infection. In addition to its impact on CD4⁺ T cells, T-bet was highly up-regulated in GC B cells elicited by infection, and limited the magnitude of the GC response in a B cell-intrinsic manner. Strikingly, T-bet expression in the B cell compartment modulated the transcriptional landscape of GC B cells to promote the GC dark zone program but constrained light zone development. In particular, T-bet suppressed expression of the regulator of G-protein signaling 13, which down-regulates the responsiveness of B cells to migrate towards the chemokine CXCL12, for effective dark and light zone transition within the GC. T-bet-driven dark zone skewing of the GC reaction following malaria infection associated with enhanced somatic hypermutation of GC B cells, and improved the avidity of antibodies against the parasite. Therefore, this thesis supports a model in which malaria-elicited inflammation mediated by T-bet, exquisitely modulates the dynamics of the GC reaction, promoting GC B cell dark zone polarization that promotes the generation of B cells with increased affinity for antigen, consequently enhancing affinity maturation. This provides novel insight into the cellular mechanisms that underlie the development of humoral immune responses in malaria, and has implications for other chronic infections and autoimmune disease that are characterised by a similarly potent inflammatory milieu.

Declaration

This is to certify that:

- I. this thesis comprises only my original work towards the PhD except where indicated in the Preface;
- II. due acknowledgement has been made in the text to all other material used;
- III. this thesis is less than 100,000 words in length, exclusive of tables, bibliographies, and appendices.

Ann Ly

Preface

The work presented in this thesis was undertaken at The Walter and Eliza Hall Institute of Medical Research (WEHI) in the laboratory of Dr. Diana S. Hansen. I was supported by an Australian Postgraduate Award through the Department of Medical Biology at The University of Melbourne, and Edith Moffat Scholarship from WEHI.

In accordance with the regulations set out by The University of Melbourne, I assess my contribution to the work presented in this thesis and duly acknowledge the important contributions of the following individuals:

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Abbreviations

A

ABCs	Age-associated B cells
AID	Activation-induced cytidine deaminase
AMA-1	Apical membrane antigen-1
ASC	Antibody-secreting cell
ATAC	Assay for transposase-accessible chromatin

B

Blimp-1	B lymphocyte-induced maturation protein-1
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin

C

CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD40L	CD40 ligand
CM	Cerebral malaria
CPM	Counts per million mapped fragments
CXCL	C-X-C-chemokine ligand
CXCR	C-X-C-chemokine receptor

D

DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DZ	Dark zone

E

EBI2	Epstein-barr virus-induced molecule 2
ELISA	Enzyme-linked immunosorbent assay

F

FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FcRL5	Fc receptor-like 5
FDC	Follicular dendritic cell
FDR	False discovery rate
Foxo1	Forkhead box protein O1
FPKM	Fragments per kilobase per million

G

GC	Germinal centre
gMFI	Geometric mean fluorescence intensity

H

HRP	Horseradish peroxidase
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I

ICOS	Inducible T cell costimulatory
ICOSL	Inducible T cell costimulatory ligand
Id	Inhibitors of DNA-binding/differentiation
IFN	Interferon
IFN- γ R	Interferon- γ receptor
Ig	Immunoglobulin

<i>IghV</i>	Immunoglobulin variable region	R	
IL	Interleukin	RBC	Red blood cell
IRF	Interferon regulatory factor	RGS	Regulator of G protein signaling
i.p.	Intraperitoneally	RNA	Ribonucleic acid
i.v.	Intravenously	RPMI	Roswell Park Memorial Institute
L			
LCMV	Lymphocytic choriomeningitis virus	S	
LT	Lymphotoxin	SAP	SLAM-associated protein
LZ	Light zone	SEM	Standard error of the mean
		SHM	Somatic hypermutation
		SLAM	Signaling lymphocytic activation molecule
M			
MBC	Memory B cell	SLE	Systemic lupus erythematosus
MEF	Myocyte-specific enhancer factor	SNP	Single nucleotide polymorphism
MSP	Merozoite surface protein	STAT	Signal transducer of activation and transcription
MSP-1 ₁₉	19-kDa fragment of MSP-1		
mTORC1	Mechanistic target of rapamycin complex 1	T	
		T-bet	T-box expressed in T cells
N		Tfh	T follicular helper
NF-κB	Nuclear factor-κB	TGF	Transforming growth factor
NK	Natural killer	Th1	T helper 1
		TLR	Toll-like receptor
O		TMB	Tetramethyl benzidine
OD	Optical density	TNF	Tumour necrosis factor
P			
PD-1	Programmed cell death-1	W	
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1	WEHI	The Walter and Eliza Hall Institute of Medical Research
pi	Post-infection		
pRBC	Parasitized red blood cell		

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Chapter 1

Introduction

1. Malaria—a global killer

Malaria is a parasitic disease caused by obligate intracellular protozoa of the genus *Plasmodium*. Of more than 100 *Plasmodium* species infecting vertebrates, only five – *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*— are known to infect humans. The parasites are transmitted by female *Anopheles* mosquitoes in tropical and sub-tropical countries. *P. falciparum* is the most prevalent species in sub-Saharan Africa, while *P. vivax* is more geographically widespread, located in South-East Asia, the Eastern Mediterranean, Western Pacific, and South and Central America. *P. malariae* and *P. ovale* have low incidences in most endemic areas, while *P. knowlesi* is a zoonotic infection encountered in South-East Asia (World Health Organization, 2018).

The World Health Organization estimated that 219 million cases of malaria occurred globally with 435,000 resulting in death in 2017 (World Health Organization, 2018). *P. falciparum* accounts for the bulk of morbidity and mortality, which mostly occur in children under the age of 5 in sub-Saharan Africa. *P. vivax* is the second most significant contributor of morbidity, responsible for approximately 3.4% of reported clinical cases (World Health Organization, 2018). The true global burden of malaria is likely to exceed this, as the reliance on data from health system records may grossly underestimate the exact number of cases and deaths (Hay *et al.*, 2010). The risk of malaria depends on ecological and socio-economic factors such as environmental suitability for parasite transmission (Lawpoolsri *et al.*, 2010; Caminade *et al.*, 2014), and access to human and financial resources, and thus, is closely linked to natural disasters, poverty and social unrest (World Health Organization, 2015). Malaria-endemic countries not only suffer from serious disease and fatalities,

but they are also trapped in a vicious cycle of poverty due to increased public health expenditure, reduced education opportunities and impaired economic growth (Gallup and Sachs, 2001; Sachs and Malaney, 2002). In the early 2000s, governments and philanthropic organisations recognised the escalating problem posed by malaria, and initiated renewed global commitment to achieve malaria eradication.

Since the turn of the century, substantial funding for malaria control interventions such as long-lasting insecticide-treated bed nets, and prompt diagnosis and treatment, led to considerable reductions in the disease burden (Bhatt *et al.*, 2015). In particular, 19 countries attained zero local cases for at least three consecutive years in 2017 (World Health Organization, 2018). Nevertheless, declines in clinical cases and deaths have largely occurred in countries with low disease burdens, and minimal progress has been made in high transmission countries (Snow *et al.*, 2017). Moreover, recent trends show a plateau in global malaria reduction (World Health Organization, 2018). The continued emergence of anti-malarial drug resistance in South-East Asia (Ashley *et al.*, 2014; Ménard *et al.*, 2016), and mosquito resistance to insecticides, threaten the success of existing control and elimination tools (Strode *et al.*, 2014). As such, an effective vaccine would potentially expedite malaria eradication. Unfortunately, none of the vaccine candidates developed to date provide long-term protection at a population level, and the most advanced, RTS,S/AS01 vaccine, only protects against clinical cases by 28% and 18% in young African children and infants, respectively (RTS,S Clinical Trials Partnership, 2015). Vaccine development is overall hampered by an incomplete understanding of the mechanisms of immunity against the parasite.

1.1 The life cycle of *Plasmodium*

The *Plasmodium* parasites have a complex lifecycle of multiple stages, which occur in the mosquito vector and vertebrate hosts. Human infection ensues via the bite of a *Plasmodium*-infected female *Anopheles* mosquito that injects sporozoites into the skin (**Figure 1.1**). A proportion of these rapidly travel to the liver where they traverse through cells and actively invade hepatocytes. Over the course of 1–2 weeks, the intracellular sporozoites develop into thousands of merozoites in a clinically asymptomatic stage, before release into the bloodstream. Extracellular merozoites use a variety of surface proteins to attach to a red blood cell (RBC), reorientate their apical end, which triggers invasion into the host cell powered by the parasite’s actinomyosin motor in a fast and dynamic process (Cowman *et al.*, 2017). Once inside, the parasite remodels the RBC to provide a protected niche for its development through the ring, trophozoite and schizont stages, reproducing 16–32 new merozoites that are ready to invade new RBCs upon egress. A small fraction of merozoites differentiate into male and female gametocytes within the RBC cocoon, which can be transmitted to another mosquito. In the mosquito gut, individual gametocytes develop into female

macrogametes and male microgametes, which form a zygote upon fertilization. The zygote transforms into a motile ookinete that traverses the midgut wall and differentiates into an oocyst. For the next 2 weeks, the oocyst produces many sporozoites that migrate to the salivary glands, from where they can be injected into a human host during the next blood meal (Cowman *et al.*, 2012).

The blood-stage of infection is solely responsible for the clinical manifestations of human infection. The cyclic waves of merozoite invasion and release from parasitized RBCs (pRBCs) coincide with the periodic fevers described by classic accounts of clinical malaria episodes. Other common symptoms include chills, myalgia, headache, vomiting and diarrhoea. The mainstay of recommended treatment for uncomplicated *P. falciparum* malaria are artemisinin-based combination therapies, which consist of an artemisinin derivative that rapidly reduces parasitemia and a partner drug that removes residual parasites over time (World Health Organisation, 2015).

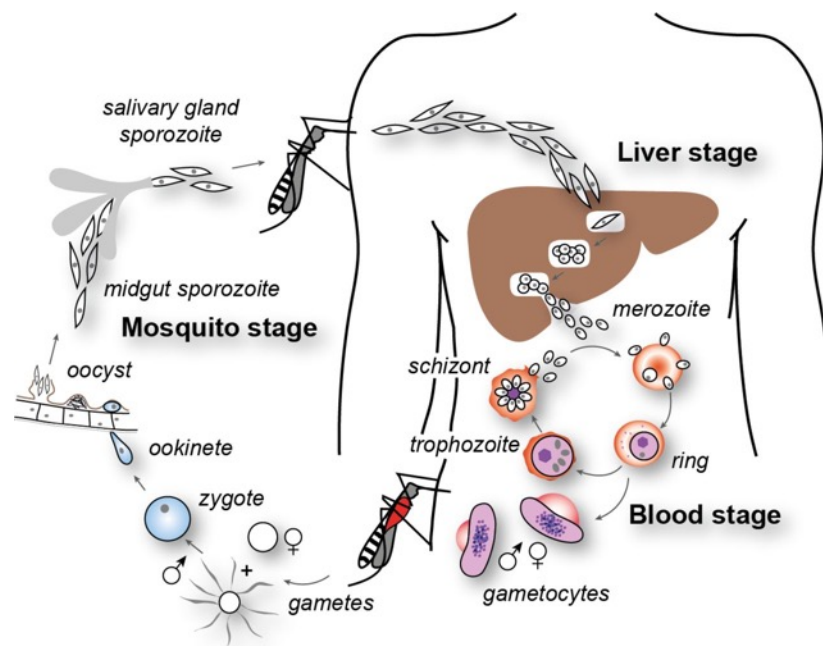


Figure 1.1 The life cycle of *P. falciparum*

A *P. falciparum*-infected mosquito injects sporozoites into an individual during a blood meal. The sporozoites invade hepatocytes in the liver and develop into merozoites that are released into the blood to invade red blood cells. They develop through ring, trophozoite and schizont stages, replicating into more merozoites that perpetuate the blood-stage cycle. Some merozoites differentiate into gametocytes that can be taken up by a feeding mosquito. In the mosquito, they develop into gametes that fuse to form a zygote. The zygote becomes an ookinete that traverses the midgut and differentiates into an oocyst, from which sporozoites develop and travel to the salivary glands for transmission to another human host (Cowman *et al.*, 2012).

1.2 Severe malaria

While most cases of symptomatic malaria are not life-threatening, they can develop into severe disease without prompt clearance of blood-stage parasites, manifesting as one or more of the following disease syndromes: cerebral malaria, severe malarial anaemia, hypoglycaemia, acidosis, acute respiratory distress, acute kidney injury, jaundice and placental malaria (World Health Organization, 2014). *P. falciparum* is the major cause of severe malaria, but both *P. vivax* and *P. knowlesi* can also contribute to complicated and fatal disease (Baird, 2013; World Health Organization, 2014; Naing *et al.*, 2014). The pattern of severe malaria is highly influenced by age and transmission intensity, with young children and non-immune adults in high transmission areas most susceptible to severe disease, while in lower transmission settings, a shift in disease incidence towards older individuals tends to occur (Snow *et al.*, 1997; Reyburn *et al.*, 2005; Roca-Feltrer *et al.*, 2010; Carneiro *et al.*, 2010; Ndungu *et al.*, 2015). Among the different syndromes, severe malarial anaemia and hypoglycaemia are more common in children, acute respiratory distress, kidney injury and jaundice are prevalent in adults, while cerebral malaria and acidosis can occur in all age groups (Dondorp *et al.*, 2008b; Seidlein *et al.*, 2012).

One of the most common severe malaria syndromes arising from *P. falciparum* infection is cerebral malaria (CM), characterised by impaired consciousness or coma, and often accompanied by convulsions and retinopathy (World Health Organization, 2014). Even with anti-malarial drug treatment, the case fatality rate is approximately 10–20% (Ranque *et al.*, 2008; Idro *et al.*, 2010; Dondorp *et al.*, 2010; Seydel *et al.*, 2015), and of the children who recover from CM, 3–15% present with long-term neurological or cognitive deficits (Dondorp *et al.*, 2010; Birbeck *et al.*, 2010; Manning *et al.*, 2011). The pathophysiology of CM is not completely understood, but extensive sequestration of pRBCs in the brain, and excessive host inflammatory responses to infection are considered to be two main drivers of disease.

1.2.1 Parasite sequestration during CM

A common feature of CM is an extensive accumulation of pRBCs in the brain microvasculature (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1985; Aikawa *et al.*, 1990; Turner *et al.*, 1994; Silamut *et al.*, 1999; Clark *et al.*, 2003; Pongponratn *et al.*, 2003; Taylor *et al.*, 2004; Dorovini-Zis *et al.*, 2011), which was first documented in post-mortem analyses conducted by Marchiafava and Bignami more than a century ago (Marchiafava and Bignami, 1894). Blood-stage *Plasmodium* parasites express adhesive proteins on the surface of pRBCs, which allow them to bind to endothelial receptors on the blood vessels of various organs, and avoid clearance by the spleen. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family is the most extensively

characterized of surface proteins, and subtypes that bind to intercellular adhesion molecule 1, and endothelial protein C receptor in the brain are associated with CM (Turner *et al.*, 2013; Lennartz *et al.*, 2017). The sequestration of pRBCs occlude blood flow (Dondorp *et al.*, 2008a), and further triggers the localized release of toxic mediators that cause endothelial cell injury (Viebig *et al.*, 2005; Tripathi *et al.*, 2006; Chakravorty *et al.*, 2007), which lead to tissue hypoxia, loss of blood-brain barrier integrity, and vascular haemorrhages (Taylor *et al.*, 2004; Francischetti *et al.*, 2007). Additionally, pRBCs cluster with uninfected RBCs to form rosettes, which may exacerbate microvascular obstruction (Carlson *et al.*, 1990; Rowe *et al.*, 1995; Doumbo *et al.*, 2009; Goel *et al.*, 2015). Importantly, histological observations from some post-mortem CM studies supported that pRBC sequestration is not the only cause of fatal disease (Silamut *et al.*, 1999; Clark *et al.*, 2003; Taylor *et al.*, 2004; Seydel *et al.*, 2006; Dorovini-Zis *et al.*, 2011), and that additional mechanisms contribute to pathogenesis.

Insight into the pathophysiology of CM in humans is limited by the analysis of peripheral blood and post-mortem tissue, which do not allow us to delineate a causal relationship between parasite- or host-derived factors, and disease induction. Consequently, murine models developed from *Plasmodium* parasites of African rodents (Vincke and Lips, 1948; Landau and Killick-Kendrick, 1966) have been extensively used to obtain mechanistic information into the immunopathogenesis of CM. Although no single model can reproduce the complexity and clinical spectrum of human malaria, infection of inbred mice with certain *P. berghei* or *P. yoelii* strains induce neurological syndromes comparable to severe disease in humans (Yoeli and Hargreaves, 1974; Rest, 1982; Curfs *et al.*, 1993). Among them, *P. berghei* ANKA malaria shares the most immunological and pathological features with human CM, and hence, the vast majority of experimental data comes from this model (Hansen *et al.*, 2012). Infection of susceptible C57BL/6 and CBA mouse strains results in the development of fatal disease within 6–10 days of infection, presenting with clinical symptoms such as ataxia, respiratory distress, convulsions and coma (Grau *et al.*, 1987; Jennings *et al.*, 1998). Importantly, this is associated with pRBC sequestration in multiple organs, including the brain (Nie *et al.*, 2009).

1.2.2 Pro-inflammatory cytokines in the induction of CM

Sequestered parasites release or express on their surface various bioactive products, such as glycosylphosphatidylinositol toxin, haemozoin and nucleic acids, which have been proposed to initiate a pro-inflammatory cytokine cascade (Sherry *et al.*, 1995; Tachado *et al.*, 1997; Naik *et al.*, 2000; Coban *et al.*, 2005; Krishnegowda *et al.*, 2005; Parroche *et al.*, 2007; Sharma *et al.*, 2011). Long-standing evidence support a role of pro-inflammatory cytokines as mediators of CM pathogenesis. In particular, tumour necrosis factor (TNF) in peripheral blood strongly correlates

with severe disease (Grau *et al.*, 1989b; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990; Molyneux *et al.*, 1993; Awandare *et al.*, 2006), and CM patients are reported with elevated levels of TNF in the brain (Brown *et al.*, 1999), and soluble TNF receptor in plasma and cerebrospinal fluid (Molyneux *et al.*, 1993; Armah *et al.*, 2007). Consistently, a significant up-regulation of TNF and TNF receptor 2 has been observed in the brains of mice with experimental CM (Lucas *et al.*, 1997; Medana *et al.*, 1997). *P. berghei* ANKA-infected CBA mice develop high serum TNF levels at the onset of disease (Grau *et al.*, 1987), while TNF neutralisation *in vivo* protects these mice from fatal infection (Grau *et al.*, 1987). Nevertheless, C57BL/6 mice remain susceptible to disease in the absence of TNF (Hermesen *et al.*, 1997a; Engwerda *et al.*, 2002b). Similarly, treatments that block TNF bioactivity or production failed to improve survival outcomes of children diagnosed with CM (Di Perri *et al.*, 1995; van Hensbroek *et al.*, 1996; Looareesuwan *et al.*, 1999), which strongly suggested the existence of other mediators of disease.

In line with this notion, related members of the TNF superfamily, lymphotoxin (LT)- α and LT- β , have been postulated to contribute to CM pathogenesis. Malaria patients appear to have increased serum levels of LT- α (Clark *et al.*, 1992), which are secreted by mononuclear cells following *P. falciparum* stimulation *in vitro* (Ferrante *et al.*, 1990). The pathogenic role of this cytokine in human CM is unclear, but murine studies suggest that LT- α may mediate pRBC sequestration in the brain in C57BL/6 mice (Engwerda *et al.*, 2002b). In support, mice deficient in LT- α alone or in combination with TNF are resistant to *P. berghei* ANKA-induced CM (Rudin *et al.*, 1997; Engwerda *et al.*, 2002b). Comparable observations have been made in the absence of LT- β or LT- β signaling (Togbe *et al.*, 2008; Randall *et al.*, 2008b).

Interferon (IFN)- γ is another cytokine that is highly abundant in the sera of acute malaria patients (Ringwald *et al.*, 1991; Ho *et al.*, 1995), and in the brain in fatal CM cases (Maneerat *et al.*, 1999). Gambian children with IFN- γ receptor (IFN- γ R) polymorphism is associated with reduced CM incidences and mortality (Koch *et al.*, 2002), which support a role of IFN- γ in disease pathogenesis. Similar to humans, IFN- γ is strongly up-regulated in the spleens and brains of susceptible mice (de Kossodo and Grau, 1993; Sexton *et al.*, 2004). Furthermore, several studies have independently shown that neutralisation, or ablation of IFN- γ signaling, protects against the development of *P. berghei* ANKA-induced CM (Grau *et al.*, 1989a; Yanez *et al.*, 1996; Amani *et al.*, 2000; Belnoue *et al.*, 2008; Villegas-Mendez *et al.*, 2011). In addition to IFN- γ , type I IFNs have also been implicated in CM pathology, with gene polymorphisms either in IFN- α or its receptor associated with protection from severe malaria in humans (Aucan *et al.*, 2003; Khor *et al.*, 2007; Ball *et al.*, 2013). In mice, type I IFN signaling variably contribute to disease (Sharma *et al.*, 2011; Haque *et*

al., 2011; Ball *et al.*, 2013; Palomo *et al.*, 2013; Haque *et al.*, 2014; Edwards *et al.*, 2015), depending on their levels at specific time points of infection (Vigário *et al.*, 2007; Morrell *et al.*, 2011).

The fine balance between pro-inflammatory and anti-inflammatory cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)- β , appears to determine the severity of disease (May *et al.*, 2000; Doodoo *et al.*, 2002; Esamai *et al.*, 2003; Walther *et al.*, 2006; Awandare *et al.*, 2006; Thuma *et al.*, 2011). Ongoing *P. falciparum* exposure has been associated with an up-regulation of cytokines that limit inflammation and enhance parasite control (Portugal *et al.*, 2014), accompanied by a progressive reduction in pro-inflammatory cytokines (Portugal *et al.*, 2014; Ademolue *et al.*, 2017). Similarly, asymptomatic *P. falciparum*-infected Malian adults exhibit dampened transcriptional activation of pro-inflammatory pathways compared to that of individuals with febrile symptoms (Tran *et al.*, 2016). Thus, protection against disease appears to involve the development of regulatory mechanisms that limit pro-inflammatory responses elicited by malaria.

1.2.3 Intravascular leukocyte infiltration in CM

Histological analyses of brain tissue from human CM cases also revealed the presence of leukocytes sequestered in the brain microvasculature (Porta *et al.*, 1993; Taylor *et al.*, 2004; Hochman *et al.*, 2015). Macrophages and monocytes (Schofield and Hackett, 1993; Sherry *et al.*, 1995; Naik *et al.*, 2000; Krishnegowda *et al.*, 2005; Robinson *et al.*, 2009; Stanisic *et al.*, 2014), natural killer (NK) and NK T cells (Artavanis-Tsakonas and Riley, 2002; Korbel *et al.*, 2005; Newman *et al.*, 2006; Horowitz *et al.*, 2010), $\gamma\delta$ T cells (Goodier *et al.*, 1995; Hensmann and Kwiatkowski, 2001; Hviid *et al.*, 2001; D'Ombrian *et al.*, 2008; Robinson *et al.*, 2009; Stanisic *et al.*, 2014), CD4⁺ and CD8⁺ T cells (Hensmann and Kwiatkowski, 2001; Walther *et al.*, 2009; Jagannathan *et al.*, 2014; 2015), readily secrete pro-inflammatory cytokines in response to *Plasmodium* parasites. Heightened levels of chemokines, including CC-chemokine ligand (CCL) 3 (MIP-1 α), CCL4 (MIP-1 β), and C-X-C-chemokine ligand (CXCL) 10 (IP-10), in the plasma or cerebrospinal fluid of patients with fatal disease are thought to attract host leukocytes to sites of parasite sequestration (Ochiel *et al.*, 2005; Armah *et al.*, 2007; Jain *et al.*, 2008; Erdman *et al.*, 2011).

In mice, a comparable accumulation of leukocytes occurs during experimental CM, with NK cells, CD4⁺ and CD8⁺ T cells being the most abundant brain-infiltrating populations (Ma *et al.*, 1996; Belnoue *et al.*, 2002; Nitcheu *et al.*, 2003; Bagot *et al.*, 2004; Hansen *et al.*, 2007; Miyakoda *et al.*, 2008; de Koning-Ward *et al.*, 2008; Villegas-Mendez *et al.*, 2012; Howland *et al.*, 2013; Pai *et al.*, 2014; Poh *et al.*, 2014; Nacer *et al.*, 2014). Up-regulation of the C-X-C-chemokine receptor (CXCR)3 has been shown to enable lymphocyte trafficking to the brains of infected mice (Nitcheu

et al., 2003; Hansen *et al.*, 2007) in response to monocyte- and neutrophil-secreted IFN- γ -inducible chemokines, CXCL9 (MIG) and CXCL10 (Nie *et al.*, 2009). In support of an important role of CXCR3 signaling in CM induction, limiting CXCR3-mediated cell migration protects *P. berghei* ANKA-infected mice from succumbing to fatal infection (Nie *et al.*, 2009). Additionally, inflammatory chemokines including CCL2 (MCP-1), CCL3, CCL4, CCL5 (RANTES), CCL9 (MIP-1 γ), have been linked to susceptibility to experimental CM (Hanum *et al.*, 2003; Sexton *et al.*, 2004; Miu *et al.*, 2008; Campanella *et al.*, 2008; Van den Steen *et al.*, 2008).

Mechanistic evidence on how NK cells, CD4⁺ and CD8⁺ T cells contribute to the pathogenesis of CM comes primarily from murine studies. NK cells dominate the IFN- γ response early during *P. berghei* ANKA infection (Hansen *et al.*, 2007; Villegas-Mendez *et al.*, 2012), promoting CXCR3⁺ T cell accumulation in the brains of infected mice (Hansen *et al.*, 2007; Ryg-Cornejo *et al.*, 2013). Polymorphisms within the NK complex loci increase CM susceptibility by enhancing IFN- γ production and CD8⁺ T cell sequestration (Hansen *et al.*, 2005; 2014). Importantly, depletion of NK cells alleviates CM induction in otherwise susceptible mice (Hansen *et al.*, 2007), supporting their crucial role in pathogenesis. Genetic ablation or antibody-mediated depletion of T cells also protects mice from CM (Grau *et al.*, 1986; Yanez *et al.*, 1996; Hermesen *et al.*, 1997b; Belnoue *et al.*, 2002; Randall *et al.*, 2008a; Claser *et al.*, 2011). Like NK cells, CD4⁺ T cells appear to play a role in the induction phase, illustrated by the finding that mice are protected from severe disease only when CD4⁺ T cells are depleted prior to the onset of neurological symptoms (Belnoue *et al.*, 2002). The precise mechanism by which CD4⁺ T cells induce CM is not clear, but they may promote parasite (Amante *et al.*, 2010; Claser *et al.*, 2011) and CD8⁺ T cell sequestration via IFN- γ production (Villegas-Mendez *et al.*, 2012). The accumulation of CD8⁺ T cells in the brain coincides with the development of acute symptoms (Belnoue *et al.*, 2002; Nitcheu *et al.*, 2003; Belnoue *et al.*, 2008; Nacer *et al.*, 2014), and depletion experiments indicated that they contribute to the effector phase of CM (Belnoue *et al.*, 2002; Randall *et al.*, 2008a). CD8⁺ T cells are thought to disrupt blood-brain barrier integrity by direct cytotoxicity on endothelial cells (Nitcheu *et al.*, 2003; Potter *et al.*, 2006; Howland *et al.*, 2013), and may mediate monocyte (Pai *et al.*, 2014) and parasite sequestration (Amante *et al.*, 2010; Claser *et al.*, 2011) at the time of disease manifestation.

2. Naturally acquired immunity to malaria

Early epidemiological observations from malaria-endemic areas by the microbiologist Robert Koch outlined that natural immunity to malaria can be achieved, but after years of repeated *Plasmodium* exposure, and that it only reduces parasitemia and disease risk (Koch, 1900). In moderate-high transmission regions, children become immune to severe complications of malaria after relatively

few symptomatic infections (Gupta *et al.*, 1999b; Reyburn *et al.*, 2005; Griffin *et al.*, 2015), but remain at risk of uncomplicated disease (**Figure 1.2**). After repeated infections over time, the incidence of symptomatic disease declines, and protection from successive malaria episodes is acquired by the ability to substantially reduce blood-stage parasite densities, which is referred to as clinical immunity (McGregor and Smith, 1952; Baird *et al.*, 2003; Drakeley *et al.*, 2005; Michon *et al.*, 2007; Ladeia-Andrade *et al.*, 2009; Bejon *et al.*, 2009; Tran *et al.*, 2013; Ndungu *et al.*, 2015). However, this form of protection is not paralleled by sterilizing immunity that prevents re-infection, as adults are frequently susceptible to asymptomatic infections (Bejon *et al.*, 2009; Okell *et al.*, 2012; Tran *et al.*, 2013; Mosha *et al.*, 2013). Thus, in endemic settings, naturally acquired immunity to malaria varies as a function of host age that is intimately linked to parasite exposure.

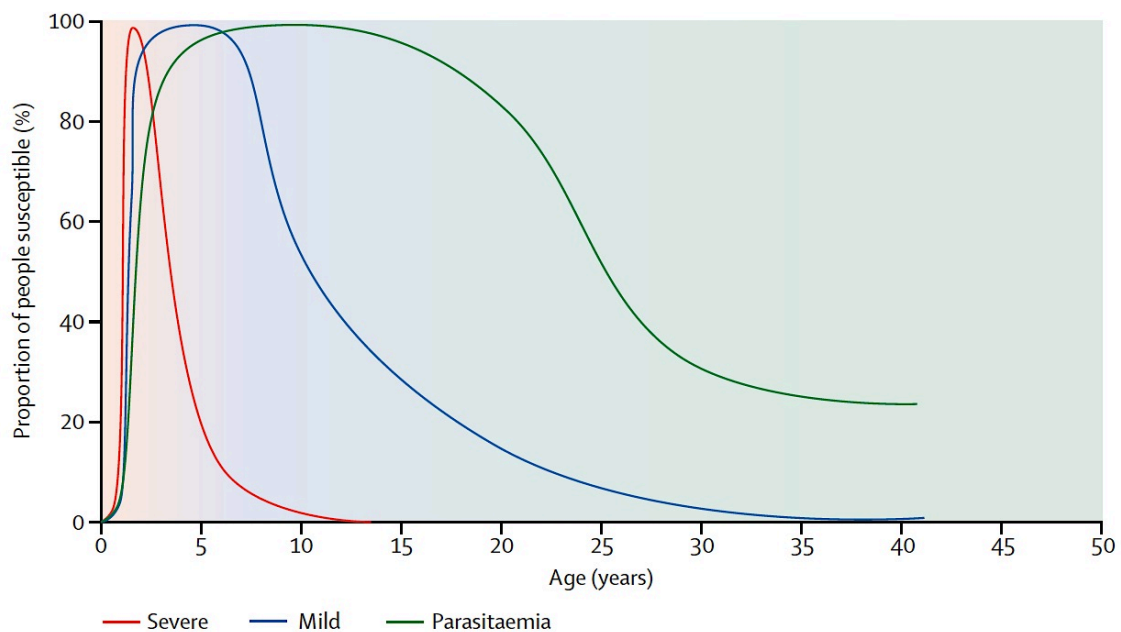


Figure 1.2 Distribution of clinical manifestations of malaria in an endemic area

Natural immunity to malaria is acquired in stages in moderate to high endemicity areas. Protection to severe malaria is acquired more rapidly than immunity to mild, uncomplicated disease. Individuals are eventually able to control parasitaemia but sterilizing immunity against re-infection is rarely, if ever achieved. Data is presented as a proportion of people susceptible for each population index in Kilifi, Kenya, adapted from (White *et al.*, 2014b).

Despite Koch's observations almost 120 years ago, many questions remain with respect to the development of naturally acquired immunity to malaria. Current knowledge about natural immunity against the liver-stage is limited, but cell-mediated mechanisms appear to be important (Malik *et al.*, 1991; Aidoo *et al.*, 1995; Kurtis *et al.*, 1999), and antibodies can block sporozoite invasion and their development within hepatocytes (Yoshida *et al.*, 1980; Mellouk *et al.*, 1990). Similarly, there is some evidence from population studies that antibodies inhibit gametocyte

transmission (Mendis *et al.*, 1987; Gamage-Mendis *et al.*, 1992; Arevalo-Herrera *et al.*, 2005); however, further research is needed to better understand the nature of protection. Much more has been learnt about immunity to blood-stage asexual parasites, which is a primary target of clinical immunity, and there is a growing consensus that it requires cell-mediated and humoral immune responses. This thesis focuses on the latter; therefore, cell-mediated immunity is briefly discussed below, but has been thoroughly reviewed elsewhere (Riley *et al.*, 2013; Crompton *et al.*, 2014; Stanisic and Good, 2016).

CD4⁺ T cells that proliferate and secrete cytokines in response to blood-stage *Plasmodium* parasites have been detected in naturally-exposed populations (Troye-Blomberg *et al.*, 1984; Mshana *et al.*, 1993; Winkler *et al.*, 1998; Ramharter *et al.*, 2005; D'Ombra *et al.*, 2008; Seth *et al.*, 2010; Stanisic *et al.*, 2014). The cells of individuals with acute malaria primarily express a pro-inflammatory T helper 1 (Th1) phenotype (Winkler *et al.*, 1998; Roetynck *et al.*, 2013; Jagannathan *et al.*, 2014; Portugal *et al.*, 2014; Boyle *et al.*, 2015b). While associations between specific CD4⁺ T cell subsets and protection from symptomatic disease are unclear, the cytokines they secrete, such as TNF and IFN- γ , have been associated with reduced parasite densities and risk of clinical episodes (Kremsner *et al.*, 1995; Mordmüller *et al.*, 1997; Luty *et al.*, 1999; Dodoo *et al.*, 2002; D'Ombra *et al.*, 2008; Robinson *et al.*, 2009; McCall *et al.*, 2010). In mice, CD4⁺ T cells act as T helper cells to promote antibody responses and secrete cytokines (Brake *et al.*, 1988; Langhorne *et al.*, 1989; Podoba and Stevenson, 1991; Meding and Langhorne, 1991; Stevenson and Tam, 1993; Langhorne *et al.*, 1998; Opata and Stephens, 2017), where IFN- γ , TNF, IL-2 and IL-12 correlate with parasite control during infection (Waki *et al.*, 1995; van der Heyde *et al.*, 1997; de Souza *et al.*, 1997; Yoneto *et al.*, 1999; Su and Stevenson, 2000; 2002; Mitchell *et al.*, 2005). In particular, T cell-derived IL-21 is necessary for effective parasitemia clearance (Pérez-Mazliah *et al.*, 2015) by supporting the development of humoral immune responses (Pérez-Mazliah *et al.*, 2015; Inoue *et al.*, 2018).

Seminal passive-transfer experiments demonstrated the paramount importance of antibodies for naturally acquired immunity to malaria, in which immunoglobulin (Ig)G transferred from *P. falciparum* immune adults protected children from high parasitemia and clinical symptoms (Cohen *et al.*, 1961; McGregor *et al.*, 1963; Sabchareon *et al.*, 1991). Numerous immuno-epidemiological studies subsequently correlated protection with antibodies to specific blood-stage parasite antigens, of which are predominantly the cytophilic IgG1 and IgG3 subclasses (Bouharoun-Tayoun and Druilhe, 1992; Braga *et al.*, 2002; Tongren *et al.*, 2006; Stanisic *et al.*, 2009; Richards *et al.*, 2010; Stanisic *et al.*, 2015). Naturally acquired antibodies to blood-stage merozoite antigens, including erythrocyte binding antigen family and reticulocyte-binding homolog 5, inhibit invasion or

parasite growth in RBCs (Persson *et al.*, 2013; Patel *et al.*, 2013), and prospectively correlate with clinical immunity (King *et al.*, 2008; Richards *et al.*, 2010; 2013; Patel *et al.*, 2013; Chiu *et al.*, 2014a; Tran *et al.*, 2014; Irani *et al.*, 2015). Antibodies against other merozoite antigens like apical membrane antigen-1 (AMA-1) and merozoite surface protein (MSP)-1 appear to have more variable associations with immunity depending on exposure and the parasite strain analysed (O'Donnell *et al.*, 2001; Braga *et al.*, 2002; Nogueira *et al.*, 2006; Roussilhon *et al.*, 2007; Stanisic *et al.*, 2009; Quelhas *et al.*, 2011; Wilson *et al.*, 2011; Mugenyi *et al.*, 2013; Moormann *et al.*, 2013; Versiani *et al.*, 2013; Stanisic *et al.*, 2013; Cutts *et al.*, 2014; Stanisic *et al.*, 2015; Murungi *et al.*, 2016). Functional antibodies directed to MSP-2 and MSP-3 have been described to opsonise merozoites for clearance by phagocytes (Sakamoto *et al.*, 2012; Osier *et al.*, 2014; Chiu *et al.*, 2015), and correlate with a reduced risk of disease (Osier *et al.*, 2014; Chiu *et al.*, 2015). Similarly, complement-fixing antibodies and those that promote neutrophil phagocytosis against MSP-2 and MSP-5 (Perraut *et al.*, 2014), respectively, are associated with protection (Joos *et al.*, 2010; Boyle *et al.*, 2015a). In addition, antibodies targeting merozoites are also suggested to mediate monocyte-dependent cellular killing (Bouharoun-Tayoun *et al.*, 1990; 1995), though its protective role has not been characterised in natural infection.

Antibodies can also be directed towards variant surface antigens expressed on the remodelled surface of pRBCs, with those against PfEMP1 most well-studied to date (Dodoo *et al.*, 2001; Chan *et al.*, 2012). PfEMP1 is a highly polymorphic family of proteins encoded by a diverse repertoire of *var* genes (Baruch *et al.*, 1995; Su *et al.*, 1995). The mutually exclusive expression of one PfEMP1 variant by *P. falciparum* at any given time (Scherf *et al.*, 1998), is thought to contribute to evasion of the antibody response (Roberts *et al.*, 1992; Biggs *et al.*, 1992; Bull *et al.*, 1998). Antibodies against selected PfEMP1 domains can inhibit pRBC rosetting with uninfected cells, and protect children from severe disease (Carlson *et al.*, 1990). They can also block pRBC adhesion to host endothelium and promote their phagocytic clearance, which associate with a reduced risk of placental malaria and improved pregnancy outcomes (Fried *et al.*, 1998; Duffy and Fried, 2003; Staaloe *et al.*, 2004; Jaworowski *et al.*, 2009; Feng *et al.*, 2009; Ataide *et al.*, 2011; Tutterrow *et al.*, 2012; Ndam *et al.*, 2015; Dechavanne *et al.*, 2015). Additionally, antibodies targeting *P. falciparum* schizont egress antigen-1 may arrest schizont rupture and egress, correlating with a decreased risk of severe disease in children (Raj *et al.*, 2014).

3. The development of T cell-dependent humoral immunity

The spleen constitutes an important secondary lymphoid organ in which the development of anti-malarial immunity to malaria takes place, exemplified by impaired parasite control following splenectomy in humans (Looareesuwan *et al.*, 1993; Boone and Watters, 1995; Le *et al.*, 1997;

Pongponratn *et al.*, 2000; Chotivanich *et al.*, 2002; Bidegain *et al.*, 2005; Bach *et al.*, 2005; Bachmann *et al.*, 2009) and mice (Oster *et al.*, 1980; Grun *et al.*, 1985; Sayles, *et al.*, 1993; Yap and Stevenson, 1994). The highly organised architecture of the spleen facilitates the induction of T cell-dependent humoral immunity upon malaria infection, which relies on the formation of germinal centre (GC) structures. The development of an effective GC response is a tightly controlled process regulated by various molecular factors and transcriptional networks at distinct stages. Here, the cellular dynamics of the GC reaction will be reviewed, with an emphasis on the molecular processes that are relevant to this thesis.

3.1 Initiation of the GC reaction

The spleen is organised into distinct micro-anatomical compartments, consisting of the red and white pulp, segregated by a marginal zone in which blood drains into (Mebius and Kraal, 2005). The red pulp is composed of a reticular cell network that filters aged or deformed RBCs, while the white pulp is organised into B cell follicles and T cell-rich zones. The GC reaction begins with the activation of naive B and T cells by cognate antigen engagement within the B cell follicle and T cell zone, respectively (**Figure 1.3**). Activated B cells bearing the chemokine receptor CXCR5 up-regulate the CC-chemokine receptor (CCR)7, which promotes their migration to the interface of the B cell follicle and T cell zone (T-B border) in response to the chemokines CCL19 and CCL21 (Garside *et al.*, 1998; Reif *et al.*, 2002; Okada *et al.*, 2005). In parallel, activated CD4⁺ T cells down-regulate CCR7 and up-regulate CXCR5, triggering their movement towards the B cell follicle that is rich in CXCL13 (Ansel *et al.*, 1999; Hardtke *et al.*, 2005; Haynes *et al.*, 2007; Katakai *et al.*, 2008). At the T-B border, B cells receive cognate signals from T cells, and begin to proliferate and localise in the outer follicle region (Coffey *et al.*, 2009) via expression of Epstein-barr virus-induced molecule 2 (EBI2) (Gatto *et al.*, 2009; Pereira *et al.*, 2009; Hannedouche *et al.*, 2011; Kelly *et al.*, 2011; Gatto *et al.*, 2011). Proliferating B cells then differentiate along one of three possible pathways. Some proliferating B cells rapidly expand into extrafollicular foci of plasmablasts along the T cell zone, which initially secrete antibodies of the IgM isotype, but later IgG (Jacob *et al.*, 1991a; Jacob and Kelsoe, 1992; Lalor *et al.*, 1992), influenced by the availability of different cytokines, such as IL-4, TGF- β and IFN- γ (Toellner *et al.*, 1998; Reinhardt *et al.*, 2009; Chan *et al.*, 2009). These early antibody-secreting cells (ASCs) provide an immediate, first wave of relatively low-affinity antibodies, but otherwise, contribute little to humoral memory (Jacob *et al.*, 1991b; Berek *et al.*, 1991; Lalor *et al.*, 1992) as they undergo apoptosis within several days of being formed (Smith *et al.*, 1996). Other proliferating B cells can develop into low-affinity memory B cells (MBCs) before the onset of GCs, which primarily express IgM (Toyama *et al.*, 2002; Inamine *et al.*, 2005; Blink *et al.*, 2005; Kaji *et al.*, 2012; Takemori *et al.*, 2014; Weisel *et al.*, 2016), and are thought to provide broad protection against antigen (Toyama *et al.*, 2002). A

selected proportion of activated B cells migrate deeper into follicle centre to establish a GC (Jacob *et al.*, 1991a; Liu *et al.*, 1991; Jacob and Kelsoe, 1992).

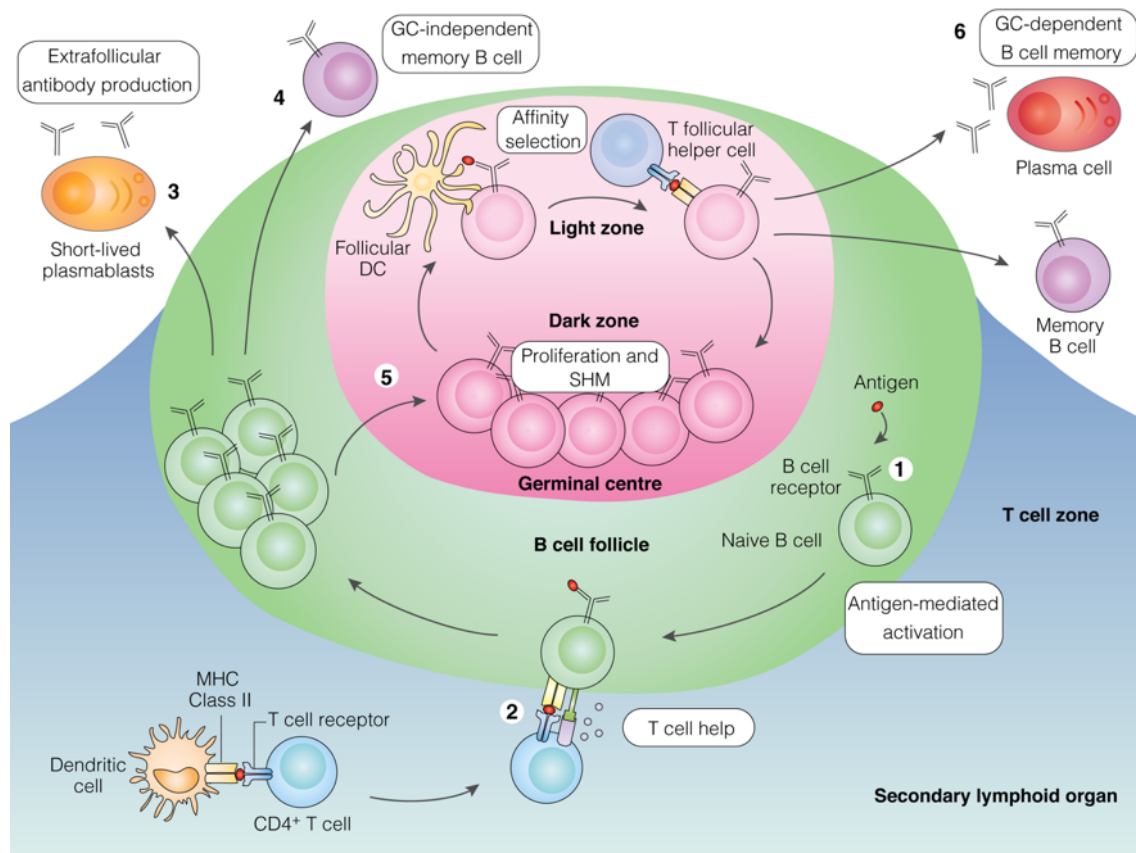


Figure 1.3 The development of T cell-dependent humoral immunity

(1) Circulating B cells are activated by recognition of cognate antigen in secondary lymphoid tissue such as the spleen. (2) Activated B cells migrate to the border of the B cell follicle and T cell zone to receive cognate CD4⁺ T cell help. (3) Some proliferating B cells differentiate into short-lived extrafollicular plasmablasts, (4) while others develop into early memory B cells prior to germinal centre (GC) induction. (5) Selected B cells aggregate in the follicle to form a GC. In the GC, B cells undergo proliferation and somatic hypermutation (SHM) in the dark zone followed by affinity-based positive selection in the light zone with the help of T follicular helper (Tfh) cells and follicular dendritic cells. (6) The GC reaction gives rise to long-lived plasma cells and memory B cells, which contribute to humoral immunity.

3.1.1 T cell help to B cells

The strength and duration of T cell help that B cells receive has been suggested to determine successful B cell differentiation, with higher affinity B cells that are able to access adequate T cell help shown to preferentially expand as extrafollicular plasmablasts (Chan *et al.*, 2009) or seed GCs (Schwickert *et al.*, 2011). T cell help comes in the form of various cell surface co-stimulatory ligand interactions and cytokine signals. CD40 stimulation by CD40 ligand (CD40L) is one of the main help signals emanating from CD4⁺ T cells. It is required for B cell proliferation (Armitage *et al.*,

1992) and GC B cell survival *in vitro* (Liu *et al.*, 1989). Genetic deletion of CD40 or its ligand, or inhibition of their interaction at any time during the GC reaction, results in a complete loss of GCs (Han *et al.*, 1995; Takahashi *et al.*, 1998). Other contact-dependent CD4⁺ T cell help is delivered by inducible T cell co-stimulator (ICOS) and signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), and deletion of either molecules results in defective GC development (McAdam *et al.*, 2001; Tafuri *et al.*, 2001; Dong *et al.*, 2001; Crotty *et al.*, 2003; Qi *et al.*, 2008; Cannons *et al.*, 2010). SAP is also required for an optimal extrafollicular antibody response (Chen *et al.*, 2008). SAP couples to and balances signals downstream of SLAM receptors to maintain stable adhesion during B and T cell interactions (Qi *et al.*, 2008; Cannons *et al.*, 2010; Kageyama *et al.*, 2012). Additionally, engagement of CD28 on T cells to the co-stimulatory ligands CD80 and CD86 expressed by B cells, is also required for B cell proliferation and GC formation (Ferguson *et al.*, 1996).

IL-21 and IL-4 are key T cell-derived cytokines that support B cell differentiation and function. IL-21 induces the proliferation and differentiation of activated B cells *in vitro* (Parrish-Novak *et al.*, 2000; Pene *et al.*, 2004; Ozaki *et al.*, 2004; Ettinger *et al.*, 2005; Good *et al.*, 2006; de Wit *et al.*, 2015). *In vivo*, IL-21 promotes class-switching (Pene *et al.*, 2004; Ettinger *et al.*, 2005), and is required for optimal GC development and affinity maturation (Nurieva *et al.*, 2008; Vogelzang *et al.*, 2008; Linterman *et al.*, 2010; Poholek *et al.*, 2010; Zotos *et al.*, 2010; Eto *et al.*, 2011; McGuire *et al.*, 2015; Weinstein *et al.*, 2016). IL-4 has been shown to be an important for the generation of ASCs (Weinstein *et al.*, 2016), and supports GC expansion (Cunningham *et al.*, 2004; King and Mohrs, 2009). Studies to date support that IL-21 acts in concert with IL-4 to regulate the GC reaction, as mice deficient in both cytokines have more severe defects in GC and antibody responses compared to deletion of the individual cytokines (Ozaki *et al.*, 2002). Similarly, combined loss of IL-21 and IL-6 has been shown to blunt the GC development (Karnowski *et al.*, 2012); although IL-6 deficiency alone does not seem to affect early GC B cell responses to viral infection, and only reduces antibody production (Poholek *et al.*, 2010), suggesting IL-6 may synergise with IL-21. Collectively, T cell help promotes the survival, proliferation, and differentiation of B cells, and is important for both extrafollicular responses and GC development.

3.1.2 Molecular control of GC B cell commitment

The commitment of antigen-activated B cells to a GC fate begins prior to their localisation to the B cell follicle centre (Kerfoot *et al.*, 2011; Kitano *et al.*, 2011), initiated upon expression of B cell lymphoma-6 (Bcl-6). The transcription factor Bcl-6 of the bric-a-bric, tramtrack, broad complex-

poxvirus zinc-finger family is essential for GC formation (Dent *et al.*, 1997; Ye *et al.*, 1997; Fukuda *et al.*, 1997). Although the molecular mechanisms that induce Bcl-6 in activated B cells are not entirely clear, its expression has been found to be co-ordinated by extensive genomic architectural reorganization (Bunting *et al.*, 2016). The transcription factors interferon regulatory factor (IRF)8 (Lee *et al.*, 2006), IRF4 (Ochiai *et al.*, 2013) and myocyte-specific enhancer factor (MEF)2B (Ying *et al.*, 2013) have been implicated in its early induction, which may be enhanced by IL-21 (Ozaki *et al.*, 2004; Ettinger *et al.*, 2005; Linterman *et al.*, 2010; Zotos *et al.*, 2010; Weinstein *et al.*, 2016) and/or IL-4 (Arguni *et al.*, 2006; Nojima *et al.*, 2011). Bcl-6 directly binds to deoxyribonucleic acid (DNA) targets using six zinc finger motifs at its C-terminus, and can recruit multiple co-repressor complexes via its N-terminal BTB domain and PEST domain-containing middle region (Basso and Dalla-Favera, 2012). Studies of the direct targets of Bcl-6 repression have revealed that it exerts broad activity on GC B cells (Ci *et al.*, 2009; Basso *et al.*, 2010). Bcl-6 down-regulates their expression of migratory receptors to facilitate homing to the B cell follicle (Pereira *et al.*, 2010; Gatto *et al.*, 2011; Huang *et al.*, 2014), and enables their effective differentiation by repressing molecules involved in T cell-mediated activation (Niu *et al.*, 1998; 2003; Saito *et al.*, 2007; Basso, 2009). Bcl-6 also inhibit genes involved in the DNA damage response (Phan and Dalla-Favera, 2004; Ranuncolo *et al.*, 2007; Basso *et al.*, 2010), and cell cycle arrest (Phan and Dalla-Favera, 2004), together with IRF8 (Zhou *et al.*, 2009), thereby increasing GC B cell tolerance to remodelling of the Ig locus. Furthermore, Bcl-6 represses genes that regulate GC exit and terminal differentiation (Shaffer *et al.*, 2000; Tunyaplin *et al.*, 2004; Ci *et al.*, 2009; Basso *et al.*, 2010), achieved in part, by collaboration with the transcription factor Bach2 (Muto *et al.*, 2004; Muto *et al.*, 2010; Huang *et al.*, 2014), and lysine-specific demethylase 1A (Hatzi *et al.*, 2019). Taken together, Bcl-6 represses a gene program that may prematurely activate GC B cells and cause their terminal differentiation, while at the same time, provide a permissive environment for physiological antibody diversification.

Downstream of BCR activation, the transcription factors MEF2C (Wilker *et al.*, 2008; Khiem *et al.*, 2008), inhibitors of DNA-binding/differentiation (Id)3 (Chen *et al.*, 2016), E2A and E2-2 (Gloury *et al.*, 2016; Wöhner *et al.*, 2016), OCT2 and co-activator OCA-B (Kim *et al.*, 1996; Schubart *et al.*, 1996), have also been demonstrated to play critical roles in GC initiation. Similarly, canonical nuclear factor- κ B (NF- κ B) signaling (Jacque *et al.*, 2014), specifically the subunit, c-Rel, has been shown to be important for early GC formation (Milanovic *et al.*, 2017). Additionally, anti-apoptotic protein myeloid cell leukaemia-1 (Vikstrom *et al.*, 2010; Wensveen *et al.*, 2016) and the transcriptional regulator CCCTC-binding factor (Pérez-García *et al.*, 2017) facilitates B cell survival and proliferation, respectively, during GC establishment. Other transcription factors,

including c-Myc (Calado *et al.*, 2012) and IRF4 (Ochiai *et al.*, 2013; Willis *et al.*, 2014) are also required for GC commitment, but exhibit a bimodal pattern of expression in GC B cells, as they are subsequently re-expressed in a subset of mature GC B cells (Saito *et al.*, 2007; Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012).

3.1.3 Tfh cell differentiation and regulation

Tfh cells are the specialised providers of B cell help (Breitfeld *et al.*, 2000; Schaerli *et al.*, 2000; Kim *et al.*, 2001) for both extrafollicular (Lee *et al.*, 2011) and GC responses (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009). They express high surface levels of CXCR5, programmed cell death-1 (PD-1), and ICOS, and produce IL-21 cytokine (Chtanova *et al.*, 2004; Kim *et al.*, 2004; Vinuesa *et al.*, 2005; Dorfman *et al.*, 2006; Haynes *et al.*, 2007; Nurieva *et al.*, 2008; Luethje *et al.*, 2012). A growing number of transcriptional regulators and molecular factors have been implicated in the development of Tfh cells (Vinuesa *et al.*, 2016). In particular, intrinsic expression of Bcl-6 in CD4⁺ T cells is essential for Tfh cell differentiation (Johnston *et al.*, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009), and is rapidly upregulated after dendritic cell priming at the T-B border (Deenick *et al.*, 2010; Choi *et al.*, 2011; Kerfoot *et al.*, 2011; Kitano *et al.*, 2011; Goenka *et al.*, 2011; Baumjohann *et al.*, 2011). Dendritic cell-mediated co-stimulation promote Bcl-6 expression (Choi *et al.*, 2011; Leavenworth *et al.*, 2015), leading to CXCR5 up-regulation and CCR7 down-regulation for GC entry (Hardtke *et al.*, 2005; Arnold *et al.*, 2007; Haynes *et al.*, 2007). CXCR5 up-regulation is associated with the transcriptional function of achaete-scute homologue 2 (Liu *et al.*, 2014) and c-Maf (Kroenke *et al.*, 2012), and sustained by Bcl-6 (Liu *et al.*, 2012; Hatzi *et al.*, 2015). Additionally, effective positioning of Tfh cells in GCs is mediated by EBI2 (Li *et al.*, 2016b) and integrins (Schrock *et al.*, 2019). Furthermore, Bcl-6 promotes the Tfh cell-specific gene program by antagonizing alternative T helper cell fates (Nurieva *et al.*, 2009; Yu *et al.*, 2009; Hatzi *et al.*, 2015), and co-operates with other transcription factors, such as IRF4 (Gupta *et al.*, 1999a) to control the differentiation of Tfh cells (Bollig *et al.*, 2012).

IL-6 and IL-21 are key cytokines that have been shown to contribute to Bcl-6 induction during Tfh cell differentiation via signal transducers and activators of transcription (STAT)3 and STAT1 activation (Nurieva *et al.*, 2008; 2009; Eto *et al.*, 2011; Choi *et al.*, 2013a; b). Mice deficient in IL-6 have been described by some (Nurieva *et al.*, 2008; Karnowski *et al.*, 2012; Choi *et al.*, 2013a), but not all (Poholek *et al.*, 2010; Eto *et al.*, 2011), to exhibit delayed or reduced induction of Tfh cells, while others have suggested that IL-6 is important for their persistence (Harker *et al.*, 2011). Similarly, IL-21 deficiency in T cells has been shown to reduce Tfh cell development in some

studies (Nurieva *et al.*, 2008; Vogelzang *et al.*, 2008; McGuire *et al.*, 2015) but not others (Linterman *et al.*, 2010; Poholek *et al.*, 2010; Zotos *et al.*, 2010; Eto *et al.*, 2011) depending on the experimental model studied. Therefore, IL-21 and IL-6 is likely to act redundantly in Tfh cell differentiation, highlighted by the observation that simultaneous loss of both cytokines significantly impair Tfh cell generation (Eto *et al.*, 2011; Karnowski *et al.*, 2012). Additionally, IL-6 and ICOS signaling (Suto *et al.*, 2008; Bauquet *et al.*, 2009; de Wit *et al.*, 2015) have been described to potentiate IL-21 production by Tfh cells.

Various transcription factors inhibit Tfh cell differentiation by opposing the function of Bcl-6 and other positive drivers of their development, with B lymphocyte-induced maturation protein-1 (Blimp-1), described to be a prominent negative transcriptional regulator. Blimp-1 (encoded by *Prdm1*) is a transcription factor induced by IL-2-dependent activation of STAT5, which antagonizes Bcl-6, thereby limiting the Tfh cell program (Johnston *et al.*, 2009; 2012; Nurieva *et al.*, 2012; Oestreich *et al.*, 2012). Consistently, IL-2 signaling potently inhibits Tfh cell development (Ballesteros-Tato *et al.*, 2012; León *et al.*, 2014), and IL-2R expression negatively associates with Tfh cell differentiation (Choi *et al.*, 2011). IL-2 responsiveness and *Prdm1* expression can be further potentiated by type I IFN signals, which suppress Tfh cell differentiation (Wong *et al.*, 2010; Ray *et al.*, 2014; Schmitt *et al.*, 2014). Additionally, Foxp1 (Wang *et al.*, 2014), Id3 (Liu *et al.*, 2014; Miyazaki *et al.*, 2015; Shaw *et al.*, 2016), Forkhead box protein O1 (Kerdiles *et al.*, 2010; Xiao *et al.*, 2014; Stone *et al.*, 2015), and Krüppel-like factor 2 (Weber *et al.*, 2015; Lee *et al.*, 2015), have also been shown to antagonize Tfh cell differentiation by suppressing Tfh cell-associated gene expression.

B cells play an important role in maintaining the phenotype and survival of Tfh cells (Haynes *et al.*, 2007; Johnston *et al.*, 2009; Glatman Zaretsky *et al.*, 2009; Deenick *et al.*, 2010; Choi *et al.*, 2011; Kerfoot *et al.*, 2011; Kitano *et al.*, 2011; Goenka *et al.*, 2011; Baumjohann *et al.*, 2013). While initial Tfh cell differentiation does require cognate B cell interaction, they act as primary antigen-presenting cells during later stages of Tfh development (Deenick *et al.*, 2010; Choi *et al.*, 2011). Co-stimulatory molecules expressed by B cells, such as CD80 (Good-Jacobson *et al.*, 2012), CD86 (Salek-Ardakani *et al.*, 2011; Linterman *et al.*, 2014) and ICOS ligand (ICOSL) (Nurieva *et al.*, 2008; Choi *et al.*, 2011), also promote Tfh cell differentiation, whereas PD-1 ligands have been implicated in suppressing Tfh cell expansion (Hamel *et al.*, 2010; Hams *et al.*, 2011; Butler *et al.*, 2011) that is associated with poor GC B cell survival and impaired B cell memory (Good-Jacobson

et al., 2010). Intriguingly, non-cognate B cell interactions have been identified to promote Tfh cell recruitment to the B cell follicle by providing ICOS stimulation (Xu *et al.*, 2013).

3.2 Expansion and polarization of the GC reaction

The early GC can be identified within days of antigen exposure and grows with the rapid proliferation of activated B cells (Kerfoot *et al.*, 2011; Kitano *et al.*, 2011). Several molecular factors have been found to be dispensable for the GC initiation but required for the expansion and maintenance phases. These include the transcription factor Spi-B (Su *et al.*, 1997; Garrett-Sinha *et al.*, 1999), the closely related PU.1 (Willis *et al.*, 2017), early B cell factor 1 (Vilagos *et al.*, 2012), and dedicator of cytokinesis protein 8 by regulating B cell immune synapse organisation (Randall *et al.*, 2009). Most of these factors regulate GC development at the level of BCR signaling and co-stimulation, and their deficiency in mice results in small and/or short-lived GCs.

Maturation of the GC is characterised by its polarization into two topographically segregated compartments consisting of the dark zone (DZ) and light zone (LZ) (**Figure 1.4**), which were named based on their histological appearance (Röhlich, 1930). The DZ is considered to be the primary site of somatic hypermutation (SHM), in which random point mutations are induced in the V(D)J region of the Ig variable region (*IghV*) genes of GC B cells (Weigert *et al.*, 1970), generating GC B cell clones with highly diverse affinity and/or specificity for antigen (Jacob *et al.*, 1991b; Berek *et al.*, 1991; Tas *et al.*, 2016). This process is driven by the enzyme activation-induced cytidine deaminase (AID) (Muramatsu *et al.*, 2000; Revy *et al.*, 2000). The DZ contains highly proliferative GC B cells that express the chemokine receptor CXCR4 (Allen *et al.*, 2004; Victora *et al.*, 2010; 2012), densely packed within a network of reticular cells (Bannard *et al.*, 2013). These reticular cells are a source of CXCR4-binding CXCL12 chemokine, which positions GC B cells in the DZ (Allen *et al.*, 2004; Bannard *et al.*, 2013; Rodda *et al.*, 2015). Consistently, CXCR4 deletion confines GC B cells to the LZ (Allen *et al.*, 2004) and reduces SHM levels (Bannard *et al.*, 2013); although high-affinity antibody production may proceed unhindered (Nie *et al.*, 2004). In contrast to the DZ, the LZ is less populated by B cells, and contains follicular B cells, tingible body macrophages, follicular dendritic cells (FDCs) and T cells (Hanayama *et al.*, 2004; Schwickert *et al.*, 2007; Victora *et al.*, 2010). LZ GC B cells are characterised by expression of activation gene signatures, consistent with the LZ being the site of selection of high-affinity SHM variants (Victora *et al.*, 2010; 2012). Recirculation of GC B cells between the dark and light zones is thought to facilitate iterative rounds of SHM followed by selection that enable affinity maturation of the humoral immune response to occur (Kepler and Perelson, 1993; Oprea and Perelson, 1997), the

intricate details of which have been resolved by intravital microscopy (Allen *et al.*, 2007; Schwickert *et al.*, 2007; Victora *et al.*, 2010; Gitlin *et al.*, 2014).

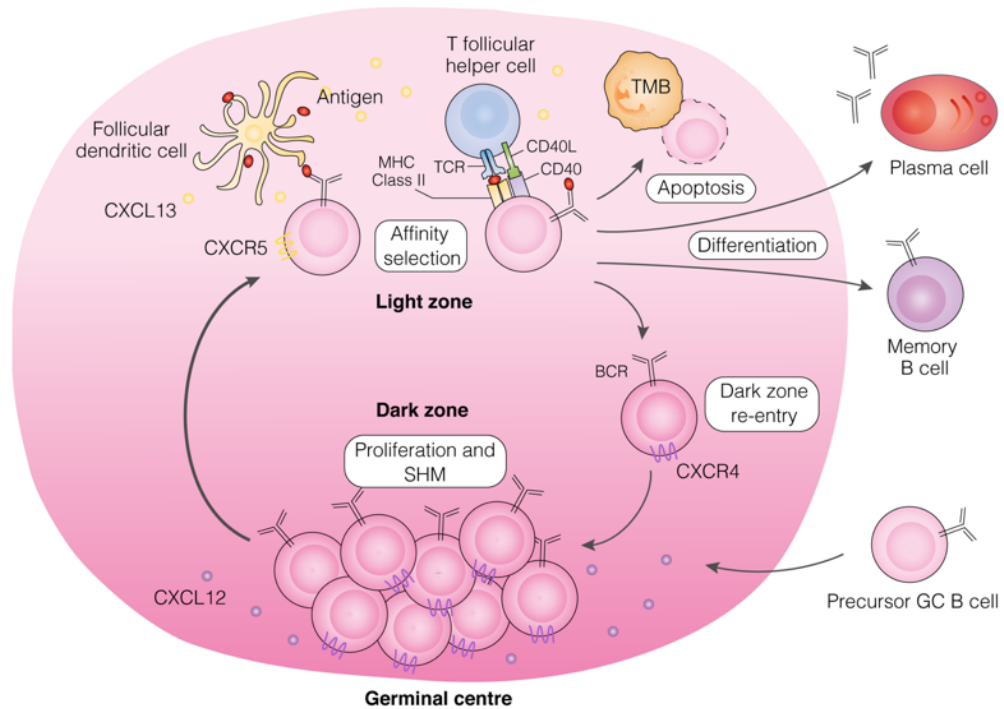


Figure 1.4 The cellular dynamics of the GC reaction

GC B cells expressing the chemokine receptor CXCR4, localise to the dark zone (DZ) rich in CXCL12 chemokine. Here, they undergo proliferation and antibody somatic hypermutation (SHM), giving rise to clones with different antigen affinities. B cells that exit the cell cycle migrate to the LZ in response to a CXCL13 chemokine gradient. In the LZ, affinity selection occurs via interaction with T follicular helper cells and antigen-presenting follicular dendritic cells. Positively-selected GC B cells recycle to the DZ for further rounds of SHM and proliferation, or differentiate into plasma cells and memory B cells. Negatively-selected GC B cells undergo apoptosis and are phagocytosed by tingible body macrophages (TBM). TCR, T cell receptor; BCR, B cell receptor.

3.2.1 Selection of high-affinity GC B cells

The positive selection of high-affinity B cell clones in the GC is widely accepted to be dictated by competition for antigen-dependent signals from Tfh cells and FDCs in the LZ. GC B cells utilise a specialised synapse organisation to acquire and test binding affinity to antigen (Nowosad *et al.*, 2016; Chen *et al.*, 2017). Antigen retained by FDCs within complement-coated immune complexes provide an ongoing source for BCR stimulation of GC B cells (Matsumoto *et al.*, 1997; Fischer *et al.*, 1998; Suzuki *et al.*, 2009; Heesters *et al.*, 2013). Additionally, FDCs promote GC retention (Wang *et al.*, 2011; Muppidi *et al.*, 2015), and survival of GC B cells through different co-stimulatory molecules including IL-6, B cell activating factor and toll-like receptor (TLR)4

(Wu *et al.*, 2009; Garin *et al.*, 2010). Additionally, BCR signaling has been suggested to play a role in GC selection, as LZ GC B cells expressing genes related to positive selection have been identified to exhibit active BCR engagement, suggesting that BCR signals may contribute to GC selection (Mueller *et al.*, 2015); however, integration of both BCR and T cell-derived signals seems to be important (Luo *et al.*, 2018).

T cell help has been recognised as a dominant factor that regulates affinity-based selection (Allen *et al.*, 2007; Victora *et al.*, 2010; Gitlin *et al.*, 2014; Shulman *et al.*, 2014; Gitlin *et al.*, 2015). Tfh cells have been visualised to transiently interact with GC B cells (Allen *et al.*, 2007; Shulman *et al.*, 2014), but can make extensive, entangled surface contact, depending on the level of B cell antigen presentation (Shulman *et al.*, 2014) and ICOSL co-stimulation, which promotes further engagement in a positive feedback mechanism (Liu *et al.*, 2015a). High-affinity GC B cells have been shown to capture more antigen that increases their surface presentation of peptide on major histocompatibility complex II, which results in a greater magnitude of T cell help (Victora *et al.*, 2010). Gitlin *et al.* demonstrated that the strength of T cell help regulates the number and speed of divisions by positively selected GC B cells in the DZ (Gitlin *et al.*, 2014; 2015). Furthermore, clonal bursts can lead to the expansion and diversification of selected high-affinity GC B cell variants that dominate GCs over time (Tas *et al.*, 2016). Importantly, mathematical analyses estimate that only 10–30% of LZ B cells are selected to re-enter the DZ (Meyer-Hermann *et al.*, 2012), with the remaining cells exiting the GC or dying by apoptosis (Mayer *et al.*, 2017). Apoptotic cells are cleared by tingible body macrophages (Hanayama *et al.*, 2004; Rahman *et al.*, 2010). Of note, DZ B cells with non-functional BCRs as a result of detrimental *IghV* gene mutations, will also undergo apoptosis (Mayer *et al.*, 2017; Stewart *et al.*, 2018). While B cells are preferentially selected for their affinity to bind foreign antigen (Sabouri *et al.*, 2014; Burnett *et al.*, 2018), self-reactive B cells that escape selection are regulated by the pro-apoptotic receptor Fas (Butt *et al.*, 2015), and may undergo further SHM to eliminate their affinity for self-antigen (Sabouri *et al.*, 2014).

3.2.2 Molecular control of GC polarization

The precise molecular mechanisms that regulate the transient DZ and LZ programs in re-circulating GC B cells are not entirely understood. To date, a few studies have identified that the transcription factors c-Myc, AP4, and forkhead box protein O1 (Foxo1), play key roles in regulating a GC B cell-intrinsic programme in coordinating polarization of the GC reaction. Following transient upregulation in precursor GC B cells, the cell cycle regulator c-Myc is re-

expressed in a subset of LZ GC B cells by BCR and CD40 co-stimulation at a time that coincides with positive selection, suggesting that c-Myc-expressing cells are primed for DZ re-entry to undergo further cycles of proliferation and mutation (Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012; Luo *et al.*, 2018). c-Myc-dependent gene expression has been described to be stabilised by the ribonucleic acid (RNA)-binding protein PTBP1, which supports cell cycle progression and DZ maintenance (Monzón-Casanova *et al.*, 2018). Moreover, c-Myc ablation leads to the dissolution of established GCs, highlighting its importance in perpetuating the GC reaction (Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012).

The transcription factor AP4 is induced by Myc and is required for selected GC B cells to expand and mutate in the DZ (Chou *et al.*, 2016). The mechanism by which AP4 functions is currently not clear, but it can directly up-regulate the epigenetic regulator Uhrf1 in GC B cells, which methylates proliferation-associated genes and promotes cell division (Chen *et al.*, 2018). Chou *et al.* found that IL-21 signals maintain AP4 expression, suggesting an important role of T cell signals in regulating AP4 activity (Chou *et al.*, 2016). Foxo1 is another key transcription factor required for DZ formation, instructing the DZ transcriptional program including CXCR4 up-regulation, while suppressing B cell activation and differentiation genes in co-operation with Bcl-6 (Sander *et al.*, 2015; Dominguez-Sola *et al.*, 2015). Genetic deletion or inhibition of Foxo1 confines GC B cells to the LZ, and impairs GC B cell SHM and proliferation (Sander *et al.*, 2015; Inoue *et al.*, 2017), supporting that Foxo1 is important for the DZ reaction. Furthermore, Foxo1 has been suggested to be induced by mechanistic target of rapamycin complex 1 (mTORC1) signaling (Lin *et al.*, 2015; Ersching *et al.*, 2017), which plays a role in GC maintenance by regulating cell metabolism (Keating *et al.*, 2013; Jones *et al.*, 2016; Ersching *et al.*, 2017). Additionally, the transcription factor Yin Yang 1 has also been described to regulate DZ formation (Trabucco *et al.*, 2016), possibly linked to GC B cell survival (Banerjee *et al.*, 2016; Trabucco *et al.*, 2016). Overall, a better understanding of the transcriptional regulators of GC polarization is needed, and more mechanistic studies are required to understand how these transcription factors are regulated by signals in the GC and integrated for effective GC polarization.

3.3 Post-GC differentiation fate

The goal of the GC is to generate high-affinity plasma cells and MBCs that exert effector and memory functions to provide effective immune protection upon antigen re-encounter. Most plasma cells migrate to the bone marrow and continue to produce antibodies for months to years, serving as a first line of defence against re-infection (Manz *et al.*, 1997; Slifka *et al.*, 1998). While

MBCs disseminate widely via the circulation or reside within secondary lymphoid tissue (Bachmann *et al.*, 1994; Dunn-Walters *et al.*, 1995; Klein *et al.*, 1998; Joo *et al.*, 2008; Mamani-Matsuda *et al.*, 2008), rapidly re-activating upon subsequent challenge to elicit an accelerated and robust antibody response (Tangye *et al.*, 2003; Good *et al.*, 2009; Kometani *et al.*, 2013). The signals that instruct GC B cells to differentiate into these long-lived populations are not entirely clear, but B cells exit the GC reaction upon down-regulating Bcl-6 (Ise *et al.*, 2018), which can occur via multiple mechanisms, including NF- κ B-induced IRF4 repression (Saito *et al.*, 2007), mitogen-activated protein kinase-induced phosphorylation (Niu *et al.*, 1998), and ubiquitin-proteasomal degradation (Duan *et al.*, 2012).

The plasma cell transcriptional programme is established by IRF4, Blimp-1 and X-box binding protein 1 (Nutt *et al.*, 2015). LZ GC B cells expressing IRF4 and low levels of Bcl-6 have been identified to mark cells that favour the plasma cell fate over GC cycling (Ise *et al.*, 2018). High level expression of IRF4 is required for plasma cell differentiation (Klein *et al.*, 2006; Sciammas *et al.*, 2006), by directly activating and releasing Bcl-6 repression of Blimp-1, an essential transcriptional regulator of plasma cell development (Shapiro-Shelef *et al.*, 2003; Kallies *et al.*, 2007). In turn, Blimp-1 represses GC B cell gene transcription (Lin *et al.*, 2002; Shaffer *et al.*, 2002), and reinforces the plasma cell program in a feed-forward loop (Ochiai *et al.*, 2013; Minnich *et al.*, 2016). The signals that regulate long-lived plasma cell versus MBC fate are not fully understood; however, there is growing consensus that BCR affinity is a key factor that controls fate commitment. Several studies indicate that the differentiation of plasma cells tends to occur in high-affinity GC B cells (Smith *et al.*, 1996; Paus *et al.*, 2006; Phan *et al.*, 2006; Taylor *et al.*, 2015). High-affinity LZ GC B cells appear to up-regulate IRF4 and Blimp-1 (Sciammas *et al.*, 2006; Ochiai *et al.*, 2013; De Silva *et al.*, 2016; Kräutler *et al.*, 2017; Ise *et al.*, 2018), where they engage antigen (Kräutler *et al.*, 2017), and subsequent receipt of Tfh cell help via stable contact-dependent adhesion enables their transition to the DZ to differentiate into mature plasma cells (Kräutler *et al.*, 2017; Ise *et al.*, 2018). In support of the dependence of precursor plasma cells on T cell help for their maturation, enhancing the magnitude of cognate T cell engagement promotes ASC generation (Victora *et al.*, 2010; Schwickert *et al.*, 2011), which may involve, but is not limited to CD40 ligation and cytokines like IL-21 (Ozaki *et al.*, 2004; Ettinger *et al.*, 2005; Bryant *et al.*, 2007; Kräutler *et al.*, 2017; Ise *et al.*, 2018). The Tfh cell-derived signals that favour seeding of GC B cells into the plasma cell compartment are yet to be fully elucidated.

The affinity requirements for MBC differentiation seems less stringent, with relatively low-affinity LZ GC B cells described to be preferentially selected into the MBC compartment (Shinnakasu *et al.*, 2016; Suan *et al.*, 2017). The transcriptional regulation of MBC development is largely unresolved; however it has been associated with a down-regulation of genes involved in GC cycling and up-regulation of the transcriptional repressor Bach2 (Shinnakasu *et al.*, 2016). Bach2 expression was found to inversely correlated with the strength of T cell help (Shinnakasu *et al.*, 2016), supporting that lower-affinity GC B cells that receive less T cell help favour MBC differentiation, which may be mediated by Tfh cell-derived IL-9 that has been shown to promote MBC development (Wang *et al.*, 2017). Additionally, the transcription factor activated B cell factor-1 has also been described to facilitate MBC formation, but is inhibited by Blimp-1 during human and mouse plasma cell differentiation (Chiu *et al.*, 2014b). T cell-derived IL-21 and CD40L signals are implicated in its induction (Chiu *et al.*, 2014b), but additional mechanistic studies will be required to understand how it regulates MBC differentiation.

4. The inefficient acquisition of humoral immunity in malaria

The cellular processes that underpin the development of immunity in malaria are poorly defined. Several mechanisms have been proposed to delay or modulate the development of effective humoral immune responses to blood-stage malaria infection, thereby contributing to the slow acquisition and non-sterilising protection of natural acquired immunity.

4.1 The slow development of a broad and high-affinity antibody repertoire

As *Plasmodium* parasites utilise a number of highly polymorphic and functionally redundant ligands to colonize various niches and to evade the immune system as discussed in Sections 1 and 3, immunity to malaria has been postulated to depend on a cumulative acquisition of antibodies to the diverse parasite antigens (Murungi *et al.*, 2013; Warimwe *et al.*, 2013; Stanisic *et al.*, 2015; Arama *et al.*, 2015; King *et al.*, 2015). Consistent with this idea, the breadth of blood-stage *Plasmodium*-specific antibody responses increases with age and exposure in endemic populations (Crompton *et al.*, 2010; Weiss *et al.*, 2010; Finney *et al.*, 2014; Torres *et al.*, 2015; King *et al.*, 2015; Dent *et al.*, 2015). In particular, protein microarray studies demonstrate that antibodies of asymptomatic individuals recognise a broader panel of antigens compared to individuals with acute disease (Torres *et al.*, 2008; Finney *et al.*, 2014). Moreover, the antibody repertoire of Malian children has been found to diversify rapidly during infancy (Wendel *et al.*, 2017). Similarly, antibody avidity to certain *P. falciparum* antigens increases with age or repeated exposure (Wipasa *et al.*, 2010; Ibison *et al.*, 2012). The exact mechanism by which this occurs is not yet clear; however, analysis of B cell clones by Lanzavecchia and colleagues revealed that antibodies with

broad reactivity against *P. falciparum* variant surface antigens, carry a gene insertion derived from the collagen-binding receptor LAIR1, and subsequently accumulate somatic hypermutations that increase binding to pRBCs (Tan *et al.*, 2016). The same group found that LAIR1 insertions represent a relatively common mechanism of antibody diversification in African individuals, in which antibodies against different *Plasmodium* antigens arise from these low-affinity precursors (Pieper *et al.*, 2017). Whether different transmission intensities and parasite exposure influences the acquisition of these antibodies, and their role in protection against disease is yet to be delineated.

4.2 Short-lived antibody responses to natural malaria infection

In endemic areas, substantial declines have been documented in *Plasmodium*-specific antibody responses following parasite clearance or at the end of the transmission season (Fruh *et al.*, 1991; Cavanagh *et al.*, 1998; Soares *et al.*, 1999; Fonjungo *et al.*, 1999; Giha *et al.*, 1999; Cavanagh *et al.*, 2004; Kinyanjui *et al.*, 2007; Akpogheneta *et al.*, 2008; Ceravolo *et al.*, 2009; Crompton *et al.*, 2010; Weiss *et al.*, 2010; Proietti *et al.*, 2013). Similarly, low levels of antibodies are detected in individuals living in regions of declining malaria transmission (Migot *et al.*, 1993; Ceesay *et al.*, 2010; Diop *et al.*, 2014), and upon emigration from an endemic area (Riley *et al.*, 1993; Moncunill *et al.*, 2013). Thus, regular exposure appears to be necessary to sustain antibody responses, and with cessation of infection, antibody levels are greatly diminished and short-lived. Further support of this notion comes from modelling studies of *P. falciparum* blood-stage-specific IgG antibodies that estimated half-lives ranging from 6–52 days in children following resolution of natural infection (Kinyanjui *et al.*, 2007; Akpogheneta *et al.*, 2008). Notably, the short-lived nature of anti-malarial antibodies contrasts with the long-lived responses induced to viral and bacterial antigens following a single exposure (Maple *et al.*, 2000; Hammarlund *et al.*, 2003; Amanna *et al.*, 2007), and is consistent with the output of short-lived plasmablasts, which typically produce a transient wave of antibodies (Jacob *et al.*, 1991b; Berek *et al.*, 1991; Lalor *et al.*, 1992). Indeed, short-lived ASCs have been detected in Ugandan children with acute malaria, which proportionally increased upon a second clinical episode (Sullivan *et al.*, 2016). The longevity of short- and long-lived ASCs have been estimated to range from 2–10 days, and 3–9 years, respectively (White *et al.*, 2014a), implying that long-lived humoral immunity can potentially be sustained after decay of the short-lived response. Intriguingly, infrequent malaria infections can induce sustained *P. falciparum*-specific antibody responses (Migot *et al.*, 1995; Singer *et al.*, 2003; Bouchaud *et al.*, 2005; Torres *et al.*, 2008; Wipasa *et al.*, 2010; Clark *et al.*, 2012; Ayieko *et al.*, 2013; Moncunill *et al.*, 2013), with half-lives projected to be substantially longer than that of individuals subjected to ongoing infections (Drakeley *et al.*, 2005; Bousema *et al.*, 2010; Wipasa *et al.*, 2010). Several independent reports also describe the maintenance of antibodies to *P. vivax* for at least a year (Braga *et al.*, 1998; Park *et al.*, 2001; Lim *et al.*, 2004; Morais *et al.*, 2006; Wipasa *et al.*, 2010; Kaneko *et al.*, 2014), although periodic boosting by relapses cannot be excluded with this parasite species. More detailed

mechanistic investigations are much needed to determine how antibody responses to malaria are sustained over time and the factors that modulate their longevity.

4.3 Modulation of B and T cell activation and function

Plasmodium parasites have been suggested to directly modulate the activation and/or function of B cells. One study showed that *P. falciparum* can directly bind to human B cells via a cysteine rich inter-domain region of PfEMP1, which induces their activation and proliferation *in vitro* (Donati *et al.*, 2004; 2006) via downstream TLR signaling (Simone *et al.*, 2011). The specific B cell subsets affected *in vivo* have not been delineated, but CD27⁺ B cells appeared to be a major responding population (Donati *et al.*, 2004; 2006), implying a potential influence on the MBC compartment. In line with this idea, MBC subsets were observed to proliferate shortly following experimental human infection (Scholzen *et al.*, 2014), suggesting that bystander activation of the humoral immune response may occur early after acute infection. In parallel, increased plasma levels of B cell activating factor have been detected in individuals with acute malaria (Nduati *et al.*, 2011; Scholzen *et al.*, 2014), which associated with disease severity in one study (Nduati *et al.*, 2011). This may have implications on B cell activation and survival during infection, but remains to be examined in naturally-exposed populations. Additionally, blood-stage malaria may modulate T helper cell function. Early work reported proliferation defects in parasite antigen- or mitogen-stimulated T cells isolated from adults with acute *P. falciparum* infection, which were ameliorated upon parasite clearance (Ho *et al.*, 1986), suggesting a direct suppressive effect by blood-stage parasites. Subsequent studies indicated that human CD4⁺ T cells undergo apoptosis (Hojo-Souza *et al.*, 2015) and up-regulate markers of T cell exhaustion, such as PD-1 and LAG-3 during infection (Butler *et al.*, 2011; Illingworth *et al.*, 2013; Zander *et al.*, 2015; Costa *et al.*, 2015), with PD-1 expression positively correlating with parasite densities (Zander *et al.*, 2015). Furthermore, simultaneous blockade of the inhibitory molecules cytotoxic T lymphocyte-associated protein-4, PD-1, and TIM-3, increased *in vitro* cytokine production of cells from individuals with uncomplicated *P. vivax* infection (Costa *et al.*, 2015). Collectively, these studies implicate malaria parasites in the modulation of B cell function.

4.4 The delayed acquisition of MBC responses in high transmission areas

Individuals exposed to high seasonal malaria transmission generate MBCs at frequencies that are often low and unstable (Dorfman *et al.*, 2005; Wipasa *et al.*, 2010; Weiss *et al.*, 2010; Nogaro *et al.*, 2011; Triller *et al.*, 2017a). Similar to the acquisition of inefficient antibody responses, the prevalence of *Plasmodium*-specific MBCs develops incrementally, after many years of parasite exposure (Weiss *et al.*, 2010; Nogaro *et al.*, 2011; Wendel *et al.*, 2017). Longitudinal analysis of the

prevalence of MBCs in Malian children identified that AMA-1- and MSP-1-specific MBCs diminished substantially following acute infection (Weiss *et al.*, 2010). While seasonal transmission prevented the longevity of these cells to be determined beyond 6 months, these observations support that the generation or maintenance of MBCs to malaria infection is sub-optimal.

In contrast to endemic areas, individuals living in areas of low transmission or subjected to fewer clinical episodes, can generate stable parasite-specific MBCs without frequent boosting (Migot *et al.*, 1995; Wipasa *et al.*, 2010; Nogaro *et al.*, 2011; Clark *et al.*, 2012; Ndungu *et al.*, 2012; Ayieko *et al.*, 2013; Ampomah *et al.*, 2014a). Wipasa *et al.* predicted no significant decline in the frequencies of *P. falciparum*-specific MBCs in Thai adults with a history of limited malaria episodes, with an estimated half-life of 7.5 years (Wipasa *et al.*, 2010). Similarly, MBCs appear to be well-maintained in individuals with a history of acute malaria but have since lived in the absence of re-infections (Ndungu *et al.*, 2012; Ayieko *et al.*, 2013). Collectively, these findings indicate that MBCs can be generated and sustained following limited malaria infections, whereas it is much more delayed and variable in high transmission areas.

4.4.1 Atypical memory B cells

The characterization of an expanded MBC population exhibiting an atypical phenotype following prolonged parasite exposure, substantiated the idea that malaria infection alters the generation of B cell memory (Weiss *et al.*, 2009; 2011; Ayieko *et al.*, 2013; Illingworth *et al.*, 2013). Originally described as an exhausted subset of MBCs implicated in humoral deficiencies in HIV infection (Moir *et al.*, 2008), a phenotypically similar circulating CD27⁺CD21⁺ MBC population accumulates in individuals following acute malaria infection in geographically diverse regions in Brazil (Figueiredo *et al.*, 2017), Gabon (Muellenbeck *et al.*, 2013), Ghana (Ampomah *et al.*, 2014b), Gambia (Nogaro *et al.*, 2011), Kenya (Ayieko *et al.*, 2013; Illingworth *et al.*, 2013), Mali (Weiss *et al.*, 2009; 2011; Portugal *et al.*, 2012; 2015; Obeng-Adjei *et al.*, 2017), Papua New Guinea (Requena *et al.*, 2014; Ubillos *et al.*, 2017), Rwanda (Subramaniam *et al.*, 2015) and Uganda (Sullivan *et al.*, 2015; 2016; Lugaajju *et al.*, 2017). Studies to date corroborate that the expansion of atypical MBCs is mediated by parasite exposure. For example, adults living in high transmission areas were found to have higher frequencies of these cells compared to children with shorter exposure histories (Weiss *et al.*, 2009) and individuals in low transmission areas (Weiss *et al.*, 2011). Whereas, a decline in the atypical MBC pool was observed during the absence of ongoing malaria transmission (Ayieko *et al.*, 2013), confirming a role of ongoing parasite exposure in their maintenance.

A few studies have assessed the function of atypical MBCs and their relationship to classical MBCs. Analysis of the IgG repertoire of atypical MBCs proposed that they share a common developmental precursor (Muellenbeck *et al.*, 2013; Portugal *et al.*, 2015; Zinöcker *et al.*, 2015), but differ phenotypically and functionally to classical MBCs. Despite one study postulating that atypical MBCs may secrete neutralizing antibodies (Muellenbeck *et al.*, 2013), subsequent work contend their lack of antibody production *ex vivo* (Portugal *et al.*, 2015; Sullivan *et al.*, 2015). Atypical MBCs appear to express lower levels of surface IgG (Requena *et al.*, 2014), and have impaired cytokine secretion *in vitro* (Portugal *et al.*, 2015; Sullivan *et al.*, 2015). Moreover, their expression of the inhibitory molecule Fc receptor-like 5 (FcRL5) has been proposed to be a marker of dysfunction, and positively correlates with parasite exposure (Sullivan *et al.*, 2015). FcRL5-expressing atypical MBCs in healthy individuals have been distinguished by their blunted proliferation and higher expression of transcription factors *Tbx21*, *Bcl-6* and *Sox5*, compared to FcRL5⁻ B cells (Li *et al.*, 2016a). Interestingly, a recent study described that atypical MBCs in Malian children express *Tbx21* (Obeng-Adjei *et al.*, 2017), which encodes the transcription factor T-box expressed in T cells (T-bet) that is required for the development of Th1 cells (Szabo *et al.*, 2000). Atypical MBCs with high levels of T-bet also expressed FcRL5, and displayed an anergic phenotype (Obeng-Adjei *et al.*, 2017). Moreover, an expansion of these cells associated with the incidence of clinical malaria in a small group of children, implicating a negative role of T-bet⁺ MBCs in protection (Obeng-Adjei *et al.*, 2017). However, a causal relationship between atypical MBCs and disease susceptibility or immunity remains unclear. The accumulation of total atypical MBCs in acutely infected children has also been reported to correlate with the control of peripheral parasitemia over time (Sullivan *et al.*, 2016). Hence, more mechanistic research is needed to delineate the role of atypical MBCs in immunity and the molecular mechanisms that drive their expansion with repeated malaria infection.

4.4.2 Insight into the generation of B cell memory from murine models

Murine models provide valuable insight into the development of humoral immunity to malaria infection, which otherwise cannot be deduced from human studies without access to lymphoid tissue. The most commonly used parasite species are *P. chabaudi* (Landau, 1965), *P. yoelii* (Landau and Killick-Kendrick, 1966), and *P. berghei* (Vincke and Lips, 1948). Phenotypic parallels between the human parasites and their rodent counterparts make them attractive models. Like in humans, antibodies play a key part in anti-malarial immunity (Diggs and Osler, 1969; Wells and Diggs, 1976; Jarra *et al.*, 1986; Spencer Valero *et al.*, 1998; Narum *et al.*, 2000; Spaccapelo *et al.*, 2010; Claser *et al.*, 2017), with B cell-deficient mice failing to clear blood-stage infection and suppress parasitemia upon re-challenge (Meding and Langhorne, 1991; van der Heyde *et al.*, 1994; Weid *et al.*, 1996; Langhorne *et al.*, 1998).

Memory-like IgG⁺ B cells have been detected between 7–20 days following non-lethal *P. chabaudi* AS infection (Castillo-Méndez *et al.*, 2007; Stephens *et al.*, 2009; Ndungu *et al.*, 2009; Nduati *et al.*, 2010). These cells may contribute to the induction of secondary GCs and antibody responses that associate with effective parasite control during re-infection (Achtman *et al.*, 2007; Stephens *et al.*, 2009). The subsequent discovery of additional MBC-specific markers and novel detection methods has allowed the phenotypic characterisation of malaria-specific MBCs in infected mice in greater depth. A recent study reported that *P. chabaudi* AS infection promotes the development of heterogeneous MSP-1-specific MBC subsets that express the surface markers CD80 and CD73, and somatically mutated BCRs, correlating with memory status (Krishnamurthy *et al.*, 2016). Moreover, B cells in *P. chabaudi* AS-infected mice have been found to up-regulate expression of the inhibitory receptor FcRL5 (Fontana *et al.*, 2016; Pérez-Mazliah *et al.*, 2018), resembling human atypical MBCs (Portugal *et al.*, 2015; Sullivan *et al.*, 2015). While these murine B cells expressed memory markers such as CD80 and PD-L2, they displayed transcriptional features of proliferation and IgG expression, and appeared to represent a short-lived population of activated B cells (Pérez-Mazliah *et al.*, 2018). Interestingly, these cells up-regulated the Th1 lineage-defining transcription factor T-bet (Pérez-Mazliah *et al.*, 2018), similar to atypical MBCs of *P. falciparum*-infected children (Obeng-Adjei *et al.*, 2017). Whether T-bet modulates MBC development and/or intrinsic function in malaria is yet to be addressed. While the aforementioned studies predominantly focused on class-switched MBCs, a few recent studies have detected IgM-expressing MBCs in malaria-exposed individuals (Krishnamurthy *et al.*, 2016; Ubillos *et al.*, 2017). Similarly in mice, IgM⁺ MBCs can be induced by *P. chabaudi* AS infection (Stephens *et al.*, 2009), express mutated BCRs, and may differentiate into IgM⁺ and IgG⁺ ASCs upon antigen re-encounter (Krishnamurthy *et al.*, 2016). Additionally, a small population of GC-independent IgM⁺ MBCs have been postulated to promote blood-stage parasite clearance by differentiating into plasmablasts in chronically infected immune-deficient mice (Borges da Silva *et al.*, 2018). Interestingly, IgM⁺ MBCs induced to bacterial infection up-regulate the transcription factor T-bet, and exhibit considerable plasticity in their differentiation potential following rechallenge (Kenderes *et al.*, 2018). In addition to immediate antibody-secreting function, IgM⁺ MBCs have also been proposed to seed secondary GC responses in immunization models (Dogan *et al.*, 2009; Pape *et al.*, 2011; Zuccarino-Catania *et al.*, 2014); whether IgM⁺ MBCs have a similar function in malaria calls for further interrogation.

4.4.3 Plasma cell responses to murine malaria infection

In addition to MBCs, murine infection also induces an early development of CD138-expressing ASCs that can be detected in the blood and spleen (Castillo-Méndez *et al.*, 2007; Stephens *et al.*,

2009; Nduati *et al.*, 2010; Inoue *et al.*, 2018). The ASC response is largely dominated by the IgG2a/c subclass (referred to as IgG2c herein) (D'Império Lima *et al.*, 1996; Castillo-Méndez *et al.*, 2007), which is considered to be the most effective IgG isotype in limiting or clearing parasitemia in murine malaria infection (White *et al.*, 1991; Waki *et al.*, 1995; Cavinato *et al.*, 2001; Elias *et al.*, 2005), attributed to its ability to bind to Fc receptors with high affinity (Nimmerjahn *et al.*, 2005; Nimmerjahn and Ravetch, 2005). The ASC response in *P. chabaudi* AS-infected mice is largely transient (Stephens *et al.*, 2009; Ndungu *et al.*, 2009; Nduati *et al.*, 2010), but mature plasma cells appeared to accumulate in the bone marrow weeks after infection (Stephens *et al.*, 2009; Ndungu *et al.*, 2009). In contrast to non-lethal *P. chabaudi* AS infection, the early differentiation of ASCs is compromised during *P. berghei* ANKA severe malaria infection, associated with strong inflammation in this model (Ryg-Cornejo *et al.*, 2016a). It is possible that severe malaria impairs the induction or maintenance of plasma cells, as malaria infection has been reported to disrupt bone marrow homeostasis (Bockstal *et al.*, 2011), and trigger apoptosis of pre-existing MSP-1-specific ASCs in the bone marrow upon lethal *P. yoelii* YM infection (Wykes *et al.*, 2005). More importantly, studies to date have not assessed the affinity of plasma cells and the antibodies they secrete, which may dictate their ability to bestow protection against malaria.

4.5 The GC response to malaria infection

While an increasing number of studies characterise B cell memory populations in malaria-infected individuals, how they are generated is much less understood. Histological analysis of the spleens of *P. falciparum* severe malaria patients revealed a dissolution of the marginal zone, a loss of B cell and T cell zone integrity, and reduced GC formation (Urban *et al.*, 2005). These findings provided novel evidence to suggest that the development of humoral immunity to acute malaria infection may be compromised. The splenic remodelling observed in humans is mirrored following blood-stage murine infection. *P. chabaudi* AS infection is accompanied by significant lymphocyte apoptosis and extensive disorganisation of the marginal zone, and B and T cell areas (Stevenson and Kraal, 1989; Helmby *et al.*, 2000; Achtman *et al.*, 2003; Krücken *et al.*, 2005; Beattie *et al.*, 2006; Cadman *et al.*, 2008). Although the alterations are dramatic, splenic structure restores upon the resolution of disease and robust GC development occurs by 3 weeks post-infection (Achtman *et al.*, 2003). In contrast to *P. chabaudi* AS, the formation of GCs is impaired following *P. berghei* ANKA severe malaria infection (Carvalho *et al.*, 2007; Ryg-Cornejo *et al.*, 2016a), and similarly, during *P. falciparum* infection of *Saimiri* monkeys (Alves *et al.*, 2015), closely resembling that of human malaria (Urban *et al.*, 2005). Importantly, GC disorganisation associated with potent pro-inflammatory responses during infection (Alves *et al.*, 2015; Ryg-Cornejo *et al.*, 2016a), suggesting that acute malaria interferes with effective development of GCs. Consistently, effective control of infection restores normal progression of the GC response (Ryg-Cornejo *et al.*, 2016a; Hahn *et al.*, 2018). In

the next sections, I will review emerging data that begin to delineate how acute malaria and the associated inflammation can modulate the development of the GC response to malaria infection.

4.5.1 The induction of Th1-like Tfh cells in malaria

Tfh cells that facilitate humoral immunity have been demonstrated to play an important role in the control of malaria infection in mice (Pérez-Mazliah *et al.*, 2015; 2017), with the transcription factor Bcl-6 (Pérez-Mazliah *et al.*, 2017), IL-21 cytokine, sustained ICOS co-stimulation and SAP-mediated interactions necessary for their development (Wikenheiser *et al.*, 2015; Pérez-Mazliah *et al.*, 2017; Inoue *et al.*, 2018). Therapeutic strategies that promote Tfh cell expansion are associated with improved B cell responses following *P. yoelii* 17XNL infection (Butler *et al.*, 2011; Zander *et al.*, 2015) and *Plasmodium* immunisation (Moon *et al.*, 2012; Liu *et al.*, 2015b; 2018). The analysis of Tfh cells in malaria-infected populations was not achievable until the discovery of circulating CXCR5⁺CD4⁺ T cells as a surrogate measure of GC-resident Tfh cells (Morita *et al.*, 2011; Locci *et al.*, 2013; He *et al.*, 2013). Recent studies characterized circulating Tfh cells in the peripheral blood of naturally-exposed individuals residing in Mali (Obeng-Adjei *et al.*, 2015) and Brazil (Figueiredo *et al.*, 2017). Circulating PD-1⁺CXCR5⁺CD4⁺ Tfh cells expressed a Tfh cell-associated phenotype, including expression of IL-21 (Obeng-Adjei *et al.*, 2015; Figueiredo *et al.*, 2017), which resembled human GC-derived Tfh cells (Locci *et al.*, 2013; He *et al.*, 2013; Schultz *et al.*, 2016). Circulating Tfh cells can be subdivided into functionally distinct subsets based on their differential expression of the chemokine receptors CXCR3 and CCR6 (Morita *et al.*, 2011; Locci *et al.*, 2013; He *et al.*, 2013; Boswell *et al.*, 2014). Intriguingly, circulating Tfh cells detected during acute *Plasmodium* malaria were polarized towards a Th1 cell phenotype (Obeng-Adjei *et al.*, 2015; Figueiredo *et al.*, 2017), expressing T-bet and CXCR3, and secreting IFN- γ following *ex vivo* stimulation (Obeng-Adjei *et al.*, 2015). These CXCR3⁺PD-1⁺ circulating Tfh cells possessed limited helper capacity to support B cell antibody production *in vitro*, and did not correlate with malaria-specific antibody responses (Obeng-Adjei *et al.*, 2015). Notably, Th1-polarized circulating Tfh cells with diminished function are implicated in humoral deficiencies associated with HIV infection and influenza immunisation (Bentebibel *et al.*, 2013; Locci *et al.*, 2013; Cubas *et al.*, 2015); therefore, it is probable that these cells cannot support optimal humoral immunity in malaria. Whether functional Tfh cell subsets are acquired as individuals develop clinical immunity remain to be investigated using longitudinal studies in different transmission settings.

Similar to humans, CD4⁺ T cells induced by acute murine infection co-express Th1 and Tfh cell features, including CXCR5, IL-21, and IFN- γ (Carpio *et al.*, 2015; Pérez-Mazliah *et al.*, 2015; Zander *et al.*, 2017). Work from our laboratory revealed that the differentiation of Tfh cells was inhibited during severe malaria infection, resulting in impaired GC development (Ryg-Cornejo *et*

et al., 2016a). Precursor Tfh cells exhibited a Th1 cell phenotype, up-regulating T-bet and CXCR3. The molecular balance between the transcription factors T-bet and Bcl-6 has been described to impart significant flexibility in Th1 and Tfh cell responses to changing environmental cues (Oestreich *et al.*, 2011; Pepper *et al.*, 2011; Nakayamada *et al.*, 2011; Oestreich *et al.*, 2012; Luethje *et al.*, 2012; Hale *et al.*, 2013; Oestreich *et al.*, 2014; Schmitt *et al.*, 2016; Shaw *et al.*, 2016), with T-bet shown to directly antagonise Bcl-6 function and allow Th1 differentiation (Oestreich *et al.*, 2011; 2012; 2014). Consistent with this concept, genetic deletion of T-bet and neutralization of the pro-inflammatory cytokines IFN- γ and TNF during malaria infection promoted Tfh cell differentiation, and restored GC formation (Ryg-Cornejo *et al.*, 2016a). Consistently, treatment of *P. yoelii* 17XNL-infected mice with exogenous IFN- γ reduces Tfh cell responses in a dose-dependent manner (Zander *et al.*, 2015), whereas deletion of CXCR3-binding CXCL10 chemokine favours the accumulation of precursor Tfh cells, leading to improved antibody responses to infection (Ioannidis *et al.*, 2016). In addition to IFN- γ , type I IFN signaling may also indirectly limit Tfh and GC B cell responses to infection, associated with reduced DC-mediated CD4⁺ T cell activation (Sebina *et al.*, 2016), and the induction of T regulatory cells that secrete IFN- γ and IL-10 (Zander *et al.*, 2016). More recent work attempt to delineate the molecular factors that fine-tune Tfh cell differentiation during infection, and implicate the damage-signal sensor P2X7 in promoting Tfh cell formation (Salles *et al.*, 2017), while IRF3 favours Th1 over Tfh cell differentiation (James *et al.*, 2018). One study utilised single-cell RNA-sequencing to identify that gene expression of transcription factors *Tcf7* and *Id2* (in addition to *Bcl6*), chemokine receptors including *Cxcr3*, and metabolic genes were associated with the bifurcation of Tfh and Th1 fates during *P. chabaudi* AS infection (Lönnerberg *et al.*, 2017). These findings strongly suggest that Tfh cell responses are modulated by the pro-inflammatory milieu of malaria infection, and the balance between Th1 versus Tfh cell commitment is likely to be a key determinant of effective humoral immunity to malaria infection.

4.5.2 Development of the GC B cell response in malaria

A few studies have begun to examine the processes that govern GC B cell responses to malaria infection, and have suggested that similar to Tfh cells, high levels of inflammatory cytokines elicited by blood-stage infection may regulate GC B cell differentiation. Although mice with non-lethal malaria appear to mount effective GC B cell responses (Achtman *et al.*, 2003; Robbiani *et al.*, 2015; Krishnamurthy *et al.*, 2016), some studies have found that enhanced Th1 pro-inflammatory responses limit the number of GC B cells induced by acute infection (Zander *et al.*, 2015; Keitany *et al.*, 2016; Ryg-Cornejo *et al.*, 2016a). In particular, neutralisation of the pro-inflammatory cytokines IFN- γ and TNF (Ryg-Cornejo *et al.*, 2016a), but not IFN- γ alone (Inoue *et al.*, 2018), improves the development of GC B cell responses following infection. Our group has further

shown that genetic ablation of T-bet, in mixed bone marrow chimeric mice, enhances the GC B cell response to *P. berghei* ANKA infection (Ryg-Cornejo *et al.*, 2016a), implicating a role of T-bet in regulating intrinsic GC B cell development. Interestingly, T-bet-deficient mice have elevated levels of IgG1 antibody that correlate with reduced *P. yoelii* 17XNL parasite burden (Oakley *et al.*, 2014). Nonetheless, how T-bet regulates a cell-intrinsic role in GC B cell differentiation during infection was not examined in these studies.

The role of T-bet in regulating B cell function has been extensively studied on its requirement for antibody class-switching to IgG2c (Peng *et al.*, 2002; Gerth *et al.*, 2003; Yoshimoto *et al.*, 2004; Rubtsova *et al.*, 2013), and the formation of IgG2c⁺ plasma cells and MBCs in mice (Mohr *et al.*, 2010; Wang *et al.*, 2012). In addition, T-bet up-regulates CXCR3 expression in B cells in response to immunisation and infection (Wang *et al.*, 2012; Serre *et al.*, 2012; Barnett *et al.*, 2016; Piovesan *et al.*, 2017), and in autoimmune conditions (Henneken *et al.*, 2005; Nicholas *et al.*, 2008; Steinmetz *et al.*, 2009; Hosomi *et al.*, 2011). Notably, T-bet is elevated in B cells (Isnardi *et al.*, 2010; Rubtsov *et al.*, 2011; 2013), characterised by a CD21^{lo/-} or CD11c⁺ phenotype (Hao *et al.*, 2011; Rubtsov *et al.*, 2011), which are associated with autoimmunity and ageing in humans (Rakhmanov *et al.*, 2009; Isnardi *et al.*, 2010; Rubtsov *et al.*, 2011; Claes *et al.*, 2016; Wang *et al.*, 2018) and mice (Rubtsov *et al.*, 2011; Koh *et al.*, 2013; Dai *et al.*, 2013; Rowland *et al.*, 2014; Jackson *et al.*, 2014; Maine *et al.*, 2015). The role of T-bet in regulating B cell responses to foreign antigen is much less understood. Barnett *et al.* proposed that T-bet may regulate other aspects of B cell function in addition to class-switching during infection based on its ability to induce a broad transcriptional program in class-switched B cells induced to chronic lymphocytic choriomeningitis virus (LCMV) (Barnett *et al.*, 2016). Yet, very limited functional data is available on how T-bet modulates GC B cell responses to pathogens. While a few studies have detected T-bet in early GC B cells induced to immunisation and infection (Wang *et al.*, 2012; Guthmiller *et al.*, 2017; Piovesan *et al.*, 2017), there is no present consensus as to whether T-bet exerts a direct impact on intrinsic GC B cell differentiation. Thus, more research is necessary to understand how T-bet modulates the dynamics and maturation of the GC B cell response for the establishment of humoral immunity to infection.

5. Thesis aims

Very little is known about the molecular mechanisms that regulate the development of the GC response in malaria. This study endeavours to investigate how the Th1 lineage-defining transcription factor, T-bet, controls the dynamics of the GC reaction to blood-stage malaria infection. This thesis is divided into three specific aims that will be addressed in the following chapters:

1. To understand the contribution of T-bet expression in CD4⁺ T cells in the control of GC development to malaria;
2. Investigate how B cell-intrinsic expression of T-bet regulates the GC B cell response to malaria infection;
3. To determine whether T-bet expression in B cells influences the generation of memory B cells and plasma cells, and the quality of antibody responses to infection.

Chapter 2

Materials and Methods

1. Media

WEHI media facility provided the following base media, which were prepared in Milli-Q H₂O and sterilised by filtration.

Dulbecco's Phosphate-Buffered Saline (DPBS)

138mM NaCl

8.1mM Na₂HPO₄

2.7mM KCl

1.5mM KH₂PO₄

pH 7.1-7.3

Roswell Park Memorial Institute (RPMI)-1640

RPMI-1640 powder

23.8mM NaHCO₃

15.4mM NaCl

1mM NaC₃H₃O₃

1x Penicillin and Streptomycin

pH 7.4

Chapter 2

RPMI-HEPES

RPMI-1640 powder

0.383 mM Hypoxanthine

25.96 mM HEPES

25 mg/L Gentamicin

pH 6.7

2. Solutions

Carbonate bicarbonate buffer

0.16M Na₂CO₃ (Merck, Darmstadt, Germany)

0.34M NaHCO₃ (ThermoFisher Scientific, MA, USA)

Deionised H₂O

pH 9.6

Fluorescence activated cell sorting (FACS) buffer

1% Foetal Bovine Serum (FBS)

2mM ethylenediaminetetra-acetic acid (Sigma-Aldrich, MO, USA)

DPBS

Foetal Bovine Serum (Sigma-Aldrich)

Heat inactivated at 56°C for 1 hour

Heparin solution

200U/ml heparin sodium injection (Pfizer, Western Australia, Australia) in RPMI-1640

Pyrimethamine and chloroquine drinking water

7 mg pyrimethamine dissolved in dimethyl sulphoxide (Sigma-Aldrich) per 100 ml H₂O

60 mg chloroquine diphosphate salt (Sigma-Aldrich) per 100 ml H₂O

pH 3.5-5.0

Red blood cell (RBC) lysis buffer

144 mM NH₄Cl (ThermoFisher Scientific)

17 mM UltraPure™ Tris (ThermoFisher Scientific)

Milli-Q H₂O

pH 7.2

3. Methods

3.1 Mice

All mice used in this study were back-crossed to a C57BL/6 background (**Table 2.1**), and maintained in specific pathogen-free conditions at WEHI. Balb/cByJ and C57BL/6J mice were bred at WEHI (Victoria, Australia). *Cxcr3*^{-/-} mice were from Jackson Laboratories (ME, USA). *Tbx21*^{fl/fl} and *Tbx21*^{fl/fl}*Cd4*^{Cre/+} (*Tbx21*^{fl/fl}*Cd4*-Cre) mice were provided by G.T. Belz (WEHI). *Tbx21*^{fl/fl}*Cd23*^{Cre/+} (*Tbx21*^{fl/fl}*Cd23*-Cre) mice were provided by K.L. Good-Jacobson (Monash University, Victoria, Australia). *Cd23*^{Cre/+} (*Cd23*-Cre) mice were provided by K.L. Good-Jacobson, A. Kallies (Peter Doherty Institute, Victoria, Australia), and S. Nutt (WEHI). All experiments were conducted in compliance with the WEHI Animal Ethics Committee requirements.

3.2 Generation of *P. berghei* ANKA stabilates

Eight to 12-week-old Balb/cByJ donor mice were inoculated intraperitoneally (i.p.) with stabilates of polyclonal *P. berghei* ANKA parasites. To generate *P. berghei* ANKA stabilates, blood was obtained from donor mice before peak parasitemia via heparinised intracardiac puncture, and used to infect separate Balb/cByJ mice with 10⁶ *P. berghei* ANKA-pRBCs i.p. The parasitemia of these mice were monitored by microscopic examination of tail vein blood smears, which were methanol-fixed and stained in Giemsa (Merck) diluted 1:10 in water. When parasitemia reached 10-15%, their blood was obtained by heparinised intracardiac puncture, diluted 1:4 in DPBS with 30% glycerol, snap frozen on dry ice, and stored at -80°C until use.

3.3 Infection of mice with *P. berghei* ANKA

The blood of Balb/cByJ donor mice infected as described above, was obtained before peak parasitemia by heparinised intracardiac or retro-orbital puncture for infection of experimental mice. Eight to 12-week-old experimental mice were infected intravenously (i.v.) with 5x10⁴ *P. berghei* ANKA in RPMI-1640. All infected experimental mice were treated with a single anti-malarial chloroquine and pyrimethamine injection i.p. (both 10 mg/kg of body weight, Sigma-Aldrich) on day 5 pi, and maintained on pyrimethamine and chloroquine drug water for 1 week until no parasites were detectable. Parasitemia was monitored by microscopic examination of Giemsa-stained blood smears.

3.4 Generation of *P. berghei* ANKA MSP-1₁₉-GST recombinant protein

The gene sequence encoding the 19-kDa fragment of MSP-1 (MSP-1₁₉) of *P. berghei* ANKA (PlasmoDB: aa glycine 1676–serine 1770) was codon-optimized for maximum expression in *Escherichia coli* (GeneArt, Germany). The synthetic gene was ligated into a pGEX-4T expression vector for expression in BL21 *E. coli* cells. Large-scale inductions were then performed in 600ml cultures (2×10^8 cells/ml) using 1mM isopropyl- β -D-thiogalactopyranoside (Sigma-Aldrich). The *P. berghei* ANKA MSP-1₁₉-GST fusion protein in the soluble supernatant fraction was purified using Glutathione Agarose beads (Sigma-Aldrich) in an Econo-column format, eluted with glutathione buffer, and collected in twelve 0.5ml fractions. The presence of the recombinant protein in each fraction was confirmed by Coomassie staining of SDS-PAGE separated proteins.

3.5 Immunofluorescence microscopy

Spleens harvested from experimental mice were snap-frozen in Tissue-Tek® Optimum Cutting Temperature compound (Sakura Finetek, Tokyo, Japan) on dry ice, and stored at -80°C until sectioning. Serial $5\mu\text{m}$ cryo-sections were made using a Microm HM525 Cryostat (ThermoFisher Scientific) and mounted on Polysine™ slides (Menzel-Gläser, ThermoFisher Scientific). Slides were fixed in ice-cold 100% acetone for 10 minutes, air-dried and stored at -80°C . Immunofluorescence staining of spleen sections was carried out in a dark, humidified box at room temperature. The sections were thawed to room temperature prior to rehydration by washing three times for 5 minutes in DPBS on a laboratory rocker. Endogenous tissue biotin was blocked using an Avidin/Biotin blocking kit (Vector Labs, CA, USA) according to manufacturer instructions, washed as before, and further blocked with DPBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich) for 45 minutes. Each section was stained with $80\mu\text{l}$ fluorescent antibodies (**Table 2.2**) diluted in DPBS 0.5% BSA. Primary antibodies were incubated for 2 hours, followed by secondary streptavidin-conjugate for 1 hour, and washed 3 times with DPBS 0.5% BSA following each incubation. Slides were mounted in Prolong Gold anti-fade reagent (Life Technologies, ThermoFisher Scientific), sealed with coverslips and stored in the dark. Confocal microscopy was performed on the Leica SP8 (Carl Zeiss, Jena, Germany) laser scanning confocal system. Images were acquired using a Plan-Apochromat 20x objective in the 488nm, 561nm and 635nm laser channels. Z-stacks of three $3.0\mu\text{m}$ sections and tile scans with 5% overlay were performed for all samples. The resulting tile images were stitched and a maximum intensity projection was applied. Quantification of stained areas in images was performed post-acquisition using Fiji (ImageJ).

3.6 Spleen and bone marrow single cell suspension preparation

The spleens and bone marrow of experimental mice were harvested at various days pi and dissociated into single cell suspensions by flushing with RPMI-1640 using a 23G needle and syringe. Spleens were further macerated through a 70µm cell strainer. Splenocytes and bone marrow were pelleted by centrifugation (350g for 5 minutes) and RBCs were lysed with RBC lysis buffer on ice for 5 minutes. After washing and resuspension in RPMI-1640, lymphocytes were counted by Trypan Blue (Sigma-Aldrich) exclusion using a haemocytometer.

3.7 Generation of bone marrow chimeras

Bone marrow was extracted from C57BL/6J (CD45.1 or CD45.2), *Cxcr3*^{-/-}, *Cd23*-Cre, *Tbx21*^{fl/fl}*Cd23*-Cre, *Tbx21*^{fl/fl} and *Tbx21*^{fl/fl}*Cd4*-Cre mice, and prepared into single cell suspensions as described above. T cells were depleted from bone marrow cells using the Dynabeads™ Mouse Pan T (Thy1.2) kit (Life Technologies, ThermoFisher Scientific) according to manufacturer's instructions. Bone marrow recipient mice, 4-7 weeks of age, were sub-lethally irradiated with 2 doses of 550 R. Each recipient mouse was injected i.v. with 4-5x10⁶ bone marrow cells in 200µL sterile DPBS. Irradiated CD45.1 mice were reconstituted with an equal ratio of bone marrow cells from CD45.1 and CD45.2 *Cxcr3*^{-/-}, C57BL/6J, *Cd23*-Cre, or *Tbx21*^{fl/fl}*Cd23*-Cre mice. Chimeras were maintained on 2mg/ml neomycin trisulfate water (Sigma-Aldrich) for 3 weeks, and infected with *P. berghei* ANKA approximately 8 weeks after reconstitution.

3.8 Flow cytometry analysis

Single cell suspensions were blocked, stained and washed in FACS buffer. To prevent non-specific binding of monoclonal antibodies to Fc receptors, cells were blocked with anti-CD16/32 antibody (FcBlock, BD Biosciences, CA, USA) for 5-10 minutes at 4°C. Cells were washed twice by centrifugation (350g for 5 minutes) and stained for surface markers with various fluorescent antibodies (**Table 2.3**) for 30-40 minutes at 4°C. Fixable Viability Dye eFluor-506 (eBioscience, CA, USA) was used to identify viable cells. Apoptotic cells were enumerated using an APC Annexin V Apoptosis Detection Kit (BioLegend, CA, USA).

For intracellular staining of immunoglobulin, cells were fixed and permeabilised after surface marker staining using the BD Cytofix/Cytoperm kit (BD Biosciences). Cells were incubated with Cytofix/Cytoperm solution for 20 minutes at 4°C, washed with PermWash buffer and stained with relevant antibodies in PermWash for 30-40 minutes at 4°C. Cells were then washed twice in PermWash buffer.

Intracellular transcription factor staining was performed after surface stains using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Cells were fixed and permeabilised with Fixation/Permeabilization solution for 20 minutes at room temperature, washed with Permeabilization buffer and stained with fluorescent antibodies for 45–60 minutes at room temperature. Cells were then washed twice in Permeabilization buffer.

To detect MSP-1₁₉-specific cells, surface-stained cells were incubated with 30nM of *P. berghei* ANKA MSP-1₁₉-GST recombinant protein for 1 hour at 4°C. Cells incubated with GST only protein was used as a control. After washing in FACS buffer, protein-bound cells were detected with an Alexa Fluor 488-conjugated polyclonal anti-GST antibody (Life Technologies, ThermoFisher Scientific) incubated for 30–40 minutes at 4°C.

Where appropriate, secondary streptavidin conjugates were incubated for 30–40 minutes at 4°C to detect staining with biotinylated antibodies. Stained cells were resuspended in FACS buffer and analysed on the BD LSRFortessa™ X-20 (BD Biosciences) fitted with Blue 488nm, Violet 405nm, Red 640nm and Yellow/Green 561nm lasers. Fluorochrome compensation was determined using BD™ CompBead Anti-rat/hamster Ig, κ/Negative control or BD™ CompBead Plus Anti-mouse Ig, κ/Negative control compensation beads (BD Biosciences) with the relevant antibodies. Data analysis was performed using FlowJo X 10.1 software (Treestar Inc., OR, USA).

3.9 BrdU incorporation assay

Mice were injected i.p. with 2.5mg of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) in DPBS and sacrificed 5 hours later. Splenocytes were stained with antibodies against GC B cell surface markers, fixed and permeabilized for detection of incorporated BrdU by flow cytometry using the FITC anti-BrdU Flow kit (BD Biosciences) according to the manufacturer's instructions.

3.10 Flow cytometry cell sorting

Splenocytes from experimental mice were stained with antibodies against CD19, CD138, CD38, GL7, and/or CD4 and CXCR3, as described in Section 3.8. In some cases, B cells were first enriched from splenocytes prior to cell staining using a negative cell isolation kit (StemCell Technologies, Victoria, Australia) following the manufacturer's instructions. GC B cells were sorted on a BD FACSAria (BD Biosciences) into DPBS with 50% FBS according to the gating strategy shown in **Figure 2.1**. The purity of sorted cells was confirmed to be >95%. Sorted cells were washed in cold DPBS and maintained on ice prior to downstream applications.

3.11 RNA-sequencing

Total RNA was purified from flow cytometry-sorted cells using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Libraries were made using a TruSeq RNA Library Preparation Kit (Illumina, CA, USA), quality checked on a bioanalyzer (Agilent Technologies, CA, USA) and sequenced on an Illumina NextSeq (81-bp paired-end reads). Libraries were mapped to the GRCm38/mm10 genome using Subread-1.5.1 (Liao *et al.*, 2013), where only uniquely mapped reads were retained. Genewise counts were obtained using featureCounts (Liao *et al.*, 2014). Genes that failed to achieve a CPM (counts per million mapped fragments) value greater than 0.5 in at least three libraries were excluded from downstream analysis. Immunoglobulin genes were also excluded from analysis. Counts were converted to log₂CPM, quantile-normalized, and precision-weighted with the voom function of the limma package (Law *et al.*, 2014; Ritchie *et al.*, 2015). Log₂CPM values were then converted to log₂FPKM (Fragments Per Kilobase per Million) values. A linear model was fitted to each gene and empirical Bayes moderated t-statistic was used to assess differences in expression. Genes were called differentially expressed if they achieved a false discovery rate (FDR) of <0.15. Gene set enrichment analysis was carried out using a GC B cell signature gene set (Gloury *et al.*, 2016) and DZ LZ signature gene set (Victora, et al 2012). The gene set enrichment plots were generated with the barcode plot function of limma. The gene enrichment p values were calculated using the roast function of limma. Gene ontology analysis of differentially expressed genes was performed using Metascape.

3.12 IghV somatic hypermutation analysis

To examine somatic hypermutation rates in the *IghV* genes of GC B cells from *Cd23-Cre* and *Tbx21^{fl/fl}Cd23-Cre* mice, replicates were merged together and mapped to the mm10 genome using Subjunc 1.6.0 (uniquely mapped fragments only). Mutations were identified from the mapped results using the exact single nucleotide polymorphism (SNP) variant caller (minimum coverage: 20; minimum alternative bases: 6; Q-value cut-off: 12). All identified mutations were assigned to exons and only mutations assigned to *IghV* genes were retained. Sequences of naive follicular B cells (Shi *et al.*, 2015) were used as controls. Mutations were identified dominant GC B cell-specific, only when their highest allele frequency in all GC B cell samples was at least 3 times higher than their frequency in the follicular B cell sequences, and if their allele frequency was lower than 3% in the follicular B cell sequences. Overall differences in frequencies of *IghV* mutation were tested with a Wilcoxon rank sum test. Differences between frequencies of individual *IghV* mutations were compared using a Fisher's exact test.

3.13 Assay for transposase-accessible chromatin (ATAC)-sequencing

ATAC-sequencing libraries were prepared as previously described (Buenrostro *et al.*, 2013). Cells isolated by flow cytometry sorting were washed with cold DPBS before treatment with cell lysis buffer containing 10mM TrisCl pH 7.4, 10mM NaCl, 3mM MgCl₂, and 0.1% IGEPAL CA-630. Nuclei were transposed in a transposition reaction mix from the Nextera DNA Library Preparation Kit (Illumina). Transposed DNA was purified using a MinElute PCR Purification Kit (Qiagen) and amplified in the presence of barcoded primers. Quality of libraries were assessed on a bioanalyzer (Agilent Technologies) and sequenced on an Illumina NextSeq 500 (75-bp paired-end reads). Libraries were mapped to the GRCm38/mm10 genome using the Subread aligner (Liao *et al.*, 2013). Only uniquely mapped reads were retained. ATAC peaks were called using the Homer program and an FDR cut-off of 1e-6 was applied for peak calling. Overlapping peaks between samples were combined to form a merged peak that covered all the original peaks. Mapped reads were then counted for these merged peaks to quantify their abundances using featureCounts (Liao *et al.*, 2014). Merged peaks were used to detect chromatin regions with changed chromatin accessibility, which were assigned to their nearest NCBI RefSeq genes for annotation. Peaks that failed to achieve a CPM value of 5 or greater in at least one library were excluded from downstream analysis. Peaks were analysed with the voom function in limma (Law *et al.*, 2014; Ritchie *et al.*, 2015). Linear models were fitted to peaks and empirical Bayes moderated t-statistic was used to assess the change of chromatin accessibility. Peaks with statistically significant changes were called using an FDR cut-off of 0.1 and a fold change cut-off of 2.

3.14 HS-Sultan cell lentivirus transfection and quantitative RT-PCR

HS-Sultan cells (ATCC® CRL1484™, American Type Culture Collection, VA, USA) were cultured in complete RPMI-HEPES (10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 4500mg/L glucose and 1500mg/L sodium bicarbonate (all from ThermoFisher Scientific)) at 37°C with 5% CO₂. Cells were transfected with mGFP-tagged human *Tbx21* LentiORF particles (OriGene Technologies, MD, USA) at a multiplicity of infection of 5 by spin infection at 37°C, for 2 hours at 1500g. Transduced clones were selected by two rounds of flow cytometry sorting of GFP⁺ cells combined with puromycin selection (2µg/mL) for 72 hours. HS-Sultan cells infected with lentivirus derived by transfection of 293T with FgH1tUTG empty vector (addgene plasmid #70183) were used as controls. Sorted GFP⁺ cells were expanded and used in experiments as described. Gene expression of transduced cells was determined by quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNeasy Plus Mini kit (Qiagen), and reverse transcribed using a SuperScript IV reverse transcriptase with Oligo(dT) primer (ThermoFisher Scientific) according to manufacturer's instructions. Quantitative RT-PCR was performed in triplicate on a ViiA 7 Real-Time PCR System (ThermoFisher

Scientific) using the following conditions: activation at 50°C for 2 minutes then 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 30 seconds. Primer sequences are listed in **Table 2.4**. *Rgs13* and *Tbx21* expression levels were determined using the Δ CT method relative to the housekeeping gene GAPDH.

3.15 Chemotaxis assay

Murine B cells were enriched from spleen cell suspensions using a negative cell isolation kit (StemCell Technologies) following the manufacturer's instructions. Cells were rested for 3 hours at 37°C prior to being plated in 5µm-pore sized Transwell inserts (2 x 10⁶ cells/well, Corning Costar, Acton, MA) in complete RPMI-1640 (5% FBS, 50µM β-mercaptoethanol (Sigma-Aldrich), 50mM L-glutamine). B cells were allowed to migrate towards recombinant mouse CXCL10 (1µg/ml), or CXCL12 (1µg/ml, all from Peprotech, Rocky Hill, NJ) or in chemokine-free media for 2 hours at 37°C with 5% CO₂. Transwells were then incubated at 4°C for 15 minutes to allow detachment of cells from the membrane. Migrated cells were collected and stained for analysis by flow cytometry. For HS-Sultan cells, a similar protocol was followed, except that cells were allowed to migrate to recombinant human CXCL12 (100ng/ml, Peprotech) in complete RPMI-HEPES for 4 hours. Migrated cells were enumerated using a haemocytometer. Chemotaxis was either expressed as the percentage of input cells that had migrated, with non-specific migration subtracted, or as a chemotaxis index, where the number of cells that migrated to chemokine was divided by the number of cells that migrated in the absence of chemokine.

3.16 Enzyme-linked immunosorbent assay (ELISA) for detection of *P. berghei* ANKA-specific antibodies

Blood obtained from experimental mice was set aside to clot at 4°C and centrifuged at 16 000g for 5 minutes to separate the serum, which was collected and stored at -20°C until analysis. Ninety-six-well u-bottom plates were coated with *P. berghei* ANKA lysate (10µg/ml) in carbonate bicarbonate buffer at 4°C overnight. Plates were washed once with DPBS and blocked with 5% skim milk in DPBS at 37°C for 1 hour. Plates were then washed 5 times with DPBS containing 0.05% Tween-20 (Sigma-Aldrich), and after each subsequent incubation. Serial 2-fold dilutions of sera (100µL/well) were incubated at room temperature for 1 hour. *P. berghei* ANKA-specific antibodies were detected by incubating with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibodies (100µL/well; **Table 2.5**) at room temperature for 2 hours. Sera and detection antibodies were diluted in DPBS with 1% skim milk and 0.01% Tween-20. Bound antibodies were revealed by reaction of peroxidase with tetramethyl benzidine (TMB) and H₂O₂ (TMB Substrate System, KPL, MD, USA), and optical density (OD) was read at 450nm on a Chameleon microplate

reader (Hidex, Turku, Finland). Antibody titres were calculated by determining the reciprocal of the lowest dilution giving a positive result. The mean plus 2 standard deviations of OD values of non-infected mouse sera was used to define a negative cut-off. To determine the avidity of antibodies, plates were coated with a gradient concentration of recombinant *P. berghei* ANKA MSP-1₁₉-GST (0.1–40 µg/ml). After blocking, sera were added at a 1:50 dilution followed by incubation with anti-mouse IgG antibody. Bound complexes were detected as described above. Antibody avidity was deduced by applying non-linear regression models to OD readings and IC₅₀ values were calculated. Best fit models were compared by extra sum-of-squares F test.

3.17 Statistics

Statistical analysis was performed using a student's two-tailed t-test in Prism 8.0 (GraphPad Software, Inc., CA, USA) unless otherwise indicated in figure legends. Results were deemed significant when $p < 0.05$.

Table 2.1 Mouse strains

Mouse Strain	Description	Reference
Balb/c ByJ	Wild-type	–
C57BL/6J (CD45.1 and CD45.2)	Wild-type	–
<i>Cd23</i> ^{Cre/+}	Cre recombinase is expressed under the control of the <i>Cd23</i> promoter; used as control mice	(Kwon <i>et al.</i> , 2008)
<i>Cxcr3</i> ^{-/-}	Mice do not express the chemokine receptor CXCR3	Deltagen, Inc.
<i>Tbx21</i> ^{fl/fl}	<i>loxP</i> sites flank exons of the <i>Tbx21</i> gene that encodes T-bet; used as control mice	(Intlekofer <i>et al.</i> , 2008; Wang <i>et al.</i> , 2012)
<i>Tbx21</i> ^{fl/fl} <i>Cd23</i> ^{Cre/+}	<i>Tbx21</i> ^{fl/fl} mice were crossed with <i>Cd23</i> ^{Cre/+} mice to conditionally delete <i>Tbx21</i> in mature B cells	Piovesan <i>et al.</i> , 2017
<i>Tbx21</i> ^{fl/fl} <i>Cd4</i> ^{Cre/+}	<i>Tbx21</i> ^{fl/fl} mice were crossed with <i>Cd4</i> ^{Cre/+} mice to conditionally delete <i>Tbx21</i> in CD4 ⁺ cells	(Kao <i>et al.</i> , 2011)

Table 2.2 Antibodies used for immunofluorescence microscopy

	Antibody	Clone	Conjugate	Source
Primary	CD3ε	145-2C11	Alexa Fluor 647	BioLegend
	GL7	GL7	Biotin	eBioscience
	IgD	11-26c.2a	Alexa Fluor 488	BioLegend
Secondary	Streptavidin	-	Alexa Fluor 568	ThermoFisher Scientific

Table 2.3 Fluorochrome-conjugated antibodies used for flow cytometry

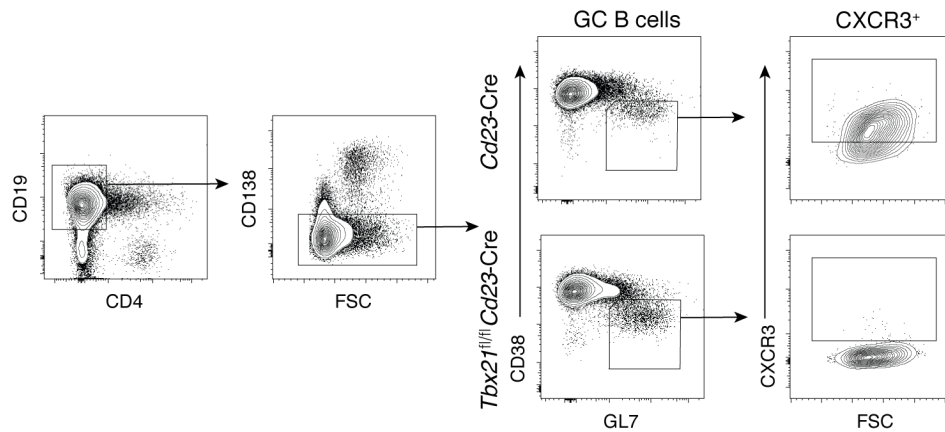
Antigen	Clone	Conjugate	Source
Bcl-6	K112-91	Alexa Fluor 647	BD Biosciences
CCR6 (CD196)	140706	Brilliant Violet 421	BD Biosciences
CD138	281-2	Brilliant Violet 421 PE	BioLegend BD Biosciences
CD19	6D5 1D3	FITC PerCP-Cy5.5 Brilliant Violet 605 Brilliant Violet 650 PE PE-Cy7	BioLegend BD Biosciences BioLegend BioLegend BD Biosciences BD Biosciences
CD38	90 NIMR-5	FITC PE-Cy7 Alexa Fluor 680	BD Biosciences BioLegend WEHI Antibody facility
CD4	RM4-5	FITC Alexa Fluor 647 PE-Cy7	BD Biosciences BioLegend BioLegend
CD45.1	A20	Alexa Fluor 647 Brilliant Violet 785 PE	BioLegend BioLegend eBiosciences
CD45.2	104	FITC	BioLegend
CD62L	MEL-14	APC-Cy7	BioLegend
CD86	GL-1	Brilliant Violet 650 PE-Cy7	BioLegend BioLegend
CXCR3 (CD183)	CXCR3-173	Brilliant Violet 421	BioLegend
CXCR4 (CD184)	2B11	PerCP-eFluor 710	eBioscience
CXCR5	2G8	PerCP-Cy5.5 Biotin	BD Biosciences BD Biosciences
Fas (CD95)	Jo2	PE-Cy7	BD Biosciences
IgD	11-26c.2a	Biotin FITC Brilliant Violet 711	eBioscience BioLegend BD Biosciences
IgG1	A85-1 X56	Brilliant Violet 421 APC	BD Biosciences BD Biosciences
IgG2c	5.7	Biotin	BD Biosciences
IgM	II/41 R6-60.2	FITC Brilliant Violet 711	BD Biosciences BD Biosciences
Ki67	SolA15	PE-eFluor 610	eBioscience
PD-1	J43	PE	BD Biosciences
T and B cell Activation Antigen (GL7)	GL7	Alexa Fluor 647 PE	BD Biosciences BD Biosciences
T-bet	4B10	PE-Cy7	BioLegend
Streptavidin	-	Brilliant Violet 605 PE-Dazzle594	BioLegend BioLegend

Table 2.4 Primers used for quantitative RT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>Gapdh</i>	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
<i>Rgs13</i>	AATTGCCTCACGGTGGAGCAGA	CCTGATGATAGTCTCTCTTGTCTG
<i>Tbx21</i>	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG

Table 2.5 Antibodies used for ELISA

Antibody	Conjugate	Source
Rabbit anti-mouse IgG	HRP	ThermoFisher Scientific
Rabbit anti-mouse IgG1	HRP	
Goat anti-mouse IgG2c	HRP	SouthernBiotech, AL, USA

**Figure 2.1 Gating strategy for flow cytometry sorting of GC B cells**

Splenocytes from *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice infected with *P. berghei* ANKA and treated with anti-malarial drugs on day 5 pi were sorted for GC B cells by flow cytometry. Representative gating strategy showing lymphocytes excluded for doublets and dead cells, gated for CD19⁺CD4⁺ B cells, and then CD138⁻ non-plasmablasts. Total CD38^{lo}GL7⁺ GC B cells were isolated for RNA-sequencing. CXCR3⁺ GC B cells of Cd23-Cre mice were further sorted for ATAC-sequencing.

Chapter 3

The role of T-bet in the control of Tfh cell differentiation and GC development in malaria

1. Introduction

The success of T cell-dependent humoral immunity depends on an effective GC reaction, during which MBCs and plasma cells are generated. The formation and maintenance of GCs is orchestrated by Tfh cells that provide crucial survival and differentiation signals to GC B cells (Vinuesa *et al.*, 2016). Tfh cell responses have not been extensively studied in malaria, and their role in governing B cell responses to infection is only beginning to be appreciated (Butler *et al.*, 2011; Opata *et al.*, 2015; Pérez-Mazliah *et al.*, 2015; Zander *et al.*, 2015; Wikenheiser *et al.*, 2015; Pérez-Mazliah *et al.*, 2017). Circulating Tfh cells characterised in humans with acute malaria have been revealed to be polarized towards a Th1 cell phenotype (Obeng-Adjei *et al.*, 2015; Figueiredo *et al.*, 2017), expressing the transcription factor T-bet and secreting IFN- γ *ex vivo* (Obeng-Adjei *et al.*, 2015). Importantly, the Th1-like circulating Tfh cells display limited helper capacity to support B cell responses *in vitro* (Obeng-Adjei *et al.*, 2015), suggesting that malaria infection may interfere with the function of Tfh cells to support effective humoral immunity.

Studies undertaken in murine models of malaria have provided more mechanistic insight into the generation of Tfh cell responses to infection. Similar to human malaria, CD4⁺ T cells induced by murine infection have also been described to express dual features of Th1 and Tfh cells, including expression of the chemokine receptor CXCR5, and cytokines IL-21 and IFN- γ (Carpio *et al.*, 2015; Pérez-Mazliah *et al.*, 2015; Zander *et al.*, 2017). Our group further discovered that Tfh cell

differentiation was suppressed during *P. berghei* ANKA severe malaria, and contributed to an impaired development of GCs to infection (Ryg-Cornejo *et al.*, 2016a). Precursor Tfh cells co-expressed Th1-associated molecules, including T-bet and CXCR3, despite normal expression of the Tfh lineage-defining transcription factor, Bcl-6. Furthermore, neutralization of the highly produced pro-inflammatory cytokines IFN- γ and TNF, promoted Tfh cell differentiation and restored GC responses (Ryg-Cornejo *et al.*, 2016a). Conversely, exogenous IFN- γ has been shown to directly limit Tfh cell responses (Zander *et al.*, 2015). These findings collectively support that Th1 inflammatory conditions associated with acute malaria can inhibit the development of Tfh cells, and thus, lead to suboptimal GC responses to infection.

A growing number of studies indicate that the environmental milieu regulates flexibility between Th1 and Tfh cell differentiation via dynamic expression of the transcriptional regulators, T-bet and Bcl-6, respectively (Oestreich *et al.*, 2011; Pepper *et al.*, 2011; Nakayamada *et al.*, 2011; Oestreich *et al.*, 2012; Luethje *et al.*, 2012; Hale *et al.*, 2013; Oestreich *et al.*, 2014; Schmitt *et al.*, 2016; Shaw *et al.*, 2016). We previously examined whether T-bet influenced the differentiation of Tfh cells during *P. berghei* ANKA infection, and found that global deletion of T-bet in mixed bone marrow chimeras favoured Tfh cell generation, which associated with enhanced GC development (Ryg-Cornejo *et al.*, 2016a). While this suggested that T-bet negatively regulated Tfh cell differentiation during infection, a cell-intrinsic role of T-bet in modulating Th1 versus Tfh cell development within the CD4⁺ T cell compartment has not been demonstrated. In this chapter, we investigated the CD4⁺ T cell-intrinsic role of T-bet on the differentiation of Tfh cells and GC development during infection of mice with *P. berghei* ANKA.

2. Results

2.1 CD4⁺ T cell-intrinsic expression of T-bet impairs Tfh cell differentiation to infection

To investigate the role of T-bet in regulating Tfh cell differentiation in response to severe malaria infection, we first assessed T-bet expression in Tfh cells over the course of *P. berghei* ANKA infection. C57BL/6 mice infected with *P. berghei* ANKA typically develop a parasitemia of 5-10%, and potent pro-inflammatory responses associated with disease within the first 6 days of infection (Ryg-Cornejo *et al.*, 2016a). As this model of malaria induces lethal disease in mice of the C57BL/6 background, infected mice were treated with anti-malarial drugs at the onset of disease on day 5 post-infection (pi) to assess responses at later time points. Parasites are rapidly cleared from the blood-stream of infected mice from day 6 onwards (not shown). Splenic Tfh cells, identified by flow cytometry as activated CD62L⁻CD4⁺ T cells expressing high levels of CXCR5 and PD-1, up-regulated T-bet by day 6 pi, coinciding with the peak of infection and onset of disease (**Figure 3.1a**). T-bet expression was maintained at high levels up to day 12 pi, and then declined to slightly lower levels by day 24 pi upon clearance of parasites. Thus, T-bet is expressed by Tfh cells early after *P. berghei* ANKA infection and is sustained at lower levels at later time points after anti-malarial treatment.

To address the precise role of T-bet expression by CD4⁺ T cells in modulating Tfh cell differentiation in response to acute malaria infection, we utilised *Tbx21^{fl/fl}Cd4-Cre* mice, which lack T-bet specifically in CD4⁺ T cells (Kao *et al.*, 2011). *Tbx21^{fl/fl}Cd4-Cre* and *Tbx21^{fl/fl}* control mice were infected with *P. berghei* ANKA pRBCs, and Tfh cells in their spleens were enumerated at various times pi by flow cytometry (**Figure 3.1b**). The absence of T-bet expression in CD4⁺ T cells increased the development of CXCR5^{hi}PD-1^{hi} Tfh cells within the activated CD62L⁻CD4⁺ T cell population, with the number of Tfh cells significantly higher in *Tbx21^{fl/fl}Cd4-Cre* mice compared to controls on day 12 pi (**Figure 3.1c,d**). The number of Tfh cells in both groups of mice peaked one week after treatment, but with a largely diminished magnitude in control mice, consistent with previous observations (Ryg-Cornejo *et al.*, 2016a). Similarly, analysis of Tfh cells expressing the Tfh lineage-defining transcription factor Bcl-6, showed a significantly higher number of CXCR5⁺Bcl-6⁺ cells in *Tbx21^{fl/fl}Cd4-Cre* mice compared to controls on day 12 pi (**Figure 3.2**). Collectively, these findings support that CD4⁺ T cell-intrinsic expression of T-bet expression negatively regulates Tfh cell differentiation after acute malaria infection.

2.2 *T-bet expression in CD4⁺ T cells limits the GC B cell response to malaria*

To assess the functional consequences of the enhanced differentiation of Tfh cells observed in the absence of T-bet in CD4⁺ T cells, the induction of GCs in *Tbx21^{fl/fl} Cd4-Cre* and control mice was examined at various time points. Spleen sections of *P. berghei* ANKA-infected mice were stained with anti-IgD to identify B cell follicles, anti-CD3ε to detect T cell zones, and GCs were revealed by staining for GL7 (**Figure 3.3**). Immunofluorescence analysis by confocal microscopy showed that on day 6 pi, the limits of the T and B cell zones were poorly segregated, and GL7⁺ cells were dispersed throughout the red pulp in both groups of mice (**Figure 3.3a**). By day 9 pi, well-defined GC structures were apparent in *Tbx21^{fl/fl} Cd4-Cre* mice but not in control mice. *Tbx21^{fl/fl} Cd4-Cre* mice displayed efficient GC formation compared to wild-type mice from day 9–24 pi, and GCs were significantly larger in size (**Figure 3.3b,c**). In agreement with these results, the frequency and number of CD38^{lo}GL7⁺ GC B cells, as enumerated by flow cytometry, showed a 3-fold increase in the spleens of *Tbx21^{fl/fl} Cd4-Cre* mice compared to controls from day 9 with the clearance of infection with anti-malarial treatment (**Figure 3.4**).

One of the key help functions of Tfh cells is to support B cell proliferation (Crotty, 2015); thus, we quantified GC B cell proliferation as a measure of T cell help during acute infection. *Tbx21^{fl/fl} Cd4-Cre* and *Tbx21^{fl/fl}* control mice were infected with *P. berghei* ANKA, and treated with the nucleoside analog BrdU for 5 hours to label proliferating GC B cells *in vivo* before harvesting their spleens for flow cytometry analysis. T-bet-deficient Tfh cells in *Tbx21^{fl/fl} Cd4-Cre* mice supported significantly higher levels of GC B cell proliferation compared to controls (**Figure 3.5**). An increased number of proliferating BrdU⁺ GC B cells were detected in the absence of CD4⁺ T cell-specific T-bet expression on day 9–12 pi, with 30–60% of GC B cells proliferating at each time point analysed. This indicates that T-bet expression in CD4⁺ T cells inhibits the capacity of Tfh cells to support the proliferation and expansion of GC B cells, thereby significantly modulating the magnitude of the GC response.

2.3 *CD4⁺ T cell-intrinsic expression of T-bet restrains the induction of MBCs and plasma cells to infection*

Our findings show that T-bet expression intrinsically in CD4⁺ T cells limits the differentiation of Tfh cells, thereby restricting the GC response to malaria infection. We thus postulated that the lack of T-bet up-regulation in CD4⁺ T cells would promote the generation of B cell memory populations. Analysis of the CD138⁺ antibody-secreting cell (ASC) response by flow cytometry revealed a significantly higher frequency and number of ASCs in the spleens of *Tbx21^{fl/fl} Cd4-Cre* mice compared to control mice on day 9 (**Figure 3.6a-c**). We also characterised the induction of

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ASCs expressing intracellular IgG2c and IgG1, antibody subclasses which have been shown to effectively control parasites during infection (Waki *et al.*, 1995; Oakley *et al.*, 2014). A significantly increased production of IgG1⁺ and IgG2c⁺ ASCs was observed in *Tbx21^{fl/fl}Cd4-Cre* mice compared to controls from day 9 to 12 pi (**Figure 3.6d,e**). Notably, the number and frequency of IgG1⁺ ASCs remained significantly higher in *Tbx21^{fl/fl}Cd4-cre* mice by day 24 pi, despite the clearance of infection at this time point.

Furthermore, we analysed the development of IgG MBCs in *Tbx21^{fl/fl}Cd4-Cre* mice by flow cytometry (**Figure 3.7a**), and found that significantly more MBCs expressing IgG1 were generated in the absence of T-bet expression in CD4⁺ T cells ($p < 0.05$). IgG1⁺ MBCs were present in a greater abundance than IgG2c⁺ MBCs in the *Tbx21^{fl/fl}Cd4-Cre* mice, whereas control mice had comparable numbers of both subclasses (**Figure 3.7b**). This contributed to an overall larger MBC pool in the *Tbx21^{fl/fl}Cd4-Cre* mice, particularly on day 24, compared to controls. These results support the idea that the enhanced development of Tfh cells in the absence of T-bet-expressing CD4⁺ T cells promotes efficient B cell differentiation into MBCs and plasma cells.

3. Discussion

Dynamic interplay between the lineage-defining transcription factors, T-bet and Bcl-6, plays a key role in regulating Tfh cell differentiation in response to environmental cytokine signals (Oestreich *et al.*, 2011; Pepper *et al.*, 2011; Nakayamada *et al.*, 2011; Oestreich *et al.*, 2012; Luethje *et al.*, 2012; Hale *et al.*, 2013; Oestreich *et al.*, 2014; Schmitt *et al.*, 2016; Shaw *et al.*, 2016). In line with Th1-polarized Tfh cells characterized in malaria-infected individuals (Obeng-Adjei *et al.*, 2015; Figueiredo *et al.*, 2017) and mice (Ryg-Cornejo *et al.*, 2016a), Tfh cells induced by *P. berghei* ANKA acute malaria rapidly up-regulated T-bet protein expression. Genetic deletion of T-bet in the CD4⁺ T cells allowed effective differentiation of mature Tfh cells, demonstrating that CD4⁺ T cell-specific expression of T-bet directly inhibits Tfh cell generation. This confirms and extends previous work from our laboratory showing that Th1 pro-inflammatory processes of malaria impede the magnitude of the Tfh cell response to infection (Ryg-Cornejo *et al.*, 2016a). Furthermore, this data provides support for an antagonistic role of T-bet on Bcl-6-driven Tfh cell differentiation during highly pro-inflammatory conditions *in vivo*, as described in LCMV and *Toxoplasma gondii* infection (Marshall *et al.*, 2011; Nakayamada *et al.*, 2011; Fang *et al.*, 2018). While a few studies have begun to delineate the molecular factors that regulate the balance between Th1 versus Tfh differentiation (Lönnberg *et al.*, 2017; Salles *et al.*, 2017; James *et al.*, 2018), more mechanistic studies are needed to delineate how pro-inflammatory signals are integrated to regulate the opposing activities of T-bet and Bcl-6. In addition to T-bet, it is possible that additional transcriptional regulators, such as Blimp-1, Foxp1, Foxo1 and KLF2, negatively modulate Tfh cell responses during infection. In particular, the IL-2-Blimp-1 signaling axis has been described as a prominent repressor of Tfh cell differentiation (Johnston *et al.*, 2012; Nurieva *et al.*, 2012; Oestreich *et al.*, 2012; Ballesteros-Tato *et al.*, 2012; Ray *et al.*, 2015). However, whether it plays a role in regulating the Tfh cell program to malaria infection has not been assessed.

An enhanced number of Tfh cells following acute *P. berghei* ANKA infection in the absence of CD4⁺ T cell-intrinsic T-bet expression boosted GC B cell development, consistent with a crucial role of Tfh cells in orchestrating the GC reaction (Victora *et al.*, 2010; Gitlin *et al.*, 2014; 2015). The effect on the GC B cell response was evident by day 9 pi, which correlated with an increased proliferation of GC B cells. This agrees with the known role of T cell help in regulating B cell clonal expansion within the GC (Gitlin *et al.*, 2014; 2015). In contrast, a lack of organised GC development was apparent in the presence of T-bet expression, in line with previous histological observations during acute malaria infection (Carvalho *et al.*, 2007; Cadman *et al.*, 2008; Ryg-Cornejo *et al.*, 2016a). Although we did not examine GC selection *per se*, the robust GC B cell response in the absence of CD4⁺ T cell-specific T-bet fuelled the export of ASC and MBC populations. Of note, *Tbx21^{fl/fl} Cd4-Cre* mice had significantly more IgG1⁺ ASCs and MBCs compared to controls by day 24, suggesting an enhanced induction of humoral memory. ASCs

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were also more abundant in *Tbx21^{fl/fl}Cd4-Cre* compared to control mice early after infection, which suggests that these ASCs may be derived from short-lived extrafollicular responses, and agree with previous work from our group showing that in malaria, Tfh cells in the spleen support the production of plasmablasts (Ioannidis *et al.*, 2016). A transient induction of ASCs has also been detected in the circulation of children at the time of acute malaria (Sullivan *et al.*, 2016). Thus, it will be interesting to examine the contribution of this increased number of ASCs in the early control of parasitemia.

Several studies indicate that Tfh cells maintain plasticity in their ability to secrete multiple cytokines including IL-21, IL-4 and IFN- γ (Johnston *et al.*, 2009; Glatman Zaretsky *et al.*, 2009; Yusuf *et al.*, 2010; Fahey *et al.*, 2011; Liang *et al.*, 2012; Luethje *et al.*, 2012; Ray *et al.*, 2014; Weinstein *et al.*, 2016; Miyauchi *et al.*, 2016; Weinstein *et al.*, 2017; Fang *et al.*, 2018), for the adaptive regulation of humoral immune responses to the pathogenic insult (King and Mohrs, 2009; Reinhardt *et al.*, 2009; Yusuf *et al.*, 2010; Perez-Shibayama *et al.*, 2014; Weinstein *et al.*, 2017; Fang *et al.*, 2018). IgG2c⁺ MBC formation largely depends on T cell-derived IFN- γ cytokine (Mohr *et al.*, 2010; Wang *et al.*, 2012), the production of which requires T-bet expression in Tfh cells (Weinstein *et al.*, 2017; Fang *et al.*, 2018). Consistent with this, MBCs of mice with T-bet-deficient Tfh cells were mostly of the IgG1 subclass. On the other hand, IgG1 class-switching is predominantly regulated by IL-4 (Snapper and Paul, 1987), and IL-4-deficient mice have impaired IgG1 responses to *P. chabaudi* AS infection (Weid *et al.*, 1994). Further analysis of the cytokine expression profiles of T-bet-deficient Tfh cells induced in response to acute malaria infection may provide greater insight into the extent of T-bet's impact on Tfh cell function and their regulation of B cell responses.

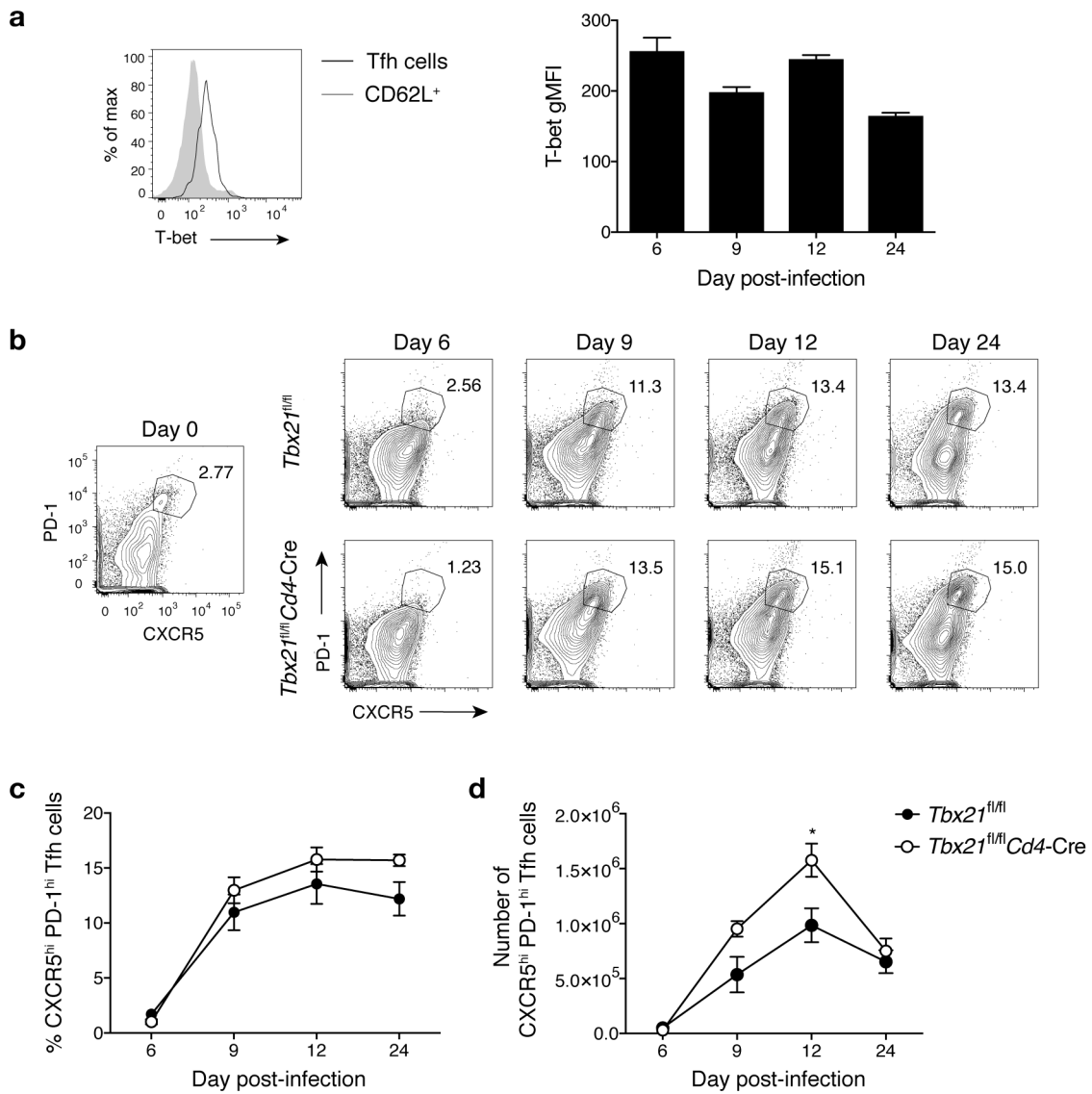


Figure 3.1 CD4⁺ T cell-intrinsic up-regulation of T-bet impairs Tfh cell responses following *P. berghei* ANKA infection

Mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs from day 5 post-infection (pi). **(a)** Representative histogram and T-bet gMFI of CXCR5^{hi}PD-1^{hi} Tfh cells among activated CD4⁺CD19⁺CD62L⁺ cells in wild-type mice was determined at different times pi. CD62L⁺ cells are shown as a naive control. **(b)** Representative flow cytometry plots showing CXCR5^{hi}PD-1^{hi} Tfh cells at different times pi in *P. berghei* ANKA-infected *Tbx21^{fl/fl}Cd4-Cre* and *Tbx21^{fl/fl}* mice. The **(c)** percentage and **(d)** absolute number of Tfh cells were enumerated. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM, * $p < 0.05$.

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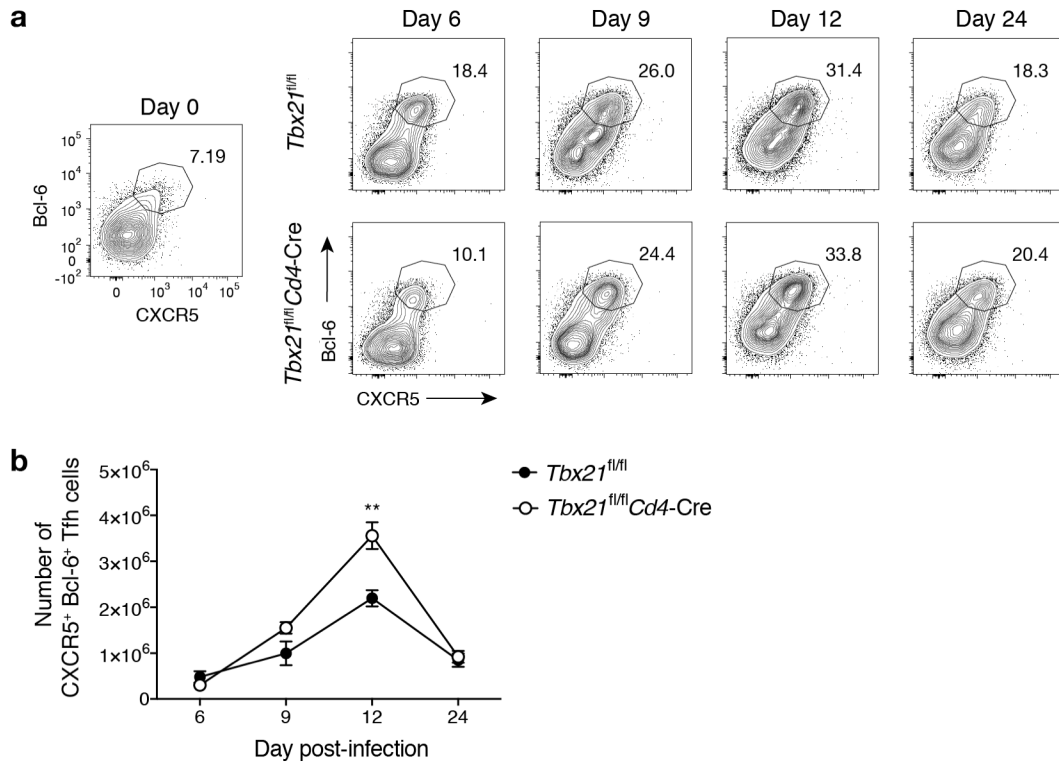
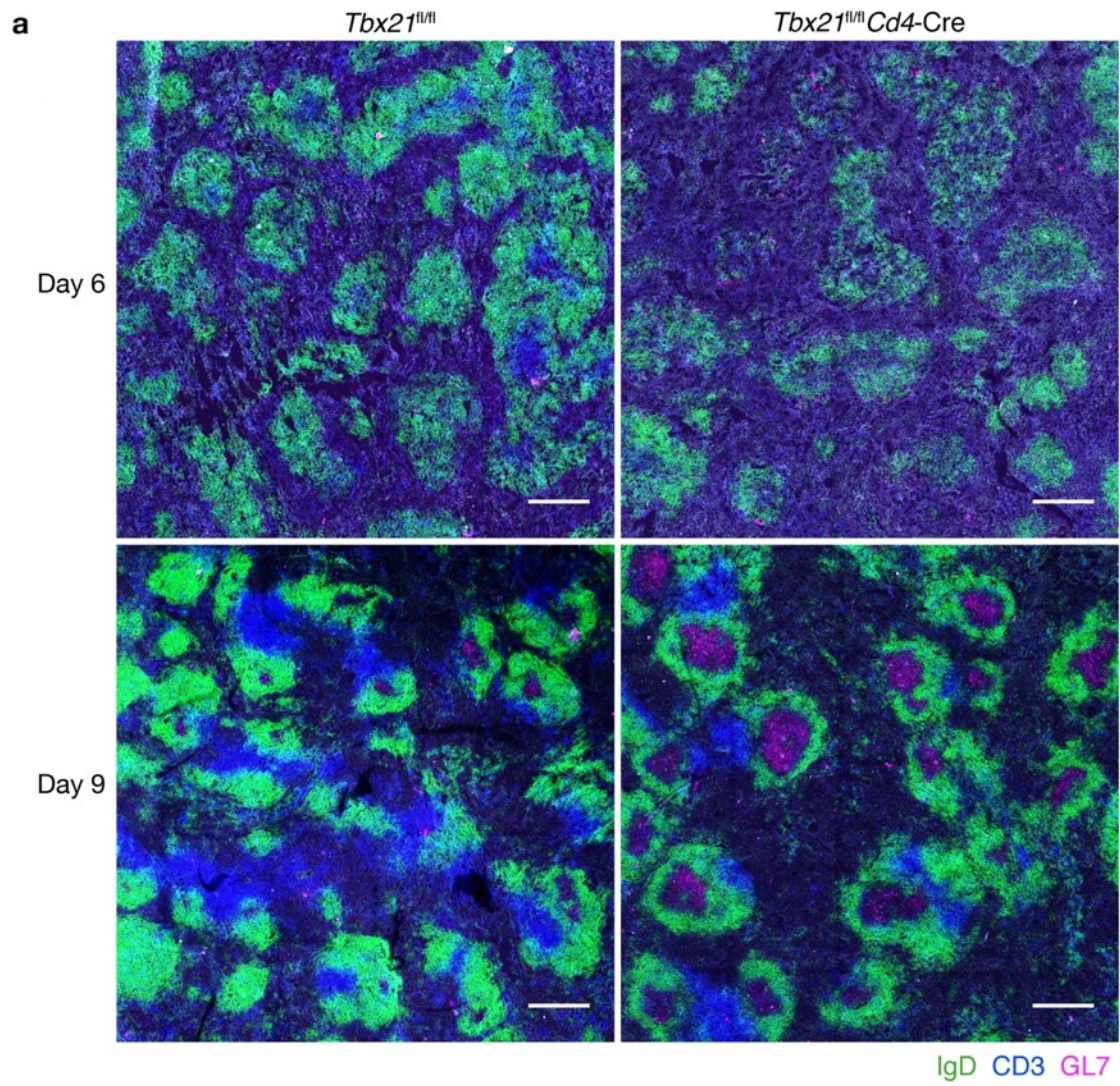


Figure 3.2 CD4⁺ T cell-intrinsic expression of T-bet impairs the differentiation of Tfh cells

(a) Representative flow cytometry plots showing CXCR5⁺Bcl-6⁺ Tfh cells among CD4⁺CD19⁻CD62L⁻ cells of *Tbx21^{fl/fl}Cd4-Cre* and *Tbx21^{fl/fl}* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. (b) The absolute number of Tfh cells were enumerated. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM, ** $p < 0.05$.



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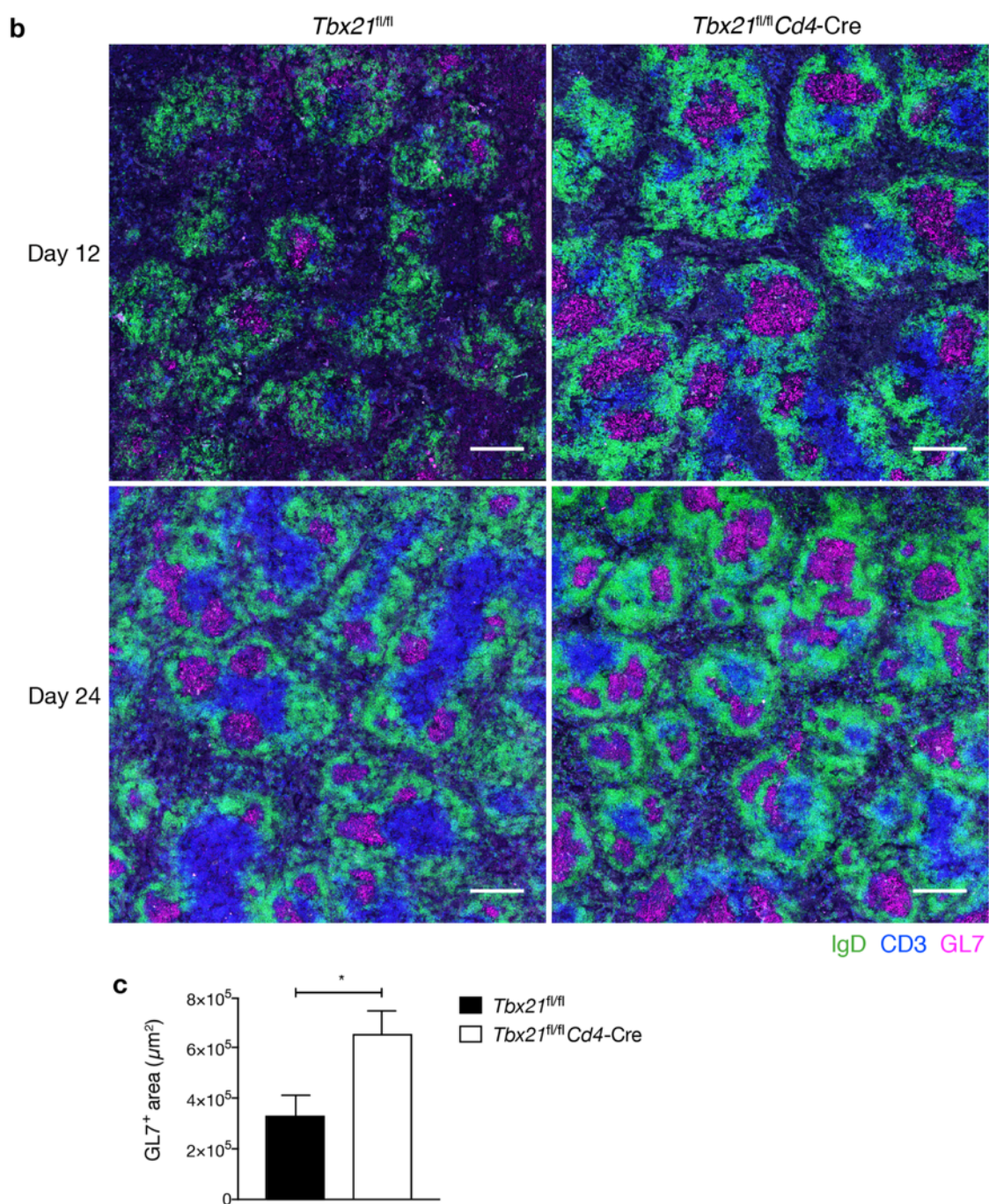


Figure 3.3 GC development is impaired in the presence of CD4⁺ T cell-specific T-bet expression

Tbx21^{fl/fl} Cd4-Cre and *Tbx21^{fl/fl}* mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Immunofluorescence analysis of spleen sections on (a) day 6 and 9, and (b) day 12 and 24 pi. B cell follicles were identified by anti-IgD (green), T cell zones with anti-CD3 (blue), and germinal centres with anti-GL7 (magenta). Images are representative of 4 mice per group. Scale bar, 300 μm . (c) Quantification of total GL7⁺ area on day 24 pi. Bars represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$.

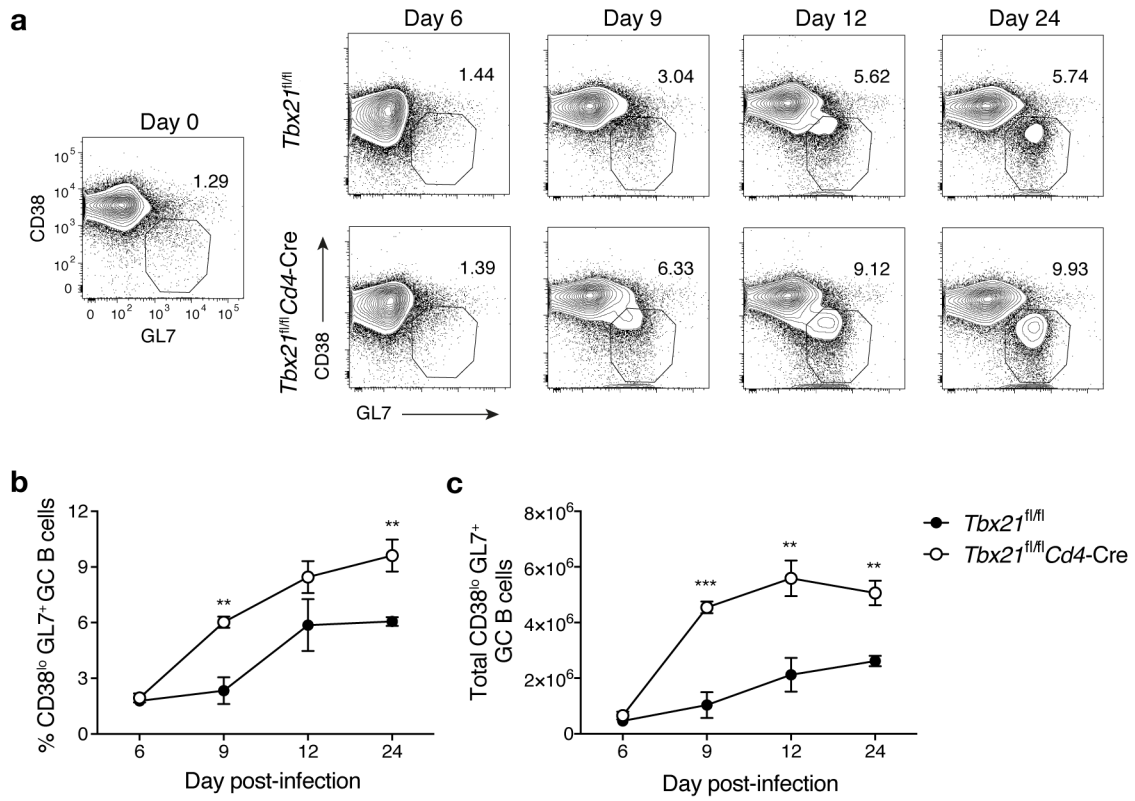


Figure 3.4 CD4⁺ T cell-intrinsic expression of T-bet impairs the magnitude of the GC B cell response to infection

(a) Representative flow cytometry plots showing CD38^{lo}GL7⁺ GC B cells among CD19⁺ cells of *Tbx21^{fl/fl}*Cd4-Cre and *Tbx21^{fl/fl}* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The percentage (b) and absolute number (c) of GC B cells were enumerated. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM, **p<0.01, ***p<0.001.

Results

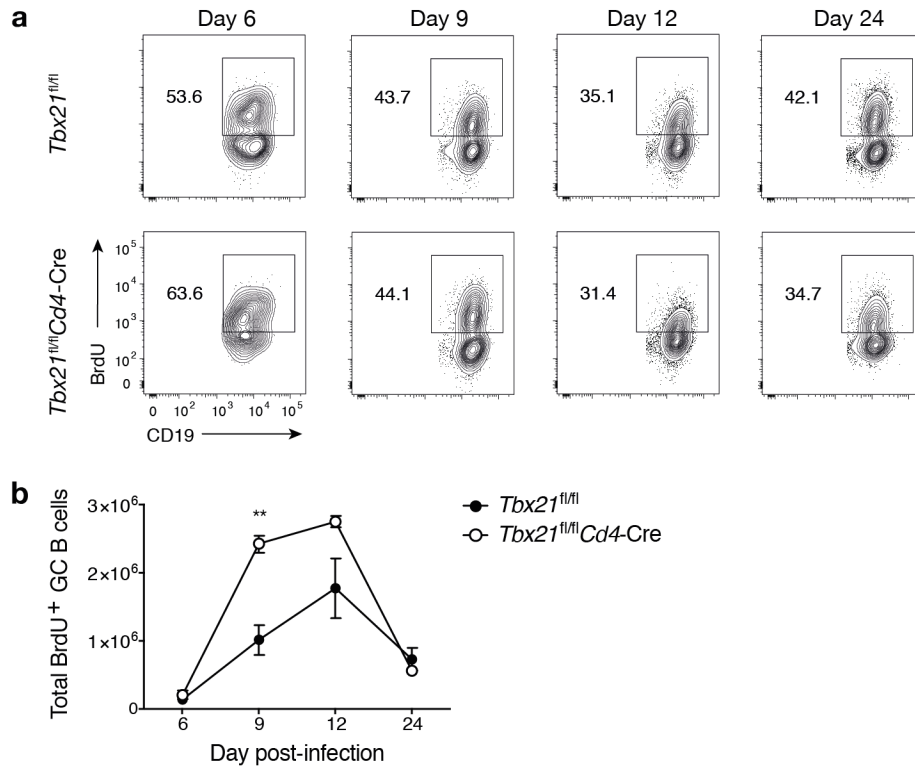


Figure 3.5 Enhanced GC B cell proliferation in the absence of T-bet expression in CD4⁺ T cells

Tbx21^{fl/fl}Cd4-Cre and *Tbx21^{fl/fl}* mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Mice were administered BrdU on various days and analyzed 5 hours later. **(a)** Flow cytometry plots showing the percentage of BrdU⁺ cells among CD38^{lo}GL7⁺ GC B cells at different times pi. **(b)** The absolute number of BrdU⁺ GC B cells were enumerated. Data represent the mean of 3-4 mice ± SEM, **p<0.01.

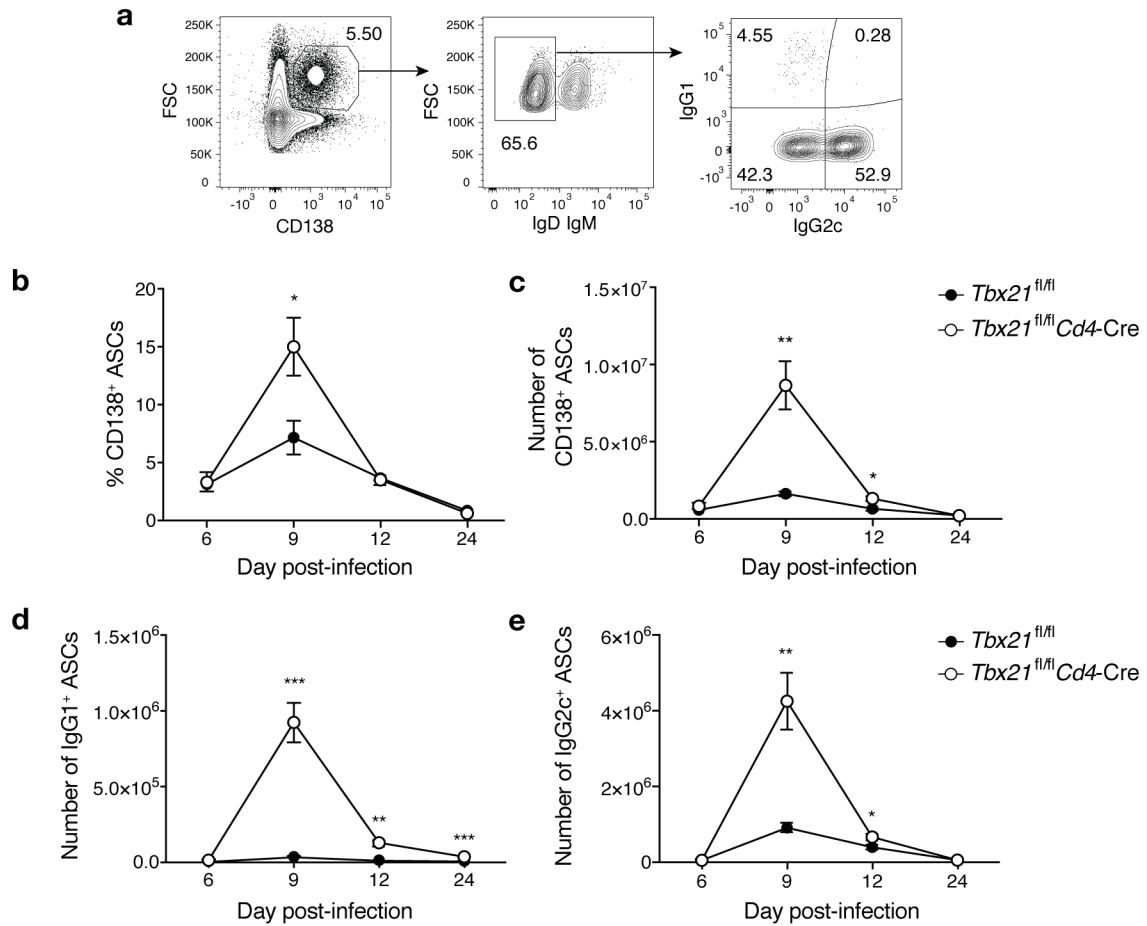


Figure 3.6 Enhanced ASC response in the absence of CD4⁺ T cell-intrinsic T-bet expression

(a) Gating strategy used to identify CD138⁺ ASCs expressing intracellular IgG1⁺ and IgG2c⁺ among class-switched (IgD⁺IgM⁺) B cells. The percentage and absolute number of (b,c) CD138⁺, (d) IgG1⁺ and (e) IgG2c⁺ ASCs were enumerated in *Tbx21*^{fl/fl} Cd4-Cre and *Tbx21*^{fl/fl} mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001.

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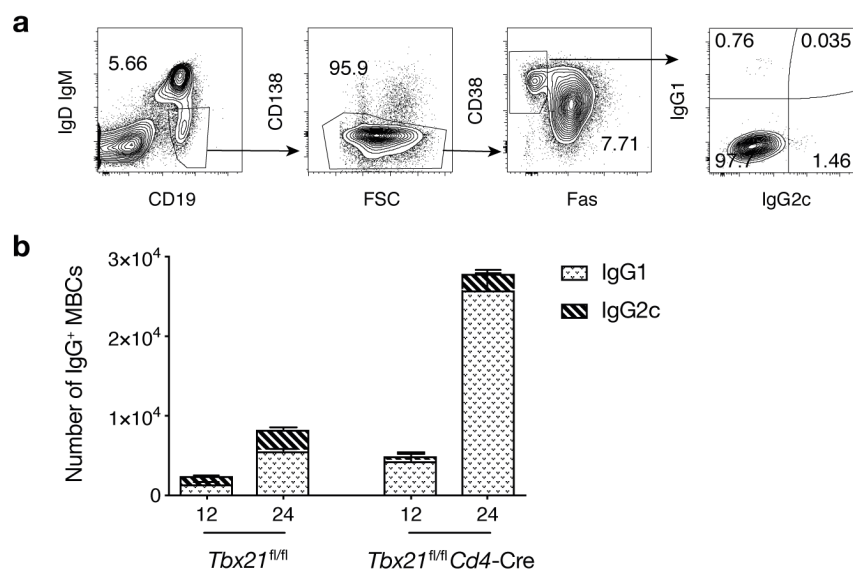


Figure 3.7 Memory B cell responses in the absence of CD4⁺ T cell-intrinsic T-bet expression

(a) Gating strategy used to identify CD38^{hi} MBCs expressing surface IgG1⁺ and IgG2c⁺ among isotype-switched (IgD⁻IgM⁻) CD19⁺ B cells, excluded for ASCs (CD138⁻). The absolute number of (b) IgG1⁺ and IgG2c⁺ MBCs were enumerated in *Tbx21^{fl/fl}Cd4-Cre* and *Tbx21^{fl/fl}* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM.

Chapter 4

The role of T-bet expression in B cells in the control of the GC B cell response in malaria

1. Introduction

Within GCs, B cells undergo iterative cycles of diversification and selection to develop into memory populations. They compete for an array of survival and differentiation cues delivered in an affinity-dependent manner by Tfh cells and FDCs in the LZ, enabling higher-affinity B cell clones to outcompete lower-affinity clones for selected re-circulation to the DZ for further rounds of SHM and clonal expansion (Allen *et al.*, 2007; Schwickert *et al.*, 2007; Victora *et al.*, 2010; Gitlin *et al.*, 2014). Many transcription factors, such as Bcl-6, c-Myc, Foxo1 and AP4 have been identified to control the expression of molecules important for the differentiation and dynamic movement of B cells during GC initiation and maturation (Basso and Dalla-Favera, 2012; Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012; Sander *et al.*, 2015; Dominguez-Sola *et al.*, 2015; Chou *et al.*, 2016; Luo *et al.*, 2018).

The previous chapter demonstrated that the transcription factor T-bet limits the magnitude of the GC response to *P. berghei* ANKA infection by inhibiting Tfh cell differentiation in a CD4⁺ T cell-intrinsic fashion. In addition to T cells, a growing body of studies identify that T-bet is also up-regulated in B cells in a diverse range of disease settings, including cancer, autoimmune disorders and infection (Karnell *et al.*, 2017). Notably, T-bet is expressed in age-associated B cells (Isnardi *et al.*, 2010; Rubtsov *et al.*, 2011; 2013; Wang *et al.*, 2018) that are associated with the development of autoreactive antibody responses in human and murine systemic lupus erythematosus (SLE)

(Wang *et al.*, 2018). Conversely, our knowledge regarding the role of T-bet in shaping B cell differentiation to foreign antigen is very limited beyond its requirement for IgG class-switching (Mohr *et al.*, 2010; Wang *et al.*, 2012; Rubtsova *et al.*, 2013; Knox *et al.*, 2017), and is only beginning to be delineated (Barnett *et al.*, 2016; Rubtsova *et al.*, 2017; Piovesan *et al.*, 2017).

In malaria, T-bet has been detected in atypical MBCs (Obeng-Adjei *et al.*, 2017) that display reduced effector function *in vitro* (Portugal *et al.*, 2015; Sullivan *et al.*, 2015), and associate with acute malaria in children (Obeng-Adjei *et al.*, 2017). Global deletion of T-bet enhances the development of GC B cell responses to *P. berghei* ANKA infection (Ryg-Cornejo *et al.*, 2016a). Despite some studies describing the expression of T-bet in GC B cells (Wang *et al.*, 2012; Guthmiller *et al.*, 2017; Piovesan *et al.*, 2017), its precise role in shaping the GC B cell program during infection remains elusive. In this chapter, we sought to investigate whether B cell-specific expression of T-bet modulates the development of the GC B cell response to *P. berghei* ANKA blood-stage malaria.

2. Results

2.1 B cell-intrinsic expression of T-bet modulates the GC B cell response to malaria

To address the B cell-intrinsic role of T-bet in the GC reaction to malaria infection, we first assessed T-bet expression in GC B cells following *P. berghei* ANKA infection and anti-malarial treatment by flow cytometry (**Figure 4.1**). As early as day 6, T-bet was highly expressed in CD38^{lo}GL7⁺ GC B cells (**Figure 4.1a,b**). T-bet expression was transient and decreased with the resolution of infection. Consistently, the number of T-bet⁺ GC B cells reached a maximum on day 12 and declined by day 24 pi (**Figure 4.1c**). Thus, T-bet is highly expressed by GC B cells early after infection and continues to be expressed at lower, but stable levels at later time points.

To determine the contribution of T-bet up-regulation in GC B cells for the development of the GC response in malaria, we utilised *Tbx21*^{fl/fl}*Cd23*-Cre mice, which lack T-bet specifically in mature B cells (Piovesan *et al.*, 2017). *Tbx21*^{fl/fl}*Cd23*-Cre and *Cd23*-Cre control mice were infected with *P. berghei* ANKA, treated with anti-malarial drugs, and their spleens analysed by immunofluorescence for GC formation (**Figure 4.2**). Deletion of T-bet in B cells improved the overall organization of B cell follicles immediately after drug-treatment on day 6 pi (**Figure 4.2a**). This was accompanied by an efficient development of GCs from day 9 pi, with GC structures significantly larger in size in *Tbx21*^{fl/fl}*Cd23*-Cre mice compared to that of controls by day 24 pi (**Figure 4.2b-c**). Furthermore, upon enumeration of GC B cells in these mice by flow cytometry (**Figure 4.3**), we found that the absence of T-bet expression in B cells gave rise to significantly higher frequencies of CD38^{lo}GL7⁺ GC B cells in the spleens of *Tbx21*^{fl/fl}*Cd23*-Cre mice compared to controls from day 9 pi (**Figure 4.3a,b**). The absolute numbers of GC B cells were also moderately higher in *Tbx21*^{fl/fl}*Cd23*-Cre mice compared to controls, reaching significance by day 24 pi (**Figure 4.3c**). Notably, the increase in GC B cell numbers in *Tbx21*^{fl/fl}*Cd23*-Cre mice was somewhat modest compared to the substantial expansion of this compartment as observed in *Tbx21*^{fl/fl}*Cd4*-Cre mice (**Chapter 3**). Analysis of GC B cells specific to *P. berghei* ANKA MSP-1₁₉, the 19-kDa fragment of the major blood-stage parasite antigen MSP-1 (Blackman *et al.*, 1991), showed that MSP-1₁₉⁺ GC B cells were more abundant in *Tbx21*^{fl/fl}*Cd23*-Cre mice than control mice on day 12 pi (**Figure 4.3d,e**), agreeing with the global GC B cell response.

Enhanced GC formation in the absence of B cell-specific T-bet expression could be attributed to improved T cell help from GC Tfh cells. To examine this, the induction of Tfh cells in *Tbx21*^{fl/fl}*Cd23*-Cre mice was enumerated by flow cytometry (**Figure 4.4a**). No significant differences were detected in the frequencies and numbers of Tfh cells induced upon infection in

the presence or absence of T-bet expression in B cells (**Figure 4.4b,c**), indicating that in *Tbx21^{fl/fl}Cd23-Cre* mice, T-bet modulates the GC B cell response to drug-treated *P. berghei* ANKA infection via a B cell-intrinsic mechanism that is independent of T cell help.

GC B cell differentiation requires antigen-activated B cells to up-regulate expression of the transcriptional repressor Bcl-6 (Dent *et al.*, 1997; Fukuda *et al.*, 1997). To determine if T-bet modulates GC B cell fate commitment, GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and control mice were assessed for their expression of Bcl-6 by flow cytometry. We found that both groups of mice expressed similar levels of Bcl-6 (**Figure 4.5a**). GC B cells were also analysed for their expression of the chemokine receptor CXCR5, which mediates B cell migration to GCs (Förster *et al.*, 1996; Voigt *et al.*, 2000; Ansel *et al.*, 2000; Allen *et al.*, 2004; Junt *et al.*, 2005). However, similar to Bcl-6, no difference in CXCR5 expression by GC B cells from both groups of mice were detected at different time points (**Figure 4.5b**). These findings suggest that T-bet expression in B cells does not affect their commitment to the GC fate.

2.2 T-bet expression during malaria infection induces CXCR3 in GC B cells

As CXCR3 is a key transcriptional target of T-bet in B cells (Beima *et al.*, 2006), its expression was examined in CD38^{lo}GL7⁺ GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* control mice (**Figure 4.6a**). We found that around 60% of all splenic GC B cells in *Cd23-Cre* mice transiently up-regulated high levels of CXCR3 on days 6–9 pi (**Figure 4.6b,c**), with the number of CXCR3⁺ GC B cells peaking on day 9 pi (**Figure 4.6d**). As expected, T-bet-deficient GC B cells of *Tbx21^{fl/fl}Cd23-Cre* mice expressed minimal surface CXCR3. Notably, the kinetics of CXCR3 expression by GC B cells strongly correlated with their expression of T-bet (**Figure 4.1**). The ability of GC B cells from infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice to migrate towards CXCR3-binding CXCL10 chemokine was then assessed using an *in vitro* chemotaxis assay (**Figure 4.6e**). GC B cells from *Cd23-Cre* mice readily migrated in response to recombinant CXCL10 compared to *Tbx21^{fl/fl}Cd23-Cre* GC B cells. These results indicate that GC B cells induced by drug-treated *P. berghei* ANKA infection express high levels of the pro-inflammatory chemokine receptor CXCR3 and respond to CXCL10-mediated migration.

Several studies showed that the strong inflammatory milieu of malaria infection induces the up-regulation of CXCR3 in lymphocytes, which enable these cells to traffic to sites of parasite sequestration in target organs, and contribute to disease pathogenesis in *P. berghei* ANKA infection (Nitchau *et al.*, 2003; Hansen *et al.*, 2007; Miu *et al.*, 2008; Campanella *et al.*, 2008; Nie *et al.*, 2009). To examine if the increased GC B cell response in *Tbx21^{fl/fl}Cd23-Cre* mice reflected a

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preferential homing of B cells to GCs due to a lack of CXCR3 expression, chimeric mice reconstituted with an equal ratio of congenically marked wild-type (CD45.1⁺) and *Cxcr3*^{-/-} (CD45.2⁺) bone marrow were infected with *P. berghei* ANKA, and GC B cell responses assessed in each compartment. Chimeras reconstituted with a mix of wild-type bone marrow (CD45.1⁺ and CD45.2⁺) were included as controls (**Figure 4.7**). CXCR3 expression was confirmed to only occur in wild-type GC B cells upon infection (**Figure 4.7a**). There were minimal differences in the frequency and number of GC B cells detected at early time points between the wild-type and *Cxcr3*^{-/-} compartments (**Figure 4.7b-d**). In contrast, on day 12 pi, the absence of CXCR3 resulted in a 3-fold reduction of GC B cell frequencies and numbers compared to wild-type cells. These findings indicate that T-bet up-regulation in B cells limits GC B cell development by mechanisms independent of CXCR3-mediated migration.

2.3 T-bet regulates the GC B cell transcriptional program to malaria infection

To further understand how the expression of T-bet impacts GC B cells in our infection model, we performed RNA-sequencing of GC B cells isolated from *Tbx21*^{fl/fl}*Cd23*-Cre and *Cd23*-Cre mice on day 10 pi. This time point was selected for analysis as the divergence in the proportion of GC B cells between both groups of mice began to occur around day 10 (**Figure 4.3a,b**). We found 407 genes differentially expressed between *Tbx21*^{fl/fl}*Cd23*-Cre and *Cd23*-Cre GC B cells (false discovery rate (FDR)<0.15). Approximately 40% of these genes were down-regulated (163) and 60% were up-regulated (244) upon T-bet deletion (**Figure 4.8a**). Gene ontology analysis of the up-regulated genes identified an enrichment in pathways that included the regulation of B cell immune processes and cell adhesion (e.g. *Clec4g*, *Cd40*, *Samsn1*, *Cd86*, *Cd209a*, *Nfkbid*, *Cd80*, *Cd83*) (**Figure 4.8a,b**). Conversely, down-regulated genes were enriched in pathways involved in cell division (e.g. *Trp73*, *Cdca8*, *Tpx2*, *Lmn1*, *Cnd3*, *Nek2*), and cell motility (*Cxcr3*, *Cxd10*, *Reln*, *Lmo4*, *Pfn2*) (**Figure 4.8a,c**). Consistent with no differences observed in Bcl-6 and CXCR5 protein expression by flow cytometry (**Figure 4.5**), the absence of T-bet did not affect the expression of these genes (**Figure 4.8a**). *Aicda* that encodes the enzyme AID for Ig somatic hypermutation (Muramatsu *et al.*, 2000; Revy *et al.*, 2000), was also not differentially expressed between GC B cells of both groups of mice.

To gain mechanistic insight into the function of T-bet in GC B cells, we compared the transcriptional shifts induced upon T-bet deletion with a transcriptional signature previously described to define the GC B cell lineage (Gloury *et al.*, 2016). In total, we identified 17 GC B cell signature genes deregulated in the absence of T-bet (p<0.05; **Figure 4.9a**). Analysis of individual genes revealed that half were associated with positive regulation of cell division (e.g. *Cdca8*, *Tpx2*, *Cnd3*, *Nek2*) and were modestly down-regulated in T-bet-deficient GC B cells.

Other notable genes including *Polh*, which encodes the DNA polymerase Pol η that regulates somatic hypermutation (Delbos *et al.*, 2007) in the GC dark zone (Victora *et al.*, 2010; McHeyzer-Williams *et al.*, 2015), was slightly up-regulated in the presence of T-bet, while genes involved in antigen presentation (*H2-Q6*) (Ohtsuka *et al.*, 2008) and cell migration (*Rgs13*) were down-regulated (**Figure 4.9b**). *Rgs13*, that encodes a negative regulator of G protein signaling (Shi *et al.*, 2002), was the only gene differentially expressed greater than 2-fold between *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* GC B cells. Collectively, these results indicate that T-bet regulates key target genes of the GC B cell transcriptional programme.

2.4 T-bet modulates GC polarization to malaria infection

As the GC expands and matures, it polarizes into the dark and light zone, where GC B cell proliferation and somatic hypermutation, and affinity-based selection occur, respectively (Allen *et al.*, 2007; Schwickert *et al.*, 2007; Victora *et al.*, 2010; Gitlin *et al.*, 2014). Recent studies have demonstrated that DZ and LZ GC B cells differ substantially in their gene expression profiles, with LZ B cells characterized by higher expression of cell activation genes, while DZ B cells upregulate genes involved in cell cycle regulation (Victora *et al.*, 2010; 2012). As our results indicated that both of these pathways were deregulated in the absence of T-bet in GC B cells (**Figure 4.8**), we sought to determine whether T-bet influences GC dark and light zone dynamics. Comparison of our RNA-sequencing data with a transcriptional signature that distinguishes DZ and LZ GC B cells (Victora *et al.*, 2012), revealed 32 DZ/LZ signature genes deregulated upon T-bet deletion (**Figure 4.10a**). DZ-defining genes involved in the control of cell migration (e.g. *Pfn2*, *Reln*, *Akap12*), and proliferation (e.g. *Rrm1*, *Cdca8*, *Nek2*), were enriched among genes that were down-regulated in the absence of T-bet, whereas LZ-defining genes associated with B cell activation (e.g. *Cd83*, *Cd86*, *Cd40*), and negative regulation of NF- κ B signaling (*Nfkbie*, *Nfkbid*) were significantly enriched among up-regulated genes (**Figure 4.10b,c**). Overall, these results suggest that T-bet modulates the GC B cell transcriptional profile to promote DZ over LZ programming.

To determine if T-bet also regulates the chromatin landscape of GC B cells, we used ATAC-sequencing to map genomic chromatin accessibility (Buenrostro *et al.*, 2013). For this analysis, we isolated GC B cells from *Tbx21^{fl/fl}Cd23-Cre* mice on day 8 pi and compared them to T-bet-expressing (CXCR3⁺) GC B cells from control mice. In total, 876 regions were differentially accessible between *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* GC B cells, with the number of regions that gained accessibility about 4-fold higher in T-bet-deficient GC B cells compared to controls (FDR<0.1; fold change>2-fold; **Figure 4.11a**). Consistent with previously described studies (Buenrostro *et al.*, 2013), ATAC-sequencing coverage in the samples was concentrated in narrow regions at gene loci, illustrated for *Cxcr3*, *Pxk*, and *Il3ra* in **Figure 4.11b**. Notably, *Cxcr3* was less

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accessible in T-bet-deficient GC B cells compared to controls. Consistent with the RNA-sequencing data, a significant enrichment of DZ and LZ signature genes was identified among differentially accessible regions between *Tbx21^{fl/fl} Cd23-Cre* and *Cd23-Cre* GC B cells ($p=0.02147$). The majority of LZ-associated genes, including *Ccr6*, *Cd83*, *Pxk* and *Nfkbiz* were more accessible in GC B cells of *Tbx21^{fl/fl} Cd23-Cre* compared to controls (**Figure 4.11a**). Interestingly, some DZ-specific genes such as *Anxa2*, *Rrm1* and *Lmo4* which had down-regulated expression levels in the absence of T-bet (**Figure 4.10b**), also remained accessible in T-bet-deficient GC B cells, suggesting that the absence of T-bet may promote increased chromatin accessibility to support plasticity in GC B cell gene expression.

2.5 A lack of T-bet expression in B cells increases the GC light zone compartment

To more precisely determine T-bet distribution within the GC B cell compartment, dark and light zone GC B cells of *P. berghei* ANKA-infected wild-type mice were analysed by flow cytometry, based on their expression of the markers CXCR4 and CD86 as previously described (Victoria *et al.*, 2010; 2012). On day 6 pi, both the expression and number of T-bet⁺ cells were around 2-fold higher in LZ GC B cells compared to the DZ GC B cells (**Figure 4.12**). Despite similar numbers of T-bet⁺ cells between the DZ and LZ compartment from day 9 pi, T-bet expression remained significantly higher in LZ than DZ B cells up to day 24 pi. To determine whether T-bet differentially impacts dark and light zone GC development, we quantitated dark and light zone GC B cells in *Tbx21^{fl/fl} Cd23-Cre* and *Cd23-Cre* mice after *P. berghei* ANKA infection (**Figure 4.13a**). In agreement with the enrichment of LZ-associated genes among T-bet-deficient GC B cells from our RNA-sequencing analysis (**Figure 4.10**), *Tbx21^{fl/fl} Cd23-Cre* mice had higher frequencies of LZ B cells, but reduced proportions of DZ B cells at various times pi (**Figure 4.13a,b**). The number of LZ B cells was also higher in *Tbx21^{fl/fl} Cd23-Cre* mice than in controls, but no significant differences were found in the numbers of DZ B cells between both groups of mice (**Figure 4.13c**). These cell fluctuations reflected in significantly reduced ratios of DZ to LZ B cells in *Tbx21^{fl/fl} Cd23-Cre* mice compared to controls, with LZ B cells over-represented in the absence of T-bet (**Figure 4.13d**).

Consistent with no effect on the number of DZ B cells in the absence of T-bet, when we quantified GC B cell proliferation, a trait that is primarily associated with the DZ reaction (Allen *et al.*, 2004; 2007; Victoria *et al.*, 2010), no significant differences were detected in the frequencies and number of BrdU⁺ GC B cells between *Tbx21^{fl/fl} Cd23-Cre* and control mice, with an average of 40–60% of GC B cells proliferating at each time point analysed (**Figure 4.14a–c**). Analysis of GC B cells for their expression of the proliferation marker Ki67, likewise revealed a similar proportion of

Ki67⁺ GC B cells between both groups of mice (**Figure 4.14d**). In contrast, analysis of apoptotic GC B cells in *Tbx21^{fl/fl}Cd23-Cre* and control mice indicated while there were no differences in the frequency of apoptotic GC B cells between both groups of mice, *Tbx21^{fl/fl}Cd23-Cre* mice had significantly more absolute numbers of apoptotic cells compared to control mice from days 6–9pi (**Figure 4.15**). This agrees with the enhanced LZ program in *Tbx21^{fl/fl}Cd23-Cre* mice, in which B cells are subjected to apoptosis during GC selection in the LZ (Mayer *et al.*, 2017).

To confirm that the effect of T-bet in the control of GC polarization was B cell-intrinsic, chimeric mice reconstituted with an equal ratio of wild-type (CD45.1⁺) and *Tbx21^{fl/fl}Cd23-Cre* (CD45.2⁺) bone marrow were infected with *P. berghei* ANKA, and the frequency of DZ and LZ GC B cells was assessed in each compartment. Chimeras reconstituted with a mix of wild-type (CD45.1⁺) and *Cd23-Cre* (CD45.2⁺) bone marrow were included as controls, and T-bet expression was only detected in wild-type GC B cells following infection (**Figure 4.16a**). Control chimeras displayed equal frequencies of DZ and LZ B cells within each of the CD45.1⁺ and CD45.2⁺ compartments; however, in the mixed chimeras, *Tbx21^{fl/fl}Cd23-Cre* GC B cells gave rise to significantly increased proportions of LZ B cells and decreased frequencies of DZ B cells compared to wild-type cells (**Figure 4.16b–d**). Collectively, our data demonstrate that T-bet controls GC polarization by limiting LZ differentiation and promoting transition to a DZ program in a B cell-intrinsic manner.

2.6 T-bet modulates GC B cell responsiveness to CXCL12-mediated migration

As our findings suggested that T-bet expression in GC B cells favours the DZ GC program, we speculated that T-bet may facilitate the cyclic re-entry of GC B cells to the DZ. GC B cell entry into the DZ requires CXCR4-guided migration (Allen *et al.*, 2004; Nie *et al.*, 2004; Bannard *et al.*, 2013). To examine if this pathway was affected by T-bet, we examined the surface expression of CXCR4 and the chemotactic response to its ligand, CXCL12, in GC B cells of *P. berghei*-ANKA-infected *Tbx21^{fl/fl}Cd23-Cre* and control mice. Flow cytometry analysis showed that CXCR4 was expressed 3–4 times higher in DZ GC B cells compared to LZ GC B cells as expected (**Figure 4.17a,b**). However, the DZ and LZ B cells of *Tbx21^{fl/fl}Cd23-Cre* and control mice expressed comparable levels of CXCR4 over the course of infection, suggesting that T-bet-modulation of GC polarization was not associated with differential CXCR4 up-regulation in GC B cells. Despite this, the capacity of T-bet-deficient GC B cells to migrate in response to CXCL12 *in vitro* was reduced by 30% compared to that of control cells (**Figure 4.17c**), signifying that T-bet modulates GC B cells responsiveness to CXCL12-mediated chemotaxis.

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One of the GC B cell-specific genes significantly up-regulated in the absence of T-bet was *Rgs13* (**Figure 4.9**), which encodes the regulator of G protein signaling (RGS)13 (Shi *et al.*, 2002). RGS proteins target chemokine receptors that signal via heterotrimeric G proteins, and limit the duration of downstream effector signaling, thereby desensitizing cells to chemotactic stimulation (Moratz *et al.*, 2004). *In vitro* studies have showed that RGS13 down-regulates cell responsiveness to CXCL12 (Shi *et al.*, 2002; Han *et al.*, 2006; Hsu *et al.*, 2007; Hwang *et al.*, 2013). To test whether increased RGS13 expression in the absence of T-bet may contribute to impaired B cell migration to the DZ, we transfected a human B cell lymphoma line (HS-Sultan) that constitutively expresses RGS13 (Shi *et al.*, 2002) with lentivirus encoding T-bet (**Figure 4.18**). Successful transfection was confirmed by flow cytometry and quantitative RT-PCR (**Figure 4.18a**). The expression of T-bet in HS-Sultan cells reduced *Rgs13* mRNA levels by 50% compared to cells transfected with control lentivirus (**Figure 4.18b**). We further found that T-bet-induced *Rgs13* down-regulation led to a 2.5-fold increase in the capacity of transfected cells to migrate to CXCL12 *in vitro* compared to controls (**Figure 4.18c**). Thus, these results indicate that T-bet directly controls B cell responsiveness to CXCL12 that is required for efficient GC polarization.

3. Discussion

An increasing number of studies detect T-bet-expressing B cells in a diverse range of disease contexts. In malaria, T-bet has been detected in human atypical MBCs (Obeng-Adjei *et al.*, 2017), and B cells induced to murine infection (Guthmiller *et al.*, 2017; Rivera-Correa *et al.*, 2017; Pérez-Mazliah *et al.*, 2018). Here, we found that T-bet is also highly expressed in GC B cells immediately following anti-malarial drug-treated *P. berghei* ANKA infection. T-bet expression was detected at an early time point, coinciding with disease and the induction of pro-inflammatory cytokines, such as IFN- γ and IL-12, in this model (Ryg-Cornejo *et al.*, 2016a). T-bet is similarly detected in GC B cells around the time of acute *P. yoelii* 17XNL infection and is regulated by IFN- γ signals (Guthmiller *et al.*, 2017), supporting a potent role of inflammatory conditions in sustaining T-bet expression in B cells in malaria. Whether other pro-inflammatory cytokines, such as IL-12, IL-18, IL-21 and IL-27 that have been described to up-regulate T-bet in B cells (Szabo *et al.*, 2000; Harris *et al.*, 2005; Larousserie *et al.*, 2006; Rubtsova *et al.*, 2013; 2016; Naradikian *et al.*, 2016a; Jackson *et al.*, 2016; Sindhava *et al.*, 2017), contribute to T-bet induction in GC B cells during malaria infection remains to be investigated.

CXCR3 is a key gene target of T-bet in B cells (Beima *et al.*, 2006). In B cells, CXCR3 upregulation has been associated with their migration to sites of inflammation in autoimmune conditions (Henneken *et al.*, 2005; Nicholas *et al.*, 2008; Hosomi *et al.*, 2011), and for the control of viral infections (Marques *et al.*, 2011; Onodera *et al.*, 2012; Gil-Cruz *et al.*, 2012; Barnett *et al.*, 2016; Knox *et al.*, 2017; Chang *et al.*, 2017). Similarly, CXCR3⁺ lymphocytes traffic to sites of parasite sequestration during *P. berghei* ANKA infection (Nie *et al.*, 2009). We found that T-bet induced CXCR3 up-regulation in GC B cells, and that the *Cxcr3* locus was more accessible in T-bet-expressing GC B cells at the chromatin level. Unlike viral infection (Marques *et al.*, 2011; Onodera *et al.*, 2012; Gil-Cruz *et al.*, 2012; Barnett *et al.*, 2016), CXCR3 up-regulation did not appear to facilitate migration of B cells away from the spleen following acute malaria infection, and instead, their expression of this chemokine receptor was associated with an expanded GC B cell pool. Whether CXCR3 ligands are produced by GC-resident stroma or haematopoietic cells is not known; although CXCR3-mediated migration to CXCL10 and CXCL9 chemokines has been shown to facilitate T cell priming in other experimental models (Groom *et al.*, 2012). A thorough characterisation of the follicular location of CXCR3-expressing GC B cells during infection, together with mechanistic experiments, will be necessary to identify CXCR3-binding chemokine gradients within GCs, and to fully understand the biological relevance of CXCR3 up-regulation in B cells for the GC reaction.

While a few studies associate T-bet to impaired GC formation in murine malaria (Ryg-Cornejo *et al.*, 2016a), a B cell-intrinsic role of T-bet in regulating the development of the GC B cell response to malaria infection is poorly defined. We show that deletion of T-bet in B cells resulted in a moderate increase in the size of the GC B cell response. The enlarged GC B cell compartment was not associated with changes in proliferation rates, and occurred independently of Bcl-6 up-regulation and CXCR5-mediated GC homing. Nevertheless, the effect was modest compared to that observed in the absence of CD4⁺ T cell-intrinsic T-bet expression, in which Tfh cells were efficiently generated (**Chapter 3**), suggesting that a lack of T-bet in T cells and enhanced induction of Tfh cells are the main drivers of the overall magnitude of the GC reaction. Conversely, the absence of B cell-specific T-bet appeared to augment GC LZ development.

Transcriptional profiling revealed that T-bet controls the expression of a large number of genes in GC B cells induced by infection, such as activation molecules, as well as regulators of cell motility and division. This suggests that T-bet induces a broad transcriptional program in GC B cells responding to malaria infection and supports a role of T-bet in regulating B cell activity beyond antibody class-switching. Our data showed that T-bet induces a transcriptional program that favours a proportionally increased DZ compartment but limits LZ development in a B cell-intrinsic

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manner. Similar to our results, other transcription factors such as AP4, c-Myc and Foxo1, have been identified to play roles in coordinating the GC DZ program (Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012; Sander *et al.*, 2015; Dominguez-Sola *et al.*, 2015; Chou *et al.*, 2016; Luo *et al.*, 2018). However, the absence of T-bet in B cells did not influence the expression of these regulators in our model of malaria infection, suggesting that T-bet controls the DZ reaction via a distinct pathway. mTORC1 and NF- κ B signaling have also been shown to regulate GC DZ re-entry of selected LZ B cells (Heise *et al.*, 2014; De Silva *et al.*, 2016; Jones *et al.*, 2016; Ersching *et al.*, 2017). Genes encoding members of the I κ B family that negatively regulate NF- κ B signaling (*Nfkbid*, *Nfkbie*), were up-regulated or accessible at the chromatin level (*Nfkbiz*) in T-bet-deficient GC B cells, which were skewed towards a LZ phenotype. These NF- κ B inhibitors bind to various NF- κ B subunits (Yamazaki *et al.*, 2001; Fiorini *et al.*, 2002), and have been independently implicated in B cell differentiation or antibody production (Doerre *et al.*, 2005; Touma *et al.*, 2011; Arnold *et al.*, 2012; Hanihara *et al.*, 2013; Hanihara-Tatsuzawa *et al.*, 2014; Hosokawa *et al.*, 2017). Whether they contribute to GC B cell development has not been defined, but I κ B- δ (*Nfkbid*) and I κ B ϵ (*Nfkbie*) have been suggested to regulate the activities of Relb (Tao *et al.*, 2014), and c-Rel (Li and Nabel, 1997; Clark *et al.*, 2011), respectively, both of which promote DZ clonal expansion (Heise *et al.*, 2014; De Silva *et al.*, 2016). Further work will be required to determine if T-bet expression influences NF- κ B-associated pathways in GC B cells to promote the GC DZ reaction in malaria.

RGS13 is a negative regulator of G-protein signaling that is expressed exclusively in GC B cells (Shi *et al.*, 2002; Hwang *et al.*, 2013), and down-regulates CXCR4 chemokine receptor signaling (Shi *et al.*, 2002; Han *et al.*, 2006; Hsu *et al.*, 2007; Hwang *et al.*, 2013). The function of RGS13 function and its regulation in disease is not entirely clear (Hsu *et al.*, 2007; Bansal *et al.*, 2008a; b; Wang *et al.*, 2013), and to date, only IL-17 has been suggested to induce RGS13 in autoimmune B cell responses (Hsu *et al.*, 2007; Wang *et al.*, 2013). Strikingly, RNA-sequencing indicated that *Rgs13* expression was increased in T-bet-deficient GC B cells during malaria infection, supporting a role of T-bet in regulating RGS13. Moreover, T-bet directly suppressed *Rgs13* expression in human B cells, which promoted their migration to CXCL12 chemokine. Whether RGS13 is a direct target gene of T-bet in GC B cells is unknown and remains to be determined. Interestingly, in contrast to our data, one study reported that malaria infection of autoimmune mice reduces B cell migration to CXCL12 *in vitro* (Badr *et al.*, 2015). This was attributed to CXCR4 down-regulation in these mice; however, we did not detect a differential regulation of CXCR4 in GC B cells by T-bet after drug-treated *P. berghei* ANKA infection. CXCL12 in the spleen has been shown to be important for parasite control in murine malaria (Garnica *et al.*, 2002). More importantly, the CXCR4-CXCL12 signaling axis is crucial for controlling B cell transition into

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the DZ during GC cycling (Allen *et al.*, 2004; Nie *et al.*, 2004; Bannard *et al.*, 2013). Therefore, the data presented in this chapter indicates that T-bet up-regulation in GC LZ B cells may promote CXCL12-mediated trafficking to the DZ by suppressing RGS13, and supports a model by which pro-inflammatory responses elicited to infection modulate GC polarization.

Results

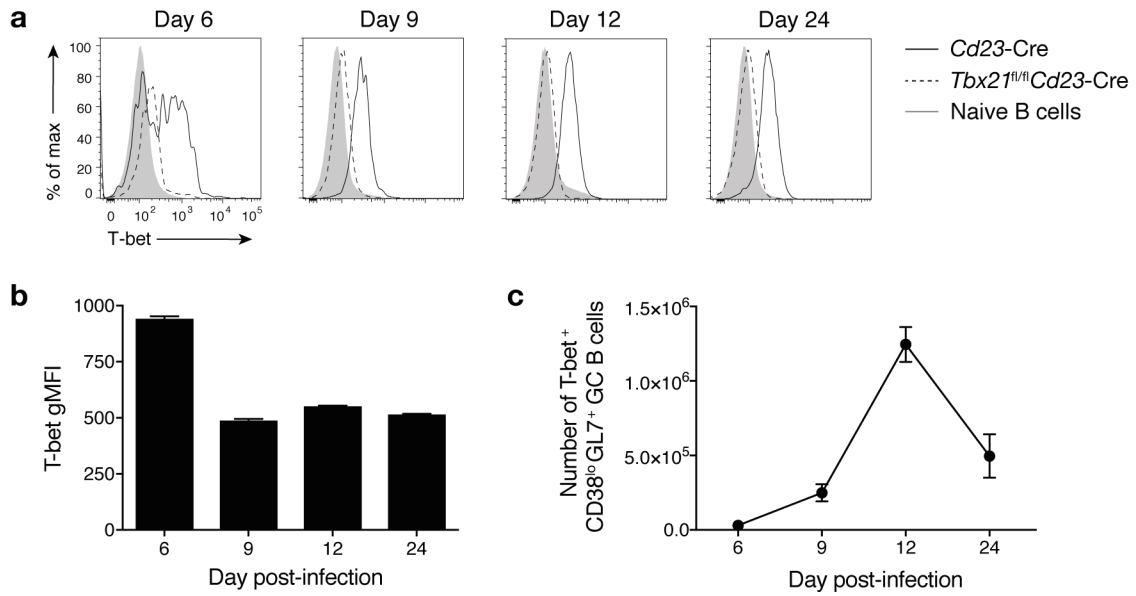
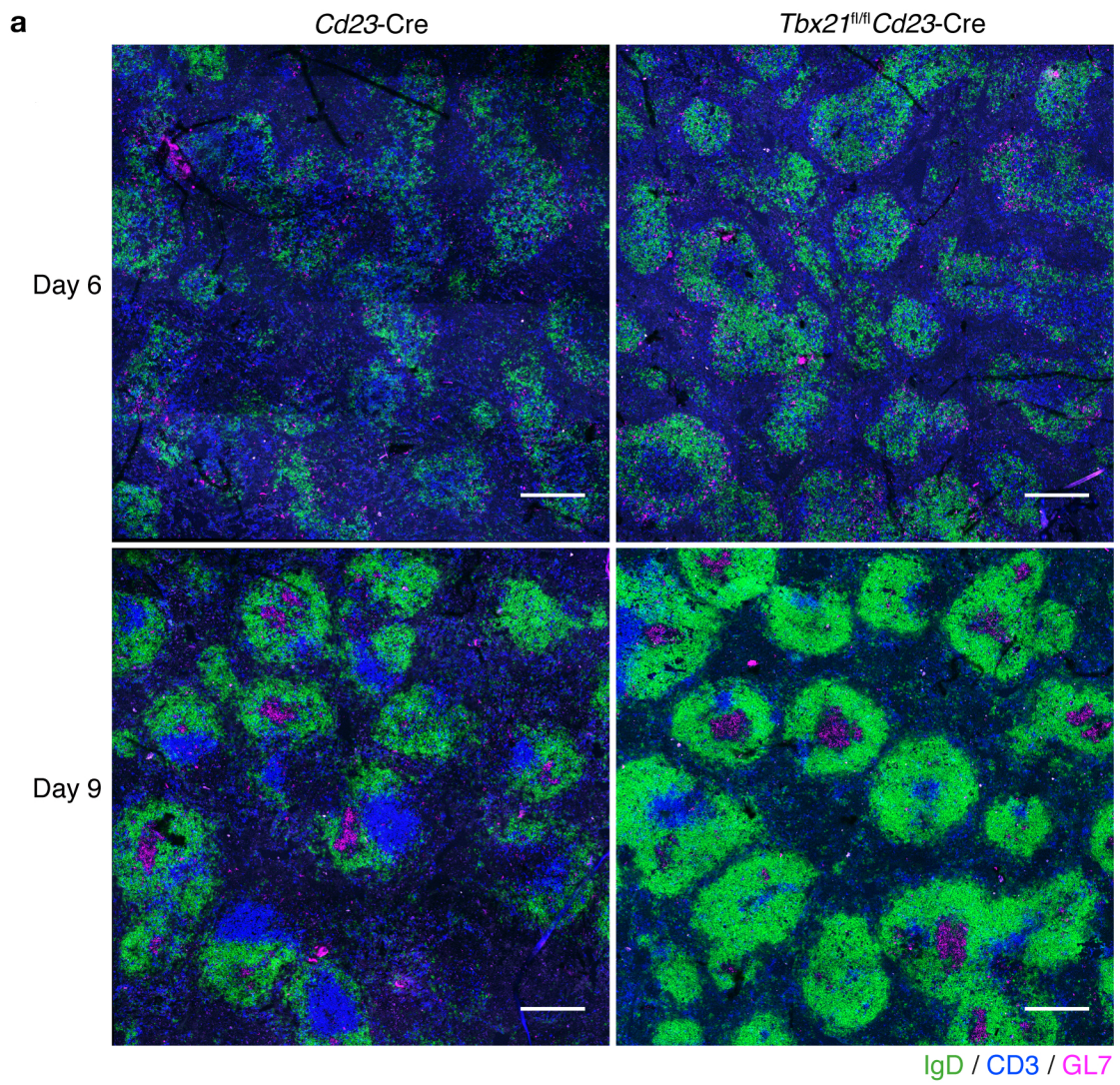


Figure 4.1 GC B cells induced to severe malaria infection express the transcription factor T-bet

Tbx21^{fl/fl}Cd23-Cre and *Cd23-Cre* mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs from day 5 post-infection (pi). **(a)** Representative flow cytometry plots showing T-bet⁺ cells among gated CD38^{lo}GL7⁺CD19⁺ GC B cells. CD38⁺GL7⁻ naive B cells are shown as a control. **(b)** T-bet gMFI and **(c)** the absolute number of T-bet⁺ GC B cells in *Cd23-Cre* mice. Data represent the mean of 4 mice ± SEM.



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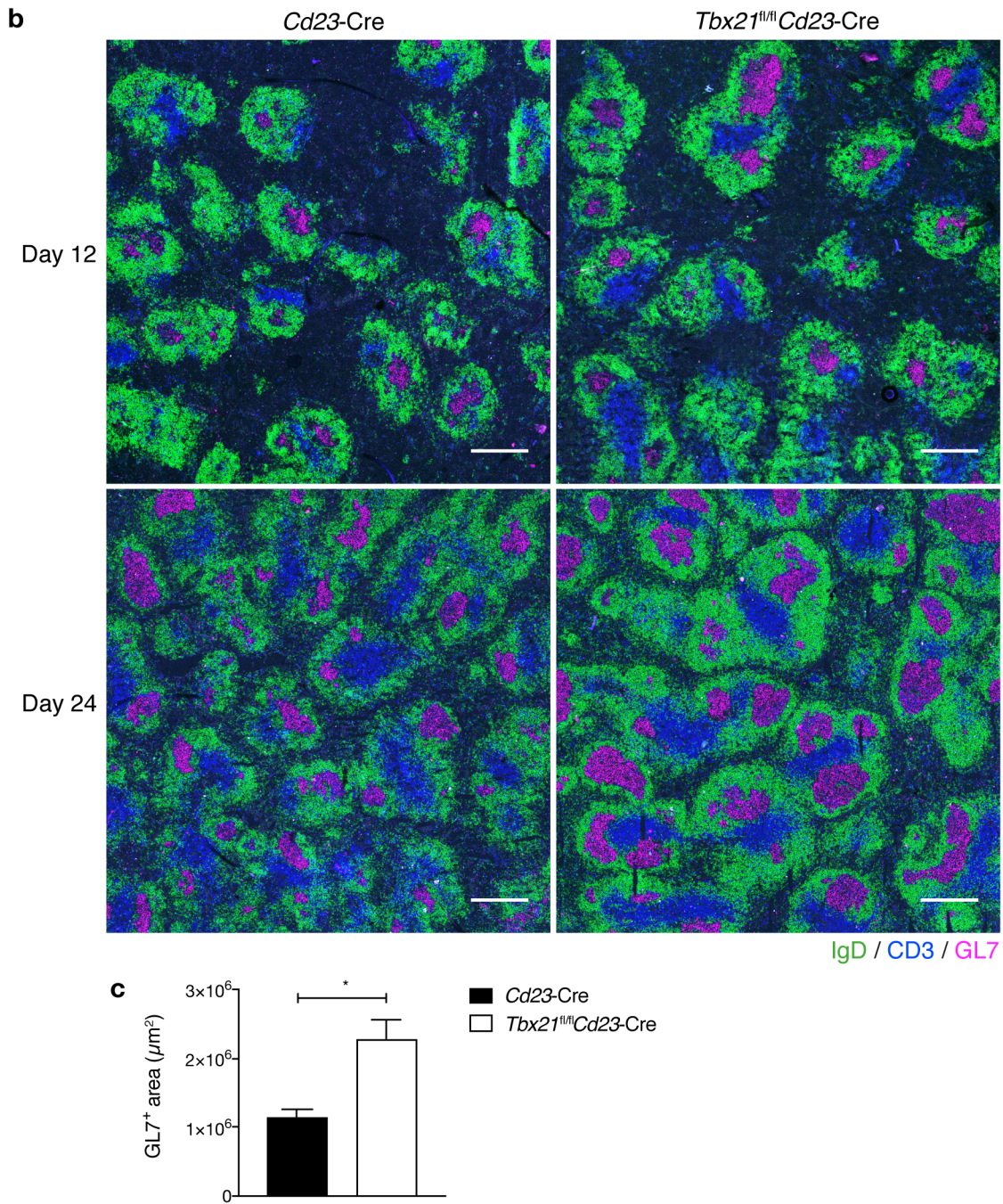


Figure 4.2 GC development is suppressed in the presence of B cell-intrinsic T-bet expression

(a, b) Immunofluorescence analysis of spleen sections of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. B cell follicles were identified by anti-IgD (green), T cell zones with anti-CD3 (blue), and germinal centres with anti-GL7 (magenta). Scale bar, 300 μm . Images are representative of 4 mice per group. (c) Quantification of total GL7⁺ area at day 24 pi. Bars represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$.

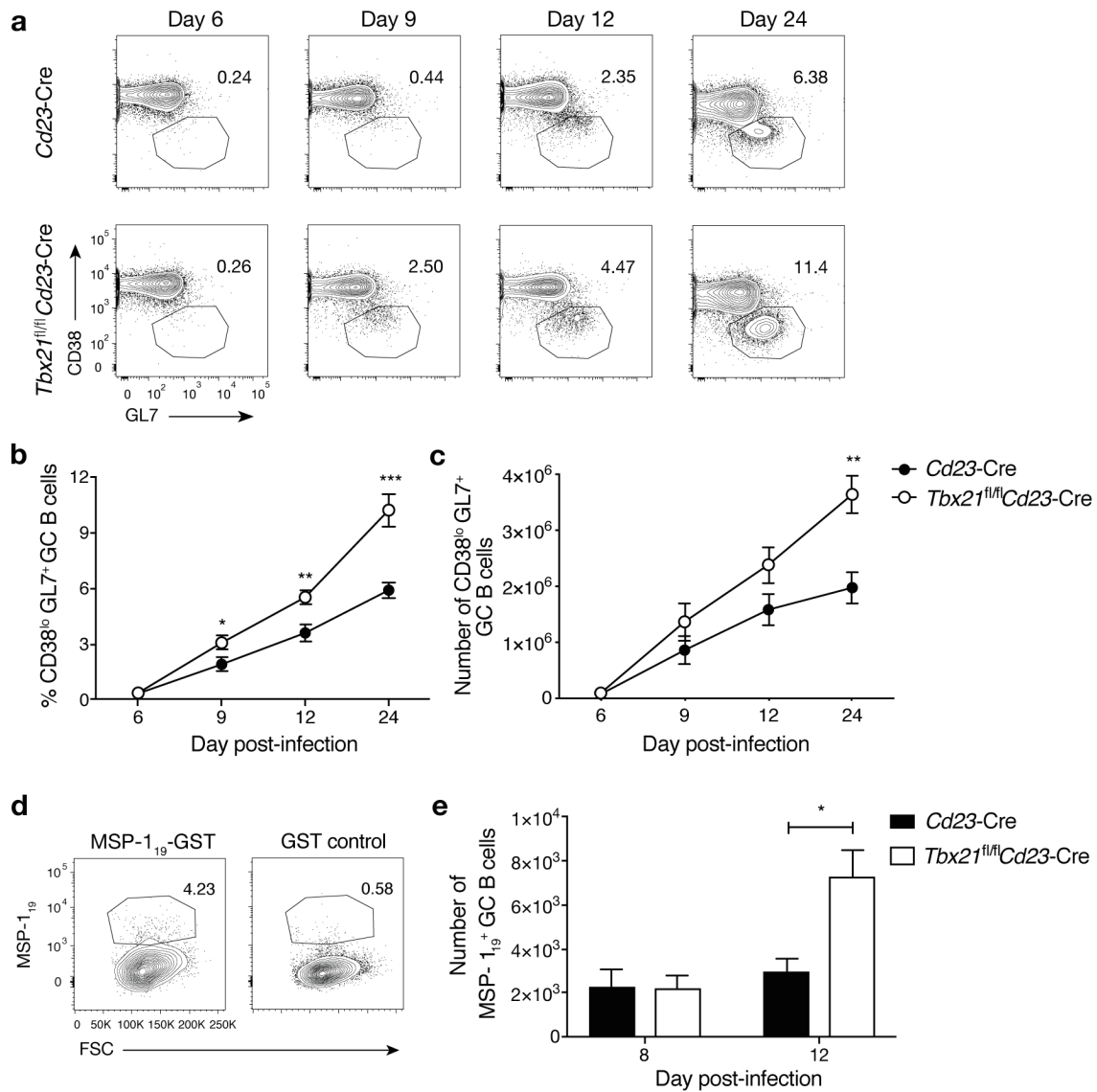


Figure 4.3 B cell-intrinsic expression of T-bet modulates the GC B cell response to malaria infection

(a) Representative flow cytometry plots showing CD38^{lo}GL7⁺ GC B cells among CD19⁺ cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The percentage (b) and absolute number (c) of GC B cells were enumerated. Representative flow cytometry plots depicting the staining (d) and absolute number (e) of MSP-1₁₉⁺ GC B cells. Cells stained with GST only served as a negative control. Data are from three (a-c) and one (d, e) experiment(s), presented as the mean of 4-12 mice $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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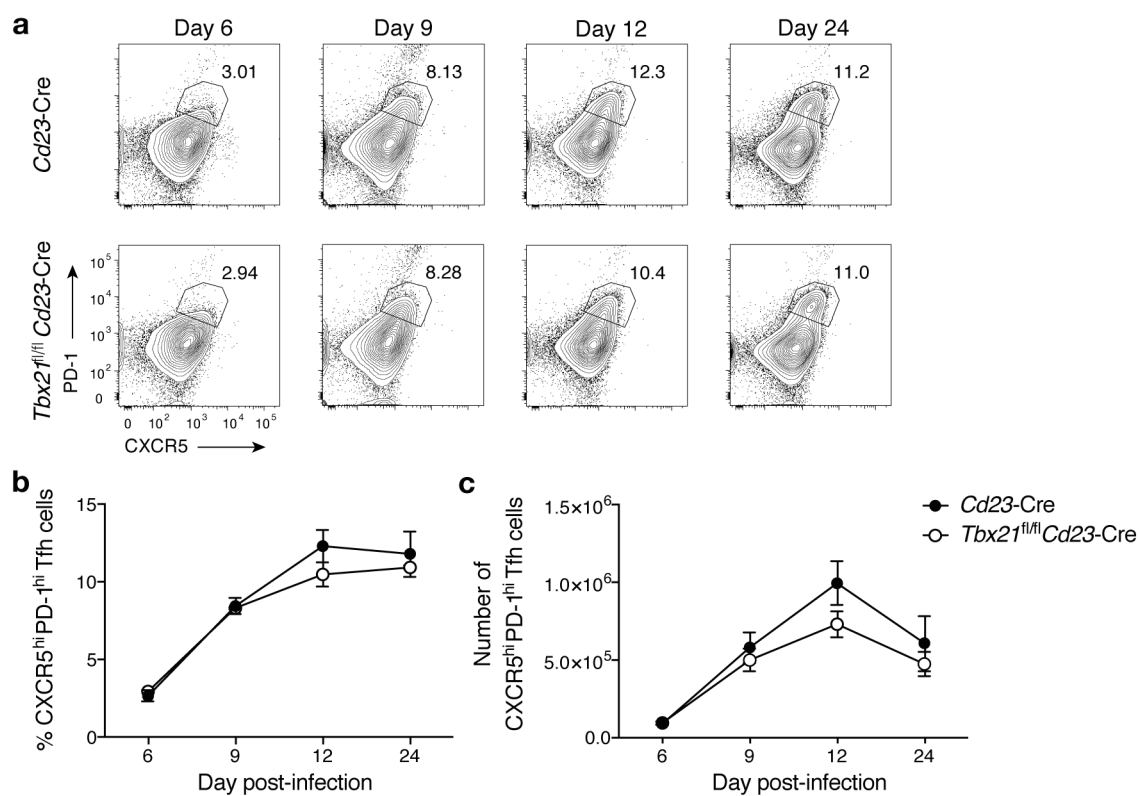


Figure 4.4 The generation of Tfh cells is not influenced by B cell-intrinsic expression of T-bet

(a) Representative flow cytometry plots showing CXCR5^{hi}PD-1^{hi} Tfh cells among CD4⁺CD19⁻CD62⁻ cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The percentage (b) and absolute number (c) of Tfh cells were enumerated. Data are representative of three independent experiments, presented as the mean of 4 mice $\pm$ SEM.

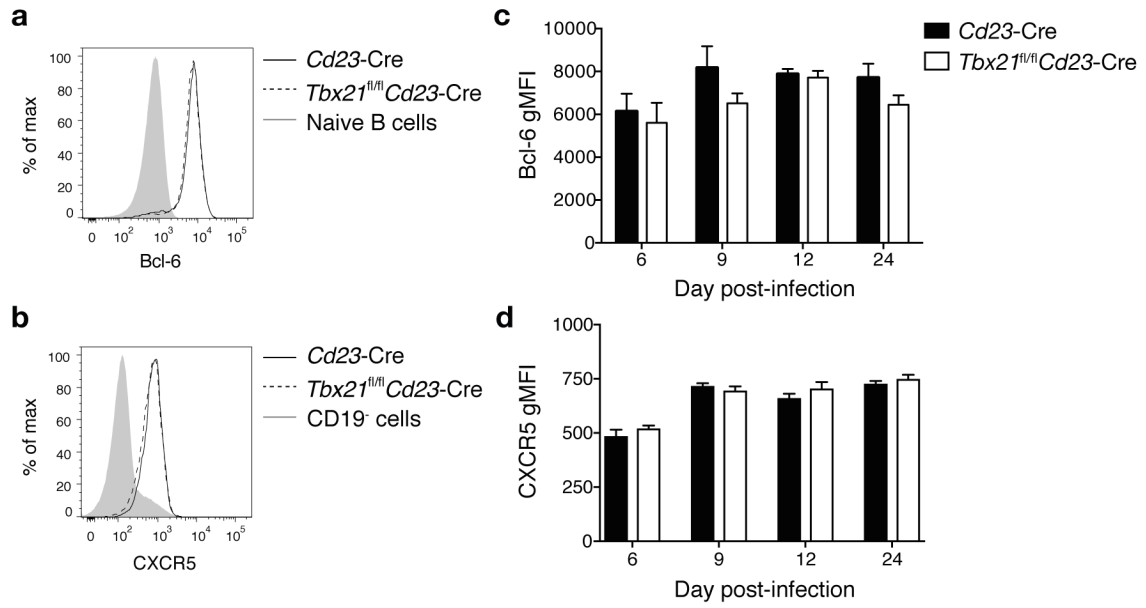


Figure 4.5 GC B cell expression of Bcl-6 and CXCR5 is not affected by T-bet co-expression

Representative histograms (**a, b**) and gMFI (**c, d**) of Bcl-6 and CXCR5 expression in CD38^{lo}GL7⁺ GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Negative controls are shaded in grey. Data are representative of 1-3 independent experiments, presented as the mean of 4 mice $\pm$ SEM.

Results

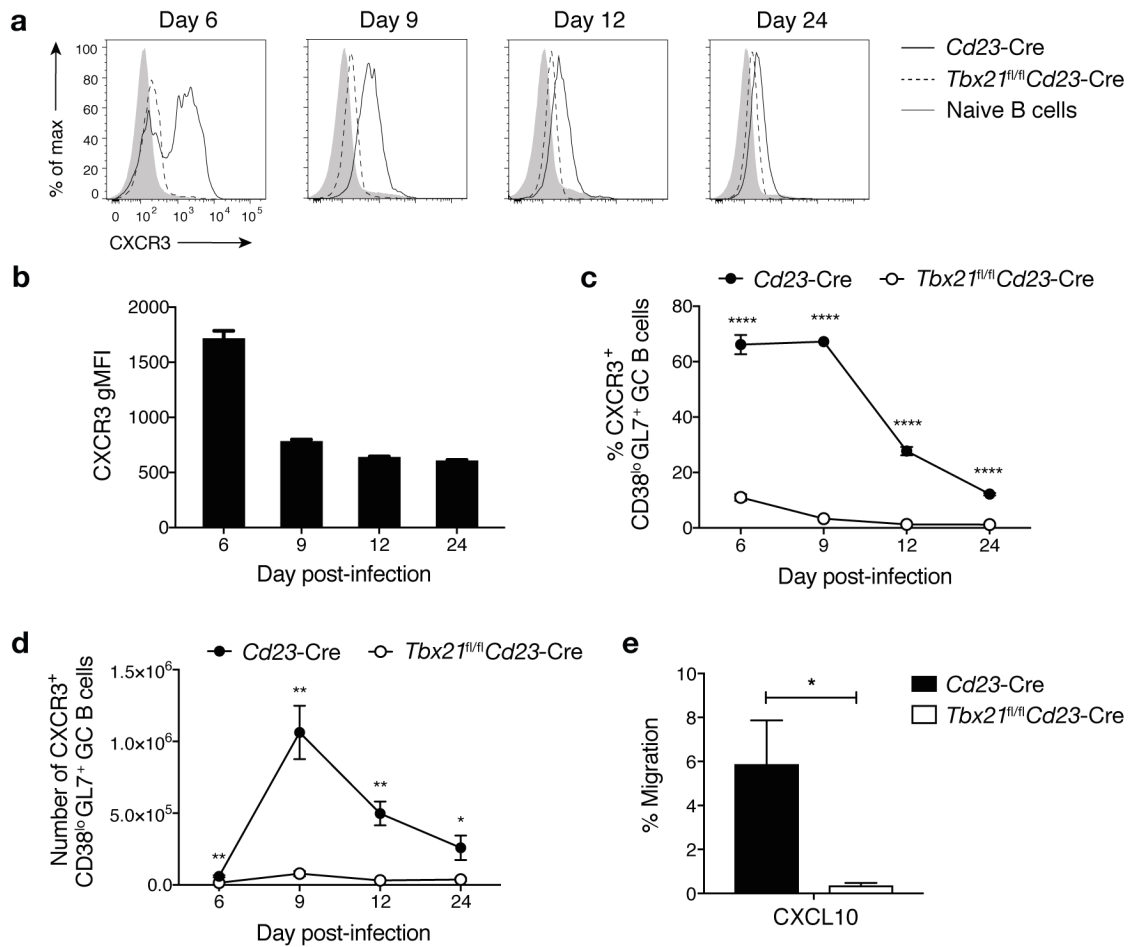


Figure 4.6 GC B cells up-regulate the chemokine receptor CXCR3 and migrate to CXCL10 chemokine

(a) Representative histograms showing CXCR3 expression among gated CD38^{lo}GL7⁺ GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Naive B cells are shown as a control. (b) CXCR3 gMFI of GC B cells of *Cd23-Cre* mice. (c) The proportion and (d) absolute number of CXCR3⁺ GC B cells of infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice. (e) Chemotaxis of GC B cells from day 8 pi to recombinant CXCL10 chemokine was assessed *in vitro*. Data are representative of 2-3 independent experiments, presented as the mean of 4 mice $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

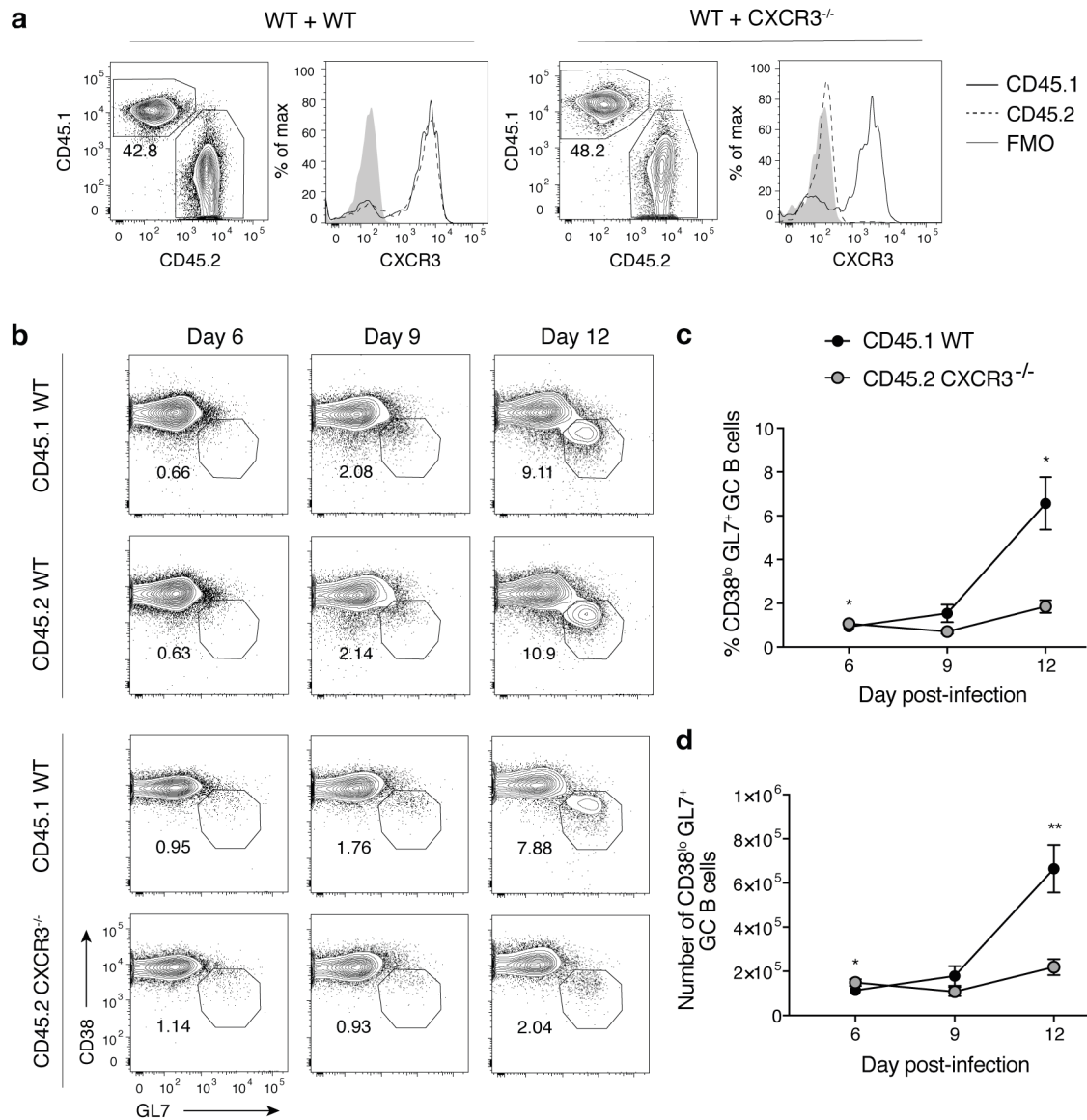


Figure 4.7 CXCR3-dependent migration does not interfere with GC B cell differentiation

Irradiated CD45.1 mice were reconstituted with a 1:1 mix of CD45.1⁺ wild-type (WT) and CD45.2⁺ *Cxcr3*^{-/-}, or CD45.2⁺ WT bone marrow. **(a)** Representative flow cytometry plots showing CD45.1/2 expression among lymphocytes in reconstituted chimeras. CXCR3 up-regulation by WT CD38^{lo}GL7⁺ GC B cells in *P. berghei* ANKA-infected chimeras on day 6 pi. Fluorescence minus one (FMO) negative control is shaded in grey. **(b)** CD38^{lo}GL7⁺ GC B cells among the CD45.1⁺ and CD45.2⁺ compartments in control (top panel) and *Cxcr3*^{-/-} mixed chimeras (bottom panel) at different times pi. **(c)** The percentage and **(d)** absolute number of GC B cells were enumerated in *Cxcr3*^{-/-} mixed chimeras. Data are representative of two experiments, presented as the mean of 3-4 mice $\pm$ SEM. Paired student's t-test was used to calculate statistical significance, * $p < 0.05$, ** $p < 0.01$.

Results

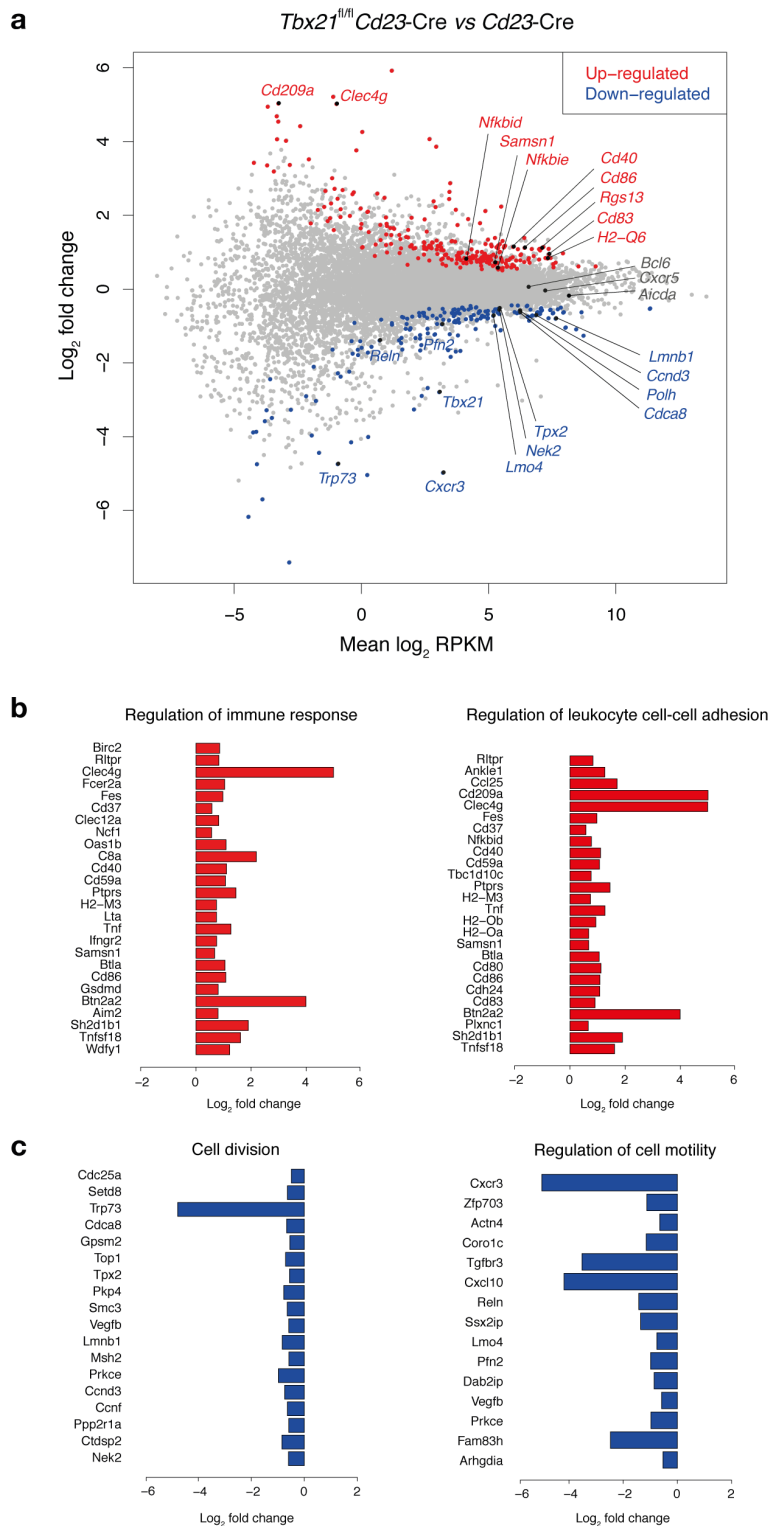


Figure 4.8 T-bet transcriptionally regulates key processes in malaria-induced GC B cells

RNA-seq analysis of CD19⁺CD38^{lo}GL7⁺ GC B cells isolated from *Tbx21^{fl/fl}* Cd23-Cre and Cd23-Cre mice on day 10 pi with *P. berghei* ANKA, after anti-malarial treatment. **(a)** Scatterplot of up-regulated (red) and down-regulated (blue) genes differentially expressed between *Tbx21^{fl/fl}* Cd23-Cre and Cd23-Cre GC B cells (FDR<0.15). RPKM, reads per kilobase per million. Log₂ fold change representation of genes in selected Metascape pathways **(b)** up-regulated and **(c)** down-regulated in *Tbx21^{fl/fl}* Cd23-Cre versus Cd23-Cre GC B cells. See Appendices I and II for a list of differentially expressed genes and enriched pathways, respectively.

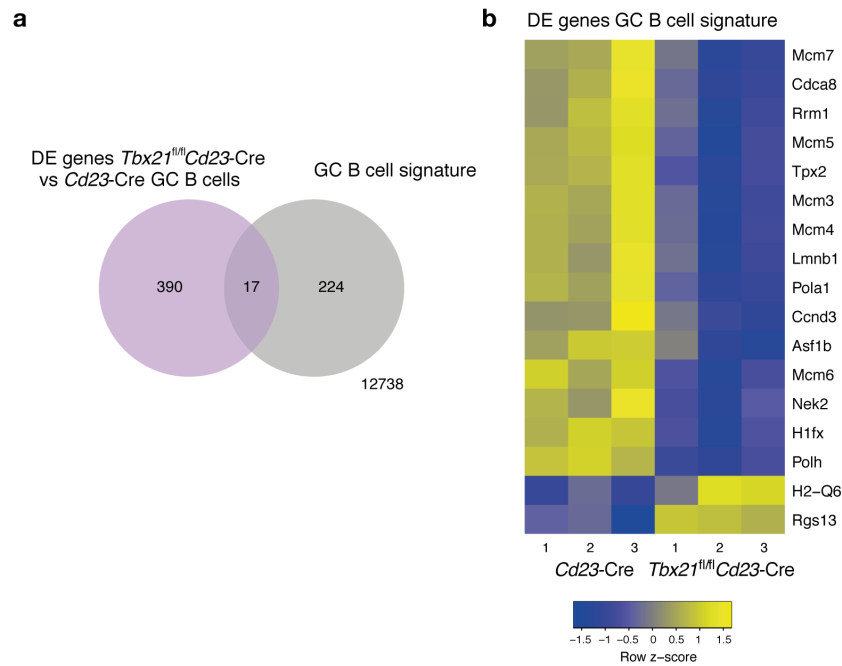


Figure 4.9 T-bet transcriptionally regulates the GC B cell transcriptional program to malaria infection

(a) Venn diagram showing overlap between genes differentially expressed (DE) in *Tbx21^{fl/fl}*Cd23-Cre versus Cd23-Cre GC B cells (purple circle), and GC B cell transcriptional signature (grey circle; Gloury *et al.*, 2016). (b) Heat-map showing relative expression levels of GC B cell signature genes deregulated upon T-bet deletion. Each column represents an independent replicate.

Results

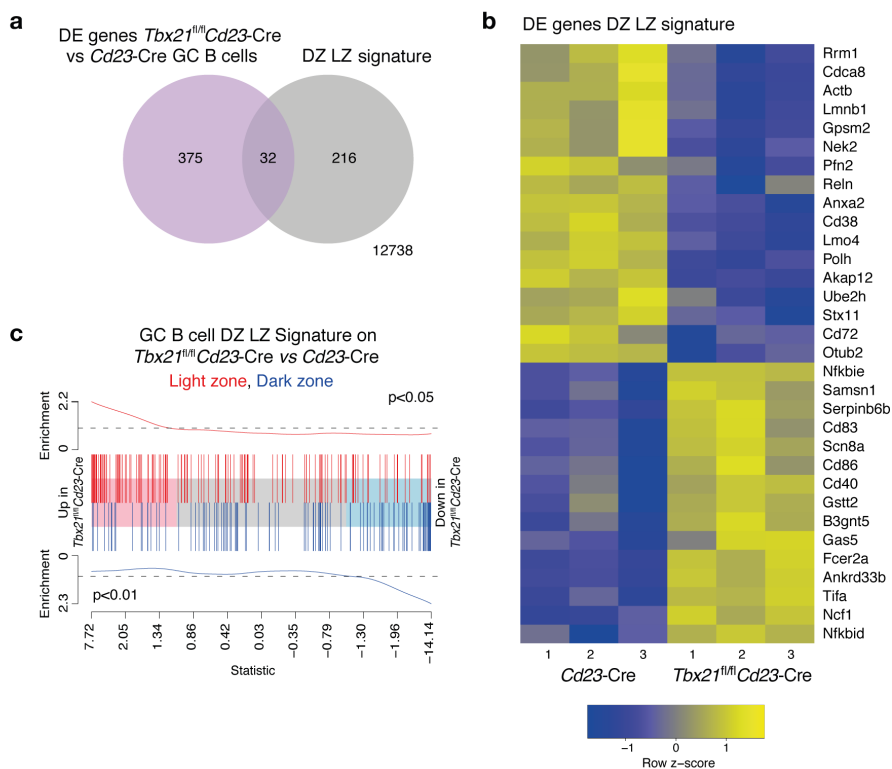


Figure 4.10 Enrichment of the DZ transcriptional program in T-bet-expressing GC B cells

(a) Venn diagram showing overlap between genes differentially expressed in *Tbx21^{fl/fl}**Cd23-Cre* versus *Cd23-Cre* GC B cells (purple circle), and dark and light zone (DZ LZ) transcriptional signature (grey circle; Vitorica *et al.*, 2012). (b) Heat-map showing relative expression levels of DZ LZ signature genes deregulated upon T-bet deletion. Each column represents an independent replicate. (c) Gene set enrichment test of DZ (blue) and LZ (red) genes that are differentially expressed between *Tbx21^{fl/fl}**Cd23-Cre* and *Cd23-Cre* GC B cells using Roast Test.

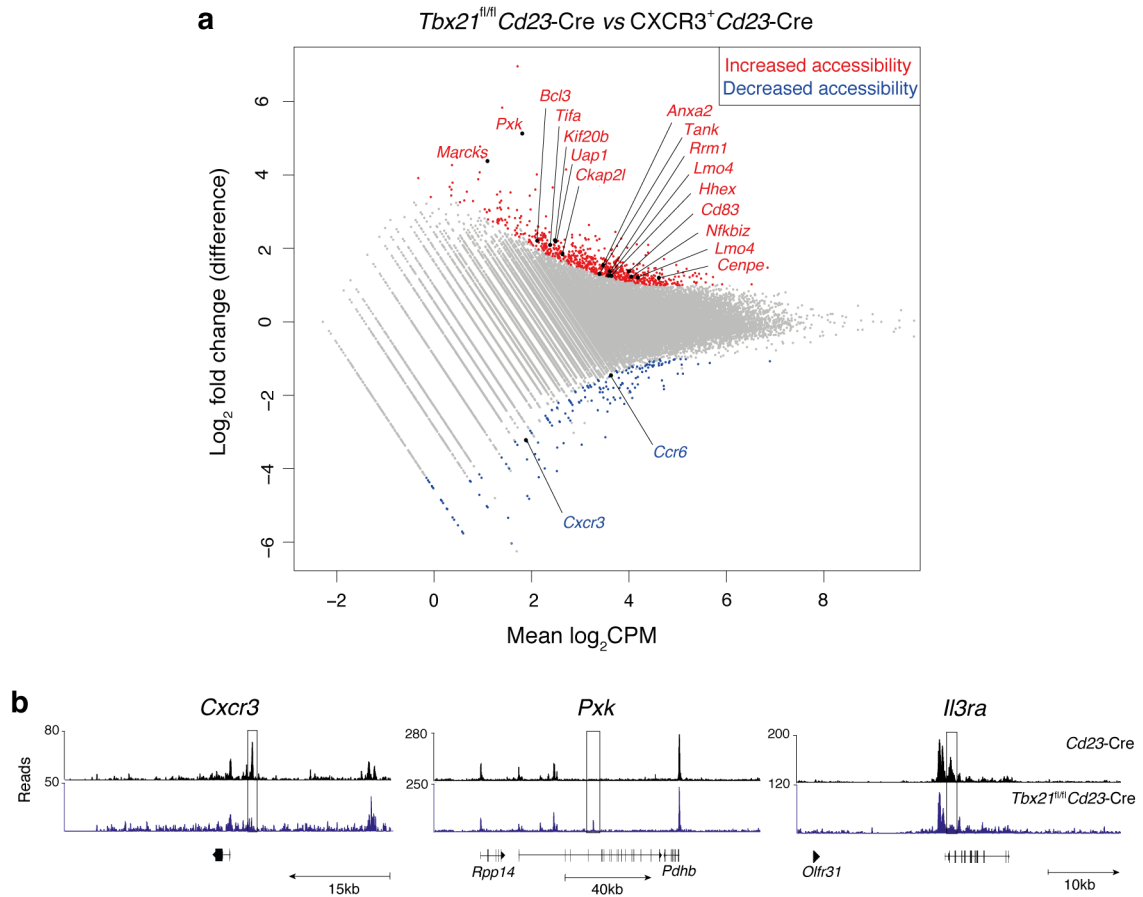


Figure 4.11 T-bet modulates the chromatin accessibility of GC B cell dark and light zone-associated genes

ATAC-seq analysis of CD19⁺CD38^{lo}GL7⁺ GC B cells isolated from *Tbx21^{fl/fl}* Cd23-Cre mice and CXCR3⁺ GC B cells from Cd23-Cre mice on day 8 pi with *P. berghei* ANKA. **(a)** Scatterplot of peaks with increased (red) or decreased (blue) accessibility in *Tbx21^{fl/fl}* Cd23-Cre versus Cd23-Cre GC B cells (FDR<0.1, fold change≥2). Each dot represents one ATAC-seq peak. CPM, counts per million mapped fragments. **(b)** Mean ATAC-seq coverage at selected gene loci. Differentially accessible peaks between *Tbx21^{fl/fl}* Cd23-Cre versus Cd23-Cre GC B cells are boxed. Arrows indicate scale and direction of transcription. See *Appendix III* for a list of differentially accessible peaks.

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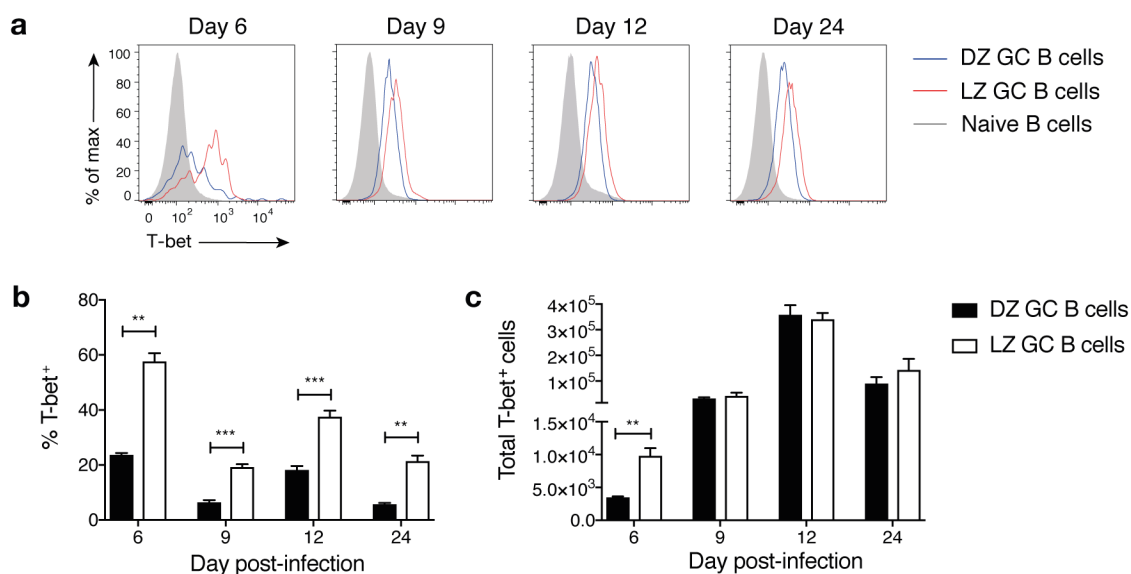


Figure 4.12 T-bet expression is enriched in light zone GC B cells following malaria infection

(a) Representative histograms showing T-bet expression of DZ (CD86^{lo}CXCR4^{hi}) and LZ (CD86^{hi}CXCR4^{lo}) CD38^{lo}GL7⁺ GC B cells of *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. CD19⁺CD38^{hi}GL7⁻ naive B cells are shown as a control. The (b) gMFI and (c) absolute number of T-bet⁺ DZ and LZ GC B cells were enumerated. Data represent the mean of 4 mice $\pm$ SEM, **p<0.01, ***p<0.001.

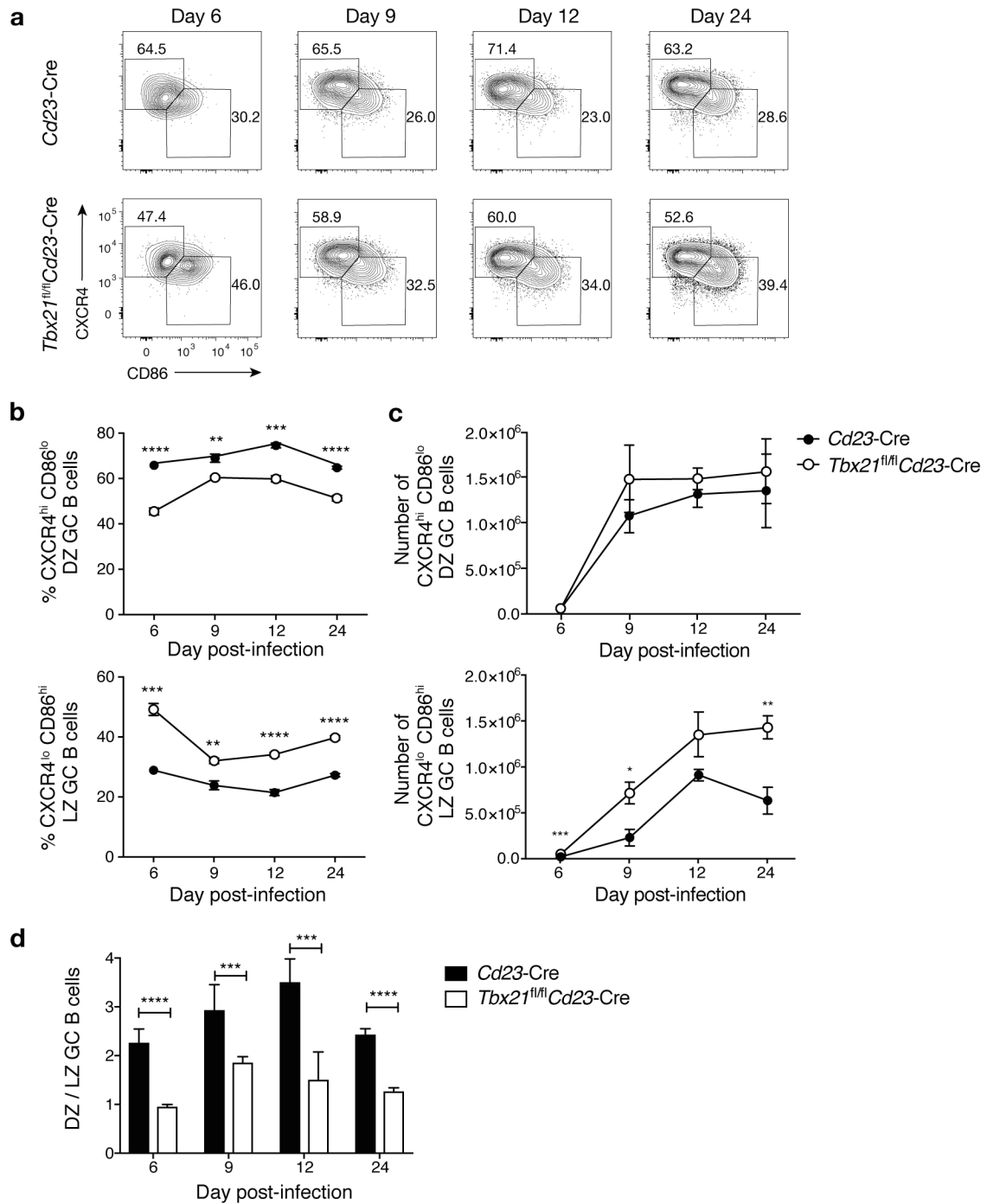


Figure 4.13 T-bet expression in B cells modulates GC B cell polarization

(a) Representative flow cytometry plots showing DZ ($CD86^{lo}CXCR4^{hi}$) and LZ ($CD86^{hi}CXCR4^{lo}$) cells among gated $CD19^{+}CD38^{lo}GL7^{+}$ GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. (b) The percentage, (c) absolute number and (d) ratio of DZ and LZ GC B cells were determined. Data are representative of three independent experiments, presented as the mean of 4 mice $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

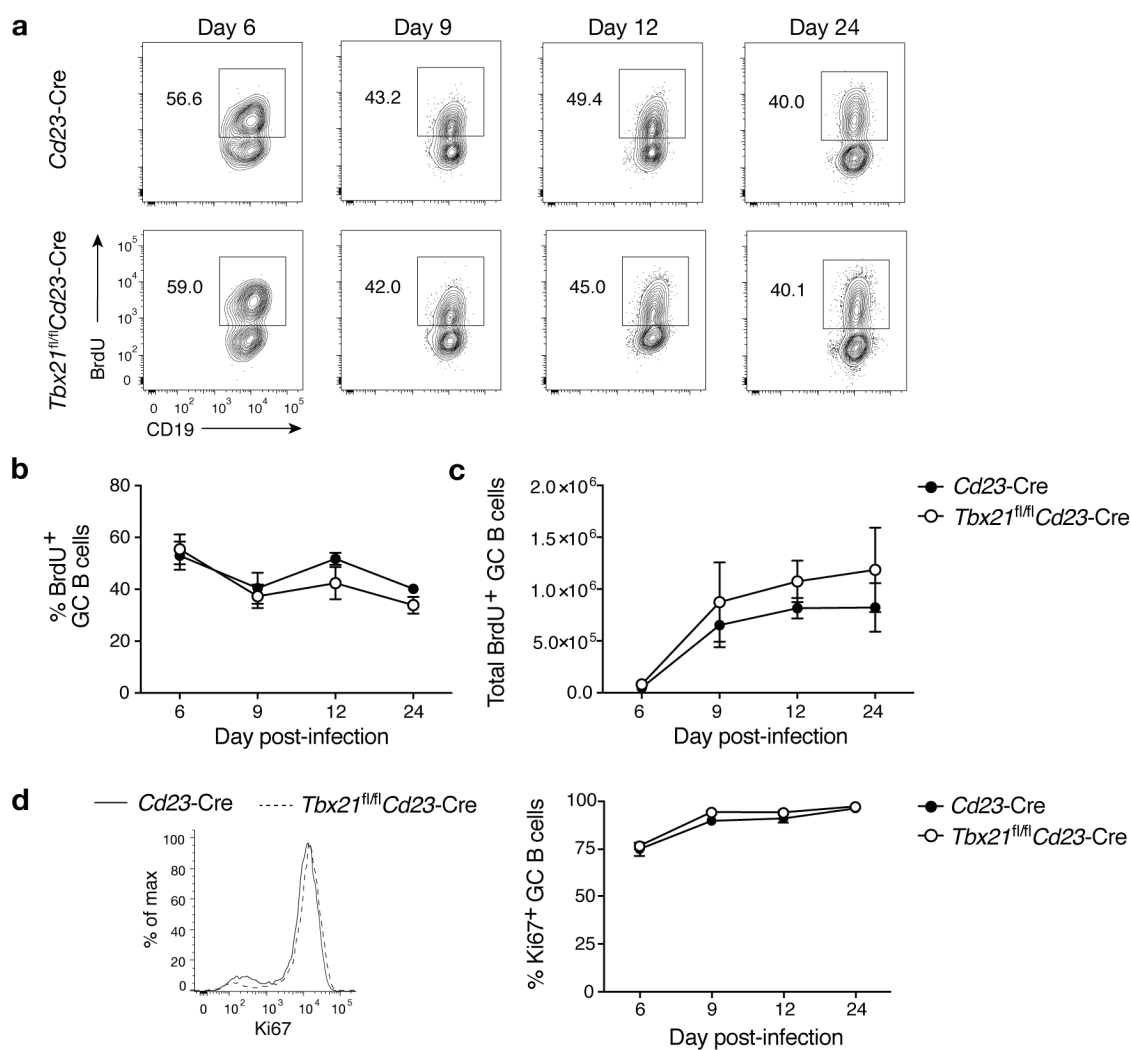


Figure 4.14 GC B cell proliferation is not affected by intrinsic T-bet expression

Tbx21^{fl/fl}Cd23-Cre and *Cd23-Cre* mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Mice were administered BrdU on different days and analyzed by flow cytometry. **(a)** Representative flow cytometry plots showing BrdU⁺ cells among CD38^{lo}GL7⁺ GC B cells. **(b)** The percentage and **(c)** absolute number of BrdU⁺ GC B cells were enumerated. **(d)** Ki67⁺ cells among CD38^{lo}GL7⁺ GC B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice. Data represent the mean of 3-4 mice $\pm$ SEM.

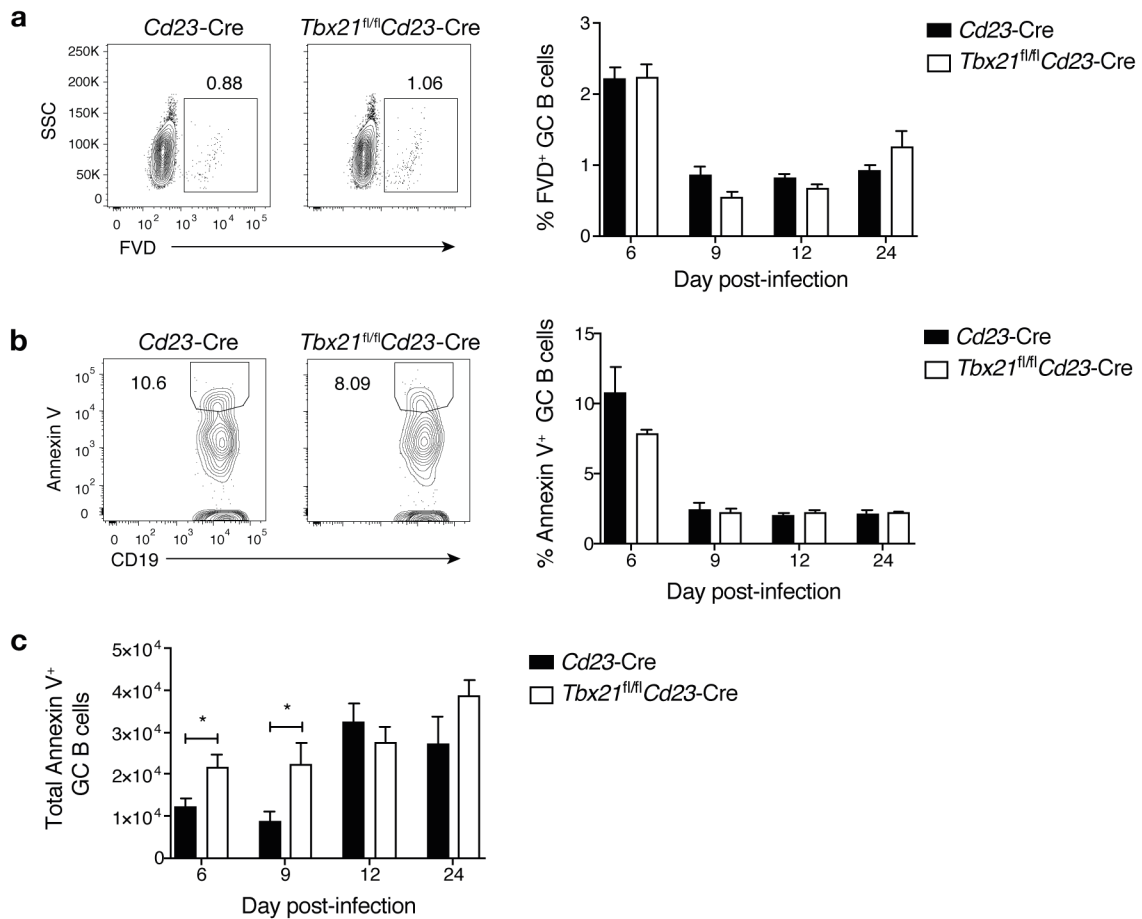


Figure 4.15 GC B cell apoptosis in the absence of T-bet expression in B cells

Tbx21^{fl/fl}Cd23-Cre and *Cd23-Cre* mice were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Representative flow cytometry plots and percentage of (a) non-viable Fixable Viability Dye (FVD)⁺ cells and (b) Annexin V⁺ cells among CD38^{lo}GL7⁺ GC B cells. (c) The absolute number of Annexin V⁺ GC B cells were enumerated. Data represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$.

Results

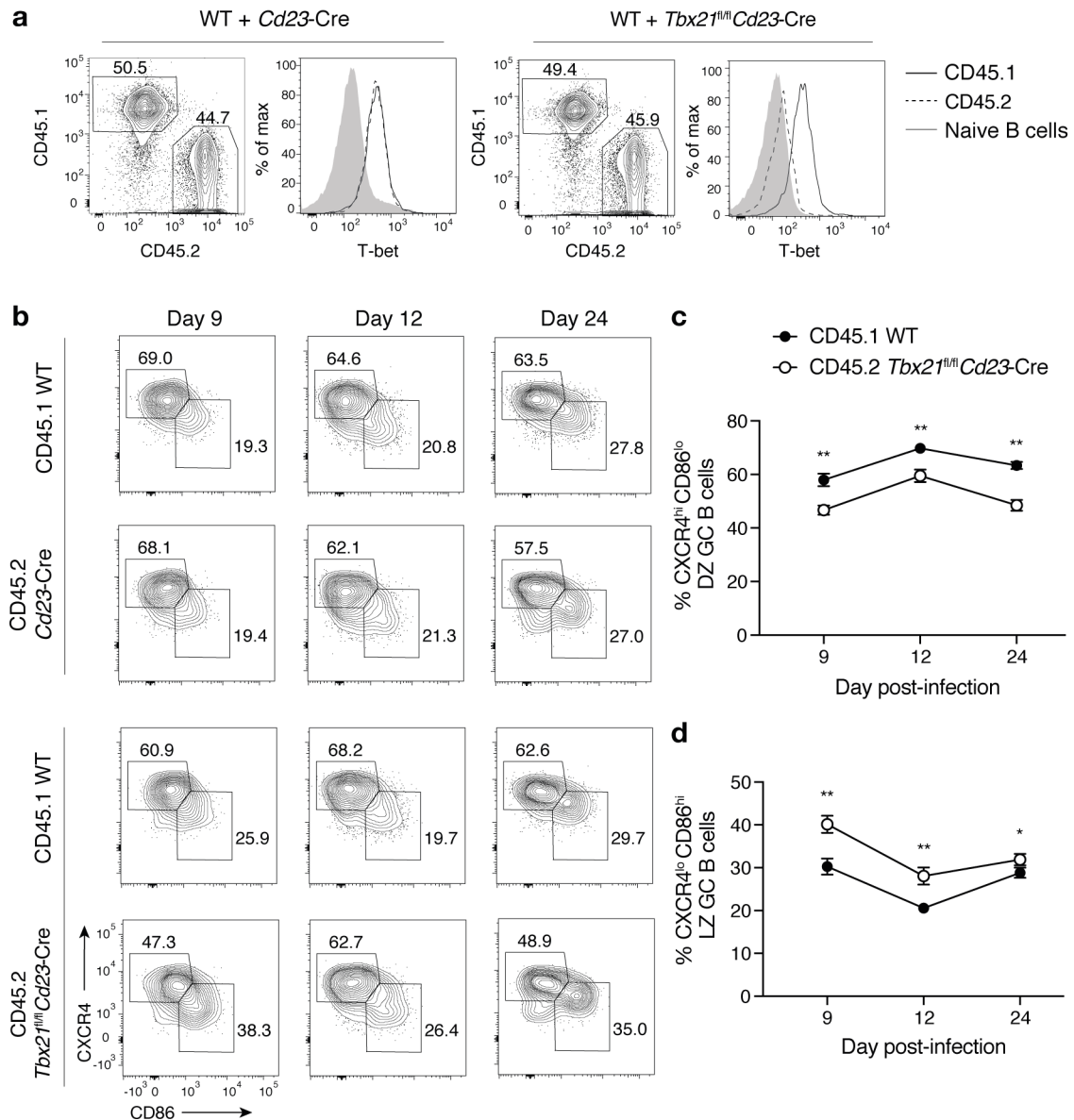


Figure 4.16 T-bet modulates GC DZ LZ dynamics in a cell-intrinsic manner

Irradiated CD45.1 mice were reconstituted with a 1:1 mix of CD45.1⁺ wild-type (WT) and CD45.2⁺ *Cd23-Cre* or CD45.2⁺ *Tbx21^{fl/fl}Cd23-Cre* bone marrow. **(a)** Representative flow cytometry plots showing CD45.1/2 expression among lymphocytes in reconstituted chimeras. T-bet expression of CD38^{lo}GL7⁺ GC B cells on day 9 pi in chimeras that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Naive B cells are shown as a control. **(b)** DZ (CD86^{lo}CXCR4^{hi}) and LZ (CD86^{hi}CXCR4^{lo}) GC B cells among the CD45.1⁺ and CD45.2⁺ compartments in control (top panel) and *Tbx21^{fl/fl}Cd23-Cre* mixed chimeras (bottom panel) at different times pi. The percentage of **(c)** DZ and **(d)** LZ GC B cells were enumerated in the *Tbx21^{fl/fl}Cd23-Cre* mixed chimeras. Data represent the mean of 4 mice $\pm$ SEM. Paired student's t-test was used to calculate statistical significance, * $p < 0.05$, ** $p < 0.01$.

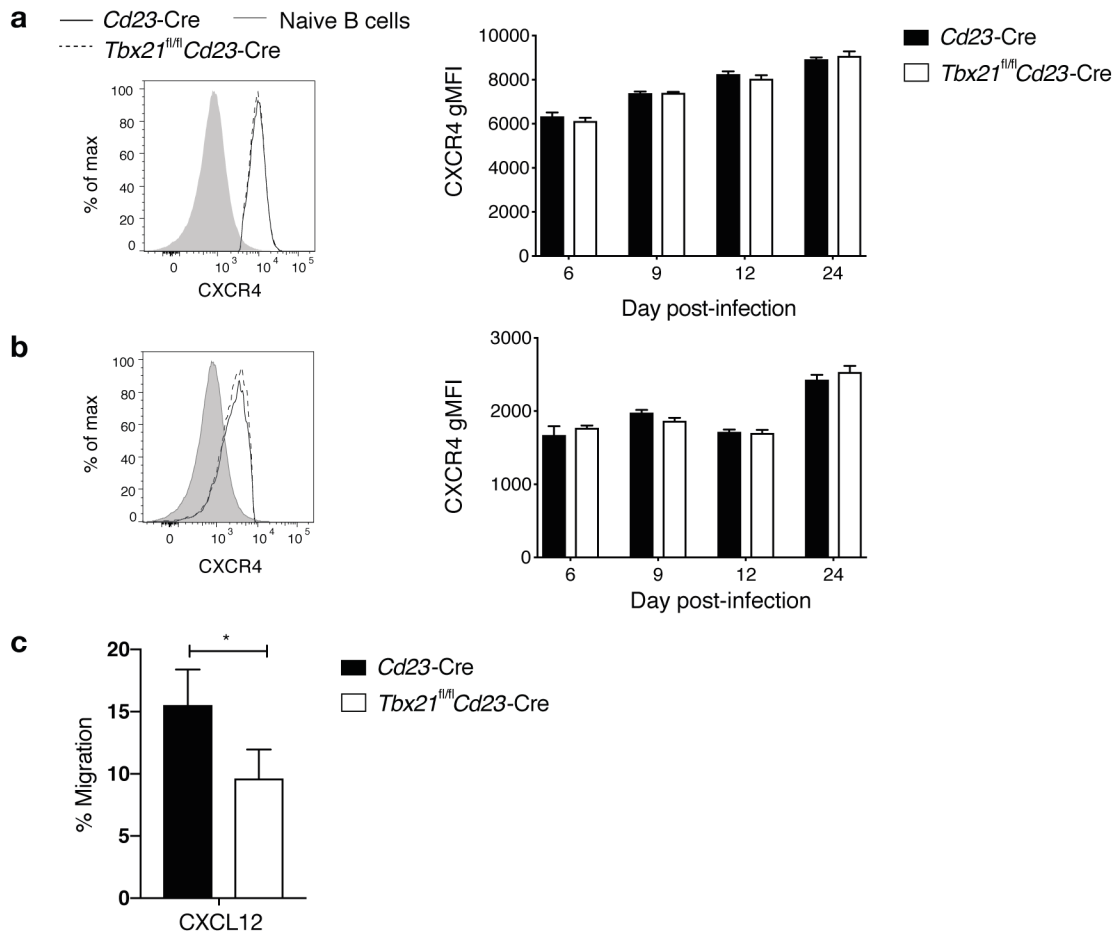


Figure 4.17 T-bet expression in GC B cells enhances migration to CXCL12 chemokine

Flow cytometry analysis of CXCR4 expression among gated **(a)** CD86^{lo}CXCR4^{hi} DZ GC B cells and **(b)** CD86^{hi}CXCR4^{lo} LZ GC B cells in *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. Naive B cells are shown as a control. **(c)** Chemotaxis of GC B cells to recombinant CXCL12 on day 12 pi was assessed *in vitro*. Data are representative of **(a, b)**, or pooled from **(c)** two experiments, presented as the mean of 4-7 mice $\pm$ SEM, * $p < 0.05$.

Results

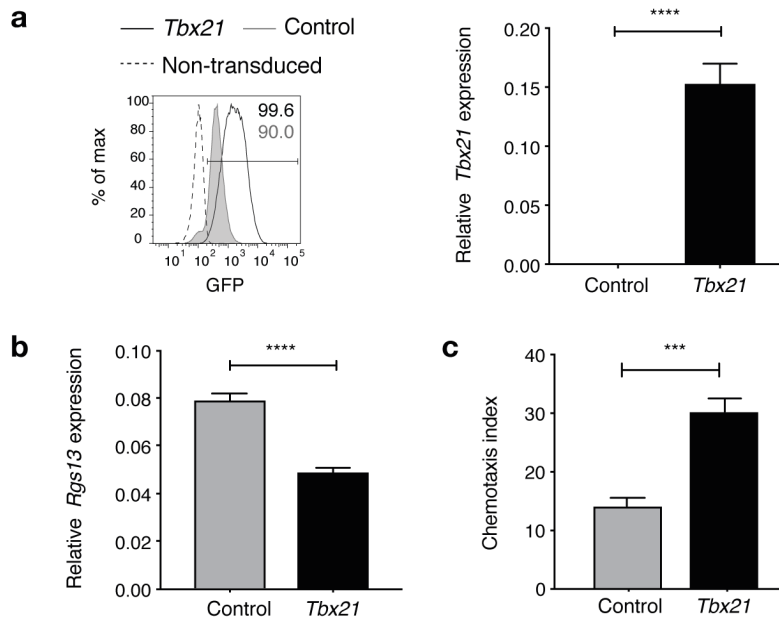


Figure 4.18 T-bet modulates CXCR4-mediated migration by regulating *Rgs13* expression

HS-Sultan cells were transfected with GFP-tagged *Tbx21*-expressing or control lentivirus. **(a)** Transduction efficiency was determined by flow cytometry analysis of GFP expression levels, and relative *Tbx21* expression was measured by quantitative RT-PCR. **(b)** Relative *Rgs13* expression in transduced HS-Sultan cells by quantitative RT-PCR. **(c)** Chemotaxis of transduced cells to recombinant human CXCL12 was assessed *in vitro*. Data are representative of two independent experiments, presented as the mean of 3-5 replicates $\pm$ SEM, *** $p < 0.001$, **** $p < 0.0001$.

Chapter 5

The effect of T-bet expression in B cells on antibody affinity maturation and the induction of B cell memory in malaria

1. Introduction

One of the hallmarks of a productive GC response is the progressive affinity maturation of the antibody repertoire over time (Eisen and Siskind, 1964), which is translated into humoral immunity by the differentiation of high-affinity antibody-secreting plasma cells and MBCs. Antigen-binding affinity and T cell-derived signals instruct the terminal differentiation of selected GC B cells into these memory populations (Shinnakasu *et al.*, 2016; Wang *et al.*, 2017; Ise *et al.*, 2018). Their generation has been suggested to occur in a temporal fashion, with IgG⁺ MBCs generated mostly in the early to peak stages of the GC response, while long-lived plasma cells that reside in the bone marrow develop much later (Weisel *et al.*, 2016; Shinnakasu *et al.*, 2016).

Epidemiological studies to date assessing B cell memory responses in malaria suggests that its generation and/or maintenance is largely impaired. This is exemplified by the short-lived nature of antibody responses against blood-stage *Plasmodium* antigens in the absence of sustained parasite exposure (Kinyanjui *et al.*, 2007; Akpogheneta *et al.*, 2008), while infrequent malaria infections can induce long-lived antibodies (Drakeley *et al.*, 2005; Bousema *et al.*, 2010; Wipasa *et al.*, 2010). Circulating MBCs are often detected at low frequencies and develop incrementally with age and exposure in high transmission areas (Dorfman *et al.*, 2005; Weiss *et al.*, 2010; Nogaro *et al.*, 2011;

Wendel *et al.*, 2017; Triller *et al.*, 2017b). Moreover, recurrent malaria infections are accompanied by an expansion of atypical MBCs that have impaired function *in vitro* (Weiss *et al.*, 2009; 2011; Portugal *et al.*, 2015; Sullivan *et al.*, 2015). One study found that atypical MBCs express elevated levels of the transcription factor T-bet, which associate with increased clinical malaria episodes in children (Obeng-Adjei *et al.*, 2017), suggesting T-bet may modulate MBC development and/or function in malaria.

T-bet is required for B cell differentiation into IgG2c⁺ plasma cells and MBCs in mice (Mohr *et al.*, 2010; Wang *et al.*, 2012), and has been implicated in the generation or function of age-associated B cells (Rubtsov *et al.*, 2013; Rubtsova *et al.*, 2017; Du *et al.*, 2019), which may contribute to pathogenic antibody production in autoimmunity and ageing (Hao *et al.*, 2011; Rubtsov *et al.*, 2011; Rubtsova *et al.*, 2013; 2017; Wang *et al.*, 2018). In malaria, T-bet has recently been detected following murine infection in short-lived B cells that share a similar phenotype to the aforementioned atypical human MBCs (Pérez-Mazliah *et al.*, 2018). However, whether T-bet plays a role in regulating the generation of B cell memory to malaria infection has not been extensively investigated. The previous chapter uncovered a B cell-intrinsic role of T-bet in regulating the dynamics of the GC reaction following *P. berghei* ANKA malaria. We propose that the altered GC polarization may influence affinity maturation of antibody responses to infection. In this chapter, we sought to investigate the consequence of B cell-specific T-bet-mediated regulation of the generation and affinity maturation of humoral immune memory.

2. Results

2.1 B cell-intrinsic expression of T-bet delays MBC differentiation

Our findings from the previous chapter indicated that T-bet restricts development of the GC LZ and promotes the DZ program in a B cell-intrinsic manner. We therefore hypothesized that T-bet may favour GC B cell cycling to the DZ instead of their terminal differentiation into memory populations. The chemokine receptor CCR6 has been described to mark LZ GC B cells poised for differentiation into MBCs, representing a population of precursor MBCs (Suan *et al.*, 2017). Hence, we sought to examine the generation of CCR6⁺ GC B cells following drug-cured malaria infection as a surrogate measure of GC B cell commitment to the MBC fate. Consistent with previous findings (Suan *et al.*, 2017), flow cytometry analysis showed that CCR6⁺ cells were enriched in MBCs and the LZ GC compartment, but not in the DZ (**Figure 5.1a**). CCR6⁺ LZ B cells exhibited phenotypic characteristics indicative of transition into memory cells, such as high CD38 and CCR6 expression, emerging transiently at an early time point, and then declined to a low frequency by day 24 (**Figure 5.1b,c**). The induction of CCR6⁺ GC LZ B cells was then compared between *P. berghei* ANKA-infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice (**Figure 5.1d,e**). Although no significant differences were detected in the frequencies of CCR6⁺ GC LZ B cells between both groups of mice, *Tbx21^{fl/fl}Cd23-Cre* mice had twice the number of these cells compared to controls as early as day 8 pi, lending support to the idea that altered GC dynamics in the presence of T-bet may restrain a premature differentiation of MBCs.

To extend these observations, we examined the production of IgG1- and IgG2c-expressing MBCs in the absence of T-bet expression in B cells at different times after infection by flow cytometry (**Figure 5.2a**). In line with the role of T-bet in driving IgG2c MBC formation (Wang *et al.*, 2012), *Tbx21^{fl/fl}Cd23-Cre* mice did not develop IgG2c⁺ MBCs, but instead, generated increased numbers of IgG1⁺ MBCs compared to control mice as early as day 9 pi (**Figure 5.2b**). The magnitude of the IgG1⁺ MBC response remained significantly higher in *Tbx21^{fl/fl}Cd23-Cre* mice than that of control mice up to day 24 ($p < 0.05$). Conversely, *Cd23-Cre* mice induced MBCs primarily expressing IgG2c, and to a lesser extent, IgG1, which were only detected one week after anti-malarial treatment. Therefore, the generation of MBCs in the presence of T-bet expression appears delayed, taking several weeks to develop after clearance of infection.

The generation of ASCs was also analysed in *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice following infection (**Figure 5.3a**). The frequencies and numbers of CD138⁺ ASCs in both groups of mice peaked on day 9 pi (**Figure 5.3b,c**). While there was a trend of increased ASCs in *Cd23-Cre* mice compared to *Tbx21^{fl/fl}Cd23-Cre* mice, no significant differences were observed between both

groups at the time points analysed. However, the number of IgG1⁺ ASCs were significantly higher in *Tbx21^{fl/fl}Cd23-Cre* mice compared to controls, whereas *Cd23-Cre* mice generated more IgG2c⁺ ASCs and minimal IgG1⁺ ASCs ($p < 0.05$ for all time points; **Figure 5.4**). Similar trends were observed when IgG1 and IgG2c antibodies against *P. berghei* ANKA lysate was assessed in the serum of infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice by ELISA (**Figure 5.5**). The altered IgG subclass distribution in these mice associated with slightly reduced total parasite-specific IgG antibody titres in *Tbx21^{fl/fl}Cd23-Cre* mice compared to controls. Collectively, these data indicate that T-bet drives IgG2c class-switching and delays the kinetics of MBC differentiation.

2.2 T-bet modulates affinity maturation of the GC response to malaria infection

We then sought to investigate whether the over-representation of the GC DZ compartment in the presence of T-bet during infection may subsequently affect affinity maturation of the GC response. We first examined the SHM rates of isolated GC B cells from *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* control mice. As the locations of mutations induced by *P. berghei* ANKA infection within *IghV* genes are unknown, we utilised a bioinformatics approach to identify them within *IghV* exons by RNA-sequencing on days 10 and 15 pi. The selection of these time points enabled analysis of GC B cells at a time when GCs begin to form in the spleen, and the assessment of changes in SHM rates over time with the resolution of infection. In total, we identified 61 dominant mutations (>3-fold higher frequency than that of naive follicular B cell sequences) in the *IghV* genes of GC B cells induced by infection (**Figure 5.6**). Consistent with antigen-driven positive selection, 96% of these mutations increased in frequency from day 10 to 15 pi (**Figure 5.6a,b**). On day 10 pi, 67% of detected mutations were found at similar frequency between *Tbx21^{fl/fl}Cd23-Cre* and control mice, 23% were significantly increased and 10% were decreased in the absence of T-bet (**Figure 5.6c**). In contrast, on day 15 pi, the overall frequency of mutations was significantly higher in GC B cells of controls compared to *Tbx21^{fl/fl}Cd23-Cre* mice ($p = 0.0365$), with 36% of mutations significantly reduced in the absence of T-bet expression (**Figure 5.6d**).

To examine the change in mutation frequency over time, we concentrated on mutations that displayed clear evidence of selection over time. Two types of mutations were identified: 1) “highly selected mutations”, which showed <3% low frequency on day 10 pi. and increased by at least 5-fold to a minimum frequency of 5% from day 10-15 pi, and 2) “early mutations”, which exhibited >5% frequency on day 10 pi (**Figure 5.7a,b**). Strikingly, changes in the mutation frequency for highly selected mutations were significantly lower in GC B cells of *Tbx21^{fl/fl}Cd23-Cre* mice than controls (**Figure 5.7c**). On the other hand, changes in the early mutation frequency were significantly higher in GC B cells of *Tbx21^{fl/fl}Cd23-Cre* mice compared to controls (**Figure 5.7d**).

Results

Together, these data show that T-bet limits SHM of GC B cells early during infection, but promotes the acquisition of highly selected mutations later during the GC response.

We extended our analysis by measuring the avidity of antibodies generated against *P. berghei* ANKA MSP-1₁₉ over the course of infection using an ELISA-based approach. MSP-1₁₉ is a key target of protective antibodies against malaria infection in mice (Daly and Long, 1993; Ling *et al.*, 1994). Sera from infected mice were measured against a gradient concentration of *P. berghei* ANKA MSP-1₁₉ recombinant protein, and the concentration at which 50% of the maximal response occurred (IC₅₀) was deduced as a measure of antibody avidity (**Figure 5.8a**). Accordingly, low avidity antibodies present at day 9, were only detected at relatively high protein concentrations and had a high IC₅₀ value. Sera avidity of both *Tbx21*^{fl/fl}*Cd23*-Cre and control mice gradually increased from day 9-24 pi (**Figure 5.8b,c**). However, from day 12 onwards, antibody avidity of *Tbx21*^{fl/fl}*Cd23*-Cre mice was significantly lower than that of control mice (**Figure 5.8c**). Together, these results indicate that T-bet expression in B cells enhances the affinity maturation of the GC B cell response that give rise to high avidity antibodies against infection.

3. Discussion

GCs are the principal site of antibody diversification and affinity maturation during infection. Thus, alterations in the cellular dynamics of the GC reaction can impact the quality and magnitude of humoral memory. The absence of T-bet expression in B cells increased the generation of CCR6⁺ LZ GC B cells early following *P. berghei* ANKA infection. These cells have been described to represent a population of precursor MBCs that are selected to exit from the GC LZ (Suan *et al.*, 2017). Consistent with the enrichment of the LZ development in the absence of T-bet in B cells (**Chapter 4**), our finding suggests that T-bet favours GC B cell cycling over early MBC differentiation. In support, IgG⁺ MBCs were generated with delayed kinetics in the presence of T-bet, and only detectable in considerable numbers after infection clearance.

The absence of T-bet expression in B cells did not appear to influence the development of CD138⁺ splenic ASCs in response to drug-cured *P. berghei* ANKA infection, consistent with other murine studies of blood-stage malaria (Castillo-Méndez *et al.*, 2007; Stephens *et al.*, 2009; Nduati *et al.*, 2010). Our finding suggests that intrinsic T-bet expression in B cells does not contribute to the overall magnitude of the ASC response to *P. berghei* ANKA infection, and supports a previously described dominant role of T cell help (Ryg-Cornejo *et al.*, 2016a). Interestingly, a recent study found that elevated T-bet expression in GC B cells upon deletion of the transcription factor c-Myb, drove an aberrant differentiation of GC B cells into plasmablasts in response to protein

immunization (Piovesan *et al.*, 2017). This raises the question as to whether skewed GC polarisation towards a DZ phenotype during infection similarly results in aberrant plasma cell differentiation. Without further phenotypic characterisation of the detected ASCs in *Tbx21^{fl/fl}Cd23-Cre* mice, it is not possible to infer whether B cell-intrinsic T-bet expression controls the development of GC-derived plasma cells. Closer analysis of the ontogeny of long-lived plasma cells in the spleen and those that subsequently emigrate to the bone marrow using antigen-specific cell detection methods, and markers like Blimp-1, may allow specific tracking of short- and long-lived ASCs (Kallies *et al.*, 2004) that arise from the altered GC dynamics in malaria infection. Additionally, the IgG1-biased antibody profile in the absence of T-bet expression in B cells aligns with previous findings that T-bet-deficient mice display increased IgG1 antibody responses to infection (Oakley *et al.*, 2014), and further supports the B cell-intrinsic requirement of T-bet for IgG2c class-switching of plasma cells (Mohr *et al.*, 2010; Wang *et al.*, 2012).

T-bet-expressing GC B cells induced by malaria infection showed evidence of enhanced affinity maturation, as they were acquired at higher frequency of positively selected *IghV* gene mutations over time, and produced antibodies with higher avidity to *P. berghei* ANKA. This was not likely to stem from enhanced AID activity, as *Aicda* expression was unaltered in the absence of T-bet (**Chapter 4**). Instead, as discussed in Chapter 4, our results are consistent with the notion that T-bet supports a transcriptional program that facilitates DZ re-entry of GC B cells for further cycles of SHM that enhance high affinity antibody production. Thus, our results imply that T-bet may drive a gradual increase in antibody affinity over time. Interestingly, incremental increases in antibody avidity to MSP-1 have previously been detected following *P. chabaudi* AS infection (Achtman *et al.*, 2007). In agreement with our data, increasing age or repeated exposure in malaria-exposed populations associate with increases in antibody avidity to *P. falciparum* blood-stage antigens (Ferreira and Katzin, 1995; Wipasa *et al.*, 2010; Ibison *et al.*, 2012). Furthermore, *IghV* genes isolated from MBCs of malaria-exposed individuals contain evidence of SHM (Muellenbeck *et al.*, 2013; Zinöcker *et al.*, 2015; Triller *et al.*, 2017a) that appear to undergo further diversification upon acute malaria infection in children (Wendel *et al.*, 2017).

Results

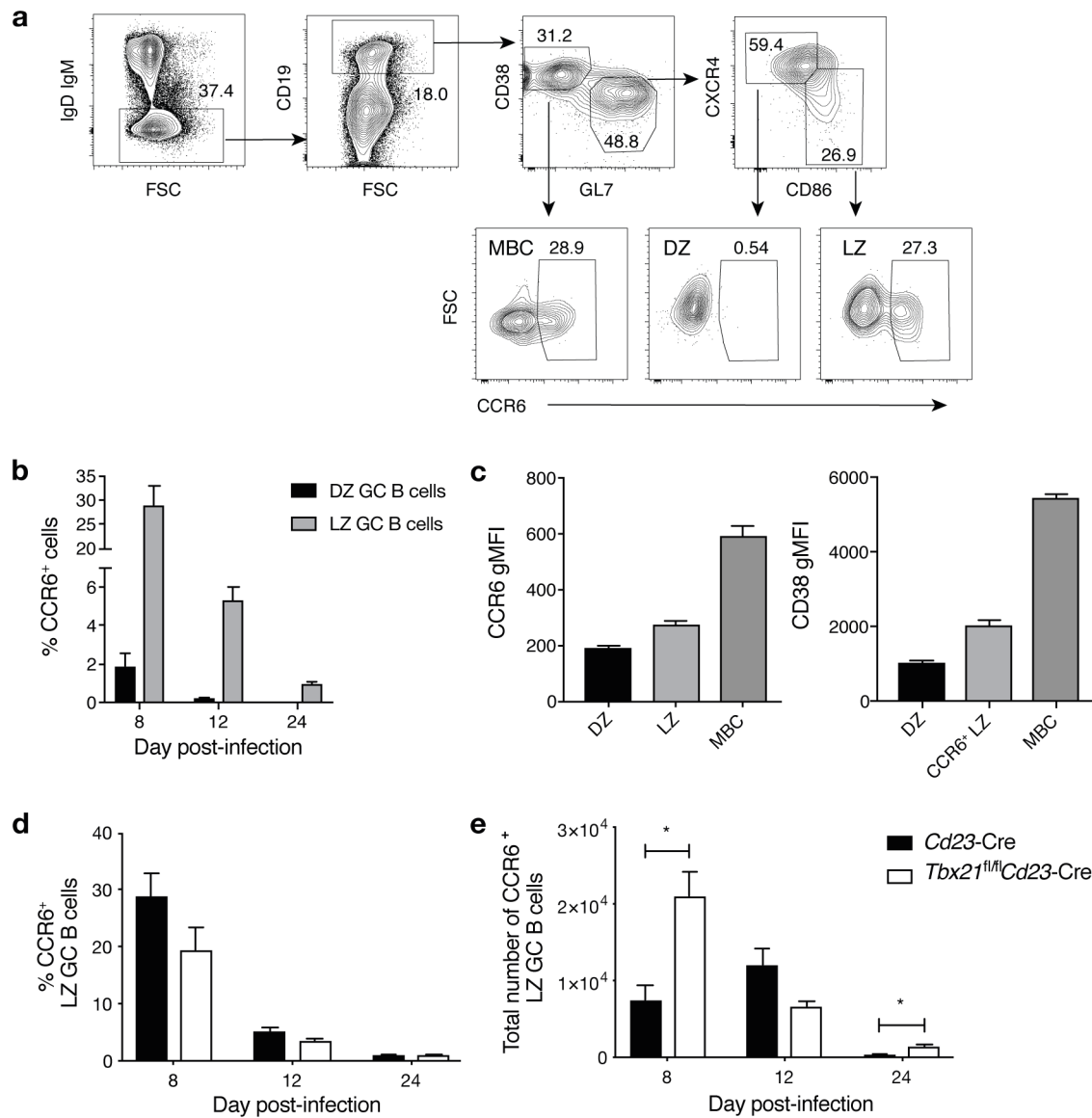


Figure 5.1 The development of CCR6⁺ LZ GC B cells following *P. berghei* ANKA infection

(a) Gating strategy used to identify CCR6⁺ cells in gated CD38^{hi}GL7⁻ MBCs, DZ (CD86^{lo}CXCR4^{hi}) and LZ (CD86^{hi}CXCR4^{lo}) CD38^{lo}GL7⁺ GC B cells among IgD⁻IgM⁻CD19⁺ B cells. (b) The percentage of CCR6⁺ DZ and LZ cells in wild-type mice infected with *P. berghei* ANKA and treated with anti-malarial drugs. (c) CCR6 and CD38 gMFI were assessed among the B cell subsets. (d) The percentage and (e) absolute number of CCR6⁺ LZ GC B cells were enumerated in *P. berghei* ANKA-infected *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice that were treated with anti-malarial drugs. Data represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$.

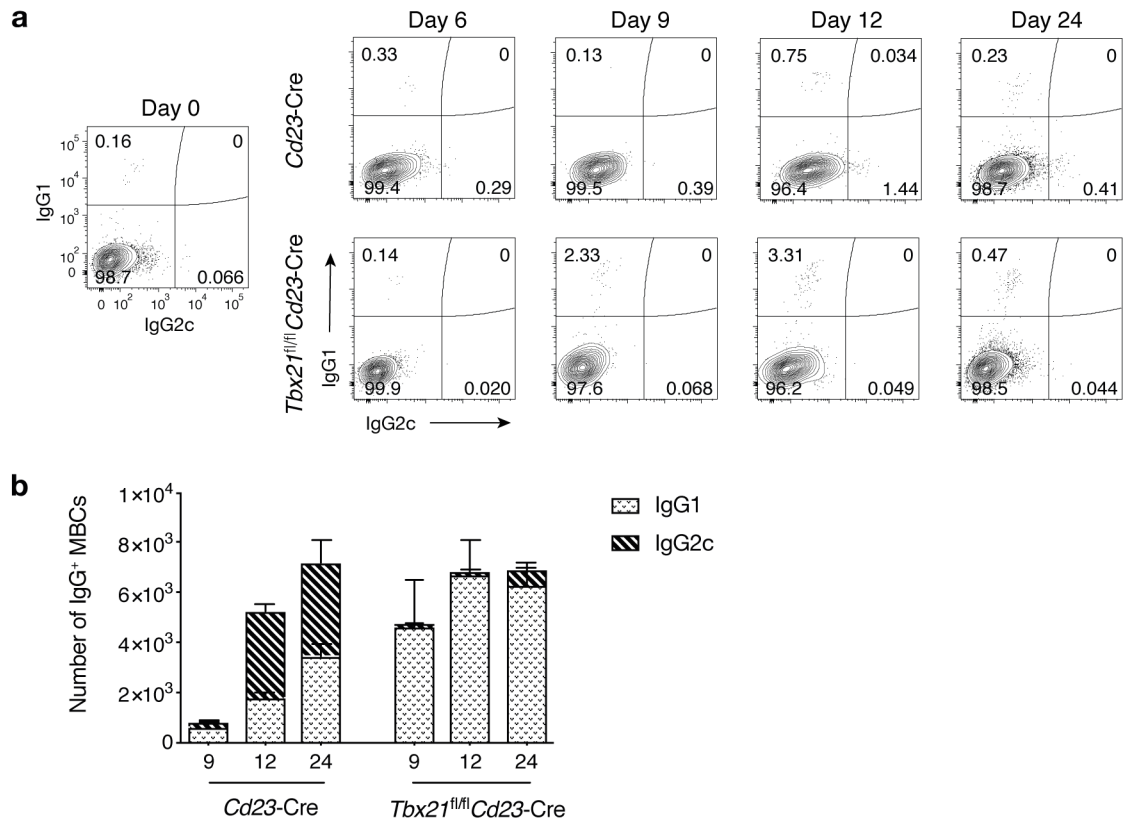


Figure 5.2 B cell-intrinsic T-bet expression influences the induction of IgG⁺ memory B cells

(a) Representative flow cytometry plots showing IgG1⁺ versus IgG2c⁺ expression of gated CD38^{hi}FasIgD⁺IgM⁺CD138⁺CD19⁺ MBCs of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The absolute number of (b) IgG1⁺ and (c) IgG2c⁺ MBCs were enumerated on different days. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM.

Results

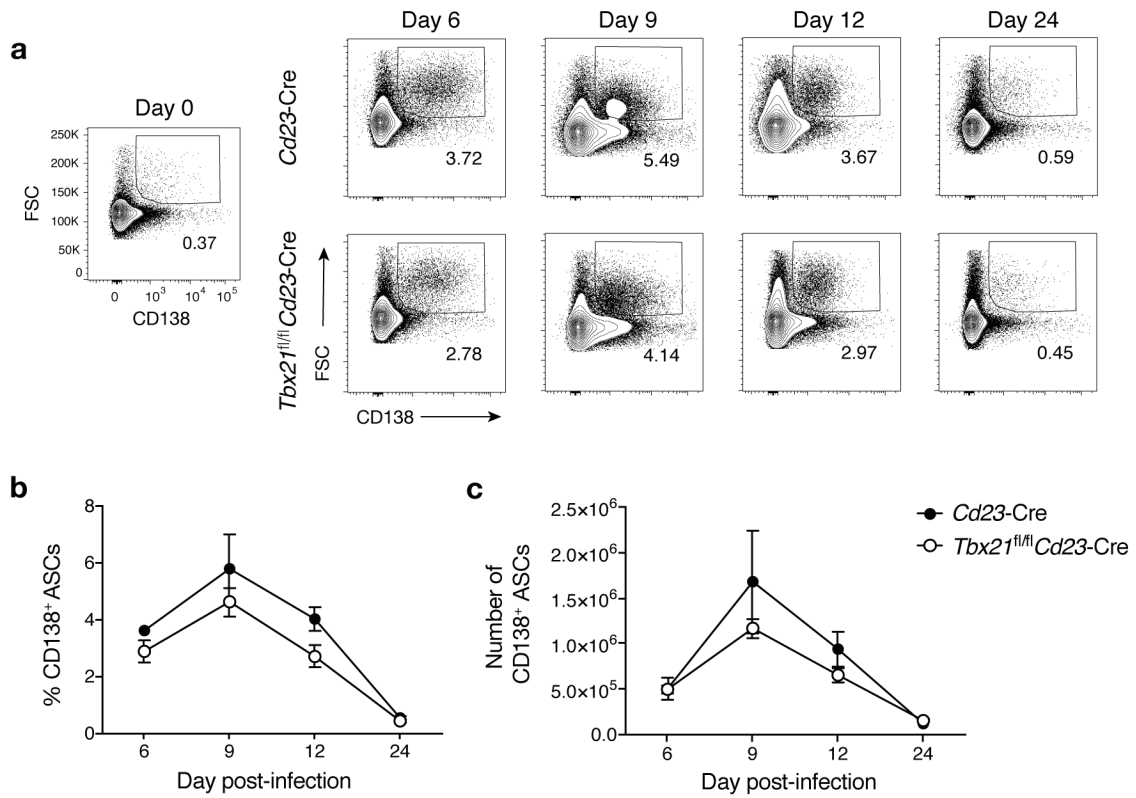


Figure 5.3 The generation of ASCs in the absence of B cell-intrinsic T-bet expression

(a) Representative flow cytometry plots showing CD138⁺ ASCs among gated CD19⁺ B cells of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The percentage (b) and absolute number (c) of CD138⁺ ASCs were enumerated. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM.

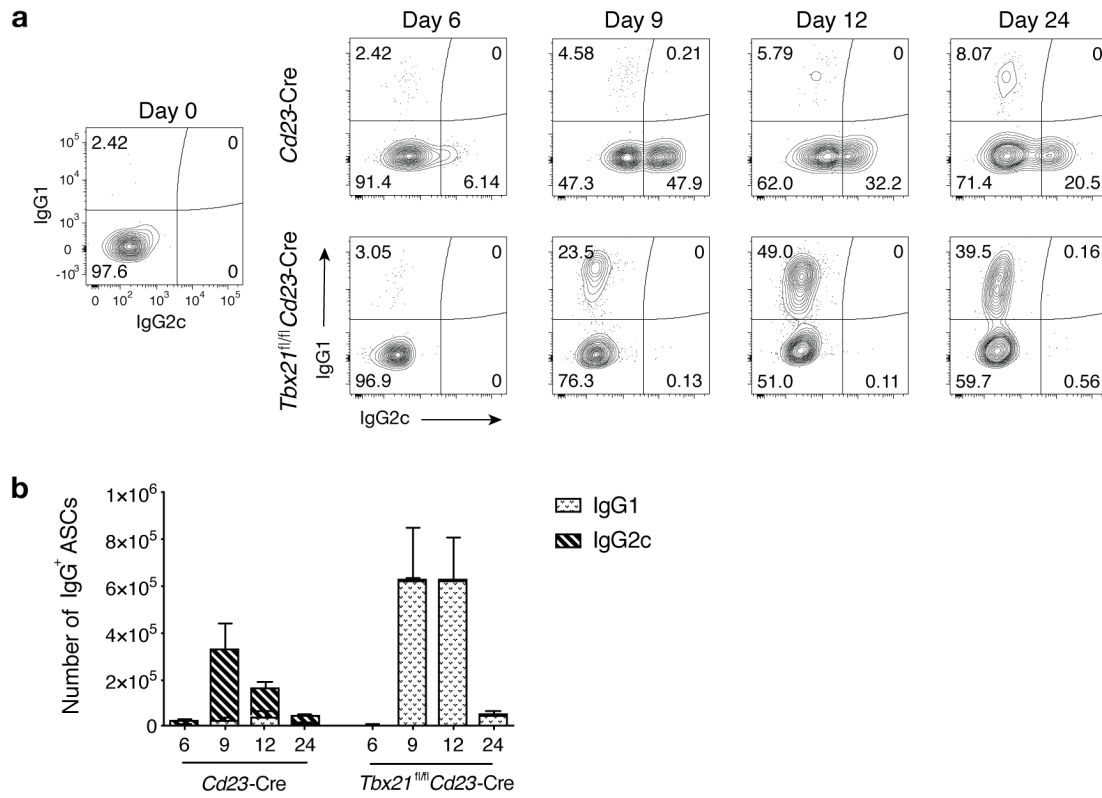


Figure 5.4 B cell-intrinsic T-bet regulates the class switching of ASCs

(a) Representative flow cytometry plots showing intracellular IgG1⁺ versus IgG2c⁺ expression of gated CD138⁺IgD⁻IgM⁻CD19⁺ ASCs of *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice that were infected with *P. berghei* ANKA and treated with anti-malarial drugs. The absolute number of (b) IgG1⁺ and (c) IgG2c⁺ ASCs were enumerated on different days. Data are representative of two independent experiments, presented as the mean of 4 mice $\pm$ SEM.

Results

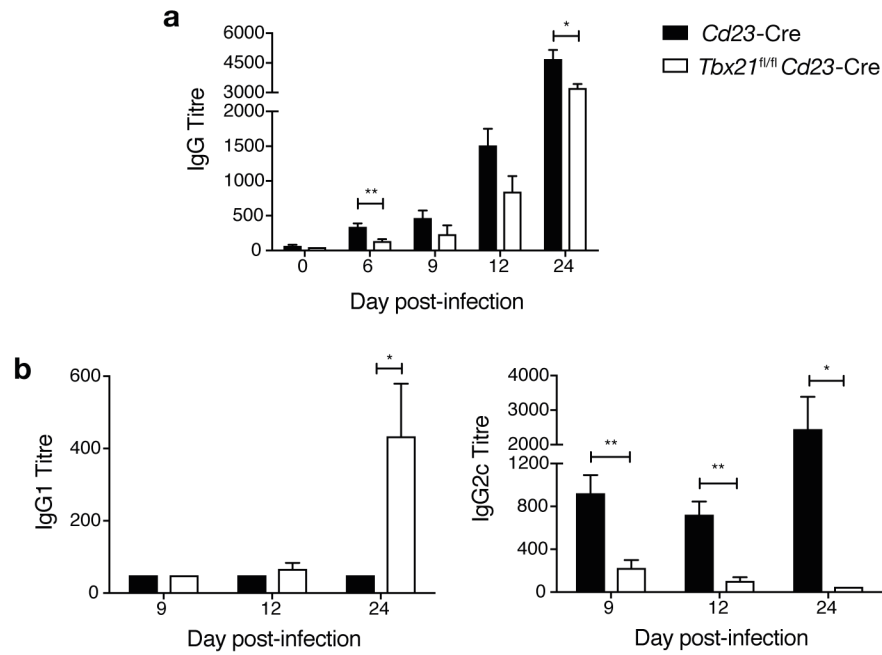


Figure 5.5 *P. berghei* ANKA-specific antibody responses in the absence of B cell-intrinsic T-bet expression

Sera were obtained from *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice at different times after infection with *P. berghei* ANKA and anti-malarial drug treatment. *P. berghei* ANKA lysate-specific (**a**) total IgG, (**b**) IgG1 and IgG2c titres were measured by ELISA. Data represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$.

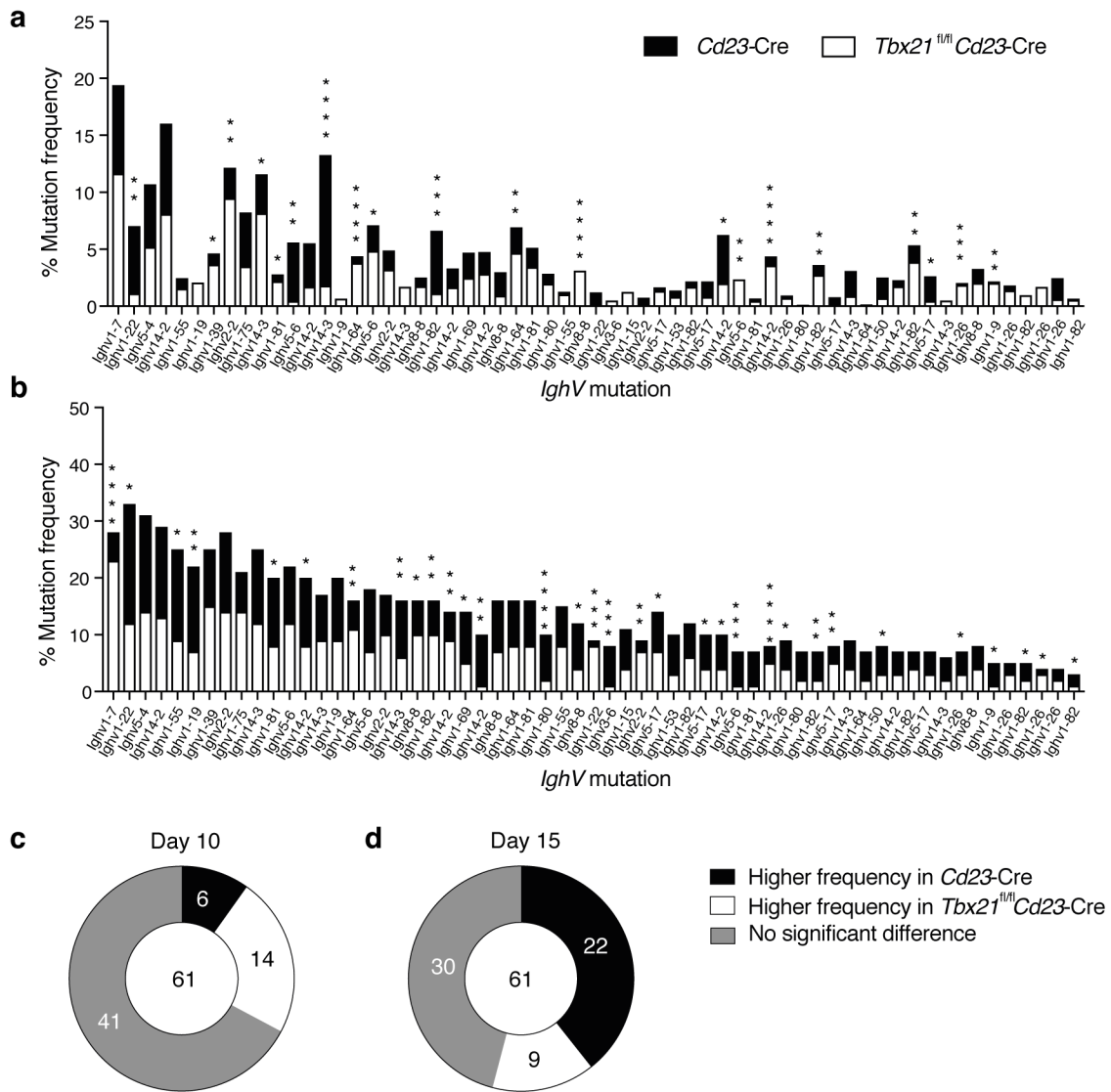


Figure 5.6 T-bet expression in B cells promotes somatic hypermutation of GC B cells

RNA sequences from sorted GC B cells of *P. berghei* ANKA-infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice were analysed for *IgHV* mutations using exact SNP 1.6.0. Frequencies of dominant mutations in individual *IgHV* genes on (a) day 10 and (b) day 15 pi. Different scaling has been used for the y-axis of the two graphs. Doughnuts show the proportion of dominant *IgHV* mutations at significantly higher frequency in *Cd23-Cre* mice, significantly higher frequency in *Tbx21^{fl/fl}Cd23-Cre* mice, or no significant difference on (c) day 10 and (d) day 15 pi. Numbers in the middle of the doughnuts indicate total mutations analysed and numbers along the perimeter denote mutations in each category. Data represent the mean of 3 independent replicates per group. Statistical significance assessed by Fisher's exact test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. See Appendix IV for a detailed list of the *IgHV* gene mutations.

Results

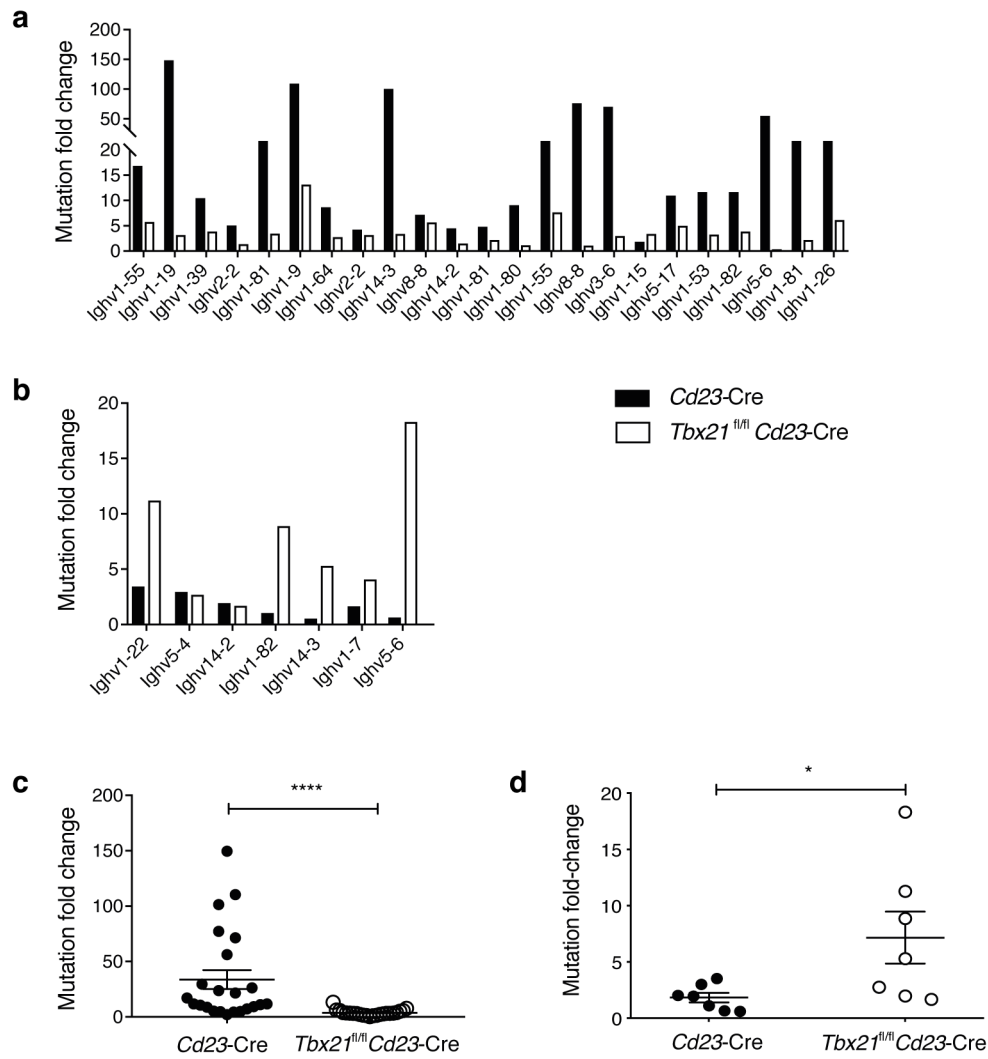


Figure 5.7 Malaria-driven B cell-intrinsic expression of T-bet modulates affinity maturation

Fold-change in *IgHV* gene mutation frequencies from day 10 to 15 pi in *P. berghei* ANKA-infected *Tbx21^{fl/fl}Cd23-Cre* and *Cd23-Cre* mice of (**a, c**) highly selected mutations and (**b, d**) early common mutations (not-highly selected). Data represent the mean of 3 mice $\pm$ SEM, * $p < 0.05$ and **** $p < 0.0001$.

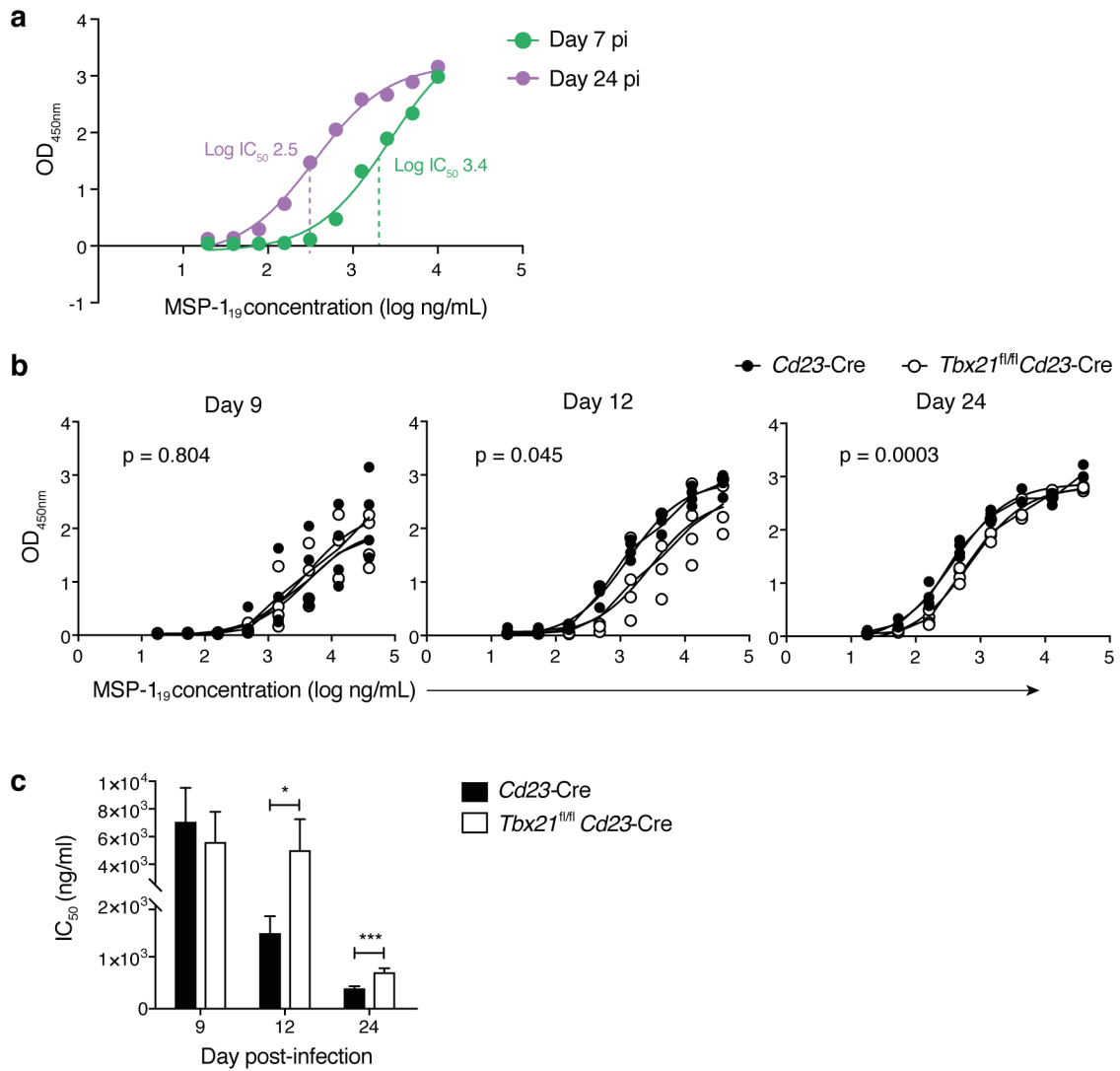


Figure 5.8 T-bet up-regulation in B cells promotes high-avidity antibody production

(a) Sera of wild-type mice infected with *P. berghei* ANKA and treated with anti-malarial drugs, were incubated with a gradient concentration of *P. berghei* ANKA MSP-1₁₉ recombinant protein. Avidity was inferred by determining IC₅₀ values of optical density (OD) curves measured by ELISA. (b) Avidity of sera from infected Tbx21^{fl/fl}Cd23-Cre and Cd23-Cre mice on different days pi were evaluated as outlined in (a). Non-linear regression models were fitted to OD values and best-fits for data compared by extra sum-of-squares F test. (c) IC₅₀ values were deduced for OD curves in (b). Data represent the mean of 4 mice $\pm$ SEM, * $p < 0.05$, *** $p < 0.001$.

Chapter 6

Concluding discussion

Long-standing evidence support that natural malaria infection is characterised by an inefficient acquisition of humoral immunity. The immune mechanisms underlying the development of protective B cell responses, however, are poorly defined. Our appreciation of acute malaria and the accompanying pro-inflammatory responses in modulating this process has grown over the last few years and is an area of intense scrutiny (Ly and Hansen, 2019). This thesis has uncovered a dual role of the Th1 transcription factor, T-bet, in regulating the dynamics of the GC reaction within CD4⁺ T and B cells to blood-stage malaria infection. T-bet up-regulation in CD4⁺ T cells directly interferes with Tfh cell differentiation, resulting in sub-optimal GC responses to infection. On the other hand, B cell-intrinsic expression of T-bet promotes its effective polarization, reinforcing commitment to the GC DZ reaction, thereby limiting early MBC differentiation and promoting somatic hypermutation, which enhance affinity maturation of the antibody response.

Dual expression of T-bet and Bcl-6 in activated CD4⁺ T cells enables flexibility in the gene expression programmes of Th1 and Tfh cells to support an immune response that is tailored against the pathogenic insult (Weinmann, 2014). While T-bet regulates the gene program unique to Th1 cells (Zhu *et al.*, 2012), strong pro-inflammatory environments that drive T-bet up-regulation in CD4⁺ T cells, have been found to antagonize Tfh cell differentiation in favour of Th1 cell responses to viral and parasitic infection (Marshall *et al.*, 2011; Nakayamada *et al.*, 2011; Fang *et al.*, 2018). A similar activation of Th1-like circulating Tfh cells that exhibit limited B cell helper function *in vitro*, have been detected in individuals with acute malaria (Hansen *et al.*, 2016; Figueiredo *et al.*, 2017). Moreover, neutralization of the pro-inflammatory cytokines IFN- γ and TNF, and global deletion of T-bet, which are highly induced during

malaria, promotes Tfh cell differentiation and improves GC responses to *P. berghei* ANKA infection (Ryg-Cornejo *et al.*, 2016a). Consistent with these data, we demonstrate that T-bet expression specifically in CD4⁺ T cells, and not B cells, inhibits Tfh cell differentiation, consequently impairing GC development. This strengthens the concept that the IFN- γ -T-bet axis compromises Tfh cell responses to support humoral immunity in malaria. Whether T-bet also influences the function of Tfh cells that persist in the memory phase (Zander *et al.*, 2017), or T follicular regulatory cells that are reported to suppress Tfh cell responses to malaria infection (Kurup *et al.*, 2017), remain to be investigated.

Enhanced Tfh cell differentiation in the absence of T-bet in CD4⁺ T cells improved splenic GC formation early following malaria infection, agreeing with a crucial role of Tfh cells in orchestrating the GC response in murine malaria (Pérez-Mazliah *et al.*, 2015; 2017). In contrast, the deletion of T-bet in B cells only modestly increased the magnitude of the GC response, and was evident only at later time points of infection, in line with a reported increase in GC B cells at a late stage of *P. yoelii* 17XNL infection upon T-bet deletion (Guthmiller *et al.*, 2017). Our findings also indicate a dispensable role of T-bet in controlling the early phase of the GC B cell response to infection, and aligns with other foreign antigen immunization and infection settings (Barnett *et al.*, 2016; Rubtsova *et al.*, 2017; Piovesan *et al.*, 2017). Thus, T-bet-mediated regulation of the magnitude of the GC response to malaria infection seems to be largely driven via T cell help, and to a lesser extent, intrinsic B cell activity, which increases the GC LZ compartment. In line with this notion, increased Tfh cells following CD4⁺ T cell-specific T-bet deletion augmented overall ASC and MBC responses to infection. Our findings provide further support for the use of immuno-therapies targeting inhibitory molecules such as PD-1 and LAG-3, to promote the induction of T cell help that may improve humoral immune responses in pre-clinical models of malaria (Butler *et al.*, 2011; Zander *et al.*, 2015; Karunarathne *et al.*, 2016; Kurup *et al.*, 2017; Zander *et al.*, 2017) and following *Plasmodium* immunisation (Liu *et al.*, 2015b). Further research would no doubt be needed to assess the feasibility of employing these biologics in endemic areas of malaria.

Iterative cycles of B cell migration between the GC dark and light zones are required for affinity maturation of the humoral immune response (Allen *et al.*, 2007; Schwickert *et al.*, 2007; Victora *et al.*, 2010; Gitlin *et al.*, 2014). While the LZ is continuously being re-populated by emigrating DZ GC B cells, only a fraction of them are selected to re-enter the DZ (Victora *et al.*, 2010; Meyer-Hermann *et al.*, 2012), and the remainder either leave the GC (Ise *et al.*, 2018) or die by apoptosis (Mayer *et al.*, 2017; Stewart *et al.*, 2018). The DZ and LZ programme of re-circulating GC B cells is likely controlled by a complex network of transcription factors, and defining these molecular regulators is an ongoing process. A few studies have identified that transcriptional regulators including c-Myc (Calado *et al.*, 2012; Dominguez-Sola *et al.*, 2012; Luo *et al.*, 2018), AP4 (Chou *et al.*, 2016) and Foxo1 (Sander *et al.*, 2015; Dominguez-Sola *et al.*, 2015) play a role in controlling GC dynamics. c-Myc is expressed in a small

subset of LZ GC B cells primed for re-entry into the DZ (Dominguez-Sola *et al.*, 2012; Luo *et al.*, 2018). c-Myc have been shown to induce the transcription factor AP4 in positively-selected GC B cells to undergo proliferation and SHM in the DZ (Chou *et al.*, 2016; Chen *et al.*, 2018). In addition, Foxo1 expression in selected LZ GC B cells instructs DZ formation (Sander *et al.*, 2015; Dominguez-Sola *et al.*, 2015; Inoue *et al.*, 2017). Importantly, cognate T cell signals are important in inducing these transcription factors for perpetuating the GC reaction (Dominguez-Sola *et al.*, 2012; Lin *et al.*, 2015; Ersching *et al.*, 2017; Luo *et al.*, 2018). This thesis reveals that in addition to T cell help, under pro-inflammatory conditions, T-bet modulates the transcriptional landscape of GC B cells to promote the DZ program. The absence of B cell-intrinsic T-bet expression did not appear to influence the expression of the aforementioned transcription factors; however, we found that T-bet directly limits GC B cell expression of *Rgs13*, a negative regulator of CXCL12-mediated signaling (Shi *et al.*, 2002). The CXCR4-CXCL12 axis is crucial for GC B cell localisation to the DZ (Allen *et al.*, 2004; Bannard *et al.*, 2013; Rodda *et al.*, 2015). T-bet suppression of *Rgs13* resulted in enhanced CXCL12-mediated migration, suggesting that T-bet promotes GC B cells cycling to the DZ for further rounds of SHM as opposed to early MBC differentiation. In agreement with this concept, B cell-specific T-bet deletion accelerated the generation of early, memory-like CCR6⁺ LZ GC B cells, as well as IgG1⁺ MBCs. Moreover, the absence of T-bet expression in B cells reduced *IghV* SHM, which in turn, diminished antibody avidity, further supporting the notion that T-bet prevents the premature exit of B cells from the GC reaction and enhances affinity maturation. Interestingly, mice deficient in RGS13 have been shown to exhibit abnormal positioning of proliferating B cells within the GC (Hwang *et al.*, 2013), which calls into question as to whether dark and light zone GC B cell positioning is altered during malaria infection. Intensely proliferating DZ B cells have previously been observed by histology in the GCs of *P. berghei* ANKA-infected mice (Carvalho *et al.*, 2007); however, a lack of appropriate markers precluded analysis of GC organisation in that study. In light of our finding that GC B cells up-regulate CXCR3, which may modulate their migration during infection, immunofluorescence analysis of intra-GC structure by staining for the markers CXCR4 and CD86 may reveal the precise location of dark and light zone GC B cells during infection.

The production of pro-inflammatory cytokines in other infectious diseases has been shown to contribute to a loss of secondary lymphoid tissue integrity that lead to impaired B cell migration (Engwerda *et al.*, 2002a; Mueller *et al.*, 2007) and GC formation (Gaya *et al.*, 2015). Furthermore, hypoxic conditions within the GC LZ has been shown to influence the threshold for GC selection (Cho *et al.*, 2016), and reduces high-affinity antibody generation (Cho *et al.*, 2016). Low oxygen tension has been demonstrated to attenuate mTORC1 signaling in B cells (Cho *et al.*, 2016), which impairs the anabolic capacity of LZ B cells to sustain clonal expansion in the DZ (Jones *et al.*, 2016; Ersching *et al.*, 2017). Similarly, alterations in B cell metabolism have been shown to reduce GC DZ B cell differentiation (Martinez-Martin *et al.*, 2017). Interestingly, Cho *et al.* showed that both hypoxia and restrained mTORC1 activity decrease T-bet expression and high-affinity IgG1 antibody responses to protein immunisation in a B

cell-intrinsic manner (Cho *et al.*, 2016). Whether inflammatory cues modulate the metabolic activity of B cells for the development of humoral immunity remains to be investigated. Further characterisation of the direct binding sites of T-bet in GC B cells induced to malaria infection may provide important insight into the extent of its activity and uncover other potential roles in GC development.

A role of inflammation-driven T-bet in reinforcing B cell affinity maturation that allows the generation of a high avidity antibodies may represent a mechanism by which highly protective antibodies to complex pathogens are generated. Although it is well-documented that antibody responses to malaria are acquired gradually over time (Ryg-Cornejo *et al.*, 2016b), our understanding of the process of affinity maturation is very limited, with a few immuno-epidemiological studies observing gradual increments in SHM load or antibody avidity in individuals living in malaria-endemic areas (Wipasa *et al.*, 2010; Ibison *et al.*, 2012; Wendel *et al.*, 2017). Antibodies with broad reactivity against *P. falciparum* have only recently been characterised to diversify from low-affinity precursors (Tan *et al.*, 2016; Pieper *et al.*, 2017). Whether these antibodies emanate from MBCs that spend extended periods of time within GC reactions, or from constantly reactivated MBCs is unknown. In HIV infection, broadly-neutralising antibodies accumulate extensive *IghV* mutations and may take years of exposure to develop (Wu *et al.*, 2011; Lynch *et al.*, 2012). It seems possible that the enhanced DZ reaction regulated by T-bet during malaria infection may increase clonal diversity in GCs that permits the generation of a broad B cell repertoire that can respond to the antigenically-variable *Plasmodium* parasites. Similar diversity has been characterised in late stage GC responses elicited to anthrax protective antigen and influenza hemagglutinin (Adachi *et al.*, 2015; Kuraoka *et al.*, 2016). Intriguingly, lower-affinity B cells persist in these GCs for extended periods of time (Kuraoka *et al.*, 2016), consistent with permissive GC selection in some circumstances (Tas *et al.*, 2016; Kuraoka *et al.*, 2016). Whether this occurs in malaria-induced GCs warrants further interrogation. As T-bet⁺ MBCs are detected in individuals with acute malaria (Obeng-Adjei *et al.*, 2017), more mechanistic research will be needed to determine if antibodies secreted by T-bet-expressing B cell memory populations control parasite burden below the disease threshold. Furthermore, whether T-bet-mediated affinity maturation occurs with the acquisition of clinical immunity to malaria is an interesting question yet to be answered. In support, T-bet⁺CD27⁺ B cells induced to influenza vaccination have been suggested to originate from active GCs and are predisposed to differentiate into long-lived plasma cells (Lau *et al.*, 2017). Vaccine strategies that promote T-bet expression in B cells may enhance the production of antibodies with increased affinity for antigen. This is not limited to malaria, but may extend to infections with viruses, bacteria and other parasites that similarly elicit T-bet⁺ B cells (Jöhrens *et al.*, 2005; 2006; 2010; Rubtsova *et al.*, 2013; 2016; Barnett *et al.*, 2016; Naradikian *et al.*, 2016b; Papillion *et al.*, 2017; Knox *et al.*, 2017; Chang *et al.*, 2017; Kenderes *et al.*, 2018), and is worthy of further investigation.

Discussion

In contrast, T-bet driven affinity maturation may have detrimental effects in cancer and autoimmune disease. Prolonged GC B cell responses to *P. chabaudi* AS murine malaria have been described to be accompanied by widespread genomic instability, in particular at the *Ig* loci of GC B cells, which modify lymphomagenesis to promote the development of mature GC-derived B cell lymphomas (Robbiani *et al.*, 2015). Moreover, augmented SHM by T-bet may contribute to the production of self-reactive antibodies in autoimmunity. T-bet expression in B cells has been linked to disease severity in autoimmunity (Frisullo *et al.*, 2008; Becker *et al.*, 2013; Wang *et al.*, 2016). In particular, T-bet has been implicated in the development and/or function of age-associated B cells (ABCs) (Rubtsov *et al.*, 2013; Jackson *et al.*, 2016; Rubtsova *et al.*, 2017; Du *et al.*, 2019). Functional studies suggests that these cells are a key source of autoimmune B cell responses in both humans (Rakhmanov *et al.*, 2009; Claes *et al.*, 2016; Wang *et al.*, 2018) and murine models (Hao *et al.*, 2011; Rubtsov *et al.*, 2011; 2013; Jackson *et al.*, 2016; Rubtsova *et al.*, 2017). Additionally, T-bet deficiency has been shown to reduce spontaneous GC formation in SLE-prone mice (Domeier *et al.*, 2016). While at present it is not entirely clear how ABCs are generated, their BCRs contain somatic hypermutations (Russell Knode *et al.*, 2017), suggesting that they arise from chronic GC reactions over time. More mechanistic work is needed determine if T-bet regulates the development of pathogenic B cell responses that contribute to disease.

This thesis fills in important gaps in our understanding of the cell-intrinsic mechanisms that regulate the dynamics of the GC reaction in blood-stage malaria. In addition to cognate T cell help, these results support a model by which the inflammatory milieu induced in response to infection imposes selective pressure on the affinity maturation of the antibody response. Hence, as the field moves forward, it will be important to define how these factors are integrated in the overall regulatory circuitry of GC development to orchestrate effective humoral immunity. In-depth phenotypic and functional characterisation of the MBC populations induced to infection is urgently needed to interrogate the implications for long-lived protection, and may be achieved through the use of additional parasite antigen- and memory-specific markers, and novel cell tracking methods. Furthermore, it will be interesting to identify the B cell repertoire that functions to provide clinical immunity to malaria. While murine models offer valuable mechanistic insight, longitudinal studies in malaria-infected populations are needed to extend model-based findings to the human setting. The global malaria burden may increase if we continue to employ the same control interventions. Therefore, a better understanding of the intricacies of immunity against disease may accelerate the development of next generation vaccines and adjunctive therapies to defeat a parasite that has co-evolved with mankind.

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Appendices

Appendix I. Differentially expressed genes between *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre GC B cells on day 10 pi.

GC B cells were isolated from *P. berghei* ANKA-infected *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice on day 10 pi, RNA-sequenced and analysed as detailed in *Materials and Methods*. Log₂ fold-change (LogFC), average expression RPKM (AveExpr), and adjusted p value (Adj p val) are shown for the top 50 most differentially expressed genes.

EntrezID	Symbol	LogFC	AveExpr	Adj p val
<i>Genes up-regulated in T-bet-deficient GC B cells</i>				
100503231	Gm19601	5.887275231	0.731255507	1.09E-02
83558	Tex11	5.179830491	-1.903851525	1.88E-02
170786	Cd209a	4.997661988	-2.629621608	1.09E-02
75863	Clec4g	4.984987766	-0.00375307	4.31E-02
17207	Mcf2l	4.909205472	-3.678515369	1.20E-01
93835	Amn	4.65223792	-2.181271684	1.06E-01
20420	Shd	4.506546191	-4.206902113	3.84E-02
75881	4930579K19Rik	4.382164731	-2.004094506	9.57E-02
100504598	Gm20305	4.225787703	-0.283502023	9.17E-02
100217457	Snord58b	4.033019618	3.222815294	7.04E-02
53321	Cntnap1	4.027118558	-3.527957553	9.57E-02
238555	Btn2a2	3.987890533	-3.208098931	9.57E-02
170942	Erdr1	3.827931775	2.986706368	1.37E-02
622070	Gm15133	3.727164254	1.396299759	1.38E-01
22342	Lin7b	3.483759451	-2.047388689	1.32E-01
67261	2900005J15Rik	3.390335792	-3.234788927	1.34E-01
319200	Gpr82	3.331616293	-3.025372166	1.09E-01
100503311	Pifo	3.319106954	-1.74287716	1.25E-01
77521	Mtus2	3.156056155	-3.021302701	1.30E-01
233066	Syne4	2.971398042	-0.525684177	7.31E-02
100302529	Snord72	2.838190354	4.224324022	1.50E-01
74757	5830416I19Rik	2.683696864	-2.05270644	1.39E-01
100500713	9530027J09Rik	2.651212748	-0.626002807	1.13E-01
100504021	Gm17638	2.625001069	-0.931565004	1.06E-01
100862004	NA	2.601824555	3.449561059	1.59E-02
243961	Shank1	2.589163843	1.090954463	1.48E-01
100044509	Tgfb3l	2.555880273	1.111862045	9.57E-02
20299	Ccl22	2.539572195	-0.66130533	9.57E-02
78376	Sapcd1	2.441986509	1.1585936	1.06E-01
330149	Hfm1	2.415993128	-3.625904922	1.06E-01
100861696	NA	2.407914413	3.550077887	2.81E-02
653030	Arhgap27os3	2.344529388	3.62895024	1.05E-01
78801	Ak7	2.287326659	-2.206861505	1.47E-01

Appendices

<i>(Continued)</i>				
EntrezID	Symbol	LogFC	AveExpr	Adj p val
72289	Malat1	2.203968429	5.704329141	1.09E-01
230558	C8a	2.192143874	-3.755016047	1.09E-01
622665	Ccdc17	2.189580187	2.332998513	6.29E-02
100502778	Gm19365	2.153314122	0.233849973	1.20E-01
320466	A230103J11Rik	2.131694604	-0.926910099	1.15E-01
100861924	NA	2.116357881	3.590105235	3.36E-02
215772	Adgb	2.111191897	-4.515261864	1.20E-01
100628611	Mir5107	2.083811831	5.390944255	1.45E-01
67434	Ankrd33b	2.056284	0.973549625	1.37E-02
66805	Tspan1	1.952105764	-2.382447373	1.23E-01
373864	Col27a1	1.922398968	-1.02044273	4.62E-02
68490	Zfp579	1.910532553	1.524105007	1.28E-01
26904	Sh2d1b1	1.899255761	0.513963012	1.59E-02
217198	Plekhh3	1.889732993	-2.292347248	1.38E-01
16199	Il9r	1.862615867	4.827029367	1.39E-01
16336	Insl3	1.857417177	3.358026269	1.15E-01
100040766	Mroh2a	1.810069007	-3.144212686	1.49E-01
<i>Genes down-regulated in T-bet-deficient GC B cells</i>				
399609	C130046K22Rik	-7.443087498	-2.492160287	9.54E-04
212996	Wbscr17	-6.208701265	-5.556439822	3.71E-03
171168	Acer1	-5.733269922	-3.738778087	6.29E-02
72469	Plcd3	-5.075127358	-1.287695106	4.80E-04
12766	Cxcr3	-5.010742902	2.581781699	5.82E-05
236285	Lancl3	-4.778526234	-4.882516626	3.84E-02
22062	Trp73	-4.778261794	-3.114248373	1.73E-03
23937	Mab21l2	-4.472516861	-3.019749571	1.88E-02
15945	Cxcl10	-4.185749319	-0.271942339	1.06E-01
109979	Art3	-4.04251855	-0.700121237	3.36E-02
14733	Gpc1	-4.002323622	-2.807043858	1.70E-02
227737	Fam129b	-3.918394543	-2.401462183	1.38E-01
13844	Ephb2	-3.903464477	-5.469670784	1.09E-01
235527	Plscr4	-3.614881162	-3.025995929	1.45E-01
21814	Tgfbr3	-3.526918584	-3.821190908	1.02E-01
108672	Zdhhc15	-3.318484925	-4.126152658	1.23E-01
22354	Vipr1	-3.302877792	-3.409050505	1.43E-01
67425	Eps8l1	-3.297537326	2.360284778	1.32E-03
56325	Abcb9	-3.063849052	-0.791106603	1.25E-01
329934	Foxo6	-2.936119973	-4.007840135	1.09E-01
72401	Slc43a1	-2.931542924	1.975793761	1.50E-01
57765	Tbx21	-2.828890672	2.673145721	4.80E-04
269389	Tox2	-2.714146904	-0.392660727	1.37E-02
105732	Fam83h	-2.475086179	-3.832368623	1.50E-01
76166	Rsg1	-2.407721294	-0.369443915	1.23E-01

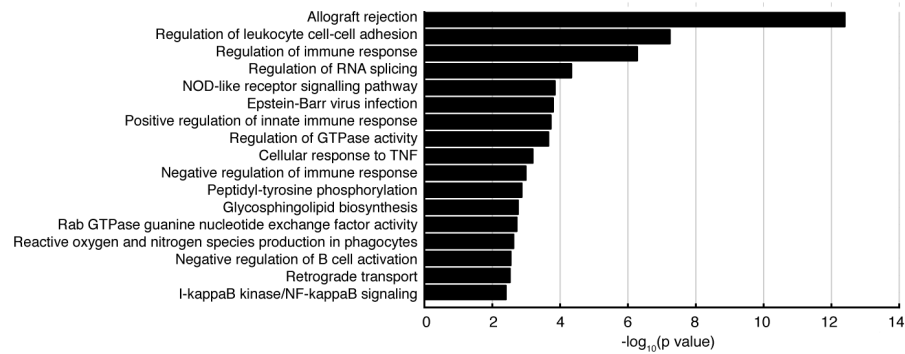
Appendices

<i>(Continued)</i>				
EntrezID	Symbol	LogFC	AveExpr	Adj p val
66972	Slc25a23	-2.313730141	-0.077509349	1.39E-01
84544	Cd96	-2.270922283	-1.246549809	1.38E-01
106763	Ttbk1	-2.139286149	-1.433355233	6.46E-02
22761	Zfpm1	-1.870369431	3.571097794	1.26E-01
74103	Nebi	-1.819571762	0.277725467	1.15E-01
27059	Sh3d19	-1.78364391	-0.956264371	6.46E-02
105651	Ppp1r3e	-1.754924648	0.945221413	1.02E-01
60361	Ms4a4b	-1.727526267	3.206265942	1.13E-01
17427	Mns1	-1.711602173	2.687108171	1.59E-02
12504	Cd4	-1.695759566	0.877338632	1.15E-01
21414	Tcf7	-1.688570259	2.130204854	1.15E-01
50780	Rgs3	-1.67580322	0.108527182	1.09E-01
22779	Ikzf2	-1.672300661	-2.787627621	1.39E-01
12511	Cd6	-1.670899591	0.343885635	1.50E-01
60505	Il21	-1.62365273	-1.518006711	1.11E-01
216438	Feb-05	-1.59852937	1.063692299	1.05E-01
68070	Pdzd2	-1.550959945	0.856649685	1.37E-02
93706	Pcdhgc3	-1.499160188	-1.161205615	1.82E-02
29810	Bag3	-1.447566278	-0.718582338	1.38E-01
215789	Phactr2	-1.441328177	-0.739613656	3.84E-02
19699	Reln	-1.425666775	-1.75940778	8.89E-02
104080	Nxph4	-1.4203379	-1.959535271	9.57E-02
16185	Il2rb	-1.376502278	2.024453137	1.31E-01
99167	Ssx2ip	-1.358623443	1.003693608	1.09E-01
69551	2310022B05Rik	-1.357141439	1.675986673	1.38E-01

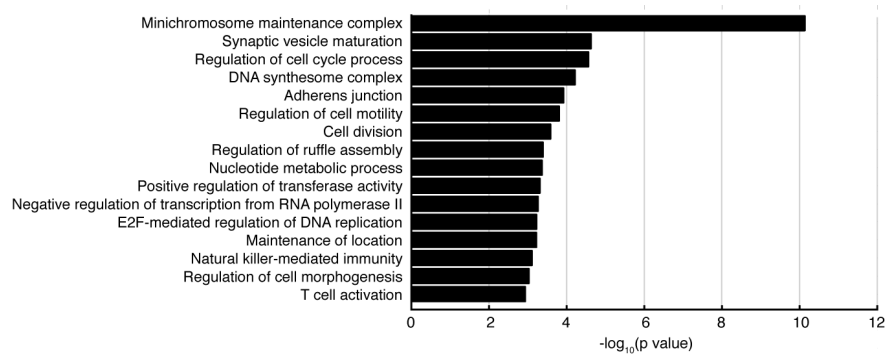
Appendix II. Enriched pathways among differentially expressed genes between *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre GC B cells.

Pathway enrichment analysis of (a) up-regulated and (b) down-regulated genes between *P. berghei* ANKA-infected *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice on day 10 pi using Metascape.

a Upregulated pathways in T-bet-deficient GC B cells



b Downregulated pathways in T-bet-deficient GC B cells



Appendix III. Differentially accessible peaks between *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre GC B cells at day 8 pi

GC B cells were isolated from *P. berghei* ANKA-infected *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice on day 8 pi, and chromatin accessibility mapped as detailed in *Materials and Methods*. Chromosome location (Chr), log₂ fold-change (LogFC), average expression CPM (AveExpr), and adjusted p value (Adj p val) are shown for the top 50 most differentially accessible peaks.

EntrezID	Symbols	Chr	Start	End	LogFC	AveExpr	Adj p val
<i>Peaks with increased accessibility in T-bet-deficient GC B cells</i>							
72003	Synpr	chr14	13361760	13361986	6.957011505	1.43506049	1.29E-05
268980	Strn	chr17	78713537	78713687	5.831295538	0.930843903	7.29E-04
218699	Pxk	chr14	8132855	8133038	5.128048784	1.388511205	1.43E-03
75429	Fam183b	chr11	58794376	58794526	4.774805341	0.051550598	1.96E-02
74430	4930452B06Rik	chr14	8453167	8453317	4.475826987	0.532829987	2.39E-02
286940	Flnb	chr14	7932985	7933135	4.473060691	-0.169383355	4.04E-02
17118	Marcks	chr10	37811889	37812058	4.382053893	1.380048238	2.81E-02
14198	Fhit	chr14	9390294	9390444	4.268245673	0.510721022	4.33E-02
71147	Oxsm	chr14	16009940	16010091	4.150013205	1.772207718	4.99E-04
72003	Synpr	chr14	13361260	13361476	4.071838469	0.83135262	4.19E-02
75985	Rab30	chr7	92784873	92785023	4.016306804	0.558803899	4.10E-03
70435	Inf2	chr12	112552403	112552553	3.915304202	0.741706794	1.03E-01
214444	Cdk5rap2	chr4	69981296	69981446	3.884318645	0.511796905	7.18E-02
270118	Maml2	chr9	13471632	13471782	3.789624173	-0.29438038	8.30E-02
100043364	Gm4392	chr3	127450959	127451109	3.674549473	-0.10051445	1.03E-01
15110	Hand1	chr11	57873068	57873231	3.660717095	1.595327555	1.87E-03
218763	Lrrc3b	chr14	15784810	15785024	3.604056419	1.594994146	2.13E-02
71900	Tmem106b	chr6	13054954	13055104	3.602505723	1.378126252	1.33E-01
230959	Ajap1	chr4	153318411	153318561	3.543188115	0.978088357	7.24E-02
73094	Sgip1	chr4	102945903	102946053	3.542648989	0.31531948	1.09E-01
216766	Gemin5	chr11	58126681	58126848	3.523840795	0.940850269	4.35E-02
103978	Gpc5	chr14	115436783	115436933	3.468399368	1.06205607	6.88E-02
56405	Dusp14	chr11	84072929	84073079	3.441674816	1.31091395	1.35E-01
76080	Ttpal	chr2	163620504	163620654	3.434562587	1.219914934	2.13E-02
751556	Mir682	chr13	75478251	75478401	3.399026852	0.526144143	1.70E-01
100502653	C130060C02Rik	chr19	16040751	16040901	3.307231951	0.567412069	1.56E-01
674895	Nek10	chr14	14971226	14971376	3.300552256	1.073406097	6.90E-02
242259	Slc44a5	chr3	154206506	154206656	3.290147226	0.583255826	1.33E-01
15379	Onecut1	chr9	74913072	74913232	3.256400965	1.819734846	1.61E-01
103836	Zfp692	chr11	58301020	58301192	3.246570146	1.625982354	7.72E-02
110173	Manba	chr3	135508416	135508566	3.109481088	1.018171773	9.48E-02
237553	Trhde	chr10	114554919	114555069	3.095761882	0.712717994	1.08E-01

Appendices

(Continued)							
EntrezID	Symbols	Chr	Start	End	LogFC	AveExpr	Adj p val
21974	Top2b	chr14	16376257	16376407	3.094886008	0.843259709	7.69E-02
27078	B9d1	chr11	61505336	61505486	3.064314829	1.083260528	1.28E-01
93732	Acox2	chr14	8232356	8232506	3.062462157	0.526585075	1.03E-01
73916	Ift57	chr16	49775420	49775570	3.060799275	1.707947031	5.85E-02
68801	Elovl5	chr9	77990342	77990492	3.050391475	1.442853072	1.21E-01
75985	Rab30	chr7	92806778	92807027	3.0452452	1.634762901	6.88E-02
329154	Ankrd44	chr1	54832829	54832979	3.025111988	1.234508779	1.32E-01
207521	Dtx4	chr19	12514742	12514892	2.987571032	1.252243416	1.45E-01
78830	Slc25a12	chr2	71327228	71327406	2.876510993	1.602472001	8.08E-02
11841	Arf2	chr11	103965341	103965491	2.871789705	1.856074889	2.69E-02
17128	Smad4	chr18	73643163	73643313	2.864399824	1.121016239	1.05E-01
100040667	Gm2895	chr15	67027866	67028016	2.825212503	1.320185734	8.08E-02
226823	Kctd3	chr1	189000543	189000693	2.823119321	1.200263671	1.01E-01
23872	Ets2	chr16	95703070	95703220	2.806964882	1.428783747	1.25E-01
225791	Zadh2	chr18	84132676	84132826	2.806964882	1.330936507	1.26E-01
407823	Baz2b	chr2	60124594	60124744	2.788485562	1.398674575	1.61E-01
233781	Xylt1	chr7	117622889	117623039	2.778778105	1.458323791	1.18E-01
210126	Lpp	chr16	24624439	24624609	2.770527808	1.624098538	1.54E-01
<i>Peaks with decreased accessibility in T-bet-deficient GC B cells</i>							
433693	Akirin2	chr4	34532431	34532597	-6.035414765	1.744255651	2.39E-02
433693	Akirin2	chr4	34528557	34528724	-5.761623762	1.489969402	2.39E-02
100041279	Gm3248	chr14	5904289	5904449	-5.713522773	2.7263569	4.91E-02
208449	Sgms1	chr19	32376880	32377042	-5.54095428	0.913922619	4.33E-02
432825	Gm5458	chr14	19609697	19609866	-5.391105671	2.475606991	6.25E-02
16600	Klf4	chr4	55623943	55624177	-5.337133563	2.017834352	4.64E-02
100041054	Gm3115	chr14	4086009	4086164	-5.325239901	2.019248877	6.30E-02
71602	Myo1e	chr9	70371679	70371875	-5.103048198	1.297659619	7.37E-02
100041279	Gm3248	chr14	5909074	5909227	-5.087365778	2.119677977	1.03E-01
625347	Gm6578	chr6	12109458	12109669	-5.05378148	0.969990565	6.86E-02
100040671	Gm2897	chr14	3130529	3130693	-5.017340339	2.378634121	8.31E-02
268780	Egflam	chr15	7206452	7206602	-4.934485381	0.68621771	8.22E-02
100038847	Gm10406	chr14	6999008	6999158	-4.881063909	1.837488851	1.27E-01
100041054	Gm3115	chr14	4081945	4082098	-4.846114755	1.310170282	1.06E-01
226527	BC026585	chr1	157440248	157440410	-4.823220632	1.939151465	5.62E-02
27386	Npas3	chr12	53973506	53973686	-4.743268542	1.640299116	5.71E-02
26388	Ifi202b	chr1	173961591	173961761	-4.710770721	1.826382796	1.08E-01
208449	Sgms1	chr19	32377856	32378011	-4.525257142	0.984218592	1.48E-01
320528	Vps13c	chr9	67907971	67908121	-4.524508803	0.752579721	1.31E-01

Appendices

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EntrezID	Symbols	Chr	Start	End	LogFC	AveExpr	Adj p val
338362	Ust	chr10	8135405	8135565	-4.492327642	0.119865635	1.39E-01
13824	Epb4.1l4a	chr18	34006503	34006653	-4.471489754	0.942148341	1.33E-01
100040671	Gm2897	chr14	3128268	3128418	-4.367411432	1.213765006	1.43E-01
100038847	Gm10406	chr14	6988711	6988922	-4.355105034	2.062084217	1.61E-01
100038847	Gm10406	chr14	7000033	7000211	-4.329114046	2.788342789	1.61E-01
432825	Gm5458	chr14	19606824	19606974	-4.31542758	1.303157294	1.53E-01
279618	Gm715	chrX	60504106	60504256	-4.247692907	0.335357449	1.54E-01
259302	Srgap3	chr6	112920848	112921052	-4.245752214	1.687526028	1.43E-01
66773	Gm17019	chr5	15040100	15040257	-4.234809552	3.607949993	8.24E-02
109979	Art3	chr5	92369175	92369368	-4.15339899	1.704880247	1.53E-01
171168	Acer1	chr17	56963569	56963720	-4.06646134	1.952071145	2.88E-02
75858	Speer7-ps1	chr5	15689840	15689990	-3.999374276	3.457659797	1.27E-01
56709	Dnajb12	chr10	59884806	59884966	-3.992696202	1.886655176	6.13E-02
360220	Speer4d	chr5	15635338	15635492	-3.875826213	2.881614461	1.37E-01
233781	Xylt1	chr7	117862755	117862912	-3.755091929	1.966583491	7.24E-02
77480	Kidins220	chr12	25043255	25043466	-3.74975448	2.163375854	1.10E-01
20970	Sdc3	chr4	130799071	130799259	-3.694007433	1.960261514	1.70E-01
623078	Gm8108	chr14	4110953	4111115	-3.600583842	2.524667078	1.27E-01
53870	Cntn6	chr6	105035748	105035907	-3.423643552	1.507680822	6.13E-02
212996	Wbscr17	chr5	131294196	131294357	-3.354093696	1.882312823	1.56E-01
66773	Gm17019	chr5	15004698	15004897	-3.345831112	4.029415163	2.69E-02
214968	Sema6d	chr2	124436677	124436838	-3.303520572	2.259095567	8.24E-02
19883	Rora	chr9	68956131	68956290	-3.288059906	2.310884543	1.10E-01
67095	Trak1	chr9	121380659	121380833	-3.269761787	1.566309961	1.71E-01
19883	Rora	chr9	68956579	68956755	-3.269543459	1.847413579	1.59E-01
12766	Cxcr3	chrX	101737244	101737450	-3.21662453	2.135988709	1.40E-01
100861702	Gm21149	chr5	15463673	15463833	-3.14526647	3.937687117	3.94E-03
211739	Vstm2a	chr11	16257589	16257739	-3.02470727	1.685827438	1.51E-01
21834	Thrb	chr14	17923256	17923443	-2.966737744	1.990335574	1.70E-01
16412	Itgb1	chr8	128645548	128645722	-2.827488386	2.756229126	1.17E-01
257633	Acsf3	chr8	122772473	122772644	-2.742582405	2.094642626	1.28E-01

Appendix IV. *IghV* mutations in *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre GC B cells

RNA sequences of flow cytometry-sorted GC B cells from day 10 and 15 *P. berghei* ANKA-infected *Tbx21^{fl/fl}*Cd23-Cre and Cd23-Cre mice were analysed for *IghV* mutations on chromosome 12 using exact SNP 1.6.0. Dominant GC B cell-specific *IghV* mutations (>3-fold above the frequency in naive follicular B cells, and <3% in naive follicular B cells) are listed.

Position	Gene	Reference Base	Alternative Bases	Coverage
114538697	Ighv1-7	C	A,G,T	831
114746391	Ighv1-22	C	A,G,T	1336
113597650	Ighv5-4	C	A,G,T	1474
113994661	Ighv14-2	C	A,G,T	2550
115208297	Ighv1-55	C	A,G,T	1205
114708766	Ighv1-19	C	A,G,T	1529
114914717	Ighv1-39	C	A,G,T	1148
113588468	Ighv2-2	C	A,G,T	1022
115834074	Ighv1-75	C	A,G,T	1701
114059957	Ighv14-3	G	A,C,T	2249
115920481	Ighv1-81	C	A,G,T	1840
113625710	Ighv5-6	C	A,G,T	1314
113994649	Ighv14-2	C	A,G,T	2298
114060037	Ighv14-3	C	A,G,T	2061
114583686	Ighv1-9	G	A,C,T	1964
115507669	Ighv1-64	C	A,G,T	2465
113625635	Ighv5-6	C	A,G,T	1710
113588393	Ighv2-2	C	A,G,T	2234
114059960	Ighv14-3	T	A,C,G	2278
115294177	Ighv8-8	G	A,C,T	2636
115952739	Ighv1-82	G	A,C,T	3159
113994660	Ighv14-2	G	A,C,T	2569
115623288	Ighv1-69	C	A,G,T	1425
113994581	Ighv14-2	G	A,C,T	2453
115294274	Ighv8-8	C	A,G,T	1796
115507642	Ighv1-64	C	A,G,T	3464
115920469	Ighv1-81	C	A,G,T	1869
115912449	Ighv1-80	C	A,G,T	3492
115208192	Ighv1-55	C	A,G,T	2416
115294186	Ighv8-8	C	A,G,T	2303
114746397	Ighv1-22	C	A,G,T	1133
114288203	Ighv3-6	T	A,C,G	2633
114657450	Ighv1-15	C	A,G,T	1853
113588422	Ighv2-2	C	A,G,T	1559
113859301	Ighv5-17	C	A,G,T	2993
115158536	Ighv1-53	C	A,G,T	1887
115952740	Ighv1-82	C	A,G,T	3174
113859276	Ighv5-17	C	A,G,T	2978

Appendices

<i>(Continued)</i>				
Position	Gene	Reference Base	Alternative Bases	Coverage
113994566	Ighv14-2	C	A,G,T	2839
113625644	Ighv5-6	C	A,G,T	1658
115920376	Ighv1-81	C	A,G,T	3465
113994517	Ighv14-2	C	A,G,T	3381
114788636	Ighv1-26	C	A,G,T	4488
115912392	Ighv1-80	C	A,G,T	3431
115952663	Ighv1-82	C	A,G,T	4078
113859311	Ighv5-17	C	A,G,T	2834
114059942	Ighv14-3	C	A,G,T	2902
115507618	Ighv1-64	T	A,C,G	3759
115119853	Ighv1-50	C	A,G,T	2089
113994549	Ighv14-2	C	A,G,T	2978
115952635	Ighv1-82	C	A,G,T	4507
113859261	Ighv5-17	G	A,C,T	3540
114059893	Ighv14-3	C	A,G,T	3551
114788606	Ighv1-26	T	A,C,G	5101
115294123	Ighv8-8	T	A,C,G	2846
114583674	Ighv1-9	C	A,G,T	2422
114788620	Ighv1-26	C	A,G,T	4228
115952610	Ighv1-82	T	A,C,G	4865
114788512	Ighv1-26	T	A,C,G	5436
114788518	Ighv1-26	C	A,G,T	5350
115952694	Ighv1-82	C	A,G,T	4163