Dissecting the molecular mechanisms of $CD4^+$ T cell exhaustion during malaria

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2019

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Contents

Word count 44,477

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Abbreviations

APC	Antigen presenting cell
Bcl-6	B cell lymphoma 6
CCL-	CC chemokine ligand-
CD-	Cluster of differentiation antigen-
cDC	Conventional dendritic cell
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
CXCR	C-X-C motif chemokine receptor
CX3CR-	CX3C chemokine receptor
GC	Germinal centre
eGFP	Enhanced green fluorescent protein
HLA	Human leukocyte antigen
IFNg	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin
IS	Immunological synapse
LAG3	Lymphocyte activation gene 3
LCMV	Lymphocytic choriomeningitis virus

MBC	Memory B cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MZ	Marginal zone
MZM	Marginal zone macrophages
NAI	Naturally acquired immunity
NK	Natural killer
OVA	Ovalbumin
PD-1	Programme death 1
PD-L1	Programmed death-ligand 1
P.i	Post infection
pMHC	peptide MHC
pRBC	Parasitized red blood cell
RBC	Red blood cell
RP	Red pulp
RPM	Red pulp macrophages
TCR	T cell receptor
Th	T helper

Tfh	T follicular helper
TGFβ	Tumour growth factor β
TIGIT	T cell immunoglobulin and ITIM domain
Tim3	T cell immunoglobulin and mucin domain 3
TLR	Toll like receptor
TNFα	Tumour necrosis factor-α
Treg cells	Regulatory T cells
WP	White pulp

Abstract

Malaria is a global life-threatening disease responsible for 400,000 deaths each year. Chronic infection with *Plasmodium* species drives $CD4^+$ T cell exhaustion, which is characterised by the inability of effector $CD4^+$ T cells to produce effector cytokines, proliferate and increased T cell apoptosis. T cell exhaustion significantly impairs parasite control during blood stage malaria. However, the molecular mechanisms promoting $CD4^+$ T cell exhaustion during malaria are poorly understood.

Using a model antigen-specific $CD4^+$ T cell system, we have shown that effector $CD4^+$ T cells rapidly become functionally exhausted during *P.yoelii* infection. The degradation of the effector $CD4^+$ T cell response appeared to relate to the loss of MHC II-TCR signalling, as blockade of MHC II signalling, post priming, did not exacerbate effector T cell dysfunction and attrition during malaria. However, apparent loss of MHC II activation during infection was not due to alterations in $CD4^+$ T cell compartmentalisation, or inability of effector $CD4^+$ T cells to interact with antigen presenting cells (APC) during infection. Instead, we propose that negative signals from co-inhibitory receptors subvert peptide MHC II-TCR signals in effector $CD4^+$ T cells, contributing to T cell exhaustion during blood stage malaria.

To further investigate the role of co-inhibitory receptors in promoting $CD4^+$ T cell exhaustion during malaria, we administered antagonistic antibodies against TIGIT and PD-L1. Dual blockade of TIGIT and PD-L1 significantly enhanced parasite control, which correlated with an increased level of systemic interferon gamma (IFNg) and an enhanced T follicular helper response during infection. Surprisingly, however, dual blockade of TIGIT and PD-L1 did not significantly improve effector $CD4^+$ T cell function. Thus, blockade of TIGIT and PD-1 signalling pathways cannot prevent $CD4^+$ T cell exhaustion during malaria.

We also investigated the synergistic role of Tim3 and PD-1 in promoting $CD4^+$ T cell exhaustion during malaria. Interestingly, Tim3 was transiently expressed on effector $CD4^+$ T cells and was downregulated as T cell exhaustion was established during infection. In agreement, co-blockade of Tim3 and PD-L1 failed to improve $CD4^+$ T cell functionality during *P.yoelii* infection, suggesting that Tim3 does not contribute to $CD4^+$ T cell exhaustion during malaria.

Collectively, this thesis has shown that effector $CD4^+$ T cell exhaustion is not associated with the inability of T cells to form stable interactions with APC during infection, but instead we propose that multiple immunoregulatory pathways act in parallel to orchestrate T cell exhaustion during blood stage malaria.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

All work presented in this thesis was carried out by Rebecca Dookie.

Copyright statement

Acknowledgements

I would like to thank my supervisors Dr Kevin Couper and Professor Andrew MacDonald for all their advice during my PhD. I would particularly like to thank Kevin for supporting me throughout my PhD.

I would also like to thank all the current and past members of the Couper lab, particularly Dr Patrick Strangward and Dr Ana Villegas-Mendez who have provided me with great technical and personal advice throughout my PhD.

A big thank you to all my lovely friends, especially Cat Sharpe, Dr Becca Shears, Dr Elena Owen, Dr Danielle Weaver, Dr Pint O' Cider and last but not least, Sizzles Hodge, who have all kept me smiling and laughing throughout my PhD.

Also, a big thank you to Dr Hazel England for all the personal advice and much needed Costa trips.

I would also like to thank Dr Phill Chivers, for always supporting me and making me laugh, especially when things got tough. I could not have done this without you.

Last, but not least, I would like to thank my Mum, who has always been so supportive and encouraging of me. I can't thank you enough for keeping me motivated and listening to me rant.

Character Built

Chapter 1: General Introduction

1.1 Malaria Burden

Malaria is a global life-threatening disease responsible for approximately 400,000 deaths each year, with African countries carrying a disproportionally high share of malaria-induced deaths (Cowman, Healer et al. 2016, WHO 2019). The causative agent of malaria is the protozoan parasite *Plasmodium,* which is transmitted between humans by the female *Anopheles* mosquito (Crompton, Moebius et al. 2014). As well as African countries, malaria is also transmitted in South-East Asia, western Mediterranean countries and the Americas. The tropical climates of these countries favour the transmission of malaria, as both the parasite and mosquito vector are temperature sensitive (van Lieshout, Kovats et al. 2004). In addition to environmental factors, the large population density and limited vector control and disease treatment within these regions, contributes to the high level of disease transmission (White, Pukrittayakamee et al. 2014). In 2017 there was an estimated 20 million fewer malaria cases compared to 2010, however, progression in controling malaria has stalled and malaria cases have slowly started to increase (WHO 2018). Resistance to insecticides and the front-line anti-malarial drug Artemisinin, as well as underfunding in malaria research has likely hindered eradication efforts (Cowman, Healer et al. 2016, WHO 2018). Moreover, the most promising malaria vaccine to date, RTS,S, has only demonstrated modest, short term efficacy during phase three of clinical trials (Mahmoudi and Keshavarz 2017). A clearer understanding of protective immunity to malaria, as well as improved vector control strategies will therefore greatly contribute to malaria eradication.

1.2 *Plasmodium* Parasites

1.2.1 *Plasmodium* species

There are 5 species of *Plasmodium* that infect humans; *falciparum*, *knowlesi*, *vivax*, *malariae* and *ovale* (de Koning-Ward, Dixon et al. 2016). Of the 5 species, infection with *P.falciparum*, *knowlesi* and *vivax* can lead to severe malaria in humans, characterised by three main syndromes; severe anaemia, respiratory distress and cerebral malaria. The highest mortality rates however, are associated with *P.falciparum* infection (Phillips, Burrows et al. 2017). In contrast, *P.malariae* and *ovale* are associated with uncomplicated malaria and rarely lead to mortality (Phillips, Burrows et al. 2017). The virulence of *P.falciparum* is likely due to its ability to invade all mature red blood cells (RBC) and its capacity to modify the RBC membrane and sequester to the vasculature endothelium (Buffet, Safeukui et al. 2011).

1.2.2 *Plasmodium* life cycle

The *Plasmodium* species have a complex life cycle that involves both human and mosquito hosts (Fig1.1). The cycle begins when the mosquito takes a blood meal, concomitantly injecting highly motile *Plasmodium* sporozoites into the skin (Sidjanski and Vanderberg 1997, Amino, Thiberge et al. 2006). Although sporozoites induce a local inflammatory response in the skin (Mac-Daniel, Buckwalter et al. 2014), the majority will leave the skin and migrate to the liver via the blood vessels. The small

percentage of parasites that remain in the skin, or drain into the lymph nodes at this stage die (Gueirard, Tavares et al. 2010). Once inside the liver, sporozoites traverse several different host cells, which appears to protect them from clearance by liver Kupffer cells (Tavares, Formaglio et al. 2013), before invading hepatocytes (Mota, Pradel et al. 2001). Within the hepatocytes, sporozoites undergo differentiation into the asexual form termed merozoite, which then undergoes extensive replication, increasing in number by 40,000 fold (Crompton, Moebius et al. 2014). This stage in the life cycle is known as the pre-erythrocytic phase and is clinically silent (Crompton, Moebius et al. 2014). Following replication, merozoites are released into the blood where they invade RBC initiating the blood stage (also known as the erythrocytic phase).

Within the RBC merozoites pass through a secondary developmental phase in which the parasite passes through three successive morphological stages termed: ring, trophozoite and schizont stage. The final schizont formation can generate up to 30 daughter merozoites, that upon rupture of the RBC, can invade new erythrocytes (Grüring, Heiber et al. 2011). The synchronicity of RBC rupture leads to the release of immunogenic parasite material, that perpetuates the malaria-associated pathologies, such as fever, every 24-78 hours depending on the species (Langhorne, Ndungu et al. 2008). A small number of blood stage parasites will develop into the sexual forms of the parasite, termed gametocytes.

Gametocytes are ingested by the mosquito when it feeds on an infected human. Over a 10 day period the male and female gametocytes progress through 5 distinct morphological stages, allowing them to pass into the midgut of the mosquito (de Koning-Ward, Dixon et al. 2016). Within the midgut, fertilisation of the gametocytes leads to the development of the zygote form, which then transforms into the motile ookinete that can penetrate the gut wall. Further transformations generate the oocyst form, that upon maturation forms the sporozoite which is capable of migrating to the salivary gland, ready to repeat the life cycle (de Koning-Ward, Dixon et al. 2016).

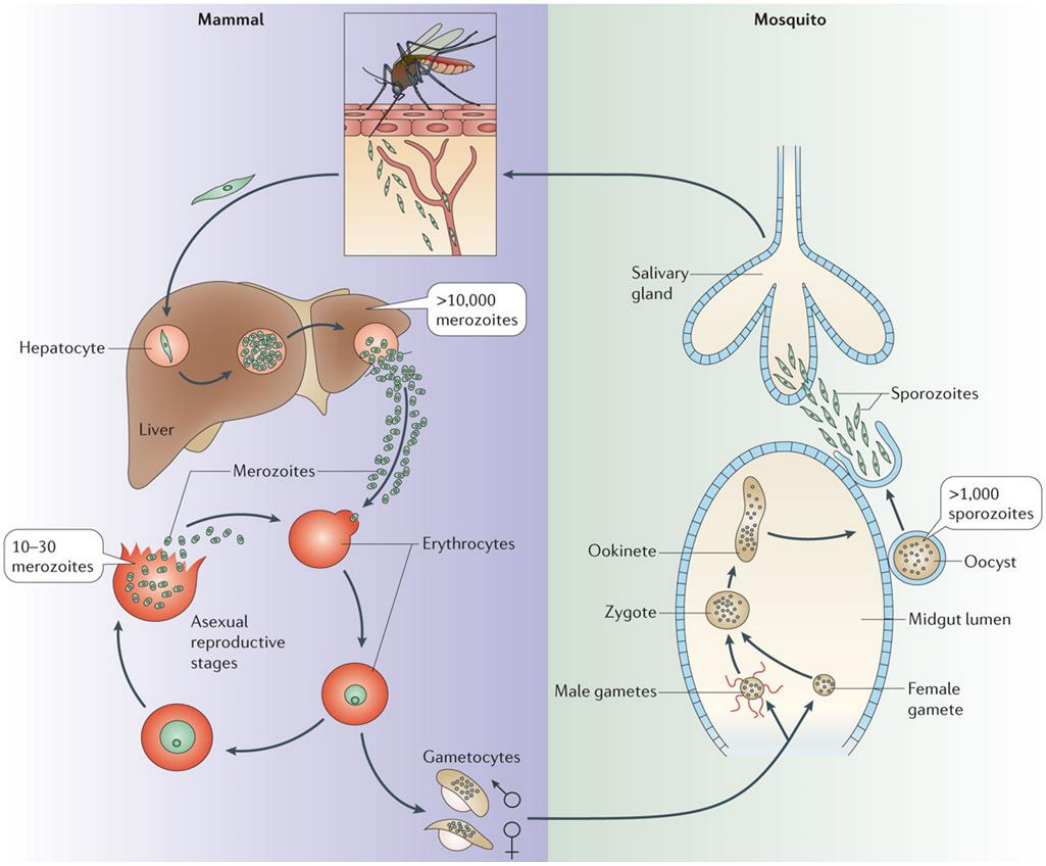

Figure1.1 Life cycle of malaria parasite in mammalian host and mosquito vector
Figure reproduced from Menard, R. *et al.*, (Menard, Tavares et al. 2013).

1.3 The role of the spleen in malaria

The spleen is a specialised secondary lymphoid organ that is highly adapted for removal of senescent RBC and blood-borne pathogens, as well as a site in which adaptive immune responses are initiated (Mebius and Kraal 2005). As malaria is a blood-borne disease, the spleen is a critical site for parasite clearance, as demonstrated by the delayed clearance of parasitized RBC (pRBC) and increased parasite burdens in splenectomised humans and rodents (Yap and Stevenson 1994, Chotivanich, Udomsangpetch et al. 2002). The unique structure of the spleen allows it to selectively filter out pRBC whilst mounting a robust immune response against the parasite.

1.3.1 Structure and function of the spleen

The spleen is a highly organised tissue, comprised of branching arterial vessels, densely packed areas of lymphocytes and is surrounded by a dense fibrous capsule of connective tissue. In both humans and rodents, the spleen is divided into two major compartments, the white pulp (WP) and the red pulp (RP), which are separated by a series of macrophage and B cell populations that comprise the marginal zone (MZ) (Mebius and Kraal 2005).

The high level of organisation and the specialised vasculature system allows the spleen to be highly effective at blood filtration and immune surveillance of blood-

borne pathogens (Mebius and Kraal 2005). The major vessel leading into the spleen is the afferent splenic artery that later branches into the central arterioles. Some of the arteriole branches terminate in the marginal sinus- the space between the WP area and MZ, whilst others traverse the MZ and end in the open chordal system in the RP. From the chords, blood collects in the venous system before exiting the spleen through the efferent splenic vein. Importantly, the chords provide a filtration system that prevents ageing erythrocytes, with reduced deformability, from passing through. Unable to pass through, these cells are phagocytosed by RP macrophages residing in the chords (Fig1.2).

The WP is a highly organised lymphoid region containing specific T and B cell compartments that surround the central arterioles (Mebius and Kraal 2005). The segregation of T cells to the periarteriolar lymphoid sheath (PALS) and B cells to the follicles is controlled by the chemokines CCL19/CCL21 (Forster, Schubel et al. 1999) and CXCL13 (Forster, Mattis et al. 1996) respectively. The surrounding MZ is an important transit area for cells entering the WP, as well as a major site for removal of bacteria and particulate matter from the blood. The MZ is comprised of several resident immune populations including; marginal zone macrophages, metallophilic macrophages and a migratory population of marginal zone B cells (Del Portillo, Ferrer et al. 2012, Arnon, Horton et al. 2013). T cell entrance into the WP is not random, but instead requires a specialised network of fibroblastic reticular cells to guide T cells into the PALS via specific marginal zone bridging channels (Bajenoff, Egen et al. 2006, Bajenoff 2008). Within the PALS, a meshwork of fibroblasts and local chemokines

continue to guide T cell movement, maximising the chances of T cells interacting with dendritic cells (DC) bearing cognate antigen (Bajenoff 2008). T cells migrate out of the WP into the RP via the marginal zone bridging channels and likely exit the spleen through the efferent splenic vein (Khanna, McNamara et al. 2007).

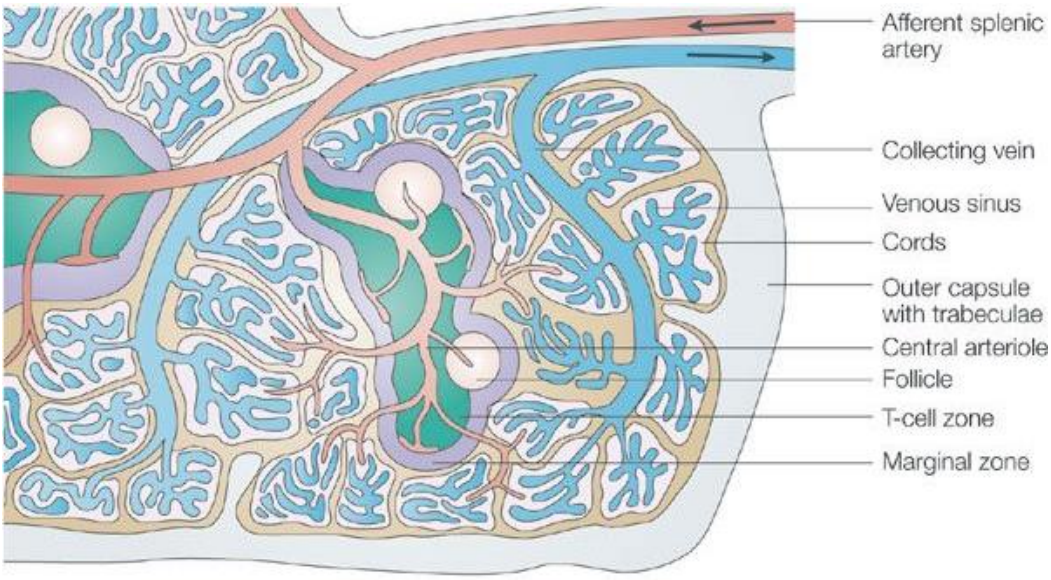

Figure1.2 Schematic of the murine spleen structure

Figure reproduced from Mebius R.E. & Kraal G. (Mebius and Kraal 2005).

1.3.2 The importance of the spleen during malaria

The RP is a major site of parasite clearance during malaria (Del Portillo, Ferrer et al. 2012). Remodelling of infected RBC through insertion of parasite-specific antigens into the membrane increases the rigidity of the pRBC (Glenister, Coppel et al. 2002). The altered pRBC becomes less deformable and thus, is retained in the RP chords where RP macrophages mediate their removal (Safeukui, Correas et al. 2008, Martin-

Jaular, Ferrer et al. 2011). Interestingly, studies have described the emergence of fibroblastic barrier cells in the spleen during malaria, that restrict blood flow into the open system of the red pulp, as such pRBC are protected from macrophage clearance (Engwerda, Beattie et al. 2005, Del Portillo, Ferrer et al. 2012). Once the parasitemia peaks, remodelling of the barrier cells enables blood flow into the red pulp and subsequent phagocytosis of pRBC (Engwerda, Beattie et al. 2005).

Plasmodium infection induces dramatic alterations in the splenic architecture, with splenomegaly being one of the most striking features of malaria (Engwerda, Beattie et al. 2005). Notably, segregation of T cell and B cell compartments is lost as extrafollicular foci of plasmablasts form in the PALS (Achtman, Khan et al. 2003). Moreover, this disruption in the WP is accompanied by the temporary loss of the MZ and increased erythropoiesis in the RP (Engwerda, Beattie et al. 2005, Beattie 2006). Despite significant modifications to the splenic architecture during malaria, a splenic immune response is still generated against the parasite.

1.4 Immunity to Malaria

Natural acquired immunity (NAI) to malaria develops gradually and is dependent on the level and duration of exposure (Langhorne, Ndungu et al. 2008). Humans with no previous exposure to malaria develop a febrile illness, which may develop into severe malaria and in a proportion of cases may lead to death. In malaria endemic regions young children are also highly susceptible to severe malaria (Langhorne, Ndungu et

al. 2008). However, with continuous exposure adults and older children develop protection from severe illness, despite potentially high parasite densities. Notably, clinical immunity is acquired more quickly in endemic areas with high transmission rates (Langhorne, Ndungu et al. 2008). By adulthood, infections in endemic areas are clinically silent with low parasite densities, however, NAI to malaria appears to be exposure dependent rather than age dependent, as demonstrated by unexposed adults that are highly susceptible to cerebral malaria (Doolan, Dobaño et al. 2009). The clinical manifestations of severe malaria are driven by two major process: sequestration of pRBC to endothelium and inflammation (Crompton, Moebius et al. 2014). Therefore, an array of tightly regulated pro-inflammatory and anti-inflammatory cytokines, as well as antibodies, are required for controlling *P.falciparum* with limited immunopathology (Crompton, Moebius et al. 2014).

1.4.1 Immune response to liver stage malaria

In humans, the liver stage is clinically silent and does not induce a strong hepatic or systemic inflammatory response (Crompton, Moebius et al. 2014). The absence of a robust adaptive immune response during the liver stage may be associated with the very low number of sporozoites injected by the mosquito (10-100) (Crompton, Moebius et al. 2014). Despite a 'muted' immune response, cross presentation of sporozoite-associated proteins, such as circumsporozoite protein, by DC activates $CD8^+$ T cells, which kill parasitized hepatocytes through the production of cytolytic factors and interferon gamma (IFNg) (Overstreet, Cockburn et al. 2008).

Notably, in the absence of $CD4^+$ T cells, the $CD8^+$ T cell response is impaired, therefore suggesting that $CD4^+$ T cell mediated 'help' is required for a protective $CD8^+$ T cell response during liver stage malaria (Overstreet, Cockburn et al. 2008). However, liver stage parasites are not completely neutralised, and the life cycle progresses to the blood stage (Crompton, Moebius et al. 2014). As the clinical manifestations of malaria are associated with the blood stage, this will be the focus for the rest of the review.

1.5.1 Proinflammatory immune response to blood stage malaria

Correlative studies in humans (Pombo, Lawrence et al. 2002, Crompton, Moebius et al. 2014, Obeng-Adjei, Portugal et al. 2015) and studies in murine models of malaria (Süss, Eichmann et al. 1988, Vinetz, Kumar et al. 1990) have identified a critical role for $CD4^+$ T cells in controlling blood stage parasite growth. $CD4^+$ T cell activation and differentiation is mainly governed by signals derived from conventional DC bearing cognate antigen. DC can be divided into two main subsets; the $XCR1^+$ DC subset, (also known as $CD8^+$ DC) and the $CD11b^+$ DC subset, which dictate $CD4^+$ T helper 1 (Th1) and Th2 responses respectively during inflammation (Plantinga, Guilliams et al. 2013, Martinez-Lopez, Iborra et al. 2015). During blood stage malaria, *Plasmodium* antigens; including the malarial pigment hemozoin, *Plasmodium*-specific GPI anchors and AT-rich DNA motifs are recognised by DC through toll-like receptor-2 (TLR2), TLR4, TLR9 and the scavenger receptor CD36 (Amorim, Chagas et al. 2016).

Following recognition, DC take up, process and present *Plasmodium*-specific antigens via MHC molecules (Urban, Ferguson et al. 1999), which confers early protection to blood stage malaria (Borges da Silva, Fonseca et al. 2015) and supports $CD4^+$ T cell activation and IFNg production (Luyendyk, Olivas et al. 2002, Perry, Rush et al. 2004). Conversely, ingestion of malaria parasites has been reported to suppress DC functions, resulting in reduced effector T cell functions (Scorza, Magez et al. 1999, Urban, Ferguson et al. 1999, Millington, Gibson et al. 2007). In particular, uptake of the malarial pigment hemozoin has been shown to suppress DC functions and attenuate co-stimulatory signalling in T cells, leading to reduced effector functions (Millington, Di Lorenzo et al. 2006, Millington, Gibson et al. 2007). Differences in parasitemia and levels of hemozoin may explain these conflicting results (Urban and Todryk 2006). Historically, blood stage malaria was associated with the sequential action of IFNg producing Th1 cells followed by a switch to a Th2 response that aided antibody production (Langhorne, Gillard et al. 1989, Perez-Mazliah and Langhorne 2014). It was initially reported that this switch was dictated by co-ordinated and sequential actions of $XCR1^+$ DC followed by $CD11b^+$ DC (Sponaas, Cadman et al. 2006). Following the identification of $CD4^+$ T follicular helper cells (Tfh) as the major $CD4^+$ T helper subset for activating B cells (Crotty 2011), the biphasic model of T cell activation during malaria was considered to be overly simplistic. Instead, protection is achieved through the co-ordinated actions of Th1 and Tfh cells (Perez-Mazliah and Langhorne 2014).

IFNg is a defining cytokine of Th1 cells and plays an important role in controlling blood stage *Plasmodium* parasites (Crompton, Moebius et al. 2014). In humans, the prevalence of IFNg producing $CD4^+$ T cells has been correlated with uncomplicated malaria (King and Lamb 2015). Furthermore, polymorphisms in the IFNg gene has been associated with susceptibility to disease (Langhorne, Ndungu et al. 2008). The importance of IFNg during malaria has been further established in murine models, which have shown that an absence of IFNg prolongs parasitemia and mice often succumb to infection (van der Heyde, Pepper et al. 1997). IFNg is a potent activator of macrophages and enhances phagocytosis of pRBC and production of nitric oxide, which is involved in parasite killing (King and Lamb 2015). In addition, IFNg can exert its protective effects by influencing isotype switching in B cells to produce antibodies that aid opsonin-mediated phagocytosis of parasites (Su and Stevenson 2000).

$CD4^+$ T cells are not the only source of IFNg during malaria, as natural killer (NK) cells and $CD8^+$ T cells also contribute to IFNg production (King and Lamb 2015). In response to *P.falciparum* and *P.chabaudi* infection in mice, NK cells were reported to provide a protective and rapid burst of IFNg (Kitaguchi, Nagoya et al. 1996, Horowitz, Newman et al. 2010). In contrast, depletion of NK cells during *P.yoelii* infection had no effect on early parasite control (Couper, Blount et al. 2007). These conflicting findings may highlight differences between *in vitro* and *in vivo* studies, as well different strains, therefore the importance of IFNg producing NK cells in early protection to *Plasmodium* remains unclear. In addition to NK cells, $CD8^+$ T cells provide another source of IFNg during blood stage infection (King and Lamb 2015).

For many years a protective role for $CD8^+$ T cells during blood stage malaria has not been clear (Vinetz, Kumar et al. 1990, Horne-Debets, Faleiro et al. 2013), However, *in vivo* studies have reported $CD8^+$ T cells mediated protection against chronic *P.chabaudi* infection (Podoba and Stevenson 1991, Horne-Debets, Faleiro et al. 2013). Whilst it was widely considered that pRBC do not express MHC I, which is required for $CD8^+$ T cell mediated killing, a recent study has shown that the non-lethal strain of *P.yoelii* can infect erythroblasts expressing MHC I and drive antigen-specific IFNg production from $CD8^+$ T cells (Imai, Ishida et al. 2015).

Tumour necrosis factor alpha (TNFα) is another pro-inflammatory cytokine produced by $CD4^+$ T cells and is elevated during *P.faciparum* infection (Lyke, Burges et al. 2004). Mechanistic insights from murine models of malaria have highlighted an important role for TNFα in the clearance of blood stage parasites (Stevenson, Tam et al. 1995, Hernandez-Valladares, Naessens et al. 2006). Although IFNg and TNFα greatly contribute to parasite clearance, elevated levels of pro-inflammatory cytokines have been associated with severe malaria (Lyke, Burges et al. 2004). Therefore, a tightly regulated balance of pro-inflammatory and regulatory cytokines is required for successful parasite clearance, without the induction of severe malaria.

1.4.2 Anti-inflammatory response

Despite, the clear protective role of inflammatory molecules during blood stage malaria, patients presenting with more severe infections typically have higher plasma

levels of IFNg and TNFα (Freitas do Rosario and Langhorne 2012). Indeed, high ratios of IFNg and TNFα to regulatory molecules, such as transforming growth factor β (TGFβ) and IL-10, is associated with an increased risk of clinical disease during *P.faciparum* infection (Walther, Woodruff et al. 2006). Further studies of *P.berghei* ANKA infection in mice have confirmed a role for IFNg in promoting experimental cerebral malaria (ECM) (Villegas-Mendez, Greig et al. 2012). Furthermore, in the absence of regulation, excessive IFNg and TNFα pro-inflammatory responses induce severe immune-mediated pathology (Li, Corraliza et al. 1999, Villegas-Mendez, Shaw et al. 2016). The control and resolution of blood stage malaria is therefore mediated through tightly co-ordinated effector and regulatory responses. In particular, effector T cell responses can be regulated through anti-inflammatory cytokines such as IL-10, regulatory T cells (Treg cells) and expression of inhibitory receptors (discussed in section 1.5).

IL-10 is an anti-inflammatory cytokine that acts to limit effector T cell responses ensuring optimal parasite clearance with minimal immune-mediated pathology (Couper, Blount et al. 2008). Its anti-inflammatory effects are mediated through the downregulation of MHC and co-stimulatory molecules on APC, as well inhibiting pro-inflammatory cytokines (Couper 2008). Low levels of IL-10 during *P.falciparum* infection correlates with a poor outcome, suggesting a protective role in reducing severe disease (Freitas do Rosario and Langhorne 2012). Interestingly, IL-10^{+} IFNg^{+} Th1 cells have been reported in children living in malaria endemic areas, with a higher proportion documented in children with uncomplicated malaria compared to severe

malaria (Perez-Mazliah and Langhorne 2014). Experimental murine studies have also demonstrated the importance of IL-10 during *Plasmodium* infection, as IL-10 deficient mice produce excessive levels of IFNg and TNFα leading to severe immune-mediate pathology (Li, Corraliza et al. 1999). Notably, IL-10^{+} IFNg^{+} Th1 cells have also been documented in murine models of malaria (Freitas do Rosario, Lamb et al. 2012 Villegas-Mendez, Shaw et al. 2015). Whilst IL-10 can be produced by a variety of cell types, it is the IFNg^{+} Th1 cells that provide the critical source of IL-10 that protect against IFNg mediated tissue damage (Freitas do Rosario, Lamb et al. 2012). This plasticity observed within the Th1 population is driven by IL-27 signalling and is important for limiting the Th1 response and preventing immune-mediated pathology (Freitas do Rosario, Lamb et al. 2012, Villegas-Mendez, de Souza et al. 2013).

TGF-β is another immunomodulatory cytokine that primarily acts to suppress effector T cells and promote Treg cell maintenance and differentiation (Butler, Harris et al. 2013). During *P.facliparum* infection, circulating TGF-β correlates with improved outcomes during severe anaemia and cerebral malaria, suggesting a protective role for TGF-β during malaria (Butler, Harris et al. 2013). Consistent with this, administration of TGF-β neutralising antibodies in mice transforms a self-resolving *P.chabaudi* infection into a lethal infection (Omer and Riley 1998). Moreover, the mortality observed in IL-10 deficient mice during malaria is exacerbated by neutralisation of TGF-β (Li, Sanni et al. 2003). Although IL-10 and TGF-β are required to limit potentially pathogenic pro-inflammatory cytokines, over production or mistimed production of IL-10 and TGF-β during malaria results in high

parasite burdens and morbidity (Omer, de Souza et al. 2003, Hugosson, Montgomery et al. 2004).

Treg cells also contribute to immune homeostasis by limiting effector responses through expression of inhibitory receptors and secretion of anti-inflammatory cytokines such as IL-10 and TGFβ (Deroost, Pham et al. 2016). Treg cell expansion has been described during *P.falciparum* infection, however, their relative contribution to parasite control and disease pathogenesis is currently unclear (Finney, Riley et al. 2010). Studies in *P.falciparum* infected children found no correlation between Treg cells and disease outcome (Walther, Jeffries et al. 2009), however, another study suggested that Treg cells might facilitate establishment of blood stage infection (Finney, Riley et al. 2010). Depletion of Treg cells in mice has also been highly conflicting (Hisaeda, Maekawa et al. 2004, Couper, Blount et al. 2008, Hansen and Schofield 2010). Whilst an exact role for Treg cells during malaria is unclear, studies in humans and mice have both identified that IFNg producing $CD4^+$ T cells are the critical protective source of IL-10 and not Treg cells during malaria (Couper, Blount et al. 2008, Walther, Jeffries et al. 2009).

1.4.3 The antibody response

In addition to $CD4^+$ T cells, B cells and their production of antibodies is essential for eliminating blood stage parasites. Early studies demonstrated that the passive transfer of purified immunoglobulin from immune adults to susceptible children

could greatly reduce the parasite burden (Cohen, Mc et al. 1961). Furthermore, B cell deficient mice infected with *P.chabaudi* infection are unable to clear the parasite giving rise to a chronic relapsing infection (von der Weid, Honarvar et al. 1996). Several mechanisms have been described for antibody-mediated protection including; blockade of merozoite invasion of RBC; inhibiting cytoadherence of mature pRBC and activating effector cell functions, such as opsonisation and phagocytosis (Azcarate, Marin-Garcia et al. 2014). However, *Plasmodium species* are able to evade the host immune system by altering the expression of surface molecules in a process known as antigenic variation (Deroost, Pham et al. 2016). It has been suggested that high antibody pressure could influence antigenic variation, which despite allowing for temporary escape from the immune system, could broaden the antibody repertoire against the parasite (Deroost, Pham et al. 2016). This is highly beneficial as an increased breadth of antibody specificity has been proposed to be crucial in determining protection against clinical malaria (Silveira, Dominguez et al. 2018). In line with this, memory B cells (MBC), essential for long-lived antibody mediated immunity, expand gradually with repeated infections (Weiss, Traore et al. 2010). This could therefore, in part, explain why NAI requires multiple exposures.

Initiation of the humoral immune response requires a specialised subset of T helper cells known as Tfh cells (Crotty 2011). Tfh differentiation is controlled by the expression of Bcl-6 which induces the expression of CXCR5, allowing Tfh cells to migrate into germinal centres (GC) and activate B cells (Crotty 2011). T-bet signalling in $CD4^+$ T cells is responsible for Th1 differentiation and directly antagonises Bcl-6

signalling (Crotty 2011). In line with this, type 1 cytokines such as IFNg, can negatively impact Tfh cell differentiation during infection (Zander, Obeng-Adjei et al. 2015, Ryg-Cornejo 2016). Circulating Tfh cells that co-express Th1 associated molecules such as T-bet, have been identified in children infected with *P.falciparum* and are poor activators of B cell responses compared with the traditional Tfh cell population (Obeng-Adjei, Portugal et al. 2015). Mechanistic studies in murine models of severe malaria, identified that excessive IFNg and TNF production induces this atypical Tfh cell population (Ryg-Cornejo 2016). Hyporesponsive atypical MBC are enriched in individuals repeatedly exposed to *P.falciparum*, therefore it is possible that these atypical Tfh cells compromise B cell memory formation during malaria (Silveira, Dominguez et al. 2018). Th1 cells and Tfh cells both play a critical role in protecting against blood stage malaria, however, a strong type 1 pro-inflammatory response may not favour a strong humoral immune response. Therefore, therapeutic strategies promoting Tfh cell responses may be highly beneficial for treating malaria.

1.5 T cell exhaustion

During an acute infection antigen-specific T cells activate, expand significantly and acquire effector functions, such as the production of cytokines (Wherry 2011). Following the peak of the response effector T cells die by apoptosis, or differentiate into resting memory T cells with a high proliferative capacity and can reactivate rapidly upon antigen re-encounter. During chronic infection however, repeated antigen stimulation through the T cell receptor (TCR) can lead to a significantly altered effector programme termed T cell exhaustion (Wherry 2011). Importantly,

there is growing evidence that malaria drives T cell exhaustion which impairs parasite control (Schlotmann, Waase et al. 2000, Chandele 2011, Illingworth, Butler et al. 2013) (discussed in 1.5.7).

First described in virus-specific $CD8^+$ T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection (Zajac et al., 1998), T cell exhaustion has now been observed in multiple viral and bacterial infections, cancers and more recently malaria (Jayaraman et al., 2016; Day et al., 2006; Sakuishi et al., 2010; Butler, 2012). T cell exhaustion occurs in a hierarchal manner, in which different effector functions are progressively lost (Fig1.3) (Wherry, Blattman et al. 2003, Wherry 2011). Typically, production of IL-2, high proliferative capacity and cytotoxic abilities are diminished first. As T cell exhaustion progresses, cells lose the ability to produce TNFα and in severe cases IFNg production is lost. The final stage of T cell exhaustion is physical depletion of cells. In addition to diminished T cell effector functions, T cell exhaustion also compromises the ability of effector T cells to transition into memory T cells, which could impact immunity to subsequent infections (Wherry, Barber et al. 2004). Importantly, exhausted T cells are not functionally inert, but retain some residual effector functions that contribute to ongoing pathogen and tumour control (Paley, Kroy et al. 2012, Wherry and Kurachi 2015). However, exhausted $CD8^+$ T cells are transcriptionally and metabolically distinct from effector T cells and memory T cells (Wherry, Ha et al. 2007).

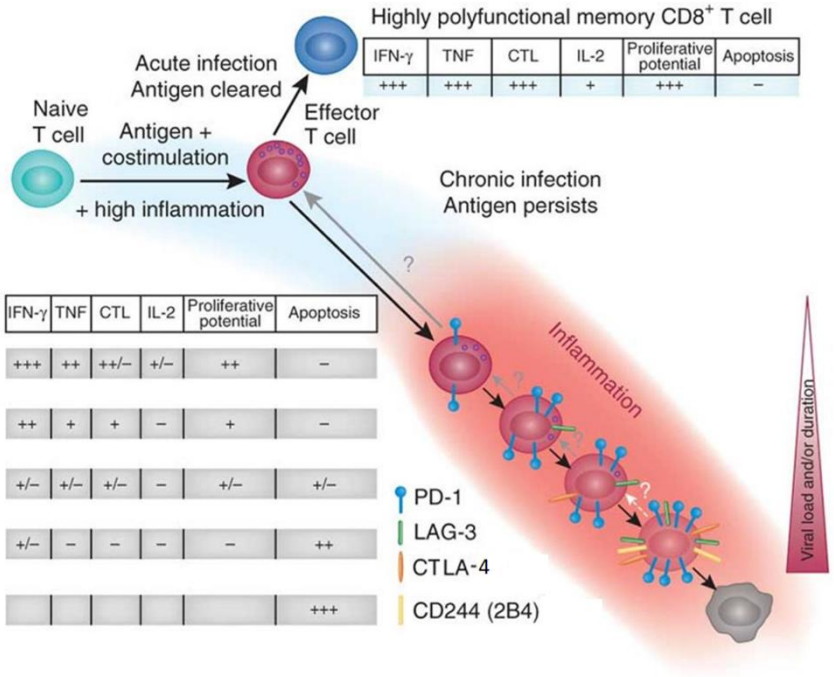

Figure1.3 Progressive development of T cell exhaustion during chronic infection

Figure modified from Wherry J. E. (Wherry 2011)

The development of T cell exhaustion is primarily driven by the continuous exposure to antigen during a chronic infection (Wherry 2011). Studies from LCMV infection demonstrated that the severity of T cell exhaustion correlated with the level of antigen stimulation (Wherry, Blattman et al. 2003). In agreement, removal of persisting antigen early during chronic infection favoured differentiation of $CD8^+$ T cells into functional memory cells, however, once T cell exhaustion was established memory formation could not be rescued (Angelosanto, Blackburn et al. 2012). Transcription factors such as nuclear factor of activated T cells (NFAT) and Blimp-1 also contribute to $CD8^+$ T cell exhaustion by directly regulating expression of exhaustion-associated genes, such as the co-inhibitory receptor PD-1 (Wherry, Ha et

al. 2007, Martinez, Pereira et al. 2015) and inhibiting differentiation into memory T cells respectively (Shin, Blackburn et al. 2009).

Initial studies characterising T cell exhaustion have primarily focussed on $CD8^+$ T cells, however, during chronic infections $CD4^+$ T cells also show diminished IL-2, IFNg and TNFα production (Butler 2012, Jayaraman, Jacques et al. 2016). Similar to $CD8^+$ T cells, exhausted $CD4^+$ T cells display a unique molecular signature compared to effector and memory $CD4^+$ T cells (Crawford, Angelosanto et al. 2014). In contrast to exhausted $CD8^+$ T cells, $CD4^+$ T cell exhaustion occurs earlier during chronic LCMV infection and cells preferential express different co-inhibitory and co-stimulatory receptors such as; CTLA-4, BTLA and OX-40 (Crawford, Angelosanto et al. 2014). In addition, the transcription factors Helios and Bcl-6 are associated with $CD4^+$ T cell exhaustion, but not $CD8^+$ T cell exhaustion. In comparison to $CD8^+$ T cells, the current understanding of the mechanisms underpinning of $CD4^+$ T cell exhaustion during infection are greatly limited.

1.5.1 Co-inhibitory receptors

Co-inhibitory receptors play a critical role in the maintenance of immune homeostasis through limiting T cell activation, ensuring proper contraction of effector T cell responses and preventing autoimmunity (Attanasio and Wherry, 2016). As such, the role of co-inhibitory receptors in regulating T cell responses have been extensively studied in the context of autoimmune diseases (Wang, Yoshida et

al. 2005, Joller 2011). More recently, the importance of co-inhibitory receptors has moved to the forefront of cancer and chronic viral infection, due to their high level of expression and role in T cell exhaustion (Fourcade et al., 2010; Barber et al., 2006; Day et al., 2006; Gruener et al., 2001). Importantly, T cell exhaustion is reversible as blocking co-inhibitory signalling pathways can restore T cell functions (Wherry 2011). Thus, transient blockade of inhibitory receptors is an attractive therapeutic strategy.

1.5.2 PD-1

Programmed death 1 (PD-1) is a member of the CD28 family that is upregulated on activated T cells and B cells (Attanasio and Wherry 2016). PD-1 binds two ligands including; PD-L1 which is expressed by haematopoietic and non-haematopoietic cells, as well as PD-L2, which is exclusively expressed on activated antigen presenting cells (APC) (McAlees, Lajoie et al. 2015). PD-1 plays an important role in immune homeostasis and peripheral tolerance as illustrated by the immunopathology that can develop in *Pdcd1*$^{-/-}$ and PD-L1$^{-/-}$ mice (Wang, Yoshida et al. 2005, Barber, Wherry et al. 2006). Interestingly, PD-1 ligation differentially regulates CD4^{+} T helper subsets, as evidenced by increased Th1 and Th17 cytokines, but significantly reduced Th2 cytokines in PD-1$^{-/-}$ mice (McAlees, Lajoie et al. 2015). PD-1 suppression may therefore play a more prominent role in type 1 associated diseases, such as cancer, viral infections and malaria.

PD-1 was the first co-inhibitory receptor shown to be markedly upregulated on the surface of exhausted $CD8^+$ T cells (Barber, Wherry et al. 2006, Day, Kaufmann et al. 2006). As such, PD-1 has come under heavy investigation as a novel cancer immunotherapy and PD-1 blockade has exhibited significant efficacy in treating several cancers (Brahmer et al., 2010; Callahan and Wolchok, 2013). PD-1 possess an ITIM (immunoreceptor tyrosine-based inhibition motif) and ITSM (immunoreceptor tyrosine-based switch motif) in its cytoplasmic tail, which is thought to play a role in PD-1-mediated suppression. Engagement of PD-1 with its ligand PD-L1 or PD-L2, leads to the phosphorylation of the two tyrosine motifs, which in turn recruits SHP2. SHP2 then dephosphorylates multiple components of the downstream TCR signalling pathway and CD28 signalling pathway (Yokosuka, Takamatsu et al. 2012, Hui, Cheung et al. 2017). However, PD-1 appears to inhibit CD28 signalling to a greater extent than TCR signalling, suggesting that PD-1 mediates T cell inhibition by blocking positive CD28 signals. This is supported by studies showing that the restoration of effector T cell function following PD-1 blockade is dependent on CD28 signalling (Hui, Cheung et al. 2017, Kamphorst, Wieland et al. 2017).

The transcription factor T-bet (expressed by $CD4^+$ Th1 cells and $CD8^+$ T cells) directly represses transcription of the *Pdcd1* gene (encoding PD-1), however, persistent antigen stimulation in chronic infection downregulates T-bet and correlates with more severe $CD8^+$ T cell exhaustion (Kao, Oestreich et al. 2011, Odorizzi, Pauken et al. 2015). Odorizzi et al. propose that PD-1 signalling limits TCR stimulation promoting the survival of the T-bet^{hi} $CD8^+$ T cells. T-bet signalling then limits PD-1 expression to

intermediary levels, preventing terminal exhaustion and promoting the survival of an exhausted pool of T cells capable of conferring partial protection against chronic infection (Odorizzi, Pauken et al. 2015). However, T cell exhaustion can occur in the absence of PD-1 (Odorizzi, Pauken et al. 2015), thus implicating a role for alternative co-inhibitory receptors in T cell exhaustion.

1.5.3 CTLA-4

Cytotoxic T lymphocyte associated protein-4 (CTLA-4) is an inhibitory receptor constitutively expressed by Treg cells and by T helper cells upon activation (Seidel, Otsuka et al. 2018). Unlike PD-1 which is a surface receptor, CTLA-4 is primarily located within intracellular vesicles, but is expressed at the cell surface following TCR and CD28 stimulation (Seidel, Otsuka et al. 2018). The importance of CTLA-4 as a negative regulator of T cell functions was demonstrated by CTLA-$4^{-/-}$ mice which succumb to lymphoproliferation disorder within 3-5 weeks of age (Tivol, Borriello et al. 1995). CTLA-4 has also been implicated in the suppression of T cell responses during cancer (Callahan and Wolchok 2013). Indeed, an anti-CTLA-4 blocking antibody was the first immune checkpoint inhibitor approved for use in treating cancer patients (Seidel, Otsuka et al. 2018).

CTLA-4 is a homolog of the activating T cell receptor CD28 (Parry, Chemnitz et al. 2005). The immunosuppressive effects of CTLA-4 are mediated through binding to CD80/CD86 with greater affinity than CD28, thereby outcompeting positive signalling

in T cells. Accordingly, the fatal phenotype observed in CTLA-4 deficient mice is lost upon concurrent deletion of either CD80/CD86 (Mandelbrot, McAdam et al. 1999) or CD28 (Tai, Van Laethem et al. 2007). Further studies have shown that CTLA-4 can also capture CD80 and CD86 from the surface of antigen presenting cells through trans-endocytosis, which can protect against fatal T cell mediated autoimmunity (Wing, Onishi et al. 2008, Qureshi, Zheng et al. 2011). Therefore, antagonising CD28 signalling is the main mechanism through which CTLA-4 supresses T cell functions.

1.5.4 LAG3

Targeting PD-1 and CTLA-4 in the clinic has shown significant efficacy against several different cancers (Seidel, Otsuka et al. 2018). However, there are still many patients who do not respond, and some tumours types remain refractory to these therapies. Additionally, improved understanding of the synergy between inhibitory receptors that contribute T cell exhaustion, has prompted investigations into other co-inhibitory receptors such as LAG-3, Tim3 and TIGIT, with the aim of broadening the therapeutic repertoire (Anderson 2016).

Lymphocyte activation gene-3 (LAG-3) is expressed on activated $CD4^+$ and $CD8^+$ T cells, NK cells and Treg cells (Anderson 2016). Although LAG-3 deficient mice display no significant phenotype (Bettini, Szymczak-Workman et al. 2011), LAG-3 deficiency in diabetes prone mice accelerates the onset of type 1 diabetes, highlighting a critical role of LAG3 in immunotolerance (Bettini, Szymczak-Workman et al. 2011). LAG-3

structurally resembles the CD4 co-receptor and suppresses cells through interactions with stable cognate peptide-MHC II complexes (pMHC II) (Maruhashi, Okazaki et al. 2018). However, direct competition with CD4 for MHC II binding is minimal. LAG-3 can also impact $CD8^+$ T cell function (Blackburn, Shin et al. 2009) which does not rely on MHC II signalling, therefore it has been speculated that LAG-3 may have additional ligands. For example, LSECtin expressed by DC and tumour cells has been reported to interact with LAG3, thus indicating an alternative mechanism through which LAG-3 can inhibit $CD8^+$ T cells (Xu, Liu et al. 2014).

LAG-3 expression has been associated with T cell exhaustion during chronic infection (Blackburn, Shin et al. 2009, Butler 2012). Interestingly, blockade of LAG3 alone has little effect on recovering T cell responses, however, co-blockade with PD-L1 can significantly improve $CD8^+$ and $CD4^+$ effector T cell responses during chronic infections (Blackburn, Shin et al. 2009, Butler 2012) and cancer (Woo, Turnis et al. 2012). Thus, LAG3 appears to act synergistically with PD-1 to negatively regulate effector T cell functions.

1.5.5 Tim3

T cell immunoglobulin-3 (Tim3) is also among the next generation of co-inhibitory receptors to receive clinical attention (Anderson 2016). It was first discovered 17 years ago and was shown to be selectively expressed on IFNg-producing Th1 and $CD8^+$ T cells (Monney, Sabatos et al. 2002). Since then, Tim3 has also been identified

on Treg cells, DC and monocytes (Anderson 2016). Tim3 was initially identified as a negative regulator of type 1 immunity, as loss of Tim3 signalling *in vivo* exacerbates experimental autoimmune encephalomyelitis (Monney, Sabatos et al. 2002) and leads to a hyperproliferative Th1 response with amplified type 1 cytokines (Sabatos 2003). Interactions between Tim3 and its ligand galectin-9 supresses type 1 responses by promoting Tim3-dependent death of $IFNg^{+}$ cells (Zhu, Anderson et al. 2005).

Tim3 has also been identified as a marker of T cell exhaustion in cancer and bacterial and viral infections (Golden-Mason 2009, Gao, Zhu et al. 2012, Jayaraman, Jacques et al. 2016). In these cases, T cells expressing Tim3 show reduced proliferation and cytokine production, however, blockade of Tim3 restores these functions. Interestingly, co-expression of Tim3 and PD-1 induces the greatest defects in T cell pro-inflammatory cytokine production, as such co-blockade of Tim3 and PD-1 is consistently more effective at restoring effector functions compared to Tim3 or PD-1 blockade alone (Jin, Anderson et al. 2010, McMahan, Golden-Mason et al. 2010).

1.5.6 TIGIT

T cell immunoglobulin and ITIM domain (TIGIT) is one of the newest receptors identified to have an inhibitory effect on T cell function (Anderson 2016). It is a member of the Ig superfamily and is expressed on activated T cells, Tfh cells, Treg cells and NK cells (Anderson 2016). TIGIT binds to its ligand, CD155, with greater

affinity than its positive counterpart CD226, thereby outcompeting the co-stimulatory signals. Furthermore, it can also directly bind CD226, disrupting homodimerization and its costimulatory functions (Johnston, Comps-Agrar et al. 2014). Interestingly, engagement between TIGIT and CD155 on DC can down regulate IL-12 production and upregulate IL-10 production, thus inducing a tolerogenic state in DC (Yu, Harden et al. 2009). It is unclear why CD226 engagement with CD155 does not induce this same tolerizing effect. However, it is known that the specific clustering of TIGIT at the membrane is necessary for CD155 phosphorylation therefore, it is possible that CD226 induces alternative clustering patterns that leads to positive signalling (Stengel, Harden-Bowles et al. 2012).

In addition to inducing tolerogenic DC, TIGIT can also inhibit immune responses by promoting Treg cell functions (Joller 2014, Kurtulus 2015). TIGIT expression on Tregs defines a specific subset of Treg cells and directs their regulatory functions. TIGIT ligation on Treg cells upregulates the effector molecule fibrinogen-like protein 2 (Fgl2), which mediates the preferential suppression of Th1 and Th17 cells, whilst sparing Th2 cells (Joller 2014). Further to this indirect inhibitory role, TIGIT can also have intrinsic inhibitory effects (Joller 2011). Genetic depletion of TIGIT or administration of an antagonistic antibody against TIGIT, causes significant hyperproliferation of $CD4^+$ T cells, exacerbating autoimmune diseases (Joller 2011). Conversely, addition of an agonising TIGIT antibody can prevent $CD4^+$ T cell proliferation (Joller 2011). Collectively, these studies show that TIGIT plays an

important role in regulating T cell effector functions and contributes to peripheral tolerance.

Tumour infiltrating $CD8^+$ T cells and NK cells express high levels of TIGIT in both humans and mice and TIGIT has been implicated in the induction of T cell exhaustion (Johnston, Comps-Agrar et al. 2014, Kurtulus 2015). Whilst PD-1 blockade can partially reverse T cell exhaustion during cancer, combined blockade of PD-1 and TIGIT significantly enhances $CD8^+$ T cell effector functions, leading to tumour rejection (Johnston, Comps-Agrar et al. 2014). TIGIT also synergises with Tim3 within the tumour microenvironment to impair effector T cell responses (Kurtulus 2015). Collectively TIGIT plays an important role in regulating multiple cell types and blockade of TIGIT in combination with other inhibitory receptors is a promising therapeutic option.

1.5.7 T cell exhaustion in malaria

There is growing evidence that blood stage *Plasmodium* infection elicits T cell exhaustion, as demonstrated by decreased IFNg production, proliferation and deletion of parasite-specific T cells prior to the peak of infection (Xu, Wipasa et al. 2002, Chandele 2011, Butler 2012, Illingworth, Butler et al. 2013, Villegas-Mendez, Shaw et al. 2016). Early findings identified increased expression of PD-1, CTLA-4 and LAG3 on $CD4^+$ and $CD8^+$ T cells isolated from children persistently exposed to *P. falciparum* (Schlotmann, Waase et al. 2000, Chandele 2011, Illingworth, Butler et al. 2013). Whilst functional responses where not measured in these studies, evidence

suggested that sustained levels of inhibitory receptors contributed to the loss of effector T cells, reduced humoral immunity and ultimately poor protection against human and murine malaria (Chandele 2011, Illingworth, Butler et al. 2013). Indeed, further investigations in mouse models of malaria identified that co-blockade of PD-1 and LAG3 could enhance $CD4^+$ T cell effector cytokine production and the antibody-mediated response, leading to accelerated parasite clearance (Butler 2012). Therefore, $CD4^+$ T cell exhaustion plays a role in the pathogenesis of malaria. Interestingly, reducing the parasitemia through drug treatment of infected mice partially reverses $CD4^+$ T cell exhaustion (Butler 2012, Zander, Obeng-Adjei et al. 2015), which is consistent with previous studies suggesting that prolonged antigen exposure drives T cell exhaustion(Wherry 2011).

In the context of malaria, PD-1 has been the most extensively studied. Genetic ablation of PD-1 in mice improved both $CD4^+$ T cell and $CD8^+$ T cell responses, which was necessary for preventing a chronic infection (Horne-Debets, Faleiro et al. 2013). Interestingly, PD-1 knock out mice infected with *P.chabaudi* did not display any alterations in their B cell and antibody-mediated responses (Horne-Debets, Faleiro et al. 2013), inconsistent with other reports showing PD-1 deficiency leads to improved humoral immunity (Liu, Lu et al. 2015). Furthermore co-blockade of PD-L1 and LAG3 during *P.yoelii* infection increased Tfh numbers, which in turn improved the parasite-specific antibody response (Butler 2012). Horne-Debets, argue that the increased numbers of regulatory follicular T cells observed in PD-$1^{-/-}$ mice are responsible for this apparent inconsistency (Horne-Debets, Faleiro et al. 2013).

Typically PD-1 is associated with inhibition of T cell responses, however, engagement between PD-1 and PD-L2 is important for establishing effective Th1 responses that protect against lethal malaria (Karunarathne, Horne-Debets et al. 2016). Moreover, a higher ratio of PD-L2:PD-L1 expressing DC correlates with reduced parasitemia in both humans and murine models of malaria (Karunarathne, Horne-Debets et al. 2016). Interestingly, it was shown that PD-L2 can out-compete PD-L1 for PD-1 binding, which prevents PD-L1 induced negative regulation and PD-1 from binding CD80. This has important implications as PD-1-CD80 ligation can recruit CTLA-4 to the immunological synapse (IS) increasing the inhibitory environment around the TCR (Pentcheva-Hoang, Egen et al. 2004). Karunarathne *et al* therefore propose that PD-1-PD-L2 engagement provides another level of positive regulation by excluding CD80 and CTLA-4 from the immunological synapse, whilst recruiting activating molecules such as ICOS and CD3 to the TCR (Karunarathne, Horne-Debets et al. 2016). Therefore, the specific arrangement of co-stimulatory and co-inhibitory receptors in the IS may be critical for regulating T cell functions during infection.

Whilst the expression of inhibitory receptors has been associated with the pathogenesis of malaria, crosstalk between T cell co-stimulatory and co-inhibitory pathways is also essential for protection to blood stage malaria. Activation of the stimulatory receptor OX40 boosts the Th1 response during *Plasmodium* infection (Zander 2017). However, disruption of PD-1 signalling concurrent with exogenous OX-40 stimulation significantly increases IFNg production, which is detrimental to the

parasite-specific Tfh cell response (Zander 2017). Therefore, low levels of PD-1 signalling in Th1 cells may be beneficial in facilitating the generation of a protective Tfh response during *Plasmodium* infection. In addition, increased CTLA-4 expression during *P.berghei* infection is protective against severe pathology in the brain and the induction of experimental cerebral malaria (Hafalla, Claser et al. 2012). Tightly co-ordinated co-stimulatory and co-inhibitory signalling in T cells may, therefore, be required to allow the development of a potent Th1 and Tfh response with limited immunopathology.

1.6 T cell behaviour

T cell interactions rely on the capacity of the TCR to interact with specific pMHC on APC, which induces a programme of proliferation and differentiation generating functional effector T cells (Dustin 2008). Disruption to these interactions could therefore be critical in altering T cell activation and effector functions.

For over a decade intravital imaging studies have explored the complex relationship between T cell behaviour and immune functions (Bousso 2008). Early studies identified essential alterations in T cell behaviour in the lymph nodes during T cell priming (Bousso and Robey 2003, Mempel, Henrickson et al. 2004). In the absence of antigen T cells are highly motile and form brief contacts with DC, promoting efficient scanning for cognate antigen by T cells (Bousso and Robey 2003, Mempel, Henrickson et al. 2004). Introduction of cognate antigen induces T cell activation, which is

highlighted by a distinct alteration in T cell behaviour (Mempel, Henrickson et al. 2004). Within 24 hours of antigen administration, T cell migration arrests and strong contacts between T cells and DC are formed and persist for hours. Importantly, these stable interactions are regulated by TCR-pMHC signalling, as administration of an MHC II blocking antibody leads to cellular dissociation of $CD4^+$ T cells and DC (Celli 2007). Following activation, T cells then regain motility and reengage in brief contacts with DC (Mempel, Henrickson et al. 2004).

T cell encounters with DC bearing cognate antigen can result in stable contacts that persist for hours, or transient migratory interactions, termed synapse and kinapse respectively (Dustin 2008). Whether a T cell forms a synapse or a kinapse appears to be dependent on antigen affinity, with high affinity antigens leading to pronounced deceleration, strong TCR signalling and synapse formation (Moreau, Lemaitre et al. 2012, Moreau 2015). In contrast, T cells preferentially engage in kinapse formation in the presence of weaker affinity antigens (Moreau, Lemaitre et al. 2012, Moreau 2015). Despite the differences in contact duration, TCR signalling occurs during both synapse and kinapse formation, however, synapses induce the strongest TCR stimulation necessary for cytokine production and proliferation (Mempel, Henrickson et al. 2004, Miller, Safrina et al. 2004, Moreau, Lemaitre et al. 2012). The interaction length can also play a role in dictating T cell differentiation (VanPanhuys 2014). Interestingly, Th1 differentiation requires longer interactions and stronger TCR signalling, whereas Th2 differentiation requires shorter interactions and weaker TCR signalling (VanPanhuys 2014).

The dynamics of T cell-DC interactions has been well studied during T cell activation, however, far less is understood about effector T cell behaviour at sites of inflammation. Similar to naïve T cells, effector T cells also require stable engagements with APC for targeted delivery of effector functions (Egen, Rothfuchs et al. 2011). Cytotoxic T cells engage with target cells and deliver lytic granules across the IS, specifically inducing cell death in the target cell without affecting bystander cells (Dustin 2008)**.** In contrast, effector $CD4^+$ T cells can extend their functions beyond the IS by generating an IFNg gradient capable of activating target cells 80µm away from the site of antigen presentation (Muller, Filipe-Santos et al. 2012). This could be highly beneficial as only a small number of T cell APC contacts are required for widespread induction of intracellular defence mechanisms. T cell effector functions are therefore, mediated in an antigen-dependent manner and require continuous engagement with APC for maintenance of effector state (Obst, van Santen et al. 2005). This is further illustrated by *in vitro* studies in which activated $CD4^+$ T cells rapidly stop producing IFNg and TNFα upon APC removal (Corbin and Harty 2005). *In vivo* studies have also demonstrated that restricted antigen presentation increases T cell motility and decreases IFNg production (Egen, Rothfuchs et al. 2011). However, administration of exogenous antigen markedly modifies T cell behaviour, leading to migration arrest and significantly increased levels of IFNg production.

Initial studies *in vitro* identified that TCR engagement with strong pMHC ligands induced a stop signal in which T cells arrested and formed a stable synaptic

connection with APC membrane (Dustin 2004). More recent studies *in vivo* support the *in vitro* data and show that in tissues effector T cells arrest in the presence of cognate antigen and importantly, there is a tight association between T cell arrest and immune functions (Azar, Lemaitre et al. 2010, Egen, Rothfuchs et al. 2011). However, co-inhibitory receptors expressed on activated T cells can inhibit the TCR stop signal, preventing migration arrest and limiting effector cytokine production (Fife 2009, Honda 2014). Disruption to PD-1 signalling however, reduces T cell motility, recovering antigen-mediated cytokine production (Honda 2014). CTLA-4 has also been reported to prevent the TCR stop signal (Schneider 2006), however, there is conflicting evidence indicating that blockade or loss of CTLA-4 does not affect T cell motility (Downey 2008, Fife 2009, Honda 2014). The reason for this discrepancy is currently unclear. Although activated T cells require continuous interactions with APC for effector functions, co-inhibitory receptor-mediated disengagement of T cells from APC may provide several benefits during infection. Firstly, limiting antigen engagement and TCR signalling can prevent over stimulation, which is thought to contribute to terminal T cell exhaustion (Odorizzi, Pauken et al. 2015). In addition, it promotes further engagements with new APC, potentially allowing for deeper migration into the tissue and activation of more target cells (Honda 2014). Finally, it provides a mechanism to prevent auto-reactive T cells from engaging with APC and inducing autoimmunity (Fife 2009). Interestingly, a study from Zinselmeyer *et al* reported a contradictory role for PD-1 in regulating T cell behaviour (Zinselmeyer, Heydari et al. 2013). Their data identified that during chronic LCMV infection, PD-L1 stabilises the IS and promotes T cell arrest, but decreases TCR signalling and cytokine production. Formation of a stable synapse may therefore be necessary for PD-1 to

successfully regulate TCR signalling during chronic infection. Inhibitory receptors can therefore, negatively regulate T cells by altering T cell APC interactions, however, our understanding of how T cell dynamics contribute to the onset of T cell exhaustion is limited.

Stable interactions between T cells and DC have been reported in the first 24 hours of *P.chabaudi* infection (Borges da Silva, Fonseca et al. 2015), consistent with previous studies examining T cell priming (Bousso and Robey 2003, Mempel, Henrickson et al. 2004). However, uptake of hemozoin by DC can prevent the formation of stable interactions with naïve T cells, which reduces effector T cell responses (Millington, Gibson et al. 2007). Although hemozoin did not alter antigen presentation, suppression of co-stimulation may modulate T cell-APC interactions during malaria. This sub-optimal $CD4^+$ T cell activation may also affect T cell migration into B cell follicles, necessary for B cell activation (Millington, Di Lorenzo et al. 2006). Therefore, alterations in T cells-APC interactions could mediate the dysfunctional $CD4^+$ T cell response observed during malaria. However, the migration and dynamic behaviour of established effector $CD4^+$ T cells is yet to be investigated during malaria. Moreover, the dynamic relationship between T cell behaviour and the induction of $CD4^+$ T cell exhaustion during a complex infection is largely unexplored.

1.8 Aims and Objectives

$CD4^+$ T cell exhaustion significantly impairs parasite control during blood stage malaria, however, the underlying mechanisms promoting $CD4^+$ T cell exhaustion during *Plasmodium* infection are poorly understood.

Therefore, the overall aims of my PhD were to utilise a model antigen-specific $CD4^+$ T cell system to further our understanding of i) the underlying mechanisms contributing to loss of $CD4^+$ T cell effector functions and ii) the role of specific co-inhibitory receptors in the development of exhausted $CD4^+$ T cells during blood stage malaria.

The specific questions to be addressed were:

i) Is $CD4^+$ T cell exhaustion associated with alterations in T cell-APC interactions during blood stage malaria?

ii) Do TIGIT and PD-1 synergistically promote $CD4^+$ T cell exhaustion during blood stage malaria?

iii) Does Tim3, in combination with PD-1 contribute to $CD4^+$ T cell exhaustion during blood stage malaria?

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Chapter 2: Antigen-specific CD4$^+$ T cell exhaustion during malaria is not due to the failure of T cells to co-localise with antigen presenting cells

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Manuscript in preparation

All work was carried out by R.S.D

Abstract

Dysregulation of $CD4^+$ T cells is a common feature of chronic infections, including malaria. $CD4^+$ T cells lose effector functions becoming functionally exhausted during blood stage malaria, which significantly impairs parasite control. However, the mechanisms that underlie $CD4^+$ T cell exhaustion during malaria are not well understood. We have shown that antigen-specific $CD4^+$ T cells rapidly develop an exhausted phenotype during blood stage malaria, which is associated with the upregulation of PD-1, LAG3 and TIGIT. Importantly, attrition of the effector $CD4^+$ T cell response appeared to correspond with loss of MHC II signals, as blockade of MHC II signalling post-priming did not significantly impact the function, or magnitude of Th1 cells. Interestingly, functional and exhausted antigen-specific $CD4^+$ T cells accumulated within the white pulp of the spleen and colocalised with antigen-presenting cells (APC). Moreover, $CD4^+$ T cell exhaustion was associated with decreased T cell motility and prolonged interactions with APC during malaria. Together, we have shown that, despite interactions with APC, subversion of MHC II signalling in Th1 cells contributes to a dysfunctional $CD4^+$ T cell response during blood stage malaria.

Introduction

$CD4^+$ T cells are essential for the protection against blood stage malaria, as depletion of $CD4^+$ T cells in mice during *Plasmodium* infection is lethal (Suss, Eichmann et al. 1988). Notably, interferon gamma (IFNg) production from $CD4^+$ Th1 cells strongly correlates with protection to blood stage malaria in both humans and mice (van der Heyde, Pepper et al. 1997, King and Lamb 2015). Additionally, $CD4^+$ follicular T helper (Tfh) cells confer protection by inducing antibody production from B cells, which is necessary for protection (von der Weid, Honarvar et al. 1996, Zander 2017). However, it has now become clear that effector $CD4^+$ T cells lose their effector capacity and become functionally exhausted during malaria (Butler 2012, Horne-Debets, Faleiro et al. 2013). Expression of co-inhibitory receptors is a hallmark feature of T cell exhaustion and is associated with decreased T cell proliferation and production of effector cytokines (Wherry and Kurachi 2015). Notably, several reports have identified that *Plasmodium* infection induces the expression of multiple co-inhibitory receptors on $CD4^+$ T cells (Schlotmann, Waase et al. 2000, Chandele 2011, Butler 2012). Moreover, mechanistic insight from experimental models of malaria identified that expression of PD-1 and LAG3 on parasite-specific $CD4^+$ T cells contributes to T cell exhaustion and impedes parasite clearance (Butler 2012). Thus, demonstrating that $CD4^+$ T cell exhaustion impairs protective immunity to malaria. However, the molecular mechanisms underlying $CD4^+$ T cell exhaustion during malaria are not well understood.

Interactions between naïve T cells and dendritic cells (DC) bearing cognate antigen within secondary lymphoid tissues activates T cells, generating effector T cells capable of clearing invading pathogens (Bousso and Robey 2003, Mempel, Henrickson et al. 2004). Recognition of peptide MHC complexes by the T cell receptor (TCR) induces a TCR stop signal, causing T cells to arrest and form a stable synapse with antigen presenting cells (APC) (Dustin 2004). During T cell priming cessation of TCR signalling terminates stable T cell-DC interactions and prevents T cell expansion and IFNg production (Celli 2007). In contrast, prolonged antigen-dependent contact supports $CD4^+$ T cell expansion and IFNg production (Celli 2007). Similarly, stable antigen-dependent T cell interactions are required for robust effector T cell responses during different infections (John 2009, Beattie, Peltan et al. 2010, Egen, Rothfuchs et al. 2011). Inability to form long-lived stable contacts promotes T cell migration, reducing TCR stimulation and limiting T cell effector functions (Egen, Rothfuchs et al. 2011, Moreau 2015). Indeed, the co-inhibitory receptors PD-1 and CTLA-4 can block the TCR stop signal, preventing stable interactions between effector T cells and APC and subsequent cytokine production (Schneider 2006, Fife 2009, Honda 2014). Elucidating the relationship between effector T cell-APC interactions and subsequent expression of effector cytokines by T cells, is therefore, critical for understanding the basis of a protective or dysfunctional effector T cell response.

Within the first 24 hours of *Plasmodium* infection, $CD4^+$ T cells form stable interactions with DC in the T cell rich areas of the spleen (Borges da Silva, Fonseca et al. 2015). However, uptake of the malaria pigment hemozoin by DC can alter cellular

interactions with naïve $CD4^+$ T cells during blood stage malaria (Millington, Gibson et al. 2007). Despite antigen presentation by DC, naïve T cells are unable to form long-lasting stable interactions with hemozoin loaded DC, which is associated with a reduced effector $CD4^+$ T cell response during malaria (Millington, Gibson et al. 2007). However, it is currently unclear whether alterations in antigen-dependent interactions between effector $CD4^+$ T cell and APC contribute to the development of T cell exhaustion during malaria.

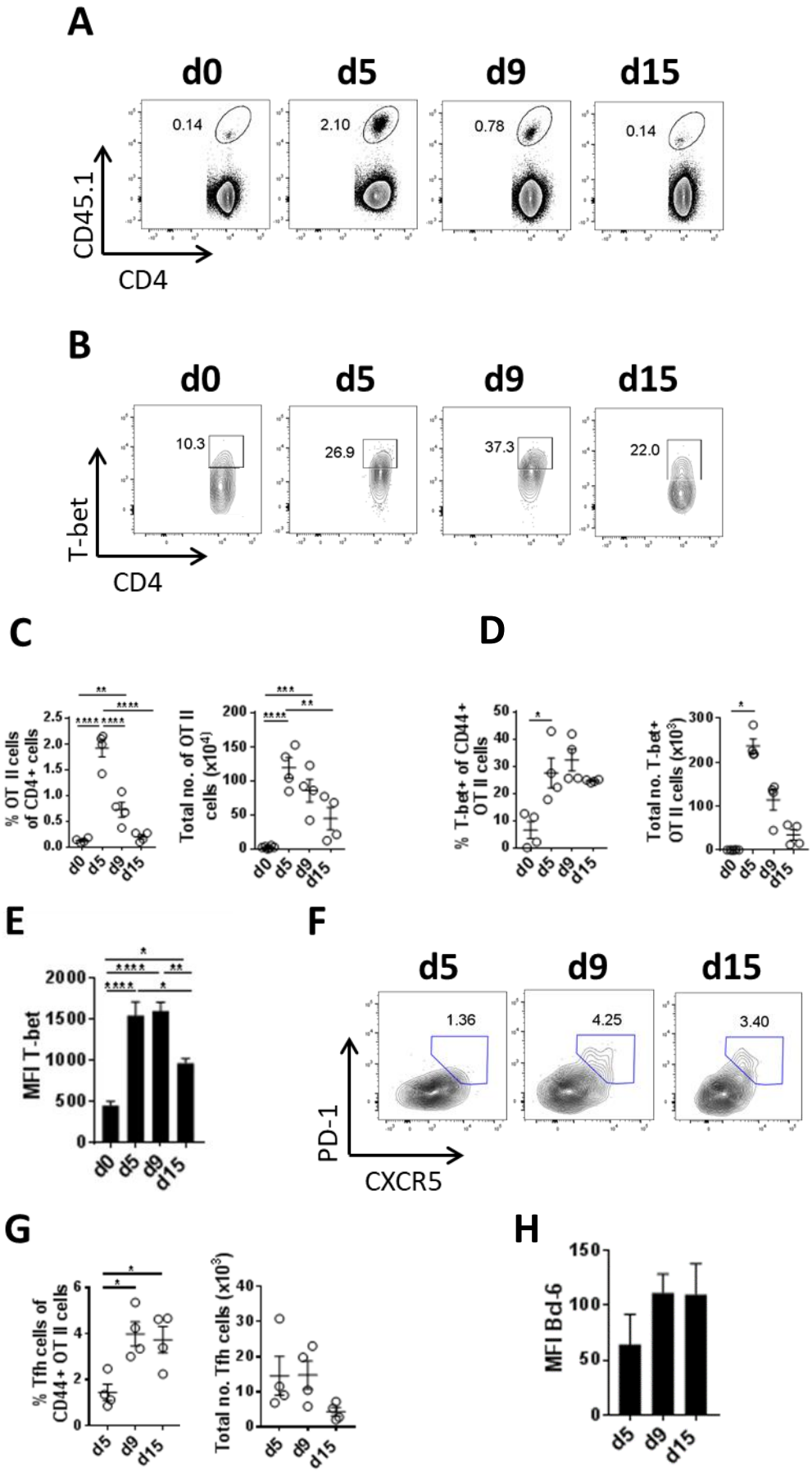
To gain novel insights into the mechanisms underlying effector $CD4^+$ T cell exhaustion during blood stage malaria, we have generated an antigen-specific system to characterise the $CD4^+$ T cell response during a non-lethal *Plasmodium yoelii (P.yoelii)* infection. We showed that antigen-specific $CD4^+$ T cells display signs of T cell exhaustion as early as day 9 of infection, significantly before peak parasitaemia. Blockade of MHC II signalling did not significantly exacerbate the magnitude or nature of Th1 cell attrition and exhaustion during established infection, suggesting that TCR signalling is inhibited. However, using dynamic 2-photon microscopy we observed that exhausted antigen-specific $CD4^+$ T cells were not defective in their ability to form stable contacts with CX_3CR1^+ APC. Therefore, we propose, that despite effector T cell interactions with APC, regulatory signals from co-inhibitory receptors subvert MHC II-TCR signalling in Th1 cells limiting effector functions during blood stage malaria.

Results

Generation of an antigen-specific CD4$^+$ T cell response to blood stage *P.yoelii* infection

To examine the mechanisms underlying malaria-induced CD4$^+$T cell exhaustion, we transferred ovalbumin-specific TCR transgenic CD4$^+$ T cells (OT II cells) into C57BL/6 mice. Mice were subsequently infected with a genetically modified non-lethal strain of *P.yoelii* that expressed ovalbumin (OVA) (hereafter referred to as *P.yoelii-OVA*). The frequencies and total numbers of OT II cells significantly increased during *P.yoelii-OVA* infection, reaching maximal numbers on day 9 of infection before significantly declining (Fig 2.1A, B). In contrast, the frequencies of OT II cells were significantly lower in mice infected with *P.yoelii*, indicating that OT II expansion was OVA-dependent (Fig S2.1). The frequencies and absolute numbers of Th1 OT II cells (indicated by T-bet expression) significantly increased between day 0 and day 5 of infection, after which, although the frequencies of T-bet$^+$ OT-II cells did not significantly change, the total numbers significantly reduced (Fig 2.1C, D). Interestingly, T-bet expression level correlates with effector function and T cell exhaustion (Odorizzi, Pauken et al. 2015) and during *P.yoelii*-OVA infection effector OT-II cells (defined as CD44$^+$) expressed significantly less T-bet between days 9 and 15 of infection (Fig 2.1E).

As well as Th1 cells, Th2, Tfh cells and regulatory T cells (Treg cells), are all subsets of $CD4^+$ T cells. The immune response to blood stage malaria has been previously described as biphasic, with a temporal change from Th1 to Th2/Tfh immunity (Perez-Mazliah and Langhorne 2014). Therefore, we also examined the dynamics of these subsets within the OT II population over the course of *P.yoelii*-OVA infection (FigS2.2). Th2 cells and Treg cells did not develop within the effector OT II population at any examined time point during *P.yoelii-OVA* infection (FigS2.3A, B). In contrast, whilst Tfh OT II cells (identified as $CXCR5^{hi}$ $PD\text{-}1^{hi}$) were not detected in naïve mice, the frequencies and total numbers of Tfh OT II cells increased during infection, until day 9, after which they slightly decreased (Fig2.1F and S2.3C). Bcl-6 is a critical transcription factor in Tfh cell differentiation (Crotty 2011). Bcl-6 expression in Tfh OT II cells was not significantly altered over the course of infection (Fig2.1H). Collectively, these data show that OT II cells activate and differentiate in an OVA-dependent manner, but are rapidly lost following day 5 of *P.yoelii*-OVA infection.


A
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26.9
37.3
22.0
T-bet
CD4
C
% OT II cells of CD4+ cells
Total no. of OT II cells (x10^4)
d0
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d9
d15
D
% T-bet+ of CD44+ OT II cells
Total no. T-bet+ OT II cells (x10^3)
E
MFI T-bet
F
d5
d9
d15
1.36
4.25
3.40
PD-1
CXCR5
G
% Tfh cells of CD44+ OT II cells
Total no. Tfh cells (x10^3)
H
MFI Bcl-6

Figure 2.1 Characterisation of *P.yoelii-OVA* induced OT II cell response

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and analysed by flow cytometry. (A) Representative flow cytometric plots of OT II cells. (B) Representative flow cytometric plots $T\text{-}bet^+$ OT II cells. (C) Percentages (left) and total numbers (right) of OT II cells. (D) Percentages (left) and total numbers (right) of $T\text{-}bet^+$ OT II cells. (E) Mean fluorescence intensity (MFI) of T-bet in the effector ($CD44^+$) OT II population. (F) Representative flow cytometric plots of Tfh OT II cells. (G) Percentages (left) and total numbers (right) of Tfh OTII cells. (H) MFI of Bcl-6 Tfh OT II cells during infection. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ***p ≤0.001 **** p ≤0.0001 (one-way ANOVA with Tukey's multiple comparison test).

Blood stage *P.yoelii* infection induces antigen-specific $CD4^+$ T cell exhaustion

Our results show that the antigen-specific OT II cell response underwent rapid collapse during *P.yoelii-OVA* infection. Whilst antigen-specific $CD4^+$ T cell apoptosis is a feature of malaria (Xu 2002), $CD4^+$ T cells also become exhausted and lose effector capacity during malaria (Butler 2012). Consistent with this, the frequencies and numbers of $IFNg^+$ effector OT II cells significantly increased on day 5 of infection, before sharply decreasing on day 9 of infection. (Fig 2.2A, C). Similarly, effector OT II cell proliferative capacity was significantly reduced from day 9 of infection, as demonstrated by the decrease in frequencies and total numbers of $Ki\text{-}67^+$ effector OT II cells between day 5 and day 9 of *P.yoelii-OVA* infection (Fig2.2B, D). Thus,

suggesting that the total effector OT II cell population was functionally exhausted following day 5 of *P.yoelii-OVA* infection.

We next investigated whether this decrease in OT II functionality was uniform across the different T helper OT II subsets during *P.yoelii-OVA* infection. Approximately 50% of Th1 OT II cells expressed IFNg on day 5 of infection (Fig2.2E). However, the frequencies and total numbers of $IFNg^+$ Th1 OT II cells significantly decreased on day 9 of infection compared with day 5 and remained low on day 15 of infection (Fig2.2E). Similarly, a high proportion of Th1 OT II cells were $Ki\text{-}67^+$ on day 5 of infection, but this was significantly reduced by day 9 of infection (Fig2.2F). Tfh OT II cells were also highly proliferative on day 5 of infection, however, this was significantly reduced by day 9 of infection (Fig2.2F). These data show that T cell exhaustion also specifically develops in the T helper OT II subsets by day 9 of *P.yoelii-OVA* infection.

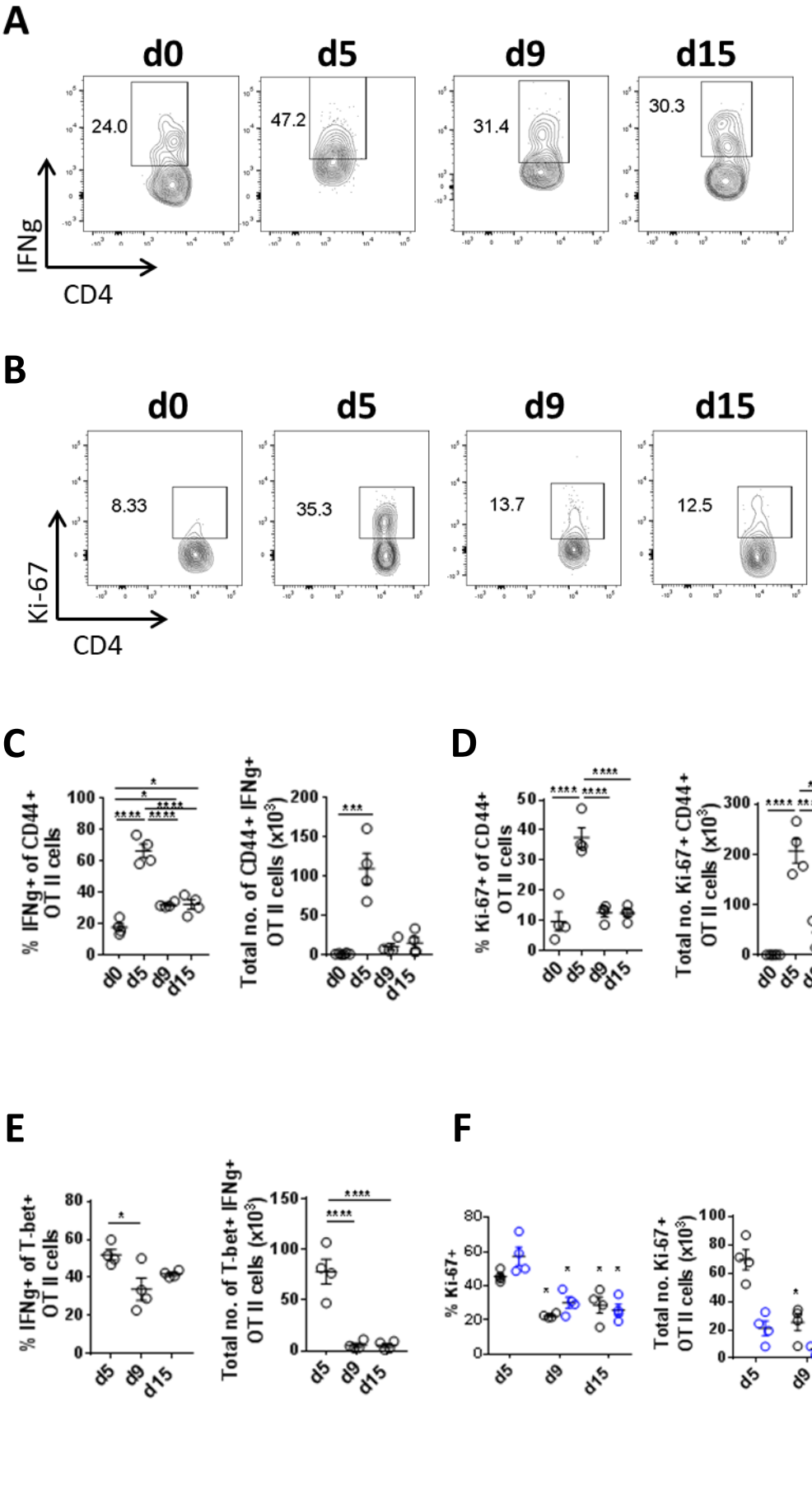
A
d0
d5
d9
d15
24.0
47.2
31.4
30.3
IFNg
CD4
B
d0
d5
d9
d15
8.33
35.3
13.7
12.5
Ki-67
CD4
C
% IFNg+ of CD44+ OT II cells
Total no. of CD44+ IFNg+ OT II cells (x10^3)
D
% Ki-67+ of CD44+ OT II cells
Total no. Ki-67+ CD44+ OT II cells (x10^3)
E
% IFNg+ of T-bet+ OT II cells
Total no. of T-bet+ IFNg+ OT II cells (x10^3)
F
% Ki-67+
Total no. Ki-67+ OT II cells (x10^3)
Th1
Tfh

Figure 2.2 *P.yoelii-OVA* induces antigen-specific T cell exhaustion

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and analysed by flow cytometry. (A) Representative flow cytometric plots of $IFNg^+$ effector OT II cells. (B) Representative flow cytometric plots of Ki-67^+ effector OT II cells. (C) Percentages (left) and total numbers (right) of $IFNg^+$ effector OT II cells. (D) Percentages (left) and total numbers (right) of Ki-67^+ effector OT II cells. (E) Percentages (left) and total number (right) of $IFNg^+$ Th1 OT II cells. (F) Percentages (left) and total numbers (right) of Ki-67^+ Th1 (black) and Tfh (blue) OT II cells. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ***p ≤0.001 ****p ≤0.0001 (one-way ANOVA with Tukey's multiple comparison test).

Prolonged expression of co-inhibitory receptors on effector T cells is a hallmark of T cell exhaustion (Wherry 2011). Therefore, we next examined the expression of multiple co-inhibitory receptors on effector OT II cells during *P.yoelii*-OVA infection. PD-1, CTLA-4, LAG3 and TIGIT expression was greatly increased on effector OT II cells at day 5 of infection, preceding T cell exhaustion (Fig2.3A). Expression of these co-inhibitory receptors remained high on day 15 of infection when T cell exhaustion was established, with the notable exception of CTLA-4, which was expressed at lower levels on effector OT II cells. (Fig2.3A). As effector functions were specifically lost in Th1 OT II cells during infection, we also examined the expression of co-inhibitory receptors on Th1 OT II cells. Interestingly, PD-1 and LAG3 expression was significantly increased on Th1 OT II cells between day 5 and day 15 of infection, whereas CTLA-4 expression was significantly decreased following day 5 of infection and TIGIT expression was comparable between day 5 and day 15 of infection (Fig2.3B).

T cell exhaustion is regulated through the synergistic actions of several inhibitory pathways and co-expression of multiple co-inhibitory receptors is associated with greater T cell exhaustion (Blackburn, Shin et al. 2009). As CTLA-4 was downregulated on effector OT II cells as T cell exhaustion was established during infection, we examined the simultaneous expression of PD-1, LAG3 and TIGIT on exhausted Th1 OT II cells. We detected all possible combinations of expression patterns on Th1 OT II cells on day 15 of infection, however, the largest proportion of Th1 OT II cells co-expressed PD-1, LAG3 and TIGIT (Fig.2.3C). Notably, expression of PD-1 and LAG3 on Th1 OT II cells inversely correlated with that of IFNg production during malaria, whereas expression of TIGIT did not appear to correlate with IFNg production. (Fig2.3D) Collectively, these data show that blood stage *P.yoelii* infection induces functional and phenotypic attributes of T cell exhaustion in antigen-specific $CD4^+$ T cells.

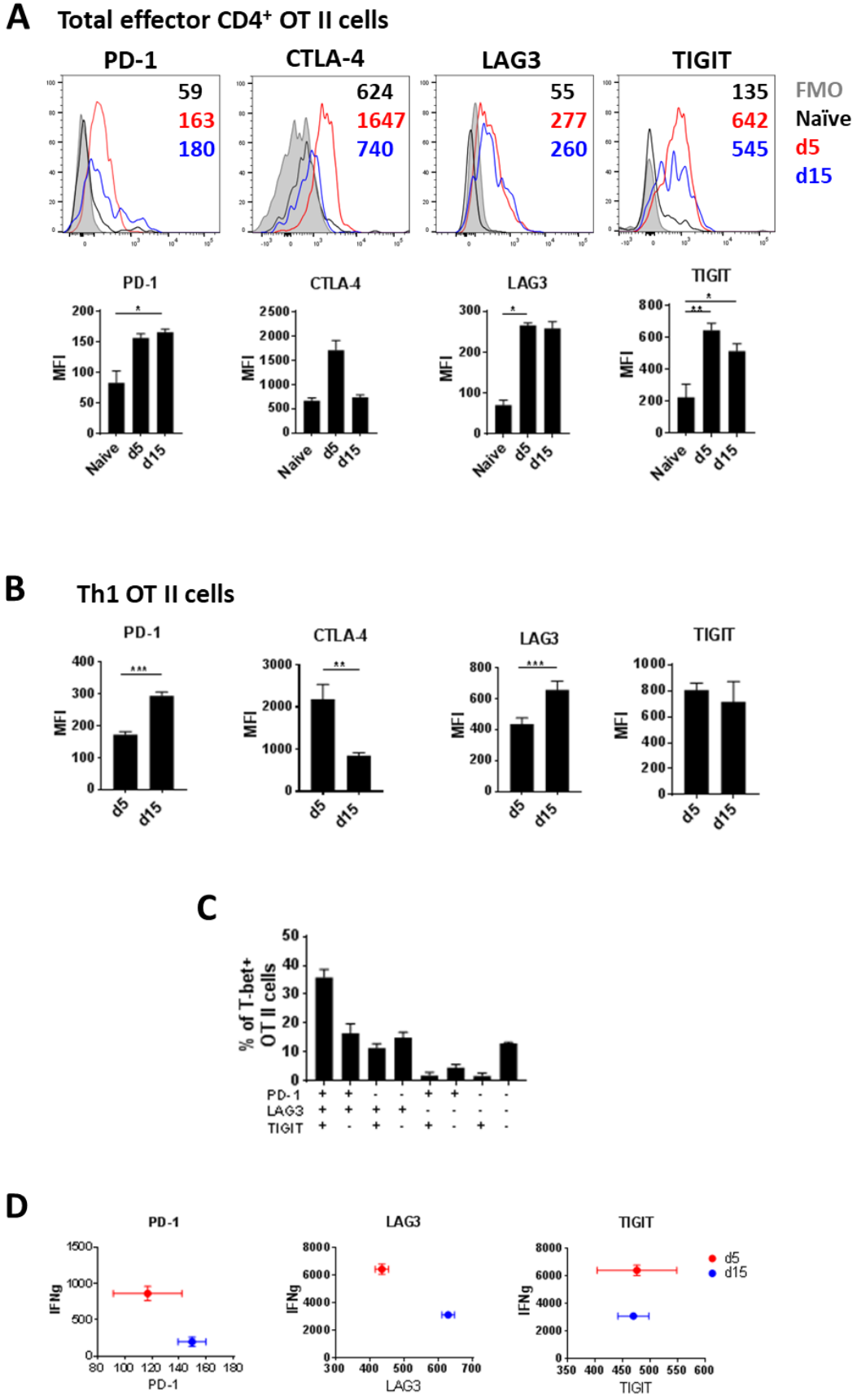
A
Total effector CD4+ OT II cells
PD-1
CTLA-4
LAG3
TIGIT
59
163
180
624
1647
740
55
277
260
135
642
545
FMO
Naïve
d5
d15
MFI
Naive
d5
d15
B
Th1 OT II cells
C
% of T-bet+ OT II cells
PD-1
LAG3
TIGIT
D
IFNg
PD-1
LAG3
TIGIT
d5
d15

Figure 2.3 *P.yoelii-OVA* infection upregulates multiple co-inhibitory receptors on effector OT II cells

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and analysed by flow cytometry. (A) Representative histograms (top) and mean fluorescence intensity (MFI) (bottom) of stated co-inhibitory receptor expression on effector OT II cells. (B) MFI of stated co-inhibitory receptors in the Th1 OT II population. (C) Boolean gating analysis of simultaneous expression of multiple inhibitory receptors on Th1 OT II cells. Graph represents relative abundance of each possible combination of co-inhibitory receptors expressed by Th1 OT II cells on day 15 of infection. (D) Correlation between IFNg expression with stated co-inhibitory receptor expression on Th1 OT II cells on day 5 and day 15. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 (two-way ANOVA with Tukey's multiple comparison test).

TCR-MHC II signalling appears to be disrupted in $CD4^+$ Th1 cells during *P. yoelii* infection

Previous reports have shown that effector $CD4^+$ T cells require constant TCR signalling for continued cytokine production and maintenance of effector state (Huppa, Gleimer et al. 2003). Consistent with this, removal of CD3 stimulation from *in vitro* generated Th1 OT II cells led to the rapid loss of IFNg production, even when CD28 co-stimulation was maintained (Fig2.4A). Removal of CD3 stimulation also significantly reduced Th1 cell proliferation, as measured by EdU incorporation

(Fig2.4A). Thus *in vitro*, TCR signalling is required to maintain Th1 cell effector functions.

Given the above data, we hypothesised that the major mechanism driving the loss of cytokine production and proliferation in antigen-specific $CD4^+$ T cells during malaria was the attenuation of peptide-MHC II activation. To investigate this, we administered an antagonistic MHC II antibody (anti-I-A^b) between day 5 and 9 of infection, ensuring T cell priming was unaffected. Administration of α-MHC II did not significantly affect the frequencies of OT II cells, or T-bet^+ OT-II cells compared with control mice (Fig2.4B-E). In addition, blockade of MHC II did not affect Th1 OT II cell function, as IFNg production and proliferation was not significantly altered compared with control mice (Fig2.4F-H). Conversely, α-MHC II treatment significantly reduced the frequencies of Tfh OT II cells compared with control mice (FigS2.4). Importantly, α-MHC II blockade from the start of infection completely blocked OT II cell activation and differentiation, demonstrating the effectiveness of α-MHC II treatment *in vivo* (FigS2.5). Therefore, as MHC II blockade did not significantly exacerbate the degradation of the Th1 OT II response, these data suggest that MHC II-TCR signalling in Th1 OTII cells is inhibited during *P.yoelii* infection.

We have shown that as T cell exhaustion is established during malaria, MHC II-TCR signalling is disrupted in Th1 OT II cells, therefore we next investigated whether OT II cell effector functions could be restored by robust TCR signalling. However, CD3

stimulation of purified OT II cells isolated from day 9 of infection, failed to restore proliferation to day 5 levels *in vitro* (Fig2.4I), suggesting that TCR signalling cannot reverse functional exhaustion in effector OT II cells during malaria.

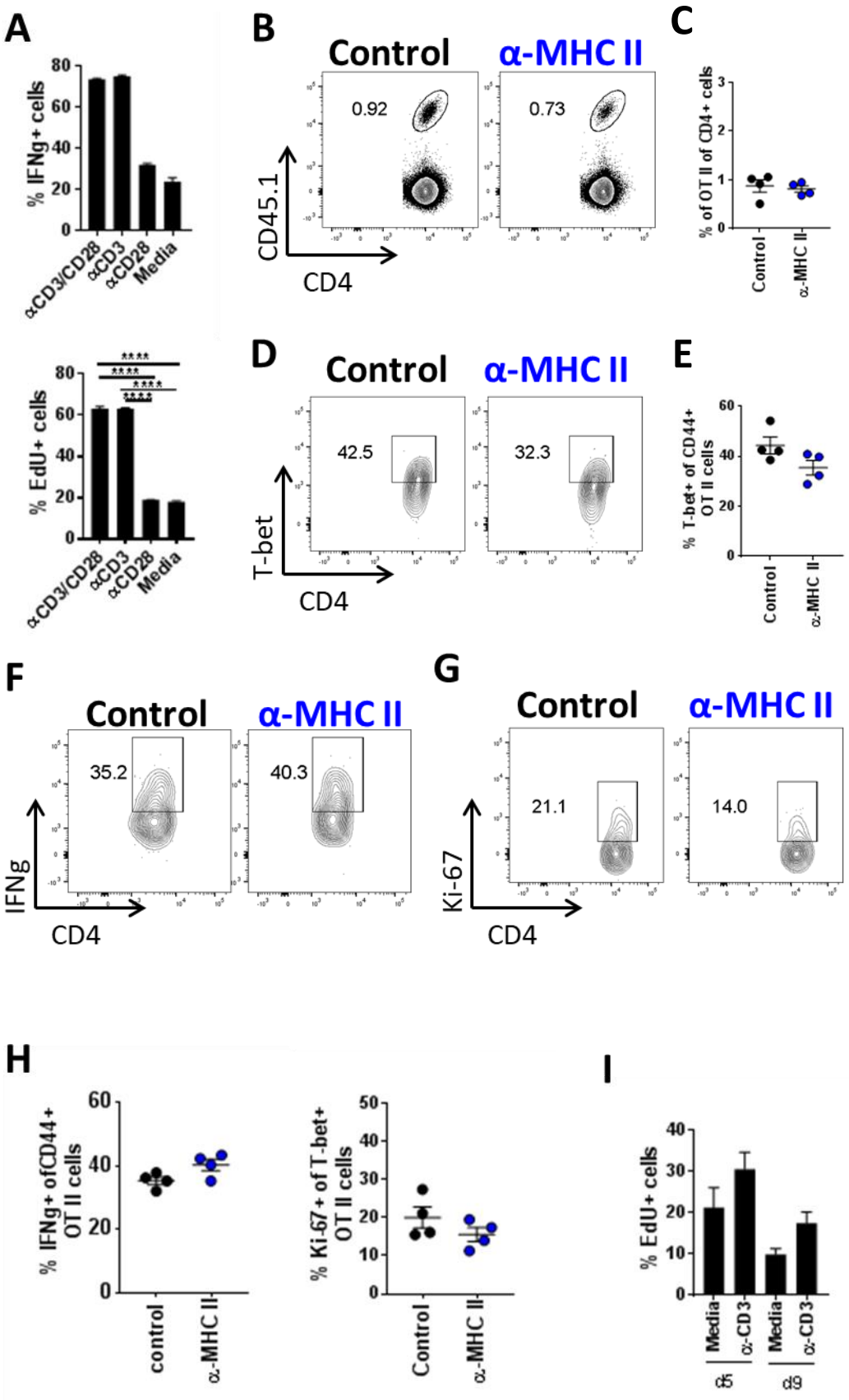
A
% IFNg+ cells
αCD3/CD28
αCD3
αCD28
Media
% EdU+ cells
B
Control
α-MHC II
0.92
0.73
CD45.1
CD4
C
% of OT II of CD4+ cells
Control
α-MHC II
D
Control
α-MHC II
42.5
32.3
T-bet
CD4
E
% T-bet+ of CD44+ OT II cells
Control
α-MHC II
F
Control
α-MHC II
35.2
40.3
IFNg
CD4
G
Control
α-MHC II
21.1
14.0
Ki-67
CD4
H
% IFNg+ ofCD44+ OT II cells
control
α-MHC II
% Ki-67+ of T-bet+ OT II cells
Control
α-MHC II
I
% EdU+ cells
Media
α-CD3
Media
α-CD3
d5
d9

Figure 2.4 *In vivo* blockade of MHC II does not alter the Th1 OT II cell response during *P.yoelii*-OVA infection

(A) *In vitro* generated Th1 OT II cells were stimulated by either α-CD3 and α-CD28, α-CD3 alone, α-CD28 alone, or left unstimulated (media). Percentages of $IFNg^+$ (top) and Edu^+ (bottom) Th1 OT II cells within stated groups. (B-H) $1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with *P.yoelii*-OVA pRBC. Mice were either treated with PBS (control) (n=4) or α-MHC II (n=4) from day 5 p.i. Spleens were taken on day 9 p.i. for flow cytometric analysis. (B) Representative flow cytometric plots of splenic OT II cells. (C) Frequencies of OT II cells on day 9 p.i. (D) Representative flow cytometric of $T\text{-}bet^+$ OT II cells. (E) Frequencies of $T\text{-}bet^+$ OT II cells. (F) Representative flow cytometric plots of $IFNg^+$ Th1 OT II cells. (G) Representative flow cytometric plots of Ki-67 expression in Th1 OT II cells. (H) Percentages of $IFNg^+$ (left) and $Ki\text{-}67^+$ (right) Th1 OT II cells. (I) Splenic $CD4^+$ T cells were purified from untreated spleens on day 5 and 9 p.i and stimulated with α-CD3 or unstimulated (media) overnight. Graph represents percentage of Edu^+ OT II cells. Results are representative of one (I), two (A) or three (B-G) independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ****p ≤0.0001 (one-way ANOVA with Tukey's multiple comparison test).

MHC II and co-stimulatory molecule expression on antigen-presenting cells is unaltered during *P.yoelii* infection

Plasmodium infection can downregulate MHC II and co-stimulatory molecule expression on DC, reducing their capacity to stimulate T cells during malaria (Millington, Di Lorenzo et al. 2006). Therefore, to examine whether the attrition of the OT II cell response and apparent loss of MHC II-mediated T cell activation was due to significant alterations within the composition and activation of the APC

compartment, we next sought to characterise APC subsets over the course of infection.

The frequencies and total numbers of red pulp macrophages (RPM), marginal zone macrophages (MZM), and the XCR1$^+$ subset of conventional DC greatly reduced on day 5 of infection compared with naïve mice, before gradually increasing following day 9 of infection (Fig2.5A and gating strategy shown in FigS2.6). In contrast, the frequencies and total numbers of monocytes sharply increased by day 5 of infection compared to naïve mice but, slightly decreased following day 9 of infection (Fig2.5A). Finally, we found that the frequencies and total numbers of CD11b$^+$ DC were not significantly altered during the course of *P.yoelii*-OVA infection (Fig2.5A). Despite significant alternations in the composition of the APC compartment, there was no significant difference in the expression of MHC II or the co-stimulatory molecule CD80 in any of the APC subsets examined over the course of infection. (Fig2.5B). PD-L1 is a major ligand of PD-1 and is expressed on APC (McAlees, Lajoie et al. 2015). Consistent with this, PD-L1 was expressed by all APC examined, but was expressed at much higher levels in CD11b$^+$ DC in both naïve and *P.yoelii*-OVA infected mice (Fig2.5C). Therefore, despite substantial alterations in the structure of the APC compartment during *P.yoelii-OVA* infection, these data suggest that loss of MHC II signalling in Th1 OT II cells is not due to the downregulation of MHC II on APC.

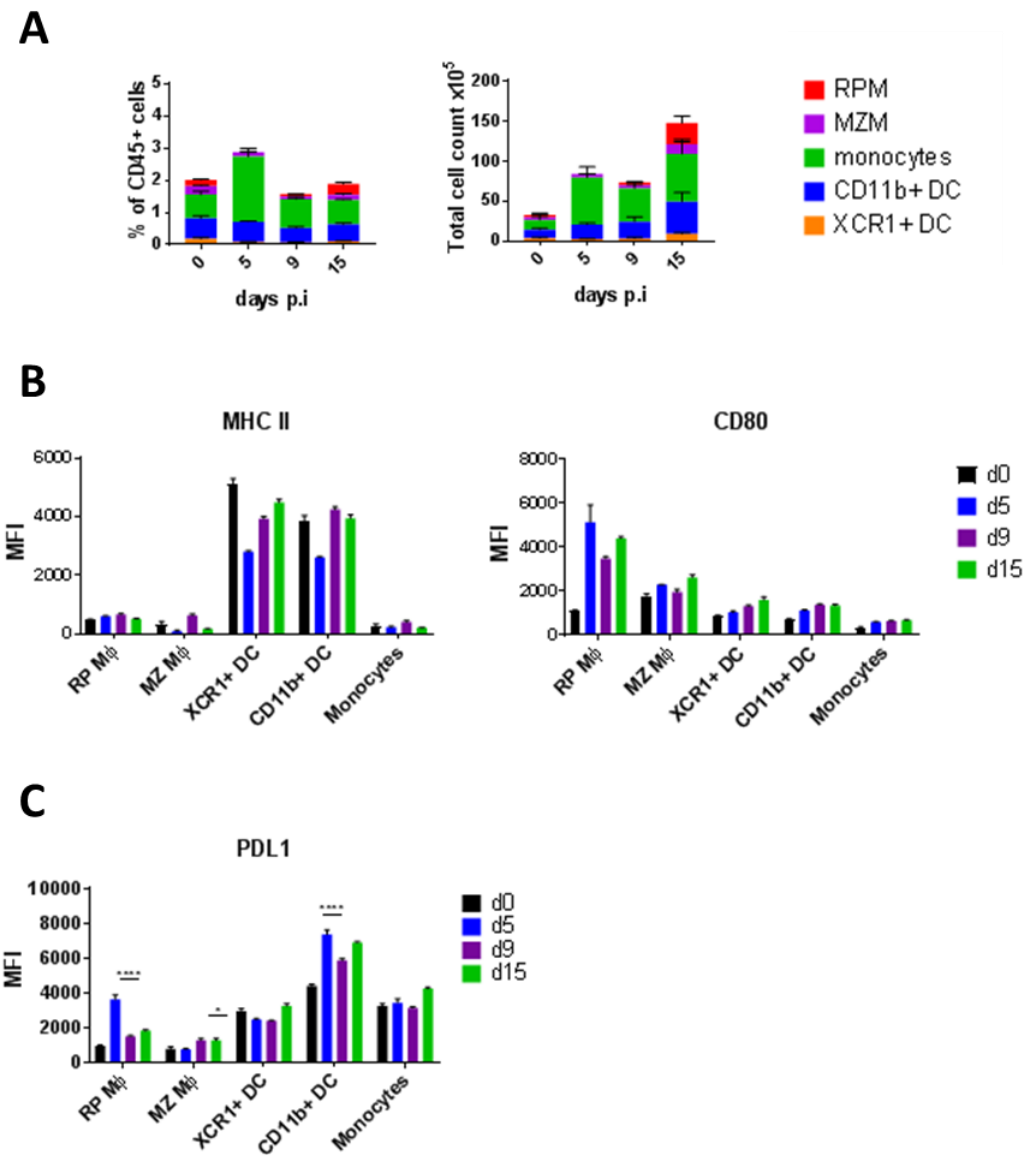

Figure 2.5 MHC II and CD80 expression on splenic APC is not altered during *P.yoelli-OVA* infection

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and digested prior to analysis by flow cytometry. (A) The frequencies (left) and total numbers (right) of red pulp macrophage (RPM), marginal zone macrophages (MZM), monocytes, $CD11b^+$ DC and $XCR1^+$ DC. (B) MFI of MHC II (left) and CD80 (right) in stated APC subsets. (D) MFI of PDL1 in stated APC populations. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ***p ≤0.001 (two-way ANOVA with Tukey's multiple comparison test)

Loss of MHC II signalling is not associated with alterations in compartmentalisation or localisation with APC during *P.yoelii* infection

MHC II was not significantly downregulated on APC during *P.yoelii-OVA* infection therefore, we next examined whether the apparent loss of MHC II signalling in OT II cells was due to alterations in T cell compartmentalisation, or failure to localise with APC during infection. To investigate this, we adoptively transferred dsRED OT II cells into $CX_3CR1eGFP^{+/-}$ mice, where GFP identifies monocytes, MZM and DC (FigS2.7). The compartmentalisation of OT II cells and localisation with APC was analysed on day 5 of infection, when OT II cells were highly functional and on day 9 of infection when OT II cell attrition was underway and functional exhaustion was established, and when OT II cells could still be detected. On day 5 of infection, OT II cells were predominately localised to the white pulp (WP) of the spleen and distributed in both the T cell zones and B cell zones (Fig 2.6A). Although OT II cells remained in the WP on day 9 of infection, their distribution appeared to be more restricted to the T cell zone, which was similar to naïve mice (Fig 2.6A). Notably, on both day 5 and day 9 of infection, OT II cells were co-localised with CX_3CR1^{+} cells that maintained MHC II expression (Fig2.6A). Collectively, these data suggest that loss of MHC II signalling in OT II cells is not due the inability to co-localise with APC expressing MHC II and that OT II activation and exhaustion likely occur within the same splenic compartments during *P.yoelii-OVA* infection.

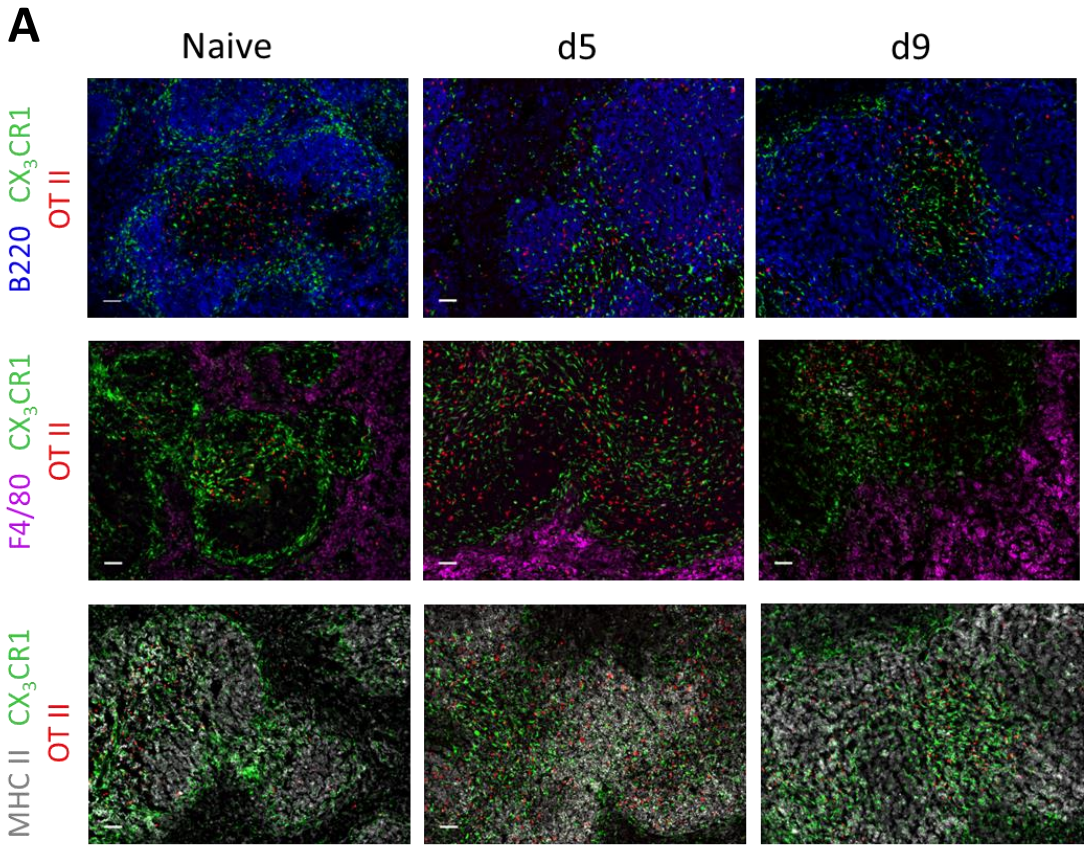

Figure 2.6 OT II cells co-localise with splenic $CX_3CR1eGFP^+$ cells during *P.yoelii*-OVA infection

$1x10^6$ dsRED OT II cells were adoptively transferred into $CX_3CR1eGFP^{+/-}$ mice prior to infection with 10^4 *P.yoelii-O*VA pRBC (n=1). (A) Spleens were removed and examined by immunofluorescence for the presence of OT II cells (red), $CX_3CR1eGFP^+$ cells (green) relative to $B220^+$ B cells (blue) (top), $F4/80^+$ red pulp macrophages (purple) (middle) and MHC II^+ cells (white) (bottom). Results are representative of one experiment. Scale bar 50µm.

OT II cell motility decreases as T cell exhaustion is established during *P.yoelii* infection

OT II cells appear to co-localise with APC as T cell exhaustion is established on day 9 of *P.yoelii* infection. However, co-inhibitory receptor expression can inhibit the arrest of T cells and the formation of a stable synapse with APC, necessary for effector function (Schneider 2006, Fife 2009, Honda 2014). We therefore used 2-photon microscopy to examine the dynamics and cellular interactions of OT II cells in the splenic WP of $CX_3CR1eGFP^{+/-}$ mice during *P.yoelii-OVA* infection. Initially, we examined OT II cell movement in the spleen and found that on day 2 of infection the majority of OT II cells were arrested (Fig2.7A, B and supplementary movie 2.1), which is consistent with T cell behaviour typically observed during T cell priming (Bousso and Robey 2003, Mempel, Henrickson et al. 2004). In contrast, OT II cell behaviour was highly heterogenous on day 5 of infection, with a proportion of cells displaying rapid movement, whilst others were arrested for the duration of the imaging window (Fig2.7A, B and supplementary movie 2.2). On day 9 of infection OT II cells displayed a similar heterogenous behaviour as seen on day 5 of infection (Fig2.7A, B and supplementary movie 2.3). Quantitative cell tracking of OT II cells showed a significant increase in mean track speed and a decrease in arrest co-efficient on day 5 compared with day 2 of infection (Fig2.7C). Surprisingly, on day 9 of infection OT II cell track speed was significantly decreased and the arrest co-efficient was significantly increased compared with day 5 of infection (Fig2.7C). These data indicated that OT II cells were more arrested following establishment of T cell exhaustion during *P.yoelii-OVA* infection.

We next examined whether the observed decrease in OT II motility on day 9 of infection was associated with increased cellular interactions with $CX_3CR1eGFP^+$ cells. Consistent with our previous immunofluorescence data, OT II cells were co-localised and interacted with $CX_3CR1eGFP^+$ cells at day 5 and 9 of infection (Fig2.7D, Fig2.6A and supplementary movies 2.4 and 2.5). On both day 5 and day 9 of infection we observed rapidly moving OT II cells that did not form stable contacts with $CX_3CR1eGFP^+$ cells during the imaging window (Fig2.7E and supplementary movie 2.6), however, fewer rapidly moving OT II cells were observed on day 9 of infection, compared to day 5 of infection (Fig2.7E). We next stratified arrested cells as in contact with $CX_3CR1eGFP^+$ cells if the distance between cells was less than 3μm away, and to be not in contact if the distance between arrested OT II cells and $CX_3CR1eGFP^+$ cells was more than 3μm. The frequencies of arrested OT II cells in contact with $CX_3CR1eGFP^+$ cells on day 5 of infection did not differ significantly to the frequencies of arrested OT II cells not in contact with $CX_3CR1eGFP^+$ cells (Fig2.7E). Importantly, the frequencies of arrested OT II cells in contact with $CX_3CR1eGFP^+$ cells was not significantly different on day 9 of infection compared with day 5 (Fig2.7E). Therefore, OT II cell exhaustion does not appear to be mediated through alterations in co-localisation or inability to form interactions with APC during *P.yoelii-OVA* infection.

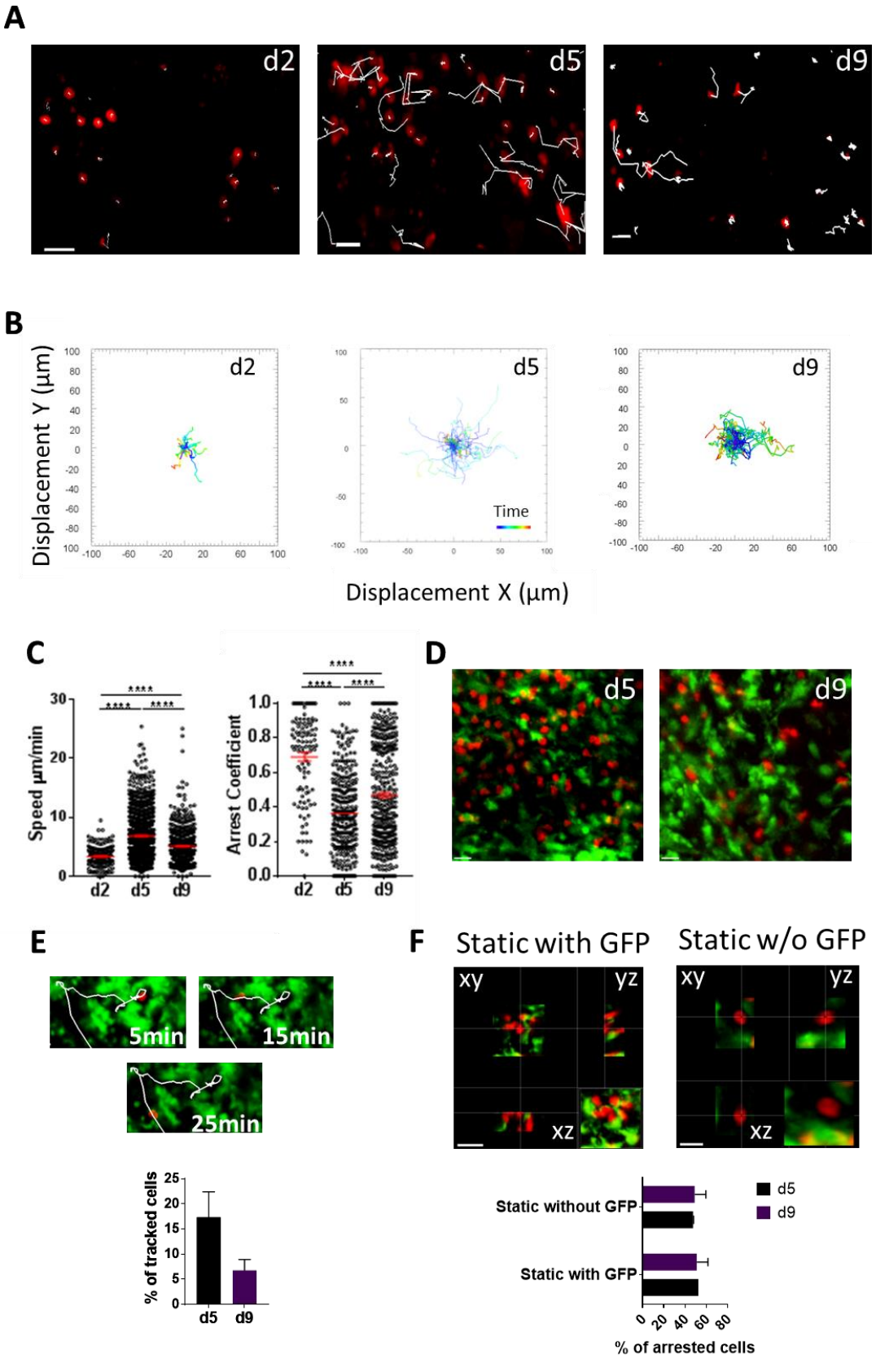
A
d2
d5
d9
B
Displacement Y (µm)
Displacement X (µm)
Time
C
Speed µm/min
Arrest Coefficient
d2
d5
d9
D
E
5min
15min
25min
% of tracked cells
F
Static with GFP
Static w/o GFP
xy
yz
xz
Static without GFP
Static with GFP
% of arrested cells

Figure 2.7 Exhausted OT II cells form stable interactions with APC during *P.yoelii*-OVA

$1x10^6$ dsRED OT II cells were adoptively transferred into $CX_3CR1eGFP^{+/-}$ mice prior to infection with 10^4 *P.yoelii*-OVA pRBC. Spleens were isolated on day 2 (n=1), day 5 (n=2) and day 9 (n=3) of infection and subjected to explant 2-photon imaging. (A) Representative images of dsRED OT II cells migrations paths during *P.yoelii-OVA* infection. Scale bar represents 20μm. (B) Flowerplots showing displacement of tracked OT II cells. (C) Quantification of OT II cell speed (left) and arrest coefficient (right). Data points represent individual cells combined from multiple experiments. (D) Representative images of OT II cells (red) localisation with $CX_3CR1eGFP^+$ cells (green) during infection. (E) Representative images (top) and frequencies (bottom) of rapidly moving OT II cells (defined as a mean speed >10μm/min) on day 5 and 9 of infection. (F) Arrested OT II cells were classified as either static in contact with $CX_3CR1eGFP^+$ cells, or static but not in contact with $CX_3CR1eGFP^+$ cells over a 15-minute period on day 5 and 9 of infection. Representative three-dimensional sections showing arrested OT II cells in contact with $CX_3CR1eGFP^+$ (t op left) and not in contact with $CX_3CR1eGFP^+$ cells (top right) in XY, XZ and YZ planes. Graph represents frequencies of stated groups (bottom). Bars represent mean ± SEM. ** $p \leq 0.01$ ***$p \leq 0.001$ **** $p \leq 0.0001$ (one-way ANOVA with Tukey's multiple comparison test).

Discussion

In this study we have used a model antigen-specific $CD4^+$ T cell system to gain mechanistic insights into $CD4^+$ T cell exhaustion during blood stage malaria. We have shown that OT II cells rapidly develop an exhausted phenotype during *P.yoelii* infection, illustrated by decreased IFNg production and proliferation and increased expression of co-inhibitory receptors. We demonstrated that blockade of MHC II signalling did not significantly exacerbate the attrition of the effector OT II response during established *P.yoelii* infection. As effector $CD4^+$ T cells require continuous peptide MHC II-TCR engagement to sustain effector functions (Huppa, Gleimer et al. 2003, Obst, van Santen et al. 2005), these data suggest that MHC II-TCR signalling is inhibited during malaria. However, this apparent loss of MHC II signalling is not due to alterations in OT II cell compartmentalisation or interactions with APC, suggesting that MHC II-TCR signalling is subverted in effector $CD4^+$ T cells contributing to T cell exhaustion and clonal deletion during malaria.

T cell exhaustion is a known feature of blood stage malaria and is associated with decreased IFNg production by effector T cells and increased expression of co-inhibitory receptors (Butler 2012, Horne-Debets, Faleiro et al. 2013, Illingworth, Butler et al. 2013). Consistent with this, we have shown that T cell exhaustion occurs within a clonal antigen-specific population of $CD4^+$ T cells during *P.yoelii*-OVA infection and is associated with high expression of PD-1, LAG3 and TIGIT. Blockade of co-inhibitory receptors has been highly efficient at restoring effector T cell functions

and accelerating parasite clearance during malaria (Butler 2012, Horne-Debets, Faleiro et al. 2013), indicating that co-inhibitory receptors actively suppress $CD4^+$ T cells during *Plasmodium* infection. However, the molecular mechanisms through which co-inhibitory pathways regulate $CD4^+$ T cell functions and promote T cell exhaustion during malaria is not fully understood.

Previous studies have shown that co-inhibitory receptors can interfere with and inhibit the TCR signalling pathway (Yokosuka, Takamatsu et al. 2012, Wei 2017, Maruhashi, Okazaki et al. 2018), which has important implications as effector $CD4^+$ T cells require continuous antigen-dependent MHC II-TCR signalling to maintain effector functions (Huppa, Gleimer et al. 2003, Obst, van Santen et al. 2005). Consistent with this, reversal of T cell exhaustion following inhibitory receptor blockade is CD28 and TCR dependent (Honda 2014, Kamphorst, Wieland et al. 2017). *In vitro,* removal of APC bearing cognate antigen results in decreased effector cytokine production (Corbin and Harty 2005). Similarly, we showed that removal of α-CD3 signalling *in vitro* rapidly decreases Th1 cell IFNg production and proliferation. Limited antigen presentation *in vivo* can also reduce effector T cell responses (Egen, Rothfuchs et al. 2011), further highlighting the importance of MHC II-TCR signalling in maintaining an effector $CD4^+$ T cell response. Interestingly, we found that *in vivo* blockade of MHC II post priming did not exacerbate the attrition and dysfunction of OT II cells during malaria, with loss of IFNg and proliferative capacity being comparable between α-MHC II treated and control mice. Therefore, our data suggests that defective MHC II-TCR signalling in effector $CD4^+$ T cells underpins the

degradation of the response during malaria. Analysis of molecules downstream of the TCR, such as ZAP70 or phosphorylated ERK, would be required to confirm the loss of TCR signalling in effector $CD4^+$ T cells during malaria.

Interestingly, the apparent loss of MHC II signalling in effector T cells was not due to alterations in compartmentalisation, or failure of OT II cells to co-localise with APC expressing MHC II and co-stimulatory molecules, despite the significant alterations in splenic architecture during malaria. Although expression of MHC II on APC was not significantly altered during the course of infection, it is unclear whether antigen was still being processed and presented. Assessing the capability of isolated splenic APC from day 5 and day 9 of infection to activate T cells *in vitro* would confirm this. Although *Plasmodium* infection has been reported to supress DC functions (Millington, Di Lorenzo et al. 2006), the ability of inhibitory receptor blockades to restore T cell effector functions (Butler 2012) and our observed maintenance of OT II cell and APC co-localisation, suggests that APC can support T cell effector functions during malaria. However, expression of inhibitory receptors may subvert pMHC II-TCR signalling in effector T cells contributing to the development of T cell exhaustion during malaria.

Following T cell priming, recently activated T cells are transiently desensitised which promotes T cell disengagement from APC and favours T cell proliferation (Bohineust, Garcia et al. 2018). In agreement with this, $CD4^+$ T cells were more motile on day 5

compared to day 2 of infection. Following this phase, effector $CD4^+$ T cells require stable interactions with APC to support effector functions (Egen, Rothfuchs et al. 2011). However, expression of co-inhibitory receptors on activated T cells modulates T cell-APC interactions by preventing the formation of a stable synapse, which limits effector functions (Schneider 2006, Fife 2009, Honda 2014). Surprisingly, we found that, whilst $CD4^+$ T cells were highly motile on day 5 of infection, as $CD4^+$ T cell exhaustion was established, T cell motility significantly decreased and $CD4^+$ T cells were observed in prolonged contacts with $CX_3CR1eGFP^+$ cells. In support of our findings, a reduction of $CD4^+$ and $CD8^+$ T cell motility has shown to be a signature of T cell exhaustion during chronic LCMV infection (Zinselmeyer, Heydari et al. 2013). Notably, in that study it was found that PD-L1 stabilises the immunological synapse, allowing PD-1 to decrease TCR signalling (Zinselmeyer, Heydari et al. 2013). Furthermore, within tumours, a site known to induce T cell exhaustion, cytotoxic lymphocytes have been observed to engage with a tumour cell for up to 6 hours (Breart, Lemaitre et al. 2008). We propose that prolonged cellular arrest on APC may facilitate $CD4^+$T cell exhaustion during malaria by exposing effector T cells to inhibitory signals, which deactivate the TCR and co-stimulatory signalling pathways leading to the loss of effector functions and deletion of cells. Further studies, measuring downstream molecules of the TCR, such as ZAP70, before and after administration of antagonistic inhibitory receptor antibodies would be required to confirm this proposal.

Collectively, our data has shown that as T cell exhaustion is established during malaria, $CD4^+$ T cells are primarily restricted to the WP of the spleen where they engage in stable interactions with APC. These interactions likely subject effector $CD4^+$ T cells to negative regulation and inhibition of TCR signalling, which contributes to the loss of effector functions during malaria. PD-L1 blockade during LCMV infection significantly increased T cell motility, which was associated with increased effector functions (Zinselmeyer, Heydari et al. 2013), therefore, it remains to be determined whether during malaria, inhibitory receptors regulate effector $CD4^+$ T cell functions through modulation of $CD4^+$ T cell behaviour.

Materials and Methods

Ethics Statement

All animal work was approved following local ethical review by the University of Manchester Animal Procedures and Ethics Committees and was performed in strict accordance with the U.K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licences 70/7293 and P8829D3B4).

Mice and Parasites

Male 7 week old C57BL/6 mice ($CD45.2^+$) were purchased from Charles River UK. RAG-1 OT II x Pep3 ($CD45.1^+$), RAG-1 OT II x β-actin dsRED mice and $CX_3CR1\text{-}eGFP^{-/+}$ mice were bred at the University of Manchester and were fully backcrossed to a C57BL/6 background. All mice were maintained in specific-pathogen free conditions in individually ventilated cages.

Cryopreserved *P.yoelii* parasites expressing mCherry OVA (under the control of the *hsp70* promoter), *P.yoelii*-OVA or *P.yoelii* were thawed and passaged once in C57BL/6 mice before being used to infect experimental mice. Animals were infected with $1x10^4$ pRBC by intravenous injection. The course of infection was monitored every other day starting from d5 p.i by peripheral parasitaemia and assessed by microscopic examination of Giemsa-stained thin blood smears. All flow cytometric analysis was carried out using the *P.yoelii:mCherry-OVA* strain and all 2-photon

microscopy and immunofluorescence experiments were carried out using *P.yoelii*-OVA parasites. Both strains were kindly supplied Leiden University Medical Centre.

In vivo blockades

α-MHC II (Y-3P [BioXcell]) was injected intraperitoneally at 500µg/mouse, every other day from time points stated. Control mice received PBS.

$CD4^+$ T cell Isolation and Adoptive Transfer

Spleens from RAG-OT II x Pep3 mice or RAG-1 OT II x β-actin dsRED mice were homogenised through a 70µm strainer (BD Biosciences) to generate a single cell suspension prior to RBC lysis (RBC lysing buffer, BD Biosciences). OVA-specific $CD4^+$ T lymphocytes (OT II cells) were then positively selected using anti-CD4 conjugated microbeads (Miltenyi Biotec) according to manufactures instructions. $1x10^6$ OT II cells and $1x10^6$ dsRED OT II cells were transferred into C57BL/6 and CX_3CR1-$eGFP^{-/+}$ mice respectively, via intravenous injection one day prior to infection.

Flow Cytometry

Spleens were removed from naïve and malaria-infected mice on stated time points p.i. Single cell suspensions were generated, and RBC lysed prior to re-suspending splenocytes in FACS buffer (HBSS with 2% FCS). For myeloid cell analysis, all or part

of the spleen was chopped into small pieces and incubated in HBSS containing 2mg/mL collagenase D (Sigma Aldric) and 50KU/mL Dnase (Sigmal Aldirch) for 30 minutes at 37^{O}C. A single cell suspension was then generated, and RBC lysed. Absolute live cell counts were calculated by trypan blue exclusion cell viability assay (Sigma).

Transferred OT II cells were identified through CD45.1 expression and where characterised using the following antibodies: CD45.1 (A20), CD4 (RM4-5), CD44 (IM7), PD-1 (RMPI-30), LAG3 (C9B7W), TIGIT (GIGD7), CD25 (PC61) CXCR5-biotin (LI38D7) for 25 minutes at 4^{O}C. Surface staining was done in the presence of FcR block (2.4G2, BioXcell). For streptavidin staining, surface antibodies were washed and then cells were incubated for 10 minutes at room temperature (RT) with streptavidin v510. For intracellular staining, cells were fixed and permeabilised using the Foxp3 fixation/permeabilisation buffer (eBioscience) for 30 minutes at 4 ^{0}C. Cells were then incubated with T-bet (4B10), GATA3 (TWAJ), Foxp3 (FJK-I6s), Bcl-6 (7D1BL), CTLA-4 (UC10-4B9) and Ki-67 (SolA15) for 30 minutes before washing and flow cytometric analysis. For analysis of the intracellular cytokines IFNg (XMG1.2), cells were stimulated *ex vivo* for 4 hours at 37 ^{0}C with 200ng/mL PMA (Sigma), 1µg/mL ionomycin (Sigma) and Brefeldin A ([1000x], eBiosceince). For splenic myeloid characterisation antibodies against the following surface markers were used: CD45 (30F1), Ly6C (HK1.4), F4/80 (BM8), MHC II (M5/114.15.2), CD80 (16-10A1), CD169 (3D6.112), PDL1 (10F9G2), CD11c (N418), CD11b (M1/70) and CD64 (X54-5/7.1). The following surface markers were stained using antibodies conjugated to the same

fluorophore to generate a lineage gate: CD3 (17A2), CD19 (6D5), NK1.1 (PK136), B220 (RA3-6B2) and Ly6G (1A8). All antibodies were acquired from eBioscience or Biolegend. Dead cells were excluded from all analyses using forward and side scatter properties and LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies).

Samples were acquired through the Fortessa (BD systems, UK) and all analysis was performed using Flowjo Software (Treestar Inc, OR, USA). Malaria infected control samples were combined to generate fluorescence minus one (FMO) samples which were used to validate the flow cytometric data.

In vitro generation of Th1 cells

For the *in vitro* generation of Th1 cells, spleens from RAG OT II x Pep3 mice were homogenised to generate a single cell suspension. Purified OT II cells were then stimulated with α-CD3 (2μg/mL, BD Biosciences) and α-CD28 (2μg/mL, eBioscience) in the presence of Th1 polarising factors, 10ng/mL rIL-12 and 10μg/mL α-IL-4 (eBioscience), in RPMI 1640 medium supplemented with 10% FCS, 55mM β-mercaptoethanol, non-essential amino acids, sodium pyruvate, L-glutamine and penicillin plus streptomycin. Cells were incubated for three days prior to use in experiments.

Tissue Processing and Immunofluorescence

Spleens were immersion-fixed in 4% paraformaldehyde (PFA)/ 30% sucrose overnight at 4 ^{0}C. Spleens were subsequently cryoprotected in PBS/30% sucrose for 48hrs, embedded in Optimum Cutting Temperature compound (OCT, Tissue-Tek), snap frozen in cold 2-Methylbutane and stored at -80 ^{0}C. 20µm sections were cut using the Leica CM3050 S cryostat, mounted onto SuperFrost slides (VWR) and stored at -80 ^{0}C until use.

For immunofluorescent staining, mounted sections were equilibrated to RT for 30 minutes and fixed in ice-cold acetone for 10 minutes prior to the start of the staining protocol. All sections were rehydrated in several changes of wash buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05% Tween in distilled water) before being blocked with 2% goat serum (Sigma Aldrich) in block buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05% Tween, 1% Bovine Serum Albumin [BSA] in distilled water) for 1.5hrs at RT. Spleen sections were then incubated with primary antibodies, diluted in block buffer overnight at 4^{0}C with the following: F4/80 (APC conjugated, clone BM8, BioLegend), B220, (rat monoclonal, clone RA3-6B2, Thermo Fisher Scientific) and MHC II (rat monoclonal antibody, clone M5/114.15.2, eBioscience). Sections were rinsed several times in wash buffer, and for fluorescent detection, sections were incubated for 1.5 hours at RT in secondary antibodies (goat anti-rat 647, or goat anti-rat 330, Life Technologies), diluted in block buffer. Sections were then washed in wash buffer and counterstained with DAPI (Sigma-Aldrich). Sections were finally sequentially rinsed

in PBS and distilled water, dried overnight in the dark at 4^0C before being coverslipped in ProLong Diamond anti-fade Mountant (Life Technologies).

Images were collected on a Zeiss Axioimager.D2 upright microscope using a 20x objective and captured using a Coolsnap HQ2 camera (Photometrics) through Micromanager software v1.4.23. Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent bleed through from one channel to the next. Images were then processed and analysed using Image J (Fiji).

Explant 2-photon microscopy and analysis

Spleens were removed from malaria infected $CX_3CR1\text{-}eGFP^{-/+}$ mice and fixed on a tissue holder and sliced into two non-symmetric pieces using a vibratome (Leica) in a bath of ice-cold PBS. Splenic tissue was then perfused with phenol-red free RPMI 1640 medium supplemented with 10mM HEPES (Sigma Aldrich) and bubbled with 95% O_2 and 5% CO_2 whilst being maintained at 37^OC.

Two-photon microscopy was performed with a Leica SP8 Upright Multiphoton microscope using a 25x dipping objective. Fluorescent excitation of eGFP and dsred was provided by a MaiTai MP laser (Spectra physics) tuned to 910nm. Emission signals were separated by the following dichroic mirrors and band pass filters: 505nm dichroic with a 680nm shortpass filter in combination with a 560 dichoric and 525/50

(eGFP) and 585/40 (dsred) bandpass filters (Semrock). Time lapse sequences were acquired over a 30µm Z stack spaced by 2 µm planes and was repeated every 30 seconds for approximately 30 minutes.

Data was processed using the software package Imaris (Bitplane) and cells were tracked by a combination of automated and manual processes. Tissue drift was corrected for using Imaris' drift correction algorithm. The arrest coefficient of a cell was defined as the percentage of time points at which the cell had an instantaneous velocity of less than 3µm/min. Tracks shorter than 2 minutes were excluded from this analysis.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, USA). Comparison between 3 or more groups was carried out using a one-way ANOVA with Tukey's test for multiple comparisons. For groups of 2, unpaired t-tests were carried out. Results were considered significant when $P<0.05$.

Supplementary Figures

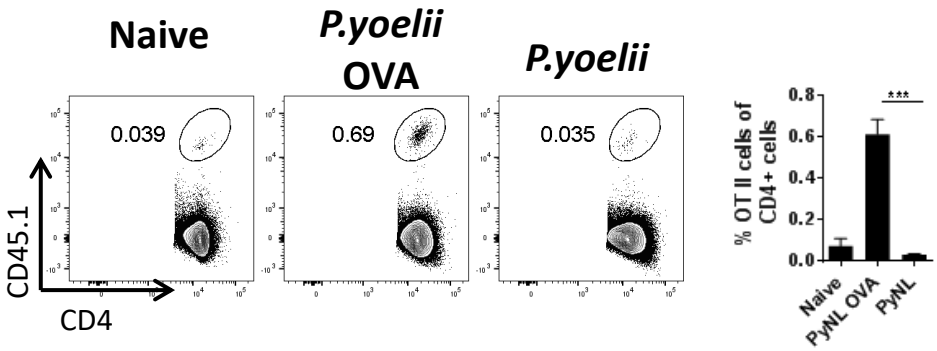

Figure S2.1 OT II expansion during infection is OVA-dependent

OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoeli*-OVA pRBC (n=4) or *P.yoelii* pRBC (n=4) . Spleens were taken at day 7 p.i. and analysed by flow cytometry. Representative flow cytometric plots of OT II cells (left) and frequencies of OT II cells (right). Bars represent mean ± SEM. ***p ≤0.001 (one-way ANOVA with Tukey's multiple comparison test).

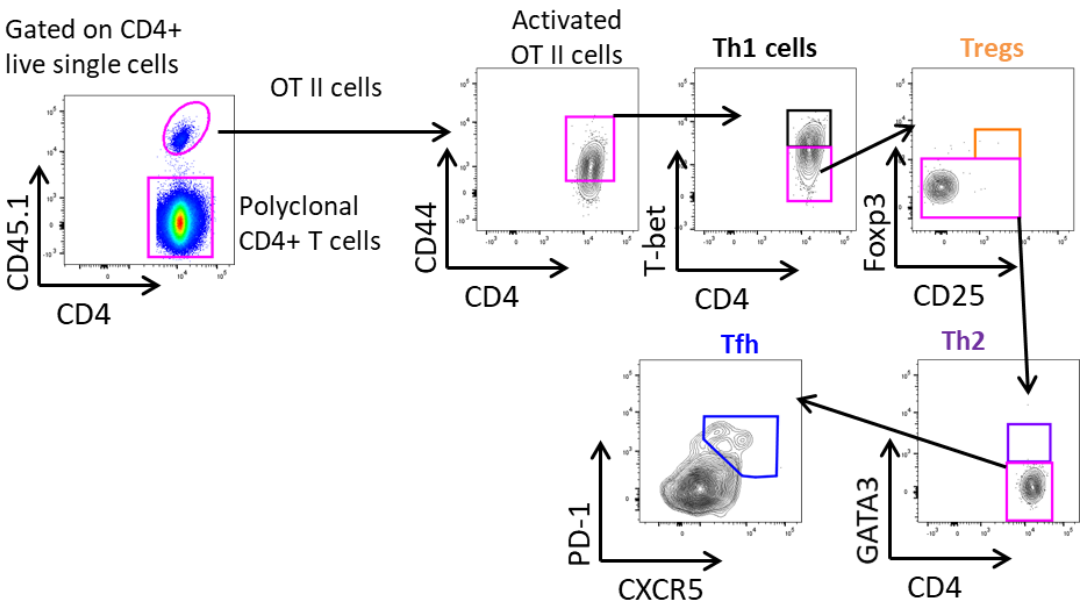

Figure S2.2 Gating scheme for OT II subsets

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with *P.yoelii*-OVA (n=4). Representative plots showing how splenic OT II cells can be sub-gated into total effector OT II cells (based on CD44 expression), Th1 OT II cells (based on T-bet expression), Treg cells (based on FOXP3 and CD25 expression), Th2 OT II cells (based on GATA3 expression) and Tfh OT II cells (based on CXCR5 and PD-1 expression). A combination of FMO controls and naïve samples were used to set appropriate gates.

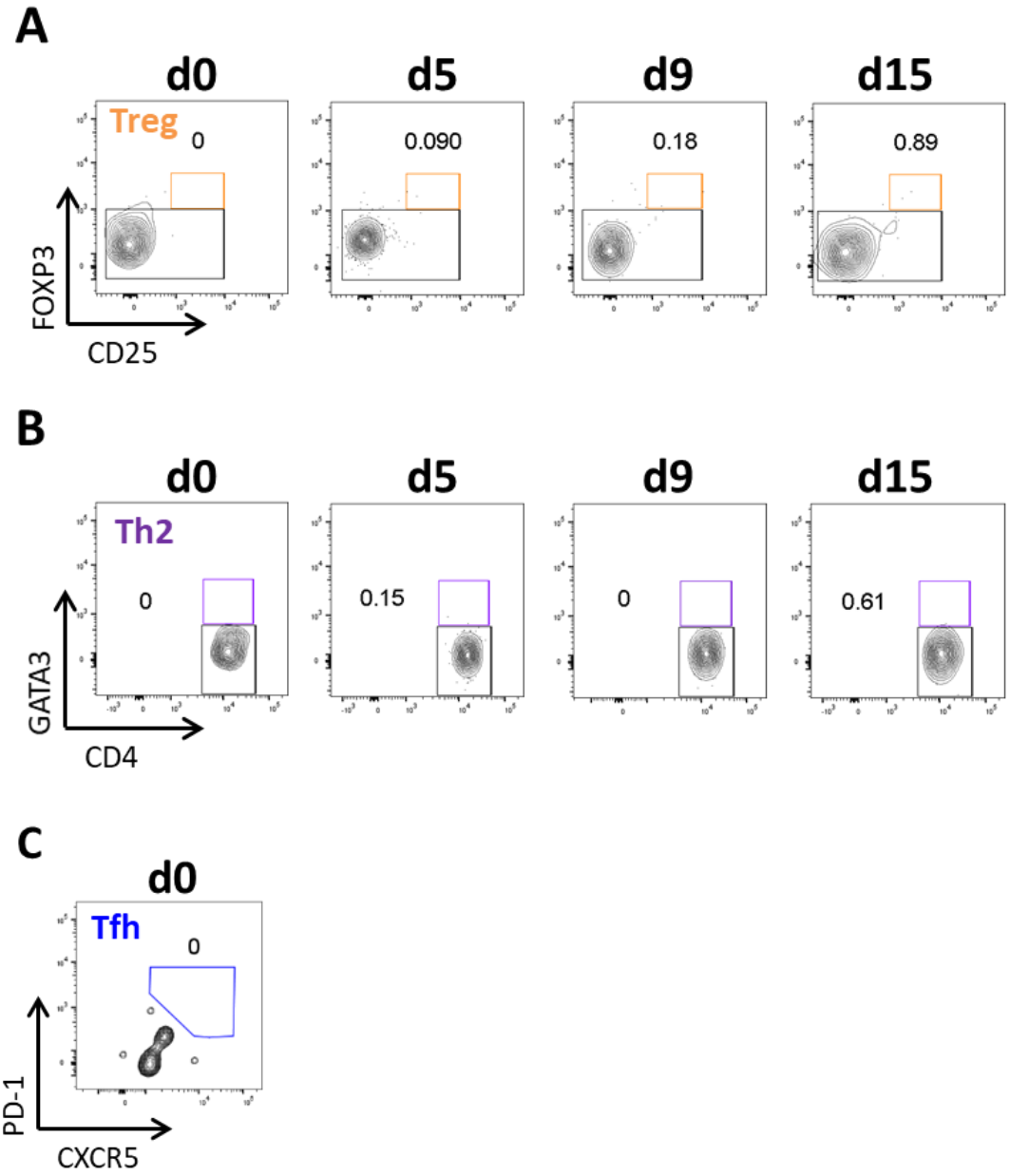

Figure S2.3 OT II subsets during the course of *P.yoelii-OVA* infection

OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and analysed by flow cytometry. (A) Representative flow cytometric plots of Treg OT II cells. (B) Representative flow cytometric plots of Th2 OT II cells. (C) Representative flow cytometric plots of Tfh OT II cells in naïve mice. Data are representative of two independent experiments.

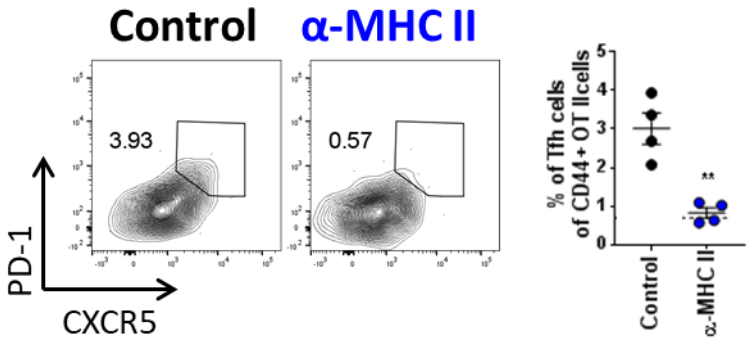

Figure S2.4 MHC II blockade significantly reduces Tfh OT II cells during *P.yoelii-OVA* infection

OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC. Mice were either treated with PBS (control) (n=4) or α-MHC II (n=4) from day 5 p.i. Spleens were taken on day 9 p.i. for flow cytometric analysis. Representative flow cytometric plots (left) and frequencies (right) of Tfh OT II cells.

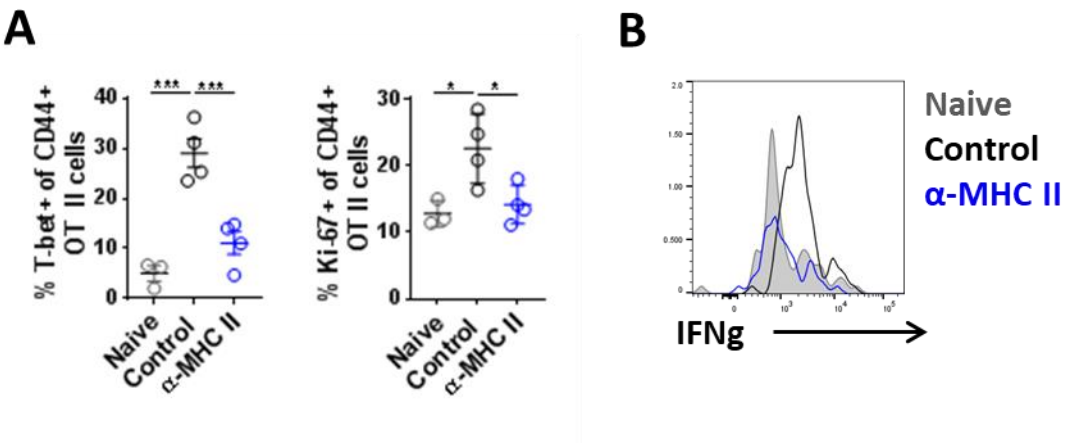

Figure S2.5 MHC II blockade during T cell priming prevents OT II activation during malaria

OT II cells were adoptively transferred into C57BL/6 mice prior to treatment either with PBS (control) (n=4) or α-MHC II (n=4) every other day. Mice were subsequently infected with 10^4 *P.yoelii*-OVA pRBC. Spleens were taken on day 3 p.i. for flow cytometric analysis. (A) Frequencies of Th1 effector OT II cells (left) and Ki-67$^+$ effector OT II cells (right). (B) Representative histogram of IFNg expression in effector OT II cells. Data are representative of two independent experiments. Bars represent mean ± SEM. *p ≤0.05, ***p ≤0.001 (one-way ANOVA with Tukey's multiple comparison test).

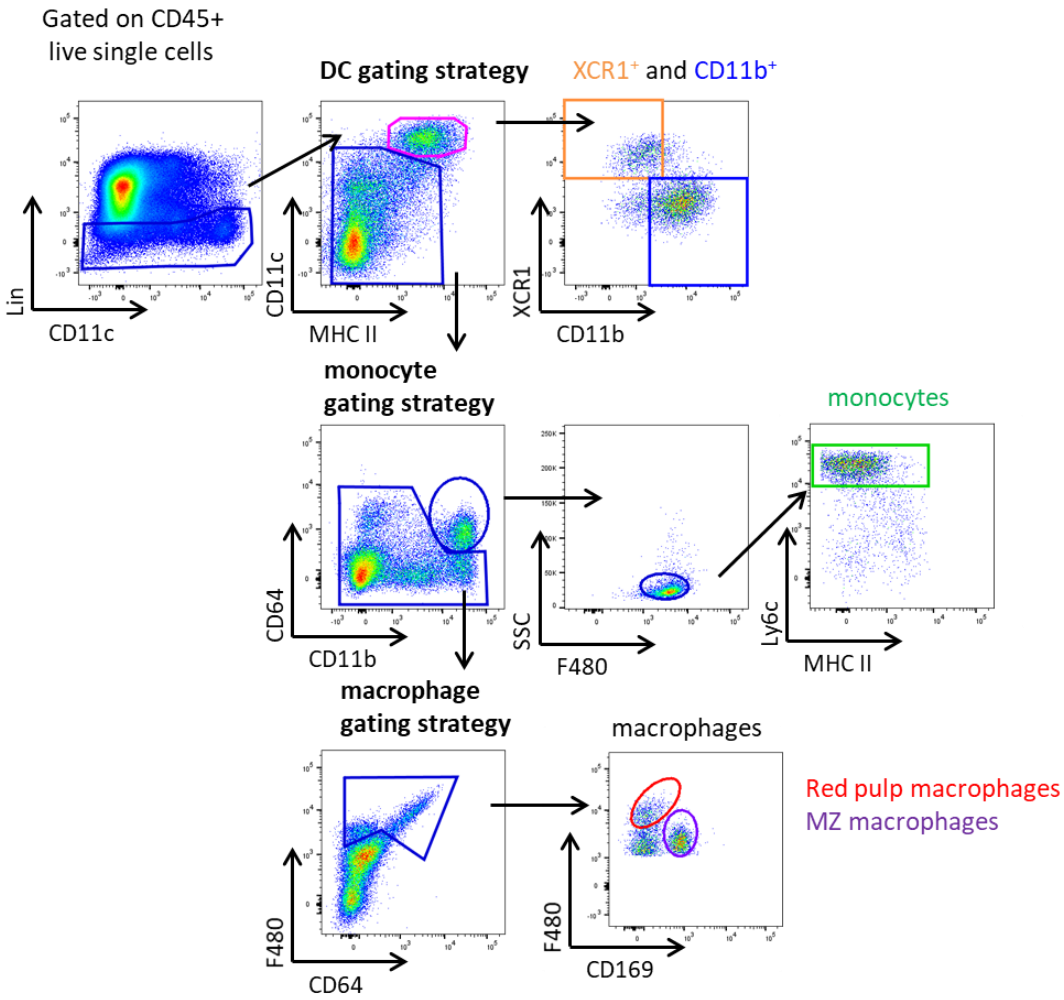

Figure S2.6 Gating scheme for splenic APC subsets

Spleens were taken from naïve mice and digested prior to flow cytometric analysis. Representative plots showing DC gating strategy, monocyte gating strategy and macrophage gating strategy. Lin gate represents CD3, CD19, B220, Ly6G and NK1.1. Data is representative of two independent experiments.

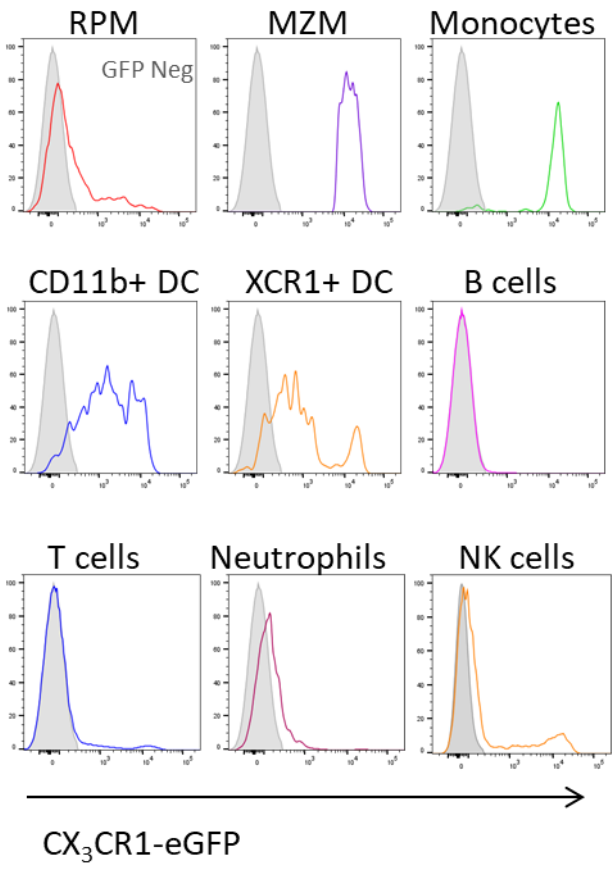

Figure S2.7 Characterisation of GFP^{+} APC subsets in CX$_3$CR1eGFP$^{+/-}$ mice

Spleens were isolated from naïve CX$_3$CR1eGFP mice and digested prior to flow cytometric analysis. Representative histograms showing GFP expression in stated APC subsets. Shaded group represents GFP negative cells.

Supplementary movies

Supplementary Movie 2.1 OT II cells are arrested on day 2 of *P.yoelii-OVA* infection

A representative time-lapse sequence showing stably arrested OT II cells on day 2 of *P.yoelii-OVA* infection. Scale bar: 20µm

Supplementary Movie 2.2 Dynamic behaviour of OT II cells on day 5 of *P.yoelii-OVA* infection

A representative time-lapse sequence showing the dynamic behaviour of OT II cells on day 5 of *P.yoelii-OVA* infection. Scale bar: 20µm

Supplementary Movie 2.3 Dynamic behaviour of OT II cells on day 9 of *P.yoelii-OVA* infection

A representative time-lapse sequence showing the dynamic behaviour of OT II cells on day 9 of *P.yoelii-OVA* infection. Scale bar: 20µm

Supplementary Movie 2.4 OT II cell-CX_3CR1^+ cell interaction on day 5 of *P.yoelii-OVA* infection

A representative time-lapse sequence showing stable interactions between OT II cells (red) and CX_3CR1^+ cells (green) in the WP of the spleen on day 5 of *P.yoelii-OVA* infection. Scale bar: 20µm

Supplementary Movie 2.5 OT II cell-CX_3CR1^+ cell interaction on day 9 of *P.yoelii-OVA* infection

A representative time-lapse sequence showing stable interactions between OT II cells (red) and CX_3CR1^+ cells (green) in the WP of the spleen on day 9 of *P.yoelii-OVA* infection. Scale bar: 20µm

Supplementary Movie 2.6 Rapidly moving OT II cells do not form stable interactions with CX_3CR1^+ cells during *P.yoelii*-OVA infection

A representative time-lapse sequence showing a rapidly moving OT II cells (red) amongst CX_3CR1^+ cells (green) in the WP of the spleen during *P.yoelii-OVA* infection. Scale bar: 20µm

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Chapter 3: Synergistic blockade of TIGIT and PD-L1 improves parasite control during blood stage *Plasmodium* infection

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Manuscript in preparation

All work was carried out by R.S.D

Abstract

Infection with *Plasmodium* species drives dysfunctional $CD4^+$ T cell and B cell responses, however the molecular mechanisms underlying this dysfunction are not fully understood. In this study, we found that the co-inhibitory receptors TIGIT and PD-1 are expressed by antigen-specific $CD4^+$ Th1 and T follicular helper (Tfh) cells, $CD8^+$ T cells and natural killer cells during *Plasmodium yoelii (P.yoelii)* infection. Importantly, synergistic blockade of TIGIT and PD-L1 improved parasite control during *P.yoelii* infection. Mechanistically, this correlated with an increase in Tfh cell numbers and increased levels of systemic interferon gamma. Taken together, our study identifies a novel role for TIGIT and PD-1 in regulating the Tfh cell response and limiting immune-mediated parasite clearance during malaria.

Introduction

Infection with *Plasmodium* species induces clinical malaria, a disease causing significant global morbidity and mortality (WHO 2018). Despite years of research, the most promising vaccine against malaria only offers short-lived protection (Mahmoudi and Keshavarz 2017, Draper, Sack et al. 2018). In addition, the emergence of drug resistant parasites may significantly impact the future treatment of *Plasmodium* infection (Crompton, Moebius et al. 2014, WHO 2018). Thus, novel approaches to combating malaria are greatly required.

The $CD4^+$ T cell response is a critical component of the protective immune response mounted against blood stage malaria (Kumar and Miller 1990, Amante and Good 1997). Notably, experimental studies in rodent models identified that interferon gamma (IFNg) production from $CD4^+$ Th1 cells is necessary to confer protection against malaria (Süss, Eichmann et al. 1988, Stephens and Langhorne 2010). B cell activation and the production of antibodies is another key component of protective immunity against malaria and is dependent on the $CD4^+$ T follicular helper (Tfh) cell response. (Cohen, Mc et al. 1961, von der Weid, Honarvar et al. 1996, Zander 2017). However, recent studies have identified that chronic *Plasmodium* infection can induce T cell exhaustion, which is characterised by decreased effector functions and increased expression of co-inhibitory receptors. (Horne-Debets, Faleiro et al. 2013, Illingworth, Butler et al. 2013, Shankar, Vignesh et al. 2018). Importantly, T cell exhaustion during malaria impedes parasite clearance (Butler 2012).

T cell exhaustion has been well characterised in $CD8^+$ T cells, however, far less is known about the mechanisms underlying $CD4^+$ T cell exhaustion (Zajac, Blattman et al. 1998, Wherry, Ha et al. 2007, Wherry 2011, Wherry and Kurachi 2015). Nevertheless, prolonged expression of co-inhibitory receptors on effector T cells is a key hallmark of T cell exhaustion in both $CD4^+$ and $CD8^+$ T cells (Wherry, Ha et al. 2007, Crawford, Angelosanto et al. 2014). During malaria, a plethora of co-inhibitory receptors are upregulated on $CD4^+$ and $CD8^+$ T cells, including PD-1, CTLA-4 and LAG3 (Chandele 2011, Butler 2012, Horne-Debets, Faleiro et al. 2013, Illingworth, Butler et al. 2013, Villegas-Mendez, Inkson et al. 2016). Therapeutic co-blockade of PD-1 and LAG3 dramatically enhances the $CD4^+$ T cell IFNg and Tfh cell response, subsequently accelerating parasite clearance during *Plasmodium yoelii* (*P.yoelii*) infection (Butler 2012). This study clearly demonstrates a critical role for co-inhibitory receptors in establishing $CD4^+$ T cell exhaustion during malaria. Whilst a protective role for $CD8^+$ T cells during blood stage malaria is unclear, there is evidence that PD-1 can induce functional exhaustion in $CD8^+$ T cells during malaria, contributing to the establishment of a chronic disease (Horne-Debets, Faleiro et al. 2013, Imai, Ishida et al. 2015). Targeting co-inhibitory receptors to re-invigorate T cells could, therefore, provide a novel therapeutic strategy for combating malaria. However, an improved understanding of how different co-inhibitory receptors, independently or synergistically, contribute to T cell exhaustion during malaria is greatly needed.

TIGIT is a member of the Ig superfamily that functions as a co-inhibitory receptor on immune cells (Anderson 2016). It is expressed on activated T cells, regulatory T cells

(Tregs) and natural killer cells (NK cells) (Stanietsky, Rovis et al. 2013, Johnston, Comps-Agrar et al. 2014, Joller 2014). TIGIT has been shown to supress immune responses through direct intrinsic signalling, as well as extrinsic mechanisms (Joller 2011, Johnston, Comps-Agrar et al. 2014, Kurtulus 2015). Genetic ablation of TIGIT was shown to directly enhance $CD4^+$ T cell activation *in vitro* and *in vivo*, exacerbating autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) (Joller 2011). The interaction of TIGIT with its ligand CD155 also promotes a tolerogenic state in dendritic cells (DC), characterised by an increase in IL-10 secretion and downregulation of IL-12, which inhibits T cell activation (Yu, Harden et al. 2009). TIGIT has also been implicated in $CD8^+$ T cell and NK cell exhaustion during cancer (Johnston, Comps-Agrar et al. 2014, Zhang, Bi et al. 2018). Importantly, TIGIT appears to act synergistically with PD-1 to suppress $CD8^+$ T cell effector functions, as such, combined antibody blockade of TIGIT and PD-L1 (the ligand of PD-1), has been very successful at restoring IFNg production and improving tumour clearance (Johnston, Comps-Agrar et al. 2014, Zhang, Bi et al. 2018). TIGIT is known to be expressed during blood stage *P.yoelii* infection (Villegas-Mendez, Inkson et al. 2016), but at present the role of TIGIT during malaria is poorly understood.

In this study we explored the synergistic role of TIGIT and PD-1 in co-ordinating $CD4^+$ T cell exhaustion during a non-lethal *P.yoelii* infection. Co-blockade of TIGIT and PD-L1 through administration of antagonistic antibodies, significantly improved parasite control, which correlated with an increased level of systemic IFNg. In addition, co-blockade of TIGIT and PD-L1 significantly increased the Tfh cell response. Surprisingly,

however, co-blockade of TIGIT and PD-L1 did not affect the functional capacity of $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells, despite these cells expressing significantly elevated levels of TIGIT and PD-1 during infection. Collectively, our results show that TIGIT and PD-1 synergistically impair parasite clearance during malaria.

Results

Co-blockade of TIGIT and PD-L1 enhances parasite control during blood stage *P.yoelii* infection

To investigate whether TIGIT signalling, in combination with PD-1 contributed to the establishment $CD4^+$ T cell exhaustion during malaria, we administered antagonistic antibodies against TIGIT and PD-L1 from day 5 of *P.yoelii* infection. Therefore, T cell priming was not affected during infection. Dual blockade of TIGIT and PD-L1 rapidly reduced peripheral parasitemia compared with IgG control treated mice (Fig3.1A). In contrast, treatment with α-TIGIT or α-PD-L1 alone did not significantly affect parasite control (FigS3.1). Combinatorial treatment with α-PD-L1 and α-TIGIT significantly increased plasma levels of IFNg, TNF and IL-2 during infection, compared with IgG control treated mice (Fig3.1B). Whereas, treatment with α-TIGIT and α-PD-L1 only showed a modest increase in IL-10 (Fig3.1B). Taken together, these results indicated that dual blockade of TIGIT and PD-L1 significantly improved parasite control during *P.yoelii* infection, which was associated with enhanced pro-inflammatory responses.

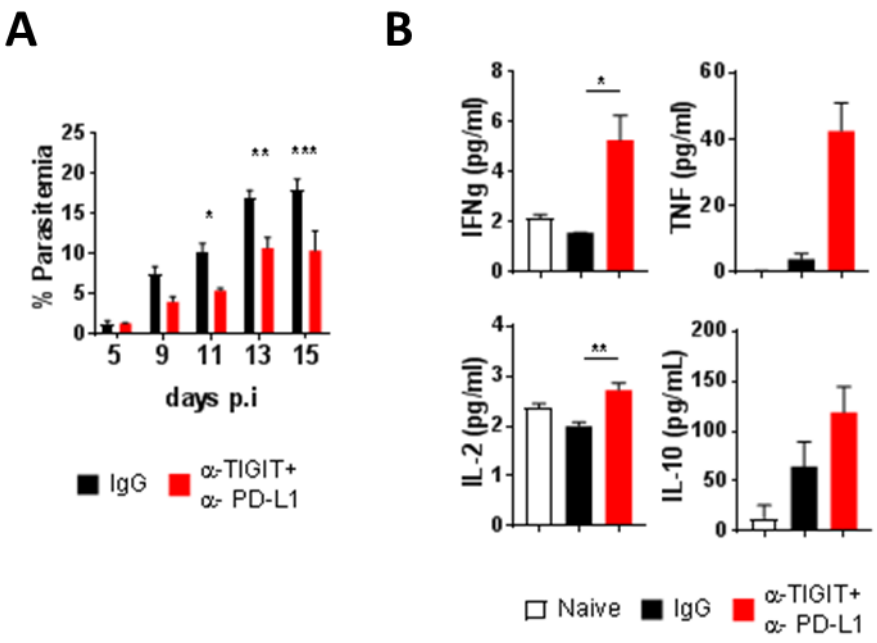


Figure 3.1 Co-blockade of TIGIT and PD-L1 significantly increases parasite control during *P.yoelii* infection

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC and then either treated with 250µg control rat IgG (n=4) or 250µg α-TIGIT and 250µg α-PD-L1 (n=4) every other day starting from day 5 p.i. (A) Peripheral parasitemia was monitored over the course of the infection. (B) Quantification of plasma cytokines IFNg, TNF, IL-10 and IL-2 on day 15 p.i. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ***p ≤0.001 (one-way ANOVA with Tukey's multiple comparison test).

Antigen-specific effector CD4$^+$ T cells co-express TIGIT and PD-1 during *P.yoelii* infection

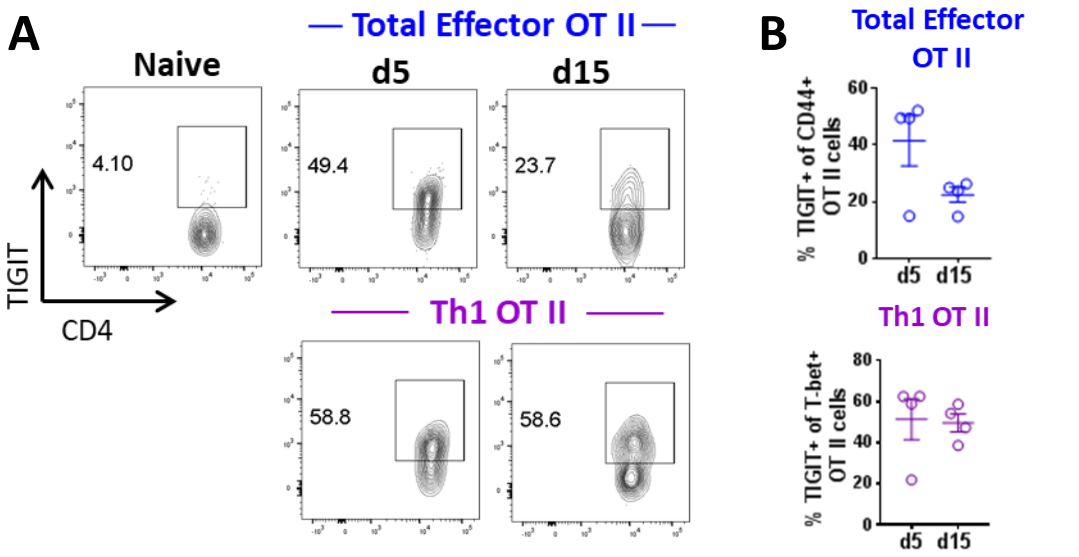
Given the effectiveness of combinatorial inhibition of TIGIT and PD-L1 during *P.yoelii* infection, we next examined the co-expression of TIGIT and PD-1 on CD4$^+$ T cells, a

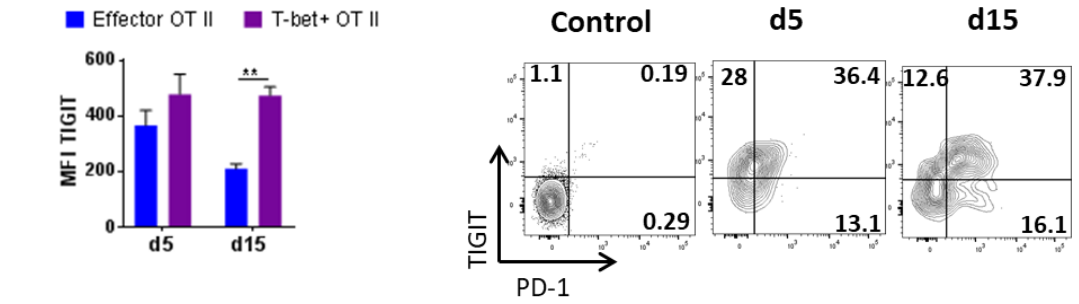
key cell type in protection against malaria. As previously described, we utilised ovalbumin (OVA) TCR-specific $CD4^+$ T cells (OT II cells) to examine an antigen-specific response during *P.yoelii*-OVA infection (Chapter 2). Spleens were isolated on day 5 post infection (p.i) when OT II cells were highly functional and day 15 p.i when OT II cells were functionally exhausted (Chapter 2).

TIGIT expression was very low on naïve OT II cells however, was rapidly upregulated on effector OT II cells (defined as $CD44^+$) during *P.yoelii*-OVA infection (Fig3.2A, B and S3.2). Interestingly, the frequencies of effector OT II cells that expressed TIGIT decreased during the course of infection. In contrast, the frequencies of $TIGIT^+$ Th1 OT II cells (based on T-bet expression) was markedly elevated at day 5 p.i and was sustained on day 15 p.i (Fig3.2A, B). Additionally, the mean fluorescence intensity (MFI) of TIGIT on $T\text{-}bet^+$ OT II cells was increased to a significantly higher level than on total effector OT II cells on day 15 of infection (Fig3.2C). Therefore, these data indicate a preferential enrichment of TIGIT expression within the Th1 subset as T cell exhaustion is established during *P.yoelii* infection.

To understand the synergistic effect of TIGIT and PD-L1 co-inhibitory treatment, we examined the relationship between TIGIT and PD-1 expression on Th1 OT II cells during *P.yoelii*-OVA infection. At day 5 of infection, similar frequencies of Th1 OT II cells were $TIGIT^+$ $PD\text{-}1^+$ and $TIGIT^+$ $PD\text{-}1^-$ (Fig3.2D). In contrast, as T cell exhaustion was established on day 15 of infection greater frequencies of Th1 OT II cells co-

expressed TIGIT and PD-1 compared to TIGIT alone ($TIGIT^+$ $PD\text{-}1^-$) or PD-1 alone ($TIGIT^-$ $PD\text{-}1^+$) (Fig3.2D, E). Moreover, $PD\text{-}1^{hi}$ Th1 OT II cells expressed significantly more TIGIT than $PD\text{-}1^{lo}$ Th1 OT II cells on day 15 of infection (Fig3.2F), further confirming that the majority of $TIGIT^+$ Th1 OT II cells co-expressed PD-1 at the point of T cell exhaustion during *P.yoelii*-OVA infection.


A
Naive
Total Effector OT II
d5
d15
4.10
49.4
23.7
TIGIT
CD4
Th1 OT II
58.8
58.6
B
Total Effector OT II
% TIGIT+ of CD44+ OT II cells
d5 d15
Th1 OT II
% TIGIT+ of T-bet+ OT II cells
d5 d15


C
Effector OT II
T-bet+ OT II
MFI TIGIT
d5 d15
D
Control
d5
d15
1.1
0.19
0.29
28
36.4
13.1
12.6
37.9
16.1
TIGIT
PD-1

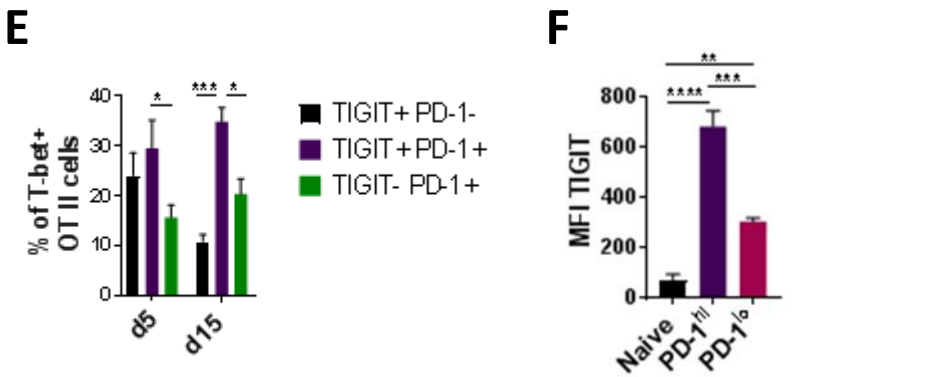

E
% of T-bet+ OT II cells
d5 d15
TIGIT+ PD-1-
TIGIT+PD-1+
TIGIT- PD-1+
F
MFI TIGIT
Naive
PD-1hi
PD-1lo

Figure 3.*2* TIGIT and PD-1 are co-ordinately expressed by Th1 OT II cells during *P.yoelii-OVA* infection

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with *P.yoelii*-OVA pRBC (n=4). Spleens were taken on stated times points for flow cytometric analysis. (A) Representative flow cytometric plots of TIGIT expression by naïve, total effector ($CD44^+$) or Th1 ($T\text{-}bet^+$) OT II cells. (B) Frequencies of $TIGIT^+$ total effector (top) and Th1 (bottom) OT II cells. (C) Mean fluorescence intensity (MFI) of TIGIT within total effector and Th1 OT II populations on days 5 and 15 p.i. (D) Representative flow cytometric plots of TIGIT and PD-1 expression on Th1 OT II cells. (E) Frequencies of TIGIT and/or PD-1 expressing Th1 OT II cells. (F) MFI of TIGIT expression within naïve and $PD\text{-}1^{hi}$ or $PD\text{-}1^{lo}$ Th1 OT II cells on day 15 p.i. Results are representative of two independent experiments. Bars represent mean ± SEM. *p≤0.05 ** p ≤0.01 ***p ≤0.001 **** p ≤0.001 (one-way ANOVA with Tukey's multiple comparison test).

Combinatorial blockade of TIGIT and PD-L1 does not alter Th1 OT II cell effector functions during *P.yoelii* infection

Given the central role of Th1 cells in producing IFNg during malaria (Perez-Mazliah and Langhorne 2014), and the preferential expression of TIGIT and PD-1 on Th1 cells, we hypothesised that blockade of TIGIT and PD-L1 prevented $CD4^+$ T cell exhaustion leading to an enhanced Th1 response during infection. However, combined blockade of TIGIT and PD-L1 during infection did not significantly alter the frequencies of total OT II cells, but surprisingly led to a small, but significant, decrease in total numbers of OT II cells compared with IgG control treated mice (Fig3.3A, B). Treatment with α-

TIGIT and α-PD-L1 also failed to increase the magnitude of the Th1 response, instead leading to a small, but significant, decrease in total Th1 OT II cell numbers compared with IgG control treated mice (Fig3.3C, D).

We next examined functional properties of Th1 OT II cells following administration of TIGIT and PD-L1 blocking antibodies during *P.yoelii*-OVA infection. Treatment with α-TIGIT and α-PD-L1 did not alter Th1 OT II proliferation, as measured by Ki-67 expression, compared with IgG control treated mice (Fig 3.3E, F). Dual blockade of TIGIT and PD-L1 led to a small increase in the frequencies of Th1 OT II cells co-expressing IFNg and TNFα (from 20 to 30%) during infection. However, treatment did not significantly increase the total numbers of $IFNg^{+}$ $TNFα^{+}$ Th1 OT II cells, compared with IgG control treated mice (Fig 3.3G, H). Additionally, combinatorial TIGIT and PD-L1 blockade did not significantly alter IFNg and TNFα expression on a per cell basis, as demonstrated by MFI analysis (Fig 3.3I). Consistent with these results using the OT II TCR transgenic system, blockade of TIGIT and PD-L1 failed to significantly modify the strength and function of the endogenous Th1 cell response during *P.yoelii*-OVA infection (data not shown).

TIGIT expressing regulatory T cells (Treg cells) have been shown to selectively inhibit Th1 cells (Joller 2014, Kurtulus 2015). Thus, we questioned whether $TIGIT^{+}$ Treg cells may play a role in limiting IFNg production and parasite control during *P.yoelli* infection. We detected a small proportion of endogenous $TIGIT^{+}$ Treg cells in the

spleens of naïve mice, which upon infection with *P.yoelii-OVA*, significantly increased (FigS3.3A, B). $TIGIT^+$ Treg cells expressed higher levels of PD-1 compared with $TIGIT^-$ Treg cells during *P.yoelii*-OVA infection, suggesting that $TIGIT^+$ Treg cells have a greater suppressive capacity than $TIGIT^-$ Treg cells (FigS3.3C, D). Although blockade of TIGIT and PD-L1 led to a minor decrease in the frequencies of Treg cells during infection, it significantly decreased the total numbers of Treg cells compared with IgG control treated mice (FigS3.3E, F). Thus, although reduction in Treg cell numbers in mice treated with α-PD-L1 and α-TIGIT did not influence the Th1 response, reduction in Treg cells may have affected the overall plasma IFNg production during *P.yoelii-OVA* infection.

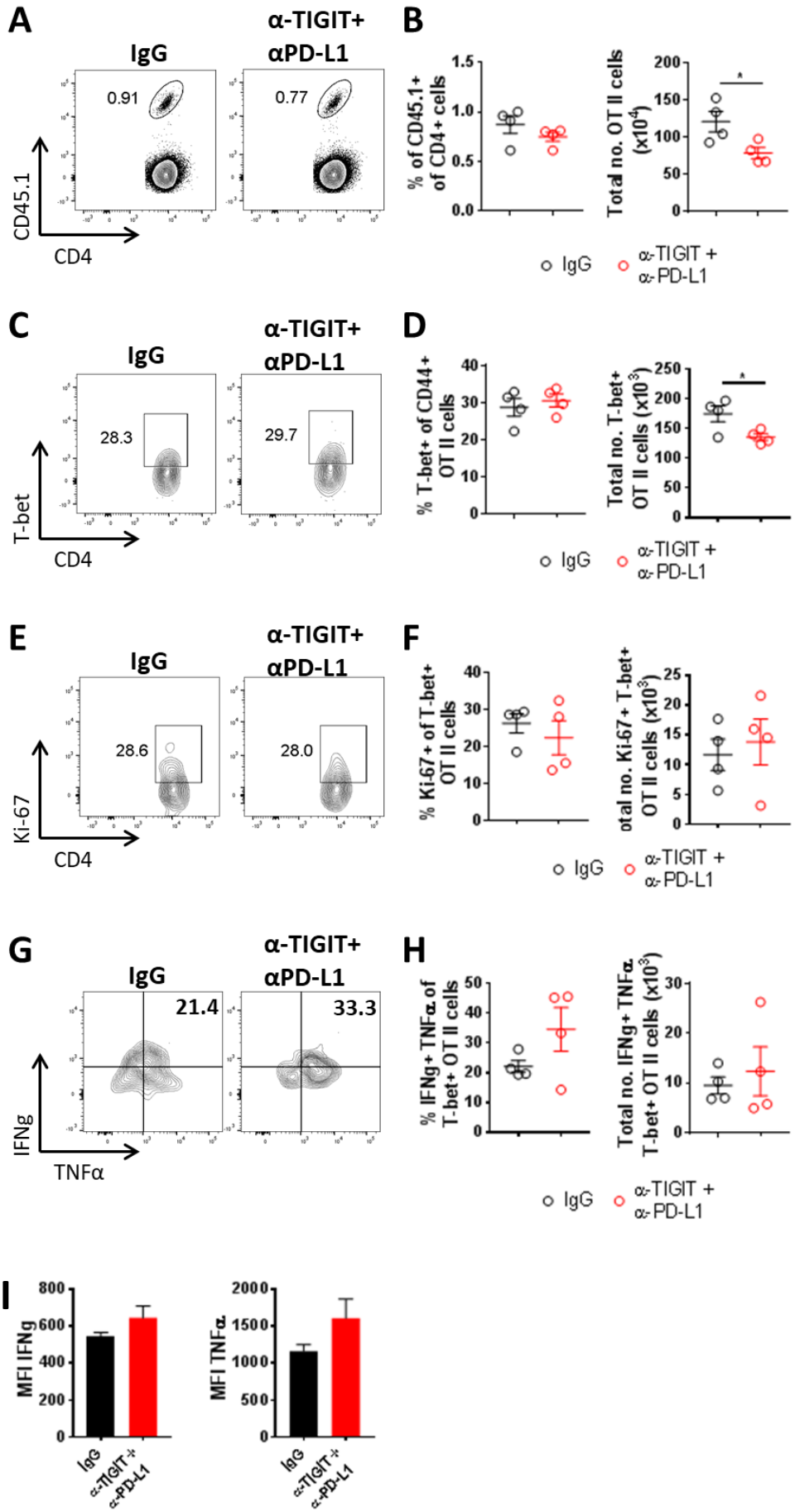

A
IgG
α-TIGIT+ αPD-L1
0.91
0.77
CD45.1
CD4
B
% of CD45.1+ of CD4+ cells
Total no. OT II cells (x10^4)
IgG
α-TIGIT + α-PD-L1
C
IgG
α-TIGIT+ αPD-L1
28.3
29.7
T-bet
CD4
D
% T-bet+ of CD44+ OT II cells
Total no. T-bet+ OT II cells (x10^3)
IgG
α-TIGIT + α-PD-L1
E
IgG
α-TIGIT+ αPD-L1
28.6
28.0
Ki-67
CD4
F
% Ki-67+ of T-bet+ OT II cells
otal no. Ki-67+ T-bet+ OT II cells (x10^3)
IgG
α-TIGIT + α-PD-L1
G
IgG
α-TIGIT+ αPD-L1
21.4
33.3
IFNg
TNFα
H
% IFNg+ TNFα of T-bet+ OT II cells
Total no. IFNg+ TNFα T-bet+ OT II cells (x10^3)
IgG
α-TIGIT + α-PD-L1
I
MFI IFNg
MFI TNFα
IgG
α-TIGIT+ α-PD-L1

Figure 3.3 Co-blockade of TIGIT and PD-L1 does not improve the CD4$^+$ Th1 cell response during *P.yoelii-OVA* infection

CD45.1$^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoeli*-OVA pRBC. Mice were either treated with control rat IgG (n=4) or α-TIGIT and α-PD-L1 (n=4) from day 5 p.i. Spleens were taken on day 15 p.i. for flow cytometric analysis. (A) Representative flow cytometric plots of OT II cells from IgG control and α-TIGIT and α-PD-L1 treated mice. (B) Frequencies (left) and total numbers (right) of splenic OT II cells. (C) Representative flow cytometric plots of T-bet$^+$ OT II cells from control and α-TIGIT and α-PD-L1 treated mice. (D) Frequencies (left) and total numbers (right) of T-bet$^+$ OT II cells. (E) Representative flow cytometric plots of Ki-67 expression by Th1 OT II cells. (F) Frequencies (left) and total numbers (right) of Ki-67$^+$ Th1 OT II cells. (G-I) Splenocytes from control and α-TIGIT and α-PD-L1 treated mice were stimulated with PMA and ionomycin and stained for IFNg and TNFα. (G) Representative flow cytometric plots of IFNg and TNFα expression by Th1 OT II cells. (H) Frequencies (left) and total numbers (right) of co-expressing IFNg and TNFα Th1 OT II cells. (I) MFI of IFNg (left) and TNFα (right) within Th1 OT II cells. Results are representative of two independent experiments. Bars represent mean ± SEM. * p≤0.05 (Unpaired t-test).

Blockade of TIGIT and PD-L1 enhances CD4$^+$ Tfh cells during *P.yoelii*-OVA infection

Surprisingly, co-blockade of TIGIT and PD-L1 signalling had no effect on the Th1 response during *P.yoelii*-OVA infection. The production of high affinity antibodies is also critical for protection against blood stage *Plasmodium* infection (Silveira, Dominguez et al. 2018), therefore we next examined the effect of TIGIT and PD-L1 blockade on the Tfh OT II cell response. Throughout the course of infection, nearly all

Tfh OT II cells expressed TIGIT (Fig3.4A, B and FigS3.2). As PD-1 expression is a defining feature of Tfh cells, all TIGIT^{+} Tfh cells co-expressed PD-1. Unlike Th1 cells, administration of α-TIGIT and α-PD-L1 during infection significantly increased the frequencies and total numbers of Tfh OT II cells, compared with IgG control treated mice (Fig3.4C, D). Tfh cells expressing Th1 associated molecules, such as T-bet, have been reported during malaria and associated with a dysfunctional B cell response (Obeng-Adjei, Portugal et al. 2015). We detected T-bet expression within Tfh OT II cells on day 15 of infection, however, administration of TIGIT and PD-L1 blocking antibodies did not significantly affect Tfh OT II cell T-bet expression, compared with IgG control treated mice (Fig3.4E). Tfh cells regulate germinal centre (GC) B cell reactions that are necessary for antibody responses during infection (Crotty 2011). Therefore, we next examined whether the increase in Tfh cells following dual blockade of TIGIT and PD-L1 affected the B cell response. Surprisingly, treatment with α-TIGIT and α-PD-L1 treatment, did not significantly enhance the GC B cell response, or plasmablast differentiation (Fig 3.4F-I). These data therefore showed that TIGIT and PD-L1 blockade only increased the Tfh cell response during *P.yoelii*-OVA infection.

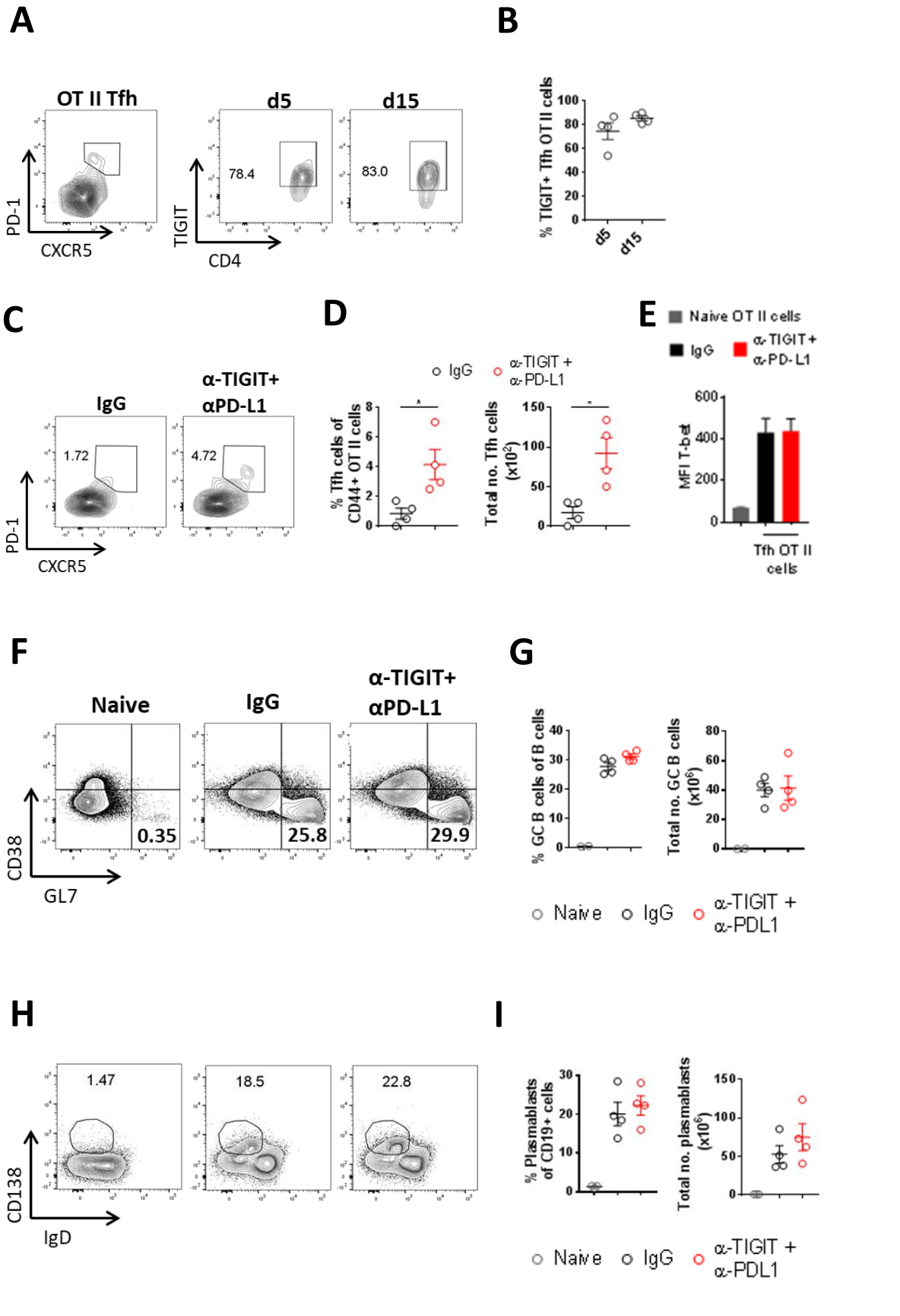

A
OT II Tfh
d5
d15
PD-1
CXCR5
78.4
83.0
TIGIT
CD4
B
% TIGIT+ Tfh OT II cells
d5
d15
C
IgG
α-TIGIT+
αPD-L1
1.72
4.72
PD-1
CXCR5
D
IgG
α-TIGIT +
α-PD-L1
% Tfh cells of CD44+ OT II cells
Total no. Tfh cells (x10²)
E
Naive OT II cells
IgG
α-TIGIT +
α-PD-L1
MFI T-bet
Tfh OT II cells
F
Naive
IgG
α-TIGIT+
αPD-L1
0.35
25.8
29.9
CD38
GL7
G
% GC B cells of B cells
Total no. GC B cells (x10⁶)
Naive
IgG
α-TIGIT +
α-PDL1
H
1.47
18.5
22.8
CD138
IgD
I
% Plasmablasts of CD19+ cells
Total no. plasmablasts (x10⁶)
Naive
IgG
α-TIGIT +
α-PDL1

Figure 3.4 Co-blockade of TIGIT and PD-L1 enhances the Tfh OT II response during *P.yoelii*-OVA infection

$1x10^6$ $CD45.1^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with *P.yoelii*-OVA pRBC. Infected mice were either left untreated (A-B) or administered with either control rat IgG (n=4) or α-TIGIT and α-PD-L1 (n=4) from day 5 p.i. (C-F). Spleens were isolated on days 5 and 15 p.i. for analysis. (A) Representative flow cytometric plots of $CXCR5^{hi}$ $PD\text{-}1^{hi}$ Tfh OT II cells (left) and TIGIT expression within Tfh OT II cells (right). (B) Frequencies of $TIGIT^+$ Tfh OT II cells. (C) Representative flow cytometric plots of Tfh OT II cells following treatment with either IgG or α-TIGIT and α-PD-L1 on day 15 of infection. (D) Frequencies (left) and total numbers (right) of Tfh OT II cells. (E) MFI of T-bet within naïve OT II cells and Tfh OT II cells on day 15 of infection. (F) Representative flow cytometric plots of $GL7^+$ $CD38^-$ GC B cells on day 15 of infection. (G) Frequencies (left) and total numbers (right) of GC B cells. (H) Representative flow cytometric plots of plasmablasts on day 15 of infection. (I) Frequencies (left) and total numbers (right) of plasmablasts. Results are representative of 2 independent experiments. Bars represent mean ± SEM. *p≤0.05 (Unpaired t-test and Mann-Whitney test).

Dual blockade of TIGIT and PD-L1 does not alter the effector $CD8^+$ T cell response during *P.yoelii*-OVA infection

$CD8^+$ T cells have been reported to co-express TIGIT and PD-1 (Johnston, Comps-Agrar et al. 2014). In addition, $CD8^+$ T cells have been suggested to a play a protective role during blood stage malaria and to be the primary responding cell population following PD-1 blockade (Horne-Debets, Faleiro et al. 2013). Therefore, we questioned whether TIGIT played a combined role with PD-1 in regulating antigen-

specific $CD8^+$ T cell responses during blood stage malaria. A strong OT I response developed during *P.yoelii*-OVA infection, as demonstrated by a significant increase in the frequencies of total OT I cells and Ki-67^+ OT I cells, as well as increased IFNg production compared to OT I cells in naïve mice (Fig 3.5A-C). A large proportion of effector OT I cells (defined as $CD44^+$) expressed TIGIT on day 15 of infection (Fig3.5D, E), with TIGIT expression positively correlating with PD-1 expression (Fig3.5F). Surprisingly, however, co-blockade of TIGIT and PD-L1 did not significantly affect the frequencies and total numbers of OT I cells, compared with IgG control treated mice (Fig3.5G, H). Moreover, TIGIT and PD-L1 blockade did not significantly influence the function of OT I cells during infection, as shown by similar co-expression of IFNg and TNFα (Fig3.5I, J), as well as granzyme B (Fig 3.5K, L) compared with IgG control treated mice. Overall, these data suggested that $CD8^+$ effector T cells were not modified following dual blockade of TIGIT and PD-L1 during *P.yoelii-OVA* infection.

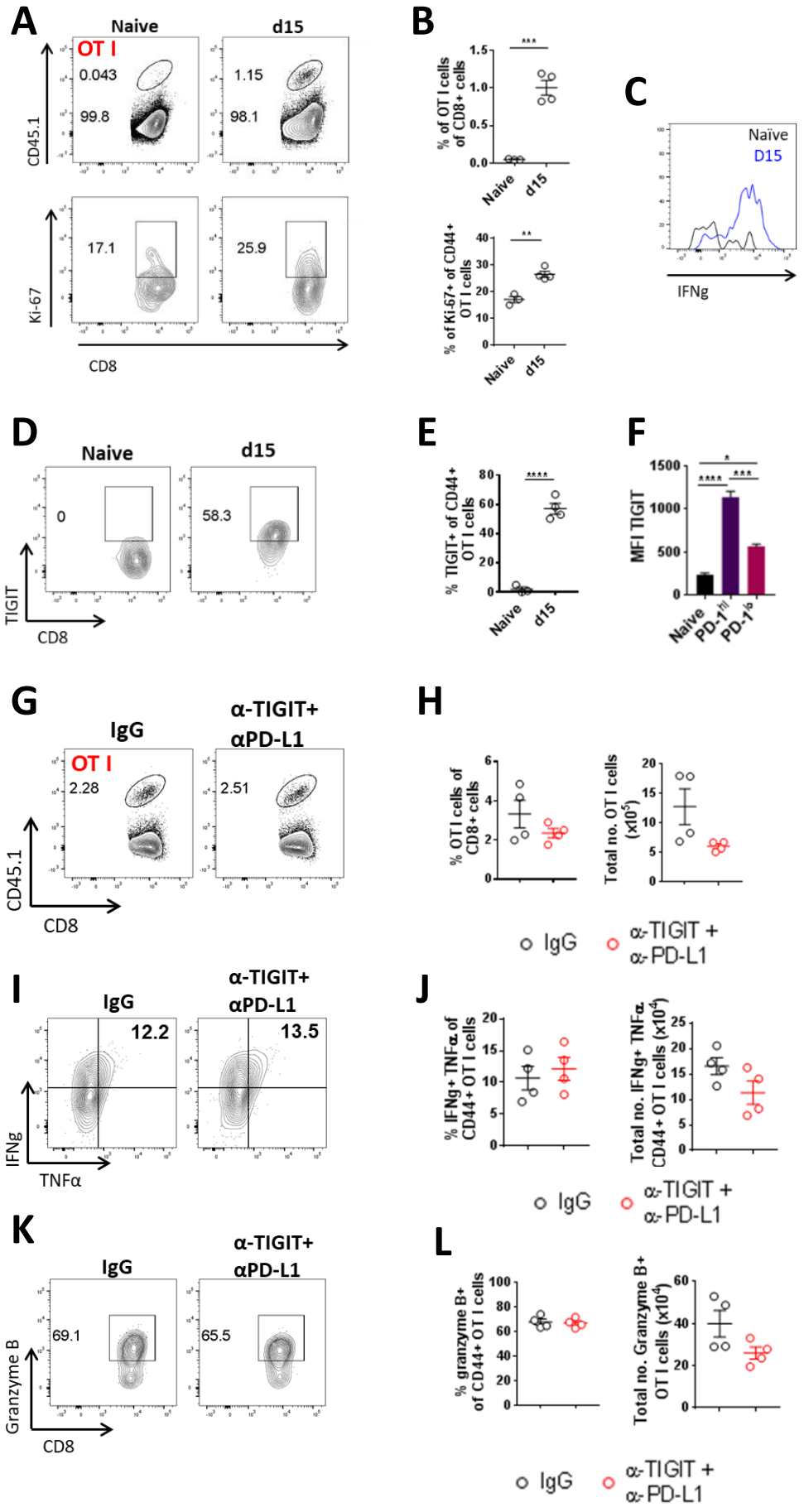

A
Naive
d15
OT I
0.043
99.8
1.15
98.1
CD45.1
17.1
25.9
Ki-67
CD8
B
% of OT I cells of CD8+ cells
Naive
d15
% of Ki-67+ of CD44+ OT I cells
C
Naïve
D15
IFNg
D
Naive
d15
0
58.3
TIGIT
CD8
E
% TIGIT+ of CD44+ OT I cells
F
MFI TIGIT
Naive
PD-1hi
PD-1lo
G
IgG
α-TIGIT+ αPD-L1
OT I
2.28
2.51
CD45.1
CD8
H
% OT I cells of CD8+ cells
Total no. OT I cells (x10^5)
IgG
α-TIGIT + α-PD-L1
I
IgG
α-TIGIT+ αPD-L1
12.2
13.5
IFNg
TNFα
J
% IFNg+ TNFα of CD44+ OT I cells
Total no. IFNg+ TNFα CD44+ OT I cells (x10^4)
IgG
α-TIGIT + α-PD-L1
K
IgG
α-TIGIT+ αPD-L1
69.1
65.5
Granzyme B
CD8
L
% granzyme B+ of CD44+ OT I cells
Total no. Granzyme B+ OT I cells (x10^4)
IgG
α-TIGIT + α-PD-L1

Figure 3.5 Co-blockade of TIGIT and PD-L1 does not improve CD8$^+$ OT I effector capacity during *P.yoelii*-OVA infection

10^5 CD45.1$^+$ CD8$^+$ OT I cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC. Spleens were taken on day 15 p.i. for flow cytometric analysis. (A) Representative flow cytometric plots of OT I cells (top) and Ki-67 expression within OT I cells (bottom). (B) Frequencies of OT I cells (top) and Ki-67+ OT I cells (bottom). (C) Representative histogram showing IFNg expression in OT I cells. (D) Representative flow cytometric plots of TIGIT expression within the effector (CD44$^+$) OT I population. (E) Frequencies of TIGIT$^+$ effector OT I cells. (F) MFI of TIGIT expression within naïve and PD-1^{hi} and PD-1^{lo} effector OT I cells. (G-L) Infected mice were either treated with control rat IgG (n=4) or α-TIGIT and α-PD-L1 (n=4) from day 5 p.i. and spleens were isolated on day 15 of infection for analysis. (G) Representative flow cytometric plots of OT I cells from IgG and α-TIGIT and α-PD-L1 treated mice on day 15 of infection. (H) Frequencies (left) and total numbers (right) of OT I cells. (I) Representative flow cytometric plots of IFNg and TNFα expressing effector OT I cells. (J) Frequencies (left) and total numbers (right) of co- expressing IFNg and TNFα effector OT I cells. (K) Representative flow cytometric plots of granzyme B expression by effector OT I cells. (L) Frequencies (left) and total numbers (right) of granzyme B$^+$ effector OT I cells. Results are representative of 2 independent experiments. Bars represent mean ± SEM. ** p ≤0.01 ***p ≤0.001 ****p≤ 0.0001 (Unpaired t-test and one-way ANOVA with Tukey's multiple comparison test).

Natural Killer cells do not contribute to the enhanced IFNg response following dual blockade of TIGIT and PD-L1 during *P.yoelii* infection

Natural killer cells (NK cells) are another source of IFNg during malaria (King and Lamb 2015). NK cells can also be regulated by TIGIT (Zhang, Bi et al. 2018). Indeed, TIGIT was highly expressed by NK cells during *P.yoelii-OVA* infection (Fig3.6A, B and

FigS3.4). Furthermore, the MFI of TIGIT on NK cells was significantly increased on day 15 of infection compared to naïve NK cells (Fig3.6C). Nevertheless, administration of TIGIT and PD-L1 blocking antibodies did not significantly affect the frequencies or total numbers of NK cells during *P.yoelii*-OVA infection, compared with IgG control treated mice (Fig3.6D). Moreover, administration of TIGIT and PD-L1 blocking antibodies did not significantly affect the frequencies or total numbers of $IFNg^{+}$ NK cells, compared with IgG control treated mice (Fig3.6E). As a result, NK cells were unlikely to have contributed to the improved parasite control observed during *P.yoelii*-OVA infection following α-TIGIT and α-PD-1 administration.

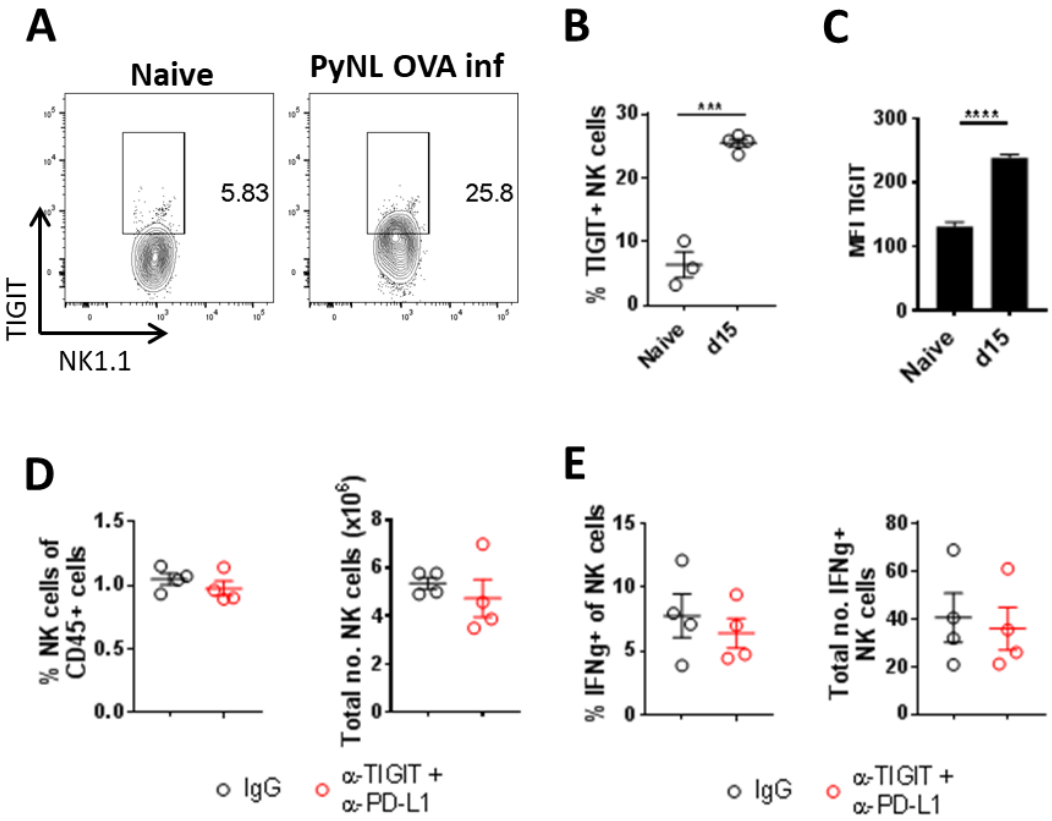


Figure 3.6 Natural killer cells are not affected by TIGIT and PD-L1 blockade during *P.yoelii* infection

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC and spleens were taken on day 15 of infection and digested prior to flow cytometric analysis. (A) Representative flow cytometric plots of TIGIT expression by natural killer cells (NK cells). (B) Frequencies of $TIGIT^+$ NK cells. (C) MFI of TIGIT within the NK cell population. (D-E) Infected mice were then either treated with control rat IgG (n=4) or α-TIGIT and α-PD-L1 (n=4) from day 5 of infection and spleens were take on day 15 of infection for analysis. (D) Frequencies (left) and total numbers (right) of NK cells. (E) Frequencies (left) and total numbers (right) of $IFNg^+$ NK cells. Results are representative of 2 independent experiments. Bars represent mean ± SEM. ***p ≤0.001 ****p≤ 0.0001 (Unpaired t-test and one-way ANOVA with Tukey's multiple comparison test).

Co-blockade of TIGIT and PD-L1 leads to an increase in red pulp macrophages during *P.yoelii*-OVA infection

The improved parasite control following administration of blocking TIGIT and PD-L1 antibodies during infection was associated with increased levels of circulating IFNg. A major role of for IFNg during malaria is to activate macrophages to improve parasite killing (King and Lamb 2015). Thus, we investigated whether the improved parasite control in α-TIGIT and α-PD-L1 treated mice was associated with an enhanced red pulp macrophage (RPM) response. Interestingly, blockade of TIGIT and PD-L1 significantly increased the frequencies and total numbers of RPM, compared with IgG control treated mice (Fig 3.7A). Whilst dual blockade of TIGIT and PD-L1 did not significantly alter CD80 and MHC II expression by RPM compared with IgG control

treatment (Fig3.7B), the expansion of RPM may facilitate increased uptake of parasitized red blood cells and contribute to improved parasite control during *P.yoelii*-OVA infection.

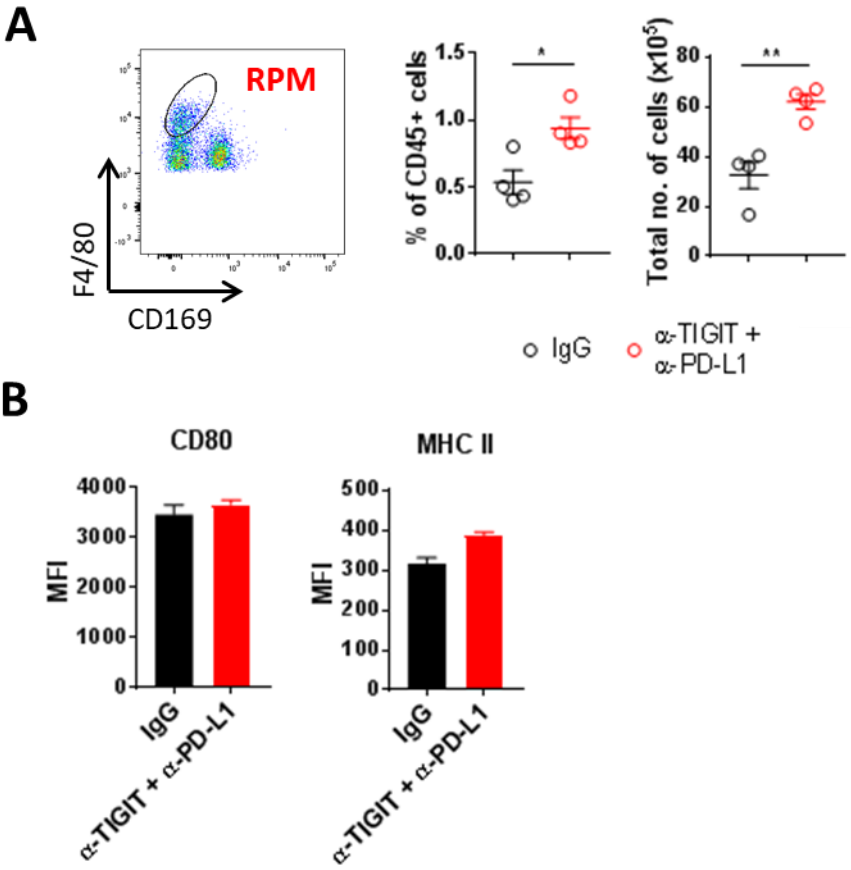


Figure 3.7 TIGIT and PD-L1 blockade increases the red pulp macrophage population

C57BL/6 mice were infected with 10^4 *P.yoelii-OVA* pRBC and were either treated with control rat IgG (n=4) or α-TIGIT and α-PD-L1 (n=4) from day 5 p.i. Spleens were taken on day 15 of infection and digested prior to flow cytometric analysis. (A) Representative flow cytometric plot of red pulp macrophage gating in naïve mice (left) and quantified frequencies (middle) and total numbers (right) of red pulp macrophages during infection. (B) MFI of CD80 (left) and MHC II (right) on red pulp macrophages. Results are representative of 2 independent experiments. Bars represent mean ± SEM. * p ≤0.05 ** p ≤0.01 (Unpaired t-test).

Discussion

In this study we have shown that TIGIT and PD-1 are highly co-expressed on functionally exhausted antigen-specific $CD4^+$ Th1 cells, as well as Tfh cells during *P.yoelii* infection. Combined blockade of TIGIT and PD-L1 markedly reduced peripheral parasitemia during infection, which was correlated with an increase in circulating IFNg and increased $CD4^+$ Tfh response.

A synergistic role for PD-1 and TIGIT in regulating $CD8^+$ T cell responses has been shown in murine cancer models (Johnston, Comps-Agrar et al. 2014, Zhang, Bi et al. 2018), however a role for TIGIT in promotion of $CD4^+$ T cell exhaustion during infection has not been previously examined. In our *P.yoelii* model, TIGIT was expressed on $CD4^+$ T cells early during infection, following T cell activation. TIGIT signalling in naïve $CD4^+$ T cells can inhibit T cell activation (Joller 2011), therefore suggesting that TIGIT may have an early regulatory role in T cell activation during malaria. Although TIGIT and PD-1 have different dynamics of expression early during infection (as shown on day 5), they appear to be highly correlated later in infection, as T cell exhaustion is established. Notably, combinatorial treatment of α-TIGIT and α-PD-L1 significantly improved parasite control, suggesting a synergistic inhibitory role in effector $CD4^+$ T cells during *P.yoelii* infection. Indeed, independent blockade of PD-L1 or TIGIT did not affect parasite control.

Our study has shown that the improved parasite control following α-TIGIT and α-PD-L1 treatment correlated with increased systemic IFNg and TNFα. Surprisingly, there was only a minor increase in the frequencies of Th1 cells co-expressing IFNg and TNFα in the spleen following α-TIGIT and α-PD-L1 treatment. This contrasts previous studies employing combinatorial inhibitory treatment during cancer or other infections, which show significant improvement in T cell polyfunctionality (Jin, Anderson et al. 2010, Butler 2012, Johnston, Comps-Agrar et al. 2014). Therefore, our data suggests that in the absence of TIGIT and PD-1 signalling, Th1 cells may still be exposed to sufficiently high levels of regulation to keep their activity supressed. Consistent with our findings, combined blockade of TIGIT and PD-L1 in a murine cancer model did not restore $CD4^+$ T cell effector functions (Johnston, Comps-Agrar et al. 2014). The immunoregulatory cytokines IL-27, IL-10 and TGFβ limit effector $CD4^+$ T cell IFNg production during malaria (Li, Corraliza et al. 1999, Li, Sanni et al. 2003, Villegas-Mendez, de Souza et al. 2013), and thus, could limit Th1 cell functions following combinatorial treatment of α-TIGIT and α-PD-L1 during *P.yoelii* infection.

To further investigate the mechanisms leading to improved parasite control following dual blockade of TIGIT and PD-L1, we examined the antigen-specific Tfh cell response. Blockade of TIGIT and PD-L1 significantly increased the number of antigen-specific Tfh cells during *P.yoelii* infection. However, dual blockade of TIGIT and PD-L1 did not influence the status of Tfh cells, as T-bet expression in Tfh cells was comparable between α-TIGIT and α-PD-L1 treated mice and IgG control treated mice. Excessive levels of IFNg and TNFα negatively regulate Tfh cell differentiation during malaria and

drive the expansion of atypical Tfh cells expressing Th1 associated molecules (Obeng-Adjei, Portugal et al. 2015, Ryg-Cornejo, Ioannidis et al. 2016, Zander 2017). Therefore, the elevated levels of circulating IFNg following TIGIT and PD-L1 blockade may not improve Tfh cell functionality and consequently the GC B cell response. However, dual blockade of PD-L1 and LAG3 during *P.yoelii* infection concomitantly enhanced the IFNg and Tfh cell response, which correlated with an improved anti-*Plasmodium* humoral response (Butler 2012). Importantly, previous studies have shown that an increase in Tfh cell numbers is associated with enhanced humoral immunity during malaria and improved parasite control (Butler 2012, Zander, Obeng-Adjei et al. 2015, Zander 2017). However, consistent with our findings, increased Tfh cell numbers is not always associated with an enhanced GC B cell reaction (Zander, Obeng-Adjei et al. 2015). Therefore, the increased Tfh cell response following combined TIGIT and PD-L1 blockade may contribute to the improved parasite control during *P.yoelii* infection. The induction of a Tfh cell response and activation of antibody-secreting B cells is key to the resolution of blood-stage malaria (von der Weid, Honarvar et al. 1996). Therefore, parasite-specific antibody titres need to be assessed to confirm that increased Tfh cell numbers improve protection to blood stage *P.yoelii* infection.

Although we did not measure any differences in the splenic Th1 response, $CD8^+$ T cell response, or NK cell response in α-TIGIT and α-PD-L1 treated mice, the short half-life of IFNg (Ando, Takahashi et al. 2014) suggested that the increased systemic IFNg in treated mice was the result of an active difference in production, compared with

control IgG treated mice. During malaria, effector $CD4^+$ T cells migrate from the spleen and accumulate in non-lymphoid tissues (Villegas-Mendez, Shaw et al. 2016). Therefore, as splenic Th1 cell cytokine production was marginally improved following administration of α-TIGIT and α-PD-L1, it is possible that treatment could partially improve IFNg production in effector $CD4^+$ T cells in multiple different tissues, which cumulatively led to an increase in systemic IFNg in treated mice during *P.yoelii* infection. Importantly, the endogenous T cell response following dual blockade of TIGIT and PD-L1 was similar to the OT II and OT I response (data not shown), demonstrating that there were no intrinsic differences preventing OT II and OT I cells from responding.

As well as directly contributing to T cell exhaustion, a major mechanism of TIGIT-mediated suppression during chronic disease is via affecting the specialised activity of Treg cells (Kurtulus, 2015). Treg cells consitutively expressing TIGIT preferntially regulate Th1 and Th17 responses whilst sparing Th2 cells (Joller 2014). We observed a significant increase in $TIGIT^+$ Treg cells during *P.yoelii* infection and dual blockade of TIGIT and PD-L1 led to a significant decrease in the number of Treg cells. However, this did not influence the splenic Th1 response. Whilst a decrease in Treg cells may suggest a major role for $TIGIT^+$ Treg cells in regulating inflammation during *P.yoelii* infection, the importance of Treg cell suppression in malaria is unclear (Couper, Blount et al. 2008, Kurup 2017). Notably, previous studies, have shown that complete depletion of Treg cells does not affect the course of infection, or the magnitude of the Th1 response (Couper, Blount et al. 2008, Villegas-Mendez, de Souza et al. 2013).

Thus, further studies will be necessary to determine whether TIGIT$^+$ Treg cells are important in regulating Tfh cells during malaria.

In murine cancer models, dual blockade of TIGIT and PD-1 restored CD8$^+$ T cell and NK cell effector functions, necessary for efficient tumour rejection and memory formation (Johnston, Comps-Agrar et al. 2014, Zhang, Bi et al. 2018) Surprisingly, we show that administration of TIGIT and PD-L1 blocking antibodies during *P.yoelii* infection did not enhance CD8$^+$ T cell or NK cell responses. At the time point examined antigen-specific CD8$^+$ T cells still produced large amounts of IFNg and granzyme B, suggesting that they were not functionally exhausted. During *P. chabaudi* infection, CD8$^+$ T cells do not develop an exhausted phenotype until day 21 of infection (Horne-Debets, Faleiro et al. 2013). Moreover, CD4$^+$ T cell exhaustion has been reported to occur more quickly in comparison to CD8$^+$ T cells (Crawford, Angelosanto et al. 2014). Thus, at the time points measured, TIGIT and PD-1 are unlikely to be driving CD8$^+$ T cell exhaustion, therefore the increased protection from TIGIT and PD-L1 blockade is unlikely to be CD8$^+$ T cell mediated. In tumour studies, TIGIT deficient NK cells did not display significant differences in IFNg production, despite significant upregulations in co-stimulatory receptors and TNFα production (Zhang, Bi et al. 2018). Therefore, IFNg may not have been the most appropriate measure of NK cell functionality.

We have also shown that the frequencies and absolute numbers of red pulp macrophages (RPM) increased in α-TIGIT and α-PD-L1 treated mice compared to IgG control mice, during malaria. As RPM play an important role in removal of pRBC (Stevenson and Riley 2004), this data suggests that increased parasite control following treatment may be mediated through increased phagocytosis of pRBC. Ligation of TIGIT with its ligand CD155 can have immunomodulatory effects on adaptive and innate immune cells (Yu, Harden et al. 2009, Johnston, Comps-Agrar et al. 2014). In particular, TIGIT signalling in macrophages can skew activation to an anti-inflammatory M2 phenotype, illustrated by increased IL-10 production (Chen 2016). Therefore, blockade of TIGIT alone may induce a pro-inflammatory phenotype in macrophages. In addition, previous studies have shown that PD-L1 blockade alone can increase macrophage proliferation, survival and expression of MHC II and CD86 (Hartley, Chow et al. 2018). However, as blockade of TIGIT or PD-L1 alone did not affect parasite control, the increased phagocytic activity may require co-removal of PD-1 and TIGIT dependent regulation. It is possible therefore, that following TIGIT and PD-L1 blockade, the enhanced RPM response may be an indirect effect of increased levels of IFNg, which is known to activate RPM effector functions such as nitric oxide and influence self-renewal, necessary for enhanced parasite killing during malaria (Yamazaki, Akiba et al. 2005, Davies 2013, Hashimoto, Chow et al. 2013, Kurotaki, Uede et al. 2015).

Collectively, we have shown that synergistic blockade of TIGIT and PD-L1 significantly improves parasite control during blood stage malaria. Whilst the exact mechanism is currently unclear, it likely involves increased IFNg and Tfh cell responses.

Materials and Methods

Ethics statement

All animal work was approved following local ethical review by the University of Manchester Animal Procedures and Ethics Committees and was performed in strict accordance with the U.K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licences 70/7293 and P8829D3B4).

Mice and parasites

Male, 7-week-old C57BL/6 ($CD45.2^+$) mice were purchased from Charles River UK or Envigo and maintained in individually ventilated cages. RAG-1 OT II x Pep3 (OT II) and RAG-1 OT I x Pep3 (OT I) mice were bred at the University of Manchester and were fully backcrossed to a C57BL/6 background.

Cryopreserved *P.yoelii* parasites expressing mCherry OVA (under the control of the *hsp70* promoter) were thawed and passaged once in C57BL/6 mice before being used to infect experimental mice. Mice were infected with 10^4 pRBC by intravenous injection. The course of infection was monitored every other day starting from day 5 of infection by peripheral parasitaemia and assessed by microscopic examination of Giemsa-stained thin blood smears.

CD4$^+$ T cell isolation and adoptive transfer

Spleens were isolated from RAG-1 OT II x Pep3 or RAG-1 OT I x Pep3 mice and then homogenised through a 70µm strainer (BD Biosciences) to generate a single cell suspension. Red blood cells (RBC) were then lysed using an RBC lysis buffer (BD Biosciences). OT II cells and OT I cells were isolated using anti-CD4 conjugated or anti-CD8 conjugated microbeads respectively (Miltenyi Biotec), according to manufacturer's instructions. $1x10^6$ OT II cells were individually transferred or co-transferred with $1x10^5$ OT I cells into C57BL/6 via intravenous injection one day prior to infection with *P.yoelii-OVA* pRBC.

In vivo blockades

For *in vivo* blockade of co-inhibitory receptors, mice received 250µg of α-PD-L1 (10F.9G2) (BioXcell) and α-TIGIT (kindly supplied by Dr Jane Grogan, Genentech) every other day from day 5 of infection via intraperitoneal injection. Control mice received 250µg of Rat IgG (Sigma Aldrich) via intraperitoneal injection.

Flow cytometry

Spleens were removed from naïve and malaria-infected mice and homogenised through a 70µm strainer and incubated with an RBC lysis buffer to generate an RBC free, single cell suspension. For analysis of the myeloid compartment, spleens were cut into small pieces and incubated with HBSS containing 2mg/mL collagenase D

(Sigma Aldrich) and 50KU/mL Dnase (Sigma Aldrich) for 30 minutes at 37^{O}C prior to RBC lysis. Absolute live cell counts were calculated by trypan blue exclusion cell viability assay (Sigma).

Splenocytes were surface stained for 25 minutes at 4 ^{O}C with CD45.1 (A20), CD4 (RM4-5), CD8a (53-6.7), CD44 (IM7), PD-1 (RMPI-30), TIGIT (GIGD7), CXCR5-biotin (L138D7), CD25 (PC61) B220 (RA3-6B2)**,** CD19 (6D5), GL7 (GL7) and CD38 (90), CD138 (281-2) and IgD (11-26c-2a). Surface staining was done in the presence of FcR block (2.4G2, BioXcell). For streptavidin staining, surface antibodies were washed, and cells were incubated for 10 minutes at room temperature (RT) with streptavidin v510. For intracellular staining, cells were incubated with Foxp3 fixation/permeabilisation buffer (eBioscience) for 30 minutes at 4 ^{O}C. Cells were then stained with the following antibodies: T -bet (4B10), Foxp3 (FJK-16s) and Ki-67 (SolA15) for 30 minutes. For granzyme B staining, cells were additionally fixed in 2% PFA prior to incubation with Foxp3 fixation/permeabilisation buffer. For analysis of the intracellular cytokines IFNg (XMG1.2) and TNFα (MP6 XT22) cells were stimulated *ex vivo* for 4 hours at 37 ^{O}C with 200ng/mL PMA (Sigma), 1µg/mL ionomycin (Sigma) and Brefeldin A ([1000x], (eBiosceince). All antibodies were acquired from eBioscience or Biolegend.

Analysis of the splenic myeloid compartment was achieved by surface staining with the following antibodies: CD45 (30F1), Ly6C (HK1.4), F4/80 (BM8), MHC II

(M5/114.15.2), CD80 (16-10A1), CD169 (3D6.112), PD-L1 (10F9G2), CD11c (N418), CD11b (M1/70), NK1.1 (PKI36) and CD64 (X54-5/7.1)The following surface markers were stained using antibodies conjugated to the same fluorophore to generate a lineage gate: CD3 (17A2), CD19 (6D5), B220 (RA3-6B2) and Ly6G (1A8). All antibodies were acquired from eBioscience or Biolegend. Dead cells were excluded from all analyses using forward and side scatter properties and LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies).

Samples were acquired through the Fortessa (BD systems, UK) and all analysis was performed using Flowjo Software (Treestar Inc, OR, USA). Malaria infected samples were combined to generate fluorescence minus one (FMO) samples which were used to validate the flow cytometric data.

Quantification of Plasma Cytokines

Plasma isolated from naïve or malaria infected mice was stored at -80 ^{O}C until further use. The concentration of IFNg, TNF, IL-10 and IL-2 were measured by a Cytometric Bead Array (CBA) mouse Th1/Th2/TH17 cytokine kit (BD Biosciences), according to manufactures instructions.

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, USA). Comparison between 3 or more groups was carried out using a one-way ANOVA with Tukey's test for multiple comparisons. For groups of 2, unpaired t-tests were carried out. Results were considered significant when $P<0.05$.

Supplementary Figures

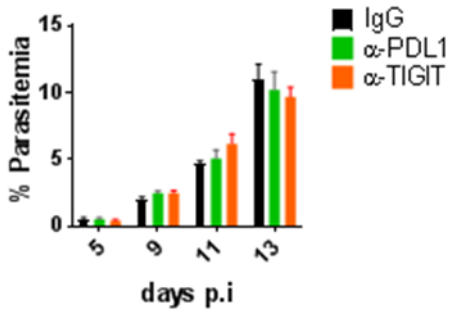


Figure S3.1 Independent blockade of TIGIT or PD-L1 does not impact parasite control during *P.yoelii*-OVA infection

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC and then either treated with rat IgG (n=4), or α-TIGIT (n=4) or α-PD-L1 (n=4) every other day starting from day 5 of infection. Peripheral parasitemia was monitored over the course of the infection.

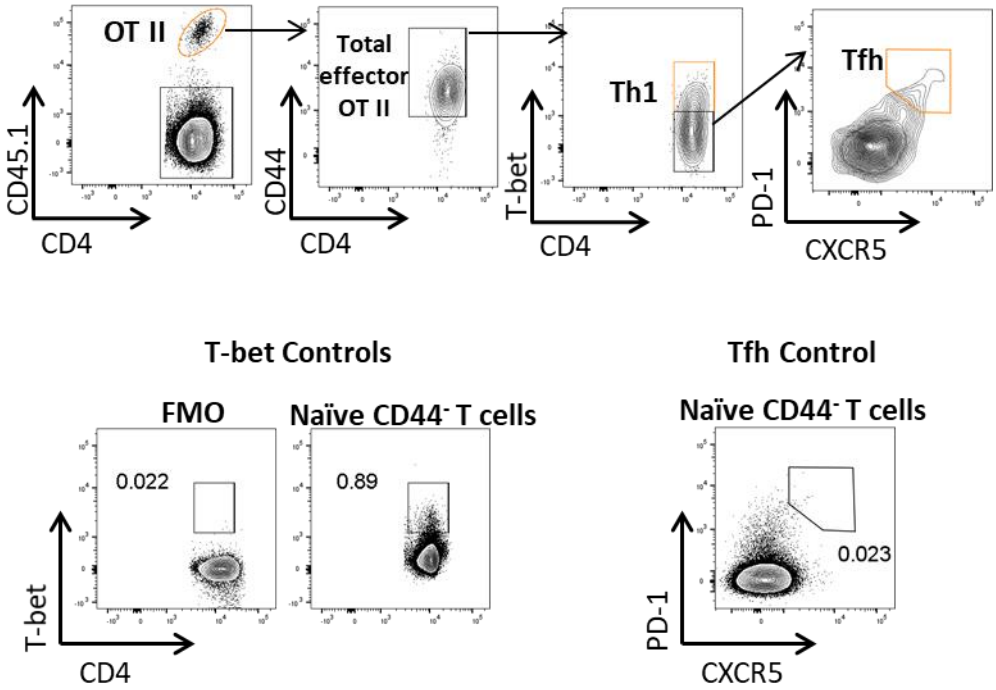


Figure S3.2 Gating strategy for effector CD4$^+$ T helper populations

1x10^6 CD45.1$^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with *P.yoelii*-OVA (n=4). Spleens from infected mice were taken for flow cytometric analysis. Representative plots showing how OT II cells can be sub-gated into total effector OT II cells (based on CD44 expression), Th1 OT II cells (based on T-bet expression) and Tfh OT II cells (based on CXCR5 and PD-1 expression). A combination of FMO controls and naïve samples were used to set appropriate gates.

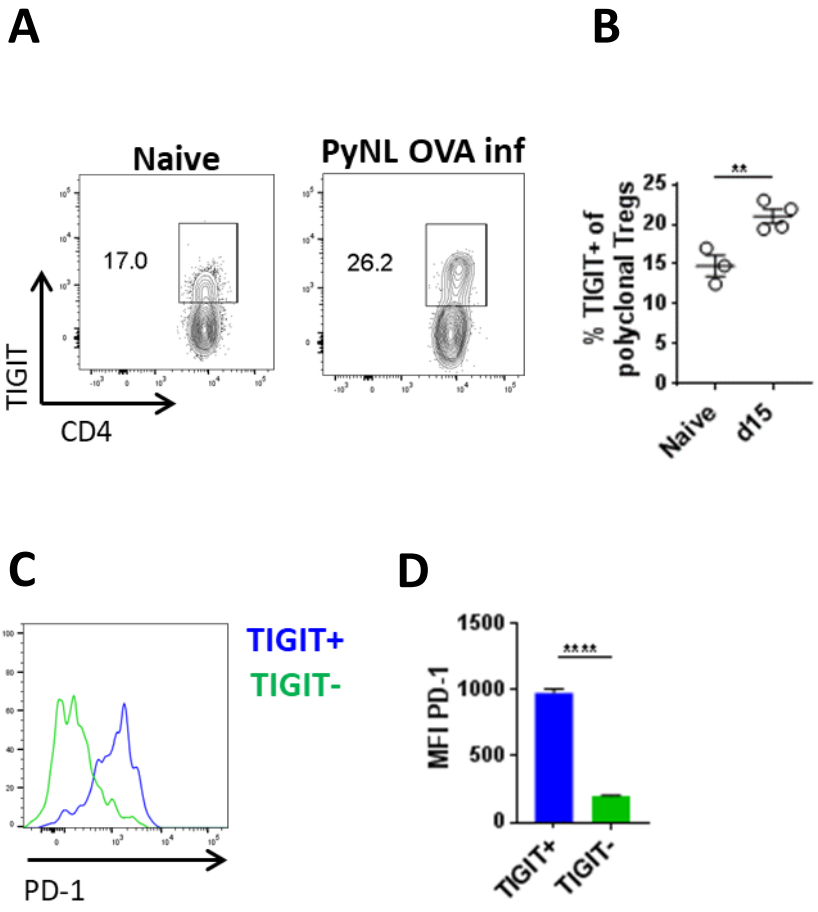

A
B
Naive
PyNL OVA inf
17.0
26.2
TIGIT
CD4
% TIGIT+ of polyclonal Tregs
**
Naive
d15
C
D
TIGIT+
TIGIT-
PD-1
MFI PD-1
****
TIGIT+
TIGIT-
E
Naive
IgG
α-TIGIT+ αPD-L1
5.94
4.40
3.89
Foxp3
CD25
F
% polyclonal Tregs of CD4+ T cells
Total no. polyclonal Tregs (x10^5)
***
*
Naive
IgG
α-TIGIT + α-PD-L1

Figure S3.3 Dual blockade of TIGIT and PD-L1 significantly reduces the number of Treg cells during *P.yoelii*-OVA infection

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC. Spleens were taken on day 15 of infection for flow cytometric analysis. (A) Representative flow cytometric plots showing TIGIT expression within the endogenous Treg cell population (B) Frequencies of $TIGIT^+$ Treg cells. (C) Representative histograms of PD-1 expression within the $TIGIT^+$ and $TIGIT^-$ Treg cell sub-populations. (D) MFI of PD-1 on $TIGIT^+$ and $TIGIT^-$ Treg cell sub-populations. (E) Representative flow cytometric plots of Treg cells in naïve mice and infected mice treated with either IgG isotype control or TIGIT and PD-L1 blocking antibodies. (F) Frequencies (left) and total numbers (right) of Treg cells.

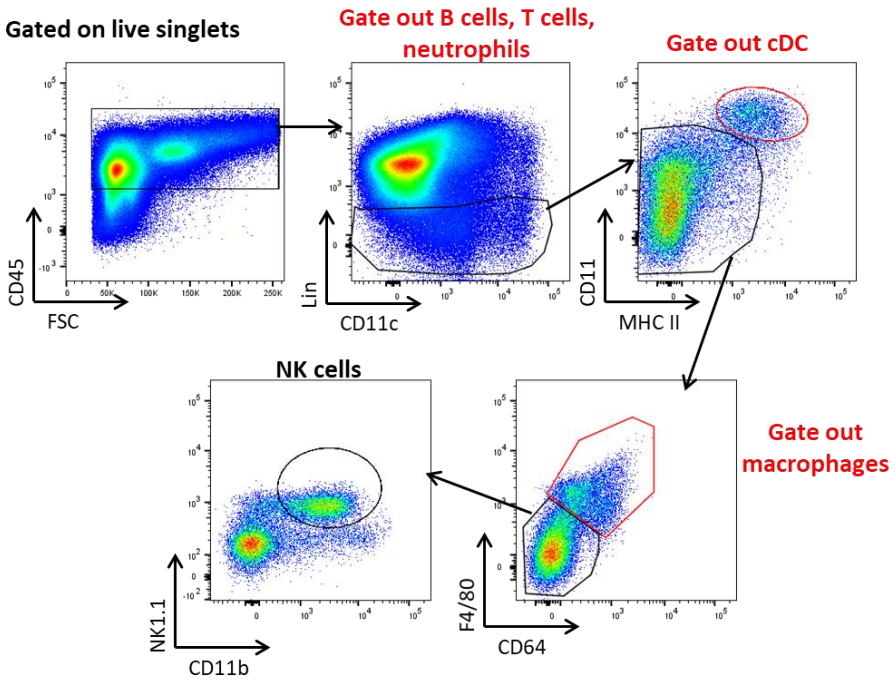


Figure S3.4 Gating strategy for natural killer cells

C57BL/6 mice were infected with 10^4 *P.yoelii*-OVA pRBC and spleens were taken for flow cytometric analysis. Representative flow plots showing the gating strategy used to identify natural killer cells (NK cells). B cells, T cells, neutrophils were gated out through a series of lineage markers including: B220, CD19, CD3 and Ly6G. Conventional dendritic cells (cDC) and macrophages were also gated out.

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Chapter 4: Tim3 signalling in combination with PD-1 does not contribute to antigen-specific CD4$^+$ T cell exhaustion during blood stage malaria

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Manuscript in preparation

All work was carried out by R.S.D

Abstract

Interferon gamma producing $CD4^+$ T cells are essential for protection against blood stage malaria, however, chronic *Plasmodium* infection can induce $CD4^+$ T cell exhaustion which contributes to the pathogenesis of the disease. Tim3 is a well-known negative regulator of T cells that can contribute to the development of T cell exhaustion, but its role in $CD4^+$ T cell exhaustion during malaria remains unclear. In this study we show that Tim3 is transiently expressed on antigen-specific $CD4^+$ T cells during *Plasmodium yoelii* infection and is downregulated as $CD4^+$ T cell exhaustion is established. Notably, combined blockade of Tim3 and PD-L1 during malaria failed to improve $CD4^+$ T cell effector functions, but instead led to a significant reduction in effector $CD4^+$ T cells. Taken together, our study has shown that Tim3 is dispensable for $CD4^+$ T cell exhaustion during malaria.

Introduction

Malaria is a leading cause of global morbidity and mortality (WHO 2018). Despite years of exposure, people living in endemic regions only generate partial immunity to malaria, frequently developing chronic infections with low to moderate levels of blood stage parasites (Langhorne, Ndungu et al. 2008). Protection against blood stage malaria requires a strong pro-inflammatory response, with $CD4^+$ T cell dependent interferon gamma (INFg) being critical for parasite control (Süss, Eichmann et al. 1988). Newly emerging evidence has shown that $CD4^+$ T cell exhaustion occurs during malaria, illustrated by loss of IFNg production and upregulation of inhibitory receptors, which contributes to infection chronicity and the pathogenesis of the disease (Butler 2012, Horne-Debets, Faleiro et al. 2013, Illingworth, Butler et al. 2013, Zander, Obeng-Adjei et al. 2015). However, the molecular mechanisms underlying $CD4^+$ T cell exhaustion during malaria are poorly understood.

Prolonged expression of co-inhibitory receptors on effector T cells is a cardinal feature of T cell exhaustion (Wherry and Kurachi 2015). Infection with *Plasmodium* species upregulates the expression of multiple co-inhibitory receptors on $CD4^+$ T cells including; PD-1, CTLA-4 and LAG3 (Schlotmann, Waase et al. 2000, Butler 2012, Illingworth, Butler et al. 2013, Mackroth, Abel et al. 2016). Blockade of PD-1 and LAG3 significantly enhances effector $CD4^+$ T cell functions and accelerates parasite clearance, demonstrating the importance of co-inhibitory receptors in $CD4^+$ T cell

dysfunction during malaria (Butler 2012). Tim3 is another co-inhibitory receptor upregulated on $CD4^+$ T cells during *P. falciparum* infection (Hou, Zou et al. 2016), however, its role in $CD4^+$ T cell exhaustion during malaria is yet to be addressed.

In non-malarial conditions, Tim3 is selectively expressed on IFNg-producing $CD4^+$ Th1 cells and $CD8^+$ T cells (Monney, Sabatos et al. 2002). Tim3 negatively regulates type 1 inflammation by promoting cell death in IFNg producing cells upon ligation with its ligand, galectin-9 (Zhu, Anderson et al. 2005). Tim3 also contributes to T cell exhaustion in cancer and bacterial and viral infections (Golden-Mason 2009, Gao, Zhu et al. 2012, Jayaraman, Jacques et al. 2016). Interestingly, Tim3 appears to act synergistically with PD-1 to induce a more severe exhaustion phenotype within effector T cells (Jin, Anderson et al. 2010). Indeed, combined blockade of Tim3 and PD-1 is consistently more effective at restoring T cell effector functions during chronic infections, compared with Tim3 or PD-1 blockade alone (Jin, Anderson et al. 2010, McMahan, Golden-Mason et al. 2010).

Given the important role of Tim3 in regulating Th1 immunity and T cell exhaustion, we hypothesised that Tim3 acts in combination with PD-1 to establish $CD4^+$ T cell exhaustion and limit parasite control during blood stage malaria. Unexpectedly, we show that antigen-specific $CD4^+$ Th1 cells only transiently upregulate and express Tim3 prior to the onset of T cell exhaustion during *P.yoelii* infection. In agreement, combined blockade of Tim3 and PD-L1 failed to restore effector $CD4^+$ T cell functions

during infection. Surprisingly, combinatorial blockade of Tim3 and PD-L1 significantly reduced the Th1 cell population, suggesting that Tim3 acts as a positive co-stimulatory molecule in the generation of effector $CD4^+$ T cells during *P.yoelii* infection. Thus, our data shows that Tim3 is not required for the establishment of $CD4^+$ T cell exhaustion and provides new information on how Tim3 influences the effector $CD4^+$ T cell response during malaria.

Results

Tim3 is transiently expressed on antigen-specific effector CD4$^+$ T cells during blood stage *P.yoelii-OVA* infection

We examined the expression of Tim3 on ovalbumin-specific TCR transgenic CD4$^+$ T cells (OT II cells), during blood stage *P.yoelii-OVA* infection (*P.yoelii-OVA*). We have previously shown that OT II cells are highly functional on day 5 post infection (p.i), but rapidly lose effector functions and develop an exhausted phenotype that is established by day 15 p.i (Chapter 2). Therefore, we first analysed Tim3 expression on splenic effector OT II cells (defined as CD44$^+$) on day 5 and day 15 p.i. We found that Tim3 expression was significantly increased on effector OT II cells on day 5 p.i., but expression significantly decreased by day 15 p.i (Fig4.1A, B). Tim3 expression was significantly higher on T-bet$^+$ effector OT II cells, than T-bet$^-$ effector OT II cells on day 5 of infection, indicating that Tim3 may preferentially regulate Th1 cell responses during malaria (Fig4.1C). Low and comparable expression of Tim3 was observed in T-bet$^+$ and T-bet$^-$ effector OT II populations on day 15 of infection. (Fig4.1C).

Tim3 has been reported to signal in combination with PD-1 and co-expression of Tim3 and PD-1 defines a distinct, severely exhausted CD8$^+$ T cell phenotype during chronic LCMV infection (Jin, Anderson et al. 2010). Consequently, we next analysed the co-expression of Tim3 and PD-1 on Th1 OT II cells, which exhibited highest Tim3 expression during *P.yoelii-OVA* infection (Fig4.1C). On day 5 of infection, the

frequencies of Tim3^{+} PD-1^{+} and Tim3^{+} PD-1^{-} Th1 OT II cells increased compared to naïve mice (Fig4.1D). However, Tim3^{+} populations did not persist to day 15 of infection, as frequencies of Tim3^{+} PD-1^{+} and Tim3^{+} PD-1^{-} Th1 OT II cells decreased to levels comparable to naïve mice (Fig4.1D). To determine if co-expression of Tim3 and PD-1 dictated the functional properties of Th1 OT II cells during infection, we measured Ki-67 expression as an indicator of proliferation. Similar frequencies of Tim3^{+} PD-1^{+} Th1 OT II cells expressed Ki-67 compared with Th1 OT II cells expressing Tim3 alone, PD-1 alone and neither Tim3 or PD-1 on day 5 of infection (Fig4.1E, F). On day 15 p.i., very few Th1 OT II cells expressed Ki-67, and proliferation was not influenced by Tim3 or PD-1 expression (Fig4.1E, F). Thus, the proliferative dysfunction within the Th1 OT II population during the course of *P.yoelii-OVA* infection did not appear to be driven by the expression of Tim3. However, Tim3 expressing T-bet^{+} OT II cells were preferentially lost at the later time points of infection.

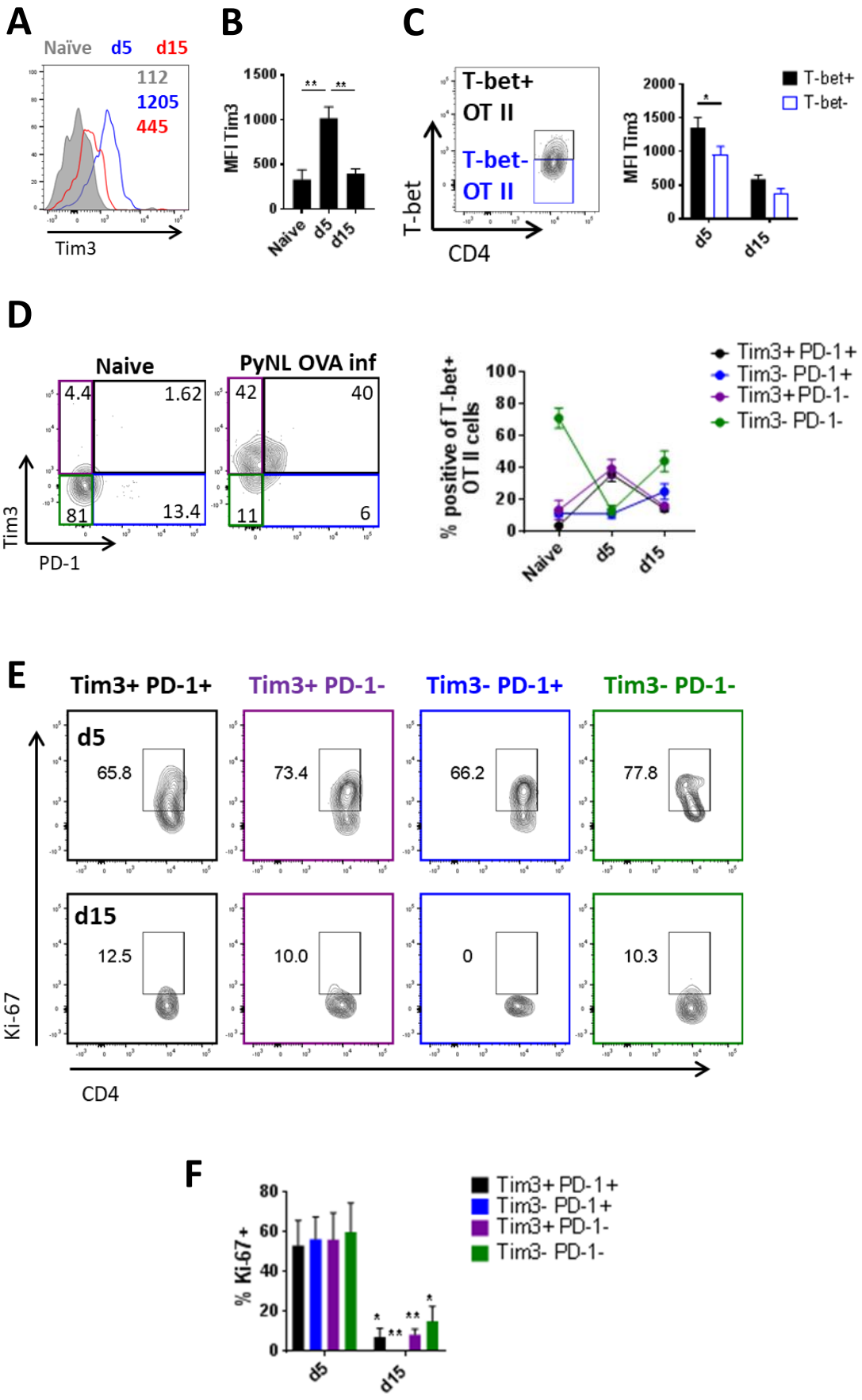
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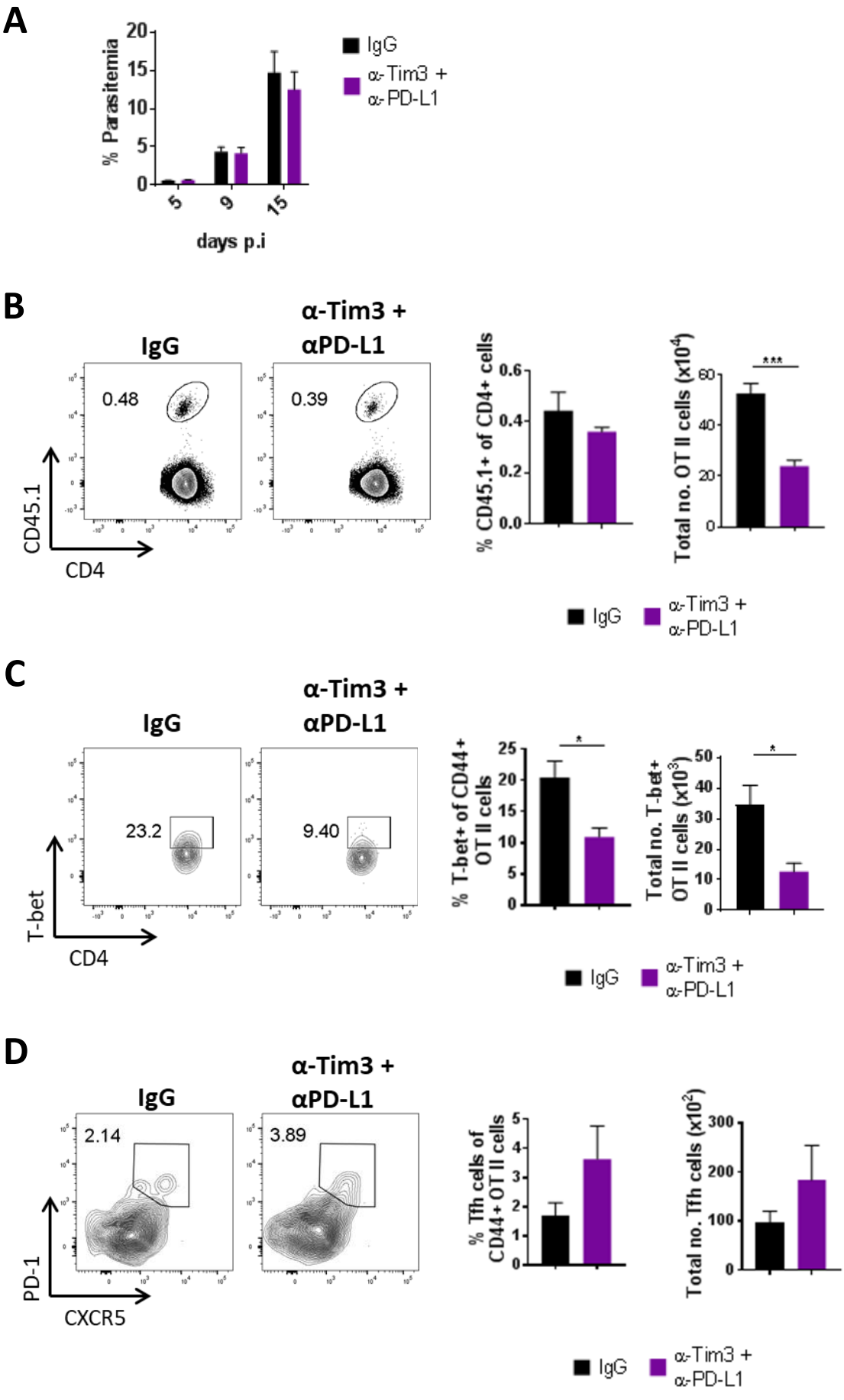
Figure 4.1 Tim3 is transiently expressed on effector OT II cells during *P.yoelii-OVA* infection

CD45.1$^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC (n=4). Spleens were taken at stated time points p.i and analysed by flow cytometry. (A) Representative histograms of Tim3 expression in naïve (grey) and CD44$^+$ OT II cells on day 5 (blue) and day 15 (red) p.i. (B) Mean fluorescence intensity (MFI) of Tim3 in effector OT II cells during *P.yoelii-OVA* infection. (C) Representative flow cytometric plots of T-bet$^+$ and T-bet$^-$ OT II sub-populations (left) and MFI of Tim3 within T-bet$^+$ and T-bet$^-$ OT II populations. (D) Representative flow cytometric plots showing Tim3 and PD-1 expression in the Th1 OT II population (left) and percentages of Tim3$^+$ and/or PD-1$^+$ OT II Th1 cells over the course of infection (right). (E) Representative flow cytometric plots of Ki-67 expression in each of the Tim3/PD-1 expressing Th1 OT II cells on d5 (top) and d15 (bottom) p.i. (F) Percentages of Ki-67$^+$ within each of the Tim3/PD-1 expressing Th1 OT II cells. Results are representative of two experiments. Bars represent mean ± SEM. **p ≤0.01 (one-way ANOVA with Tukey's multiple comparison test).

Simultaneous *in vivo* blockade of Tim3 and PD-L1 does not prevent effector OT II cell attrition during *P.yoelii* infection

Although CD4$^+$ T cell expression of Tim3 did not appear to correlate with T cell exhaustion based upon T cell proliferation, it was unclear whether Tim3 contributed to the loss of effector CD4$^+$ T cells during *P.yoelii*-OVA infection. To address this, we administered monoclonal antibodies against Tim3 and PD-L1 during *P.yoelii*-OVA infection. α-PD-L1 was administered in conjunction with α-Tim3 as previous studies have shown a strong synergy between Tim3 and PD-1, therefore, co-blockades have been significantly more effective than single blockades (Anderson 2016). Administration of α-Tim3 and α-PD-L1 from day 5p.i. did not significantly affect

peripheral parasitemia compared with IgG control treated mice (Fig4.2A). Although combined blockade did not alter the frequencies of OT II cells, treatment did significantly reduce the total numbers of OT II cells compared with IgG control treated mice (Fig4.2B). Interestingly, α-Tim3 and α-PD-L1 treatment significantly decreased the frequencies and total numbers of T-bet^{+} OT II cells during infection compared with IgG control treated mice (Fig4.2C). Whilst we focussed our investigations on the influence of Tim3 and PD-1 on T-bet^{+} OT II cells during *P.yoelii-OVA* infection, to expand our analyses we also examined whether α-Tim3 and α-PD-L1 treatment modified the T follicular helper cell (Tfh) response during infection. However, administration of α-Tim3 and α-PD-L1 only marginally increased the frequencies and absolute numbers of Tfh OT II cells compared with IgG control mice (Fig4.2D).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0
5
9
15
days p.i
IgG
α-Tim3 +
α-PD-L1
B
IgG
α-Tim3 +
αPD-L1
0.48
0.39
CD45.1
CD4
% CD45.1+ of CD4+ cells
0.6
0.4
0.2
0.0
Total no. OT II cells (x10^4)
60
40
20
0
***
IgG
α-Tim3 +
α-PD-L1
C
IgG
α-Tim3 +
αPD-L1
23.2
9.40
T-bet
CD4
% T-bet+ of CD44+ OT II cells
25
20
15
10
5
0
*
Total no. T-bet+ OT II cells (x10^3)
50
40
30
20
10
0
*
IgG
α-Tim3 +
α-PD-L1
D
IgG
α-Tim3 +
αPD-L1
2.14
3.89
PD-1
CXCR5
% Tfh cells of CD44+ OT II cells
5
4
3
2
1
0
Total no. Tfh cells (x10^2)
300
200
100
0
IgG
α-Tim3 +
α-PD-L1

Figure 4.2 Blockade of Tim3 and PD-L1 does not improve parasite control or alter the CD4+ T cell response during *P.yoelii-OVA* infection

CD45.1$^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC. Infected mice were either treated with control rat IgG (n=4) or α-Tim3 and α-PD-L1 (n=4) every 2 days starting from day 5 p.i. Spleens were taken for flow cytometric analysis on day 15 p.i. (A) Peripheral parasitemia was monitored every other day throughout the course of infection. (B) Representative flow cytometric plots (left), percentages (middle) and total numbers (right) of OT II cells. (C) Representative flow cytometric plots (left), percentages (middle) and total numbers (right) of T-bet$^+$ OT II cells. (D) Representative flow cytometric (left), percentages (middle) and total numbers (right) of OT II Tfh cells. Results are representative of 3 independent experiments. Bars represent mean ± SEM. *p≤0.05 (Unpaired t-test).

We next examined if α-Tim3 and α-PD-L1 treatment modified Th1 OT II cell effector functions during *P.yoelii-OVA* infection. Administration of α-Tim3 and α-PD-L1 did not significantly alter the frequencies or total numbers of IFNg$^+$ and TNF-α$^+$ Th1 OT II cells compared with IgG control treated mice (Fig4.3A). Treatment with α-Tim3 and α-PD-L1 slightly increased the frequencies, but not total numbers of Ki-67$^+$ Th1 OT II cells compared with IgG control treated mice (Fig4.3B). Collectively, these results indicate that in the absence of Tim3 and PD-1 signalling pathways, fewer Th1 OT II cells were maintained. However, Tim3 and PD-1 did not synergistically contribute to the loss of effector OT II cell functions and the development of T cell exhaustion during blood stage *P.yoelii* infection.

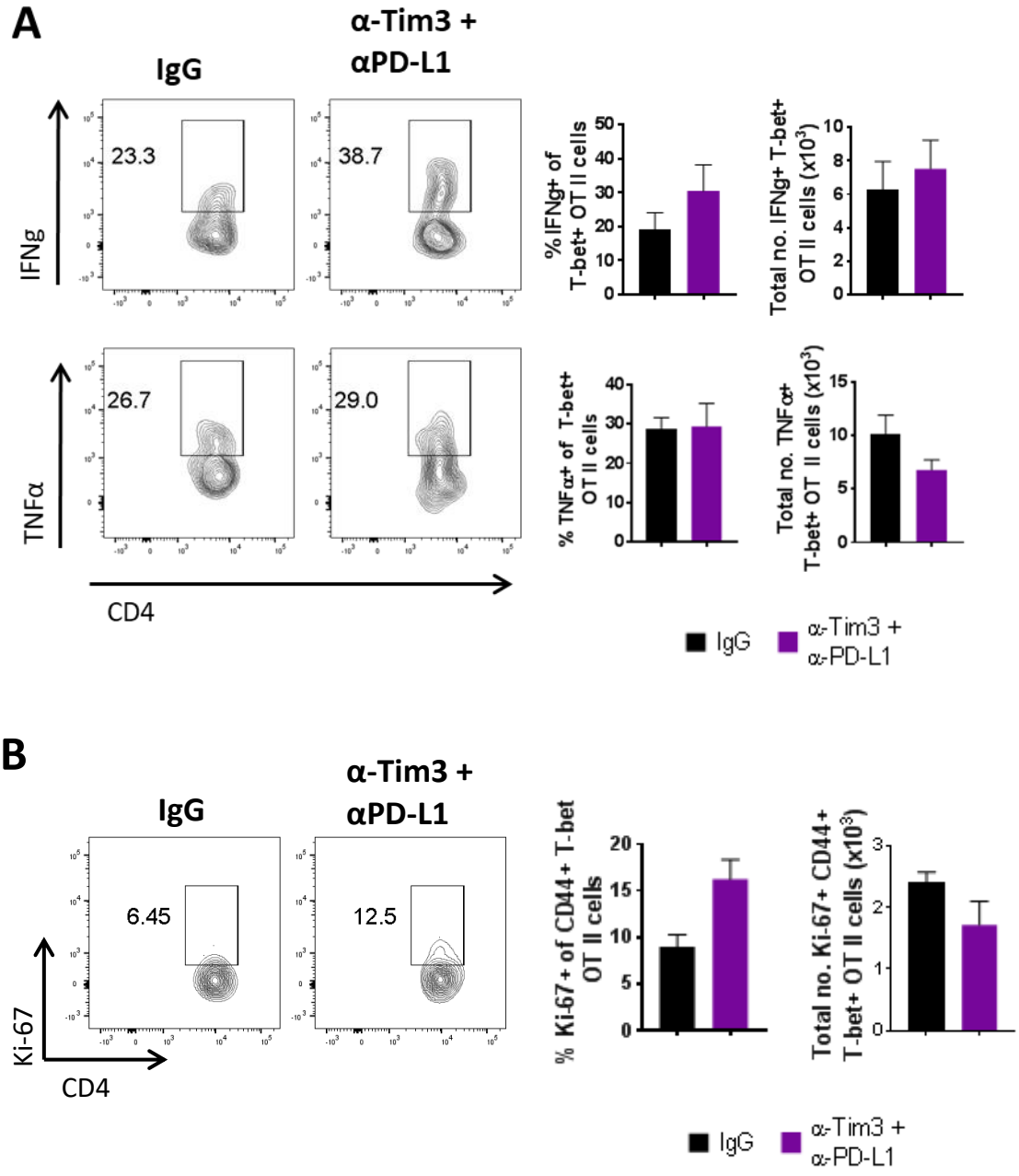

Figure 4.3 Tim3 does not impair effector Th1 cell function during *P.yoelii-OVA* infection

1x10^6 CD45.1$^+$ OT II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P.yoelii*-OVA pRBC. Infected mice were treated with either control rat IgG (n=4) or α-Tim3 and α-PD-L1 (n=4) from day 5 p.i. Spleens were taken on day 15 p.i for flow cytometric analysis. (A) Representative plots (left), frequencies (middle) and total numbers (right) of IFNg$^+$ (top) and TNFα$^+$ (bottom) Th1 OT II cells. (B) Representative flow cytometric plots (left), percentages (middle) and total numbers (right) of Ki-67$^+$ Th1 OT II cells. Results are representative of 3 independent experiments. Bars are mean ± SEM (unpaired t-test).

Discussion

In this study we have addressed the synergistic role of Tim3 and PD-1 in establishing T cell exhaustion in antigen-specific $CD4^+$ T cells during blood stage malaria. Prolonged co-expression of Tim3 and PD-1 has been associated with T cell exhaustion during chronic infections (Jin, Anderson et al. 2010, Jayaraman, Jacques et al. 2016). However, we have shown that Tim3 expression on antigen-specific $CD4^+$ T cells is transiently upregulated during early stages of *P.yoelii-OVA* infection, before being downregulated as $CD4^+$ T cell exhaustion is established. Previous studies identified that co-expression of Tim3 and PD-1 was associated with more severe $CD8^+$ T cell exhaustion during chronic LCMV infection (Jin, Anderson et al. 2010). Conversely, our data suggest that co-expression of Tim3 and PD-1 on $CD4^+$ T cells during malaria does not specifically define exhausted T cells. Based upon expression kinetics, it is therefore unlikely that Tim3 contributes to the induction of $CD4^+$ T cell exhaustion during malaria. Our blockade studies further support this conclusion, as combinatorial blockade of Tim3 and PD-L1 failed to improve $CD4^+$ T cell functionality during *P.yoelii-OVA* infection. In line with this finding, genetic ablation of Tim3 prior to chronic LCMV infection did not influence $CD8^+$ T cell exhaustion, based on the loss of IFNg and TNFα in Tim3 knockout T cells, thus demonstrating that Tim3 is not required for the development of T cell exhaustion (Avery, Filderman et al. 2018). Therefore, although Tim3 signalling may not promote T cell exhaustion, it may be an important regulator in maintaining exhausted T cells, in combination with PD-1 during chronic infections.

Previous studies have demonstrated a role for PD-1 in impairing immunity to malaria (Horne-Debets, Faleiro et al. 2013, Liu, Lu et al. 2015, Karunarathne 2016). However, as we did not observe any significant alterations in $CD4^+$ T cell effector functions following combinatorial Tim3 and PD-L1 treatment, we concluded that Tim3 and PD-1 did not synergistically or independently contribute to T cell exhaustion during *P.yoelii* infection. In line with this finding, Butler *et al.* reported that PD-L1 administration alone did not significantly affect $CD4^+$ T cell effector functions during *P.yoelii* infection (Butler 2012). Inconsistent findings regarding the role of PD-1 during malaria may arise from the use of PD-1 or PD-L1 knock out mice, compared to antibody blockade of PD-L1 during the established malaria infection. However, single treatments of α-Tim3 and α-PD-L1 is required to confirm that Tim3 and PD-1 do not independently regulate $CD4^+$ T cells during *P.yoelii* infection.

Whilst we did not identify a role for Tim3 in promoting $CD4^+$ T cell exhaustion, we found that blockade of Tim3 and PD-L1 lead to a significant reduction in Th1 OT II cells during *P.yoelii-OVA* infection. This suggests that Tim3 signalling may be required for effector $CD4^+$ T cell development or maintenance during malaria. Consistent with our data, previous studies have shown that the absence of Tim3 significantly reduces the activated, antigen-specific $CD8^+$ T cell population during *Listeria monocytogenes* infection (Gorman, Starbeck-Miller et al. 2014). However, early Tim3 signalling has been shown to enhance T cell activation and promote the generation of short-lived effector T cells, potentially by acting through mTOR, during LCMV infection (Avery, Filderman et al. 2018). Thus the observed decrease in Tim3 MFI by Th1 OT II cells on

day 15 of *P.yoelii-OVA* infection may indicate the preferential loss of short-lived effector Th1 cells. However, as dual blockade of Tim3 and PD-L1 failed to improve the maintenance of effector $CD4^+$ T cells during *P.yoelii* infection, this scenario is unlikely during malaria. Nevertheless, administration of α-Tim3 from the start of infection would be required to confirm this notion. Furthermore, examining the expression of apoptosis associated markers, such as caspase 3, in Th1 cells during malaria would help to clarify whether $Tim3^+$ Th1 cells are targeted for cell death and that Tim3 is not downregulated on Th1 cells.

IL-27 is a critical immunoregulatory molecule that upregulates IL-10 expression in Th1 cells during blood stage malaria (Freitas do Rosario, Lamb et al. 2012, Villegas-Mendez, de Souza et al. 2013). IL-27 signalling has also been reported to induce Tim3 expression in Th1 cells (Zhu 2015). Therefore, the low levels of Tim3 expression at day 15 p.i was unexpected, as this time point is associated with strong IL-27 signalling in Th1 cells (Villegas-Mendez, de Souza et al. 2013). Tim3 expression is also regulated by IL-12 signalling, which has been shown to be limited in effector $CD4^+$ T cells at the later stages of *Plasmodium* infection (Villegas-Mendez, de Souza et al. 2013), therefore, reduced IL-12 signalling could prevent the upregulation of Tim3 in effector $CD4^+$ T cells during *P.yoelii* infection.

Collectively, our data has shown that Tim3 and PD-1 do not synergistically contribute to $CD4^+$ T cell exhaustion during malaria. Instead we propose that Tim3 is required

for effector $CD4^+$ T cell development during infection. Co-ordinated signalling from other inhibitory receptors and IL-10 likely establish $CD4^+$ T cell exhaustion during malaria therefore, manipulation of these pathways may represent a more beneficial therapeutic approach for treating blood stage malaria.

Materials and Methods

Ethics Statement

All animal work was approved following local ethical review by the University of Manchester Animal Procedures and Ethics Committees and was performed in strict accordance with the U.K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O Project Licences 70/7293 and P8829D3B4).

Mice and Parasites

Male 7week old C57BL/6 mice ($CD45.2^+$) were purchased from Charles River UK. RAG-1 OT II x Pep3 ($CD45.1^+$) mice (Barnden, Allison et al. 1998) were bred at the University of Manchester and were fully backcrossed to a C57BL/6 background. All mice were maintained in specific-pathogen free conditions in individually ventilated cages.

Cryopreserved *P.yoelii* parasites expressing mCherry OVA (under the control of the *hsp70* promoter) were thawed and passaged once in C57BL/6 mice before being used to infect experimental mice. Animals were infected via intravenous injection of 10^4 pRBC. The course of infection was monitored every other day starting from d5 p.i by peripheral parasitaemia and assessed by microscopic examination of Giemsa-stained thin blood smears.

In vivo blockades

For *in vivo* blockade of co-inhibitory receptors mice received 250µg of α-PD-L1 (10F.9G2) and α-Tim3 (RMT3-23) every two days from day 5 p.i via intraperitoneal injection. Both antibodies were supplied from BioXcell. Control mice received 250µg of Rat IgG (Sigma Aldrich) via intraperitoneal injection.

$CD4^+$ T cell Isolation and Adoptive Transfer

Spleens were isolated from RAG-1 OT II x Pep3 mice, homogenised through a 70µm strainer (BD Biosciences) and incubated in RBC lysis buffer (BD Biosciences) to generate an RBC-free single cell suspension. OT II cells were isolated using anti-CD4 conjugated microbeads (Miltenyi Biotec), according to manufacturer's instructions. $1x10^6$ OT II cells were transferred into C57BL/6 via intravenous injection one day prior to infection with *P.yoelii-OVA* pRBC.

Flow Cytometry

Spleens were removed from naïve and malaria-infected mice at stated time points, homogenised through a 70µm strainer and incubated with RBC lysis buffer. Absolute live cell counts were calculated by trypan blue exclusion cell viability assay (Sigma). Splenocytes were then surface stained for 25 minutes at $4\ ^OC$ with CD45.1 (A20), CD4 (RM4-5), CD44 (IM7), PD-1 (RMPI-30), CXCR5-biotin (LI38D7), Tim3 (RMT3-23). Surface staining was done in the presence of FcR block (2.4G2, BioXcell).

For streptavidin staining, cells were initially washed before being stained with streptavidin v510 for 10 minutes at room temperature (RT). For intracellular staining, cells were incubated with Foxp3 fixation/permeabilisation buffer (eBioscience) for 30 minutes at 4 ^{O}C. Cells were subsequently stained with the following antibodies: T -bet (4B10) and Ki-67 (SolA15) for 30 minutes. For analysis of the intracellular cytokines IFNg (XMG1.2) and TNFα (MP6XT22) cells were stimulated *ex vivo* for 4 hours at 37 ^{O}C with 200ng/mL PMA (Sigma), 1µg/mL ionomycin (Sigma) and Brefeldin A ([1000x], eBiosceince). All antibodies were acquired from eBioscience or Biolegend. Dead cells were excluded from all analyses using forward and side scatter properties and LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies).

Samples were acquired through the Fortessa (BD systems, UK) and all analysis was performed using Flowjo Software (Treestar Inc, OR, USA). Malaria infected control samples were combined to generate fluorescence minus one (FMO) samples which were used to validate the flow cytometric data.

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, USA). Comparison between 3 or more groups was carried out using a one-way ANOVA with Tukey's test for multiple comparisons. For groups of 2, unpaired t-tests were carried out. Results were considered significant when $P<0.05$.

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Chapter 5: General discussion

Malaria is a leading cause of morbidity and mortality worldwide and is expected to be exacerbated by the increasing emergence of drug-resistant parasites (WHO 2018). Despite great efforts to develop a vaccine against malaria, success has been limited with the most promising vaccine candidate RTS,S, only offering short-term protection in young children (Draper, Sack et al. 2018). Efforts have likely been hampered by *Plasmodium* species ability to evade adaptive immunity and an incomplete understanding of how to induce robust antibody and memory responses during malaria (Draper, Sack et al. 2018). However, it is well established that $CD4^+$ T cells are critical for protection against blood stage malaria (Suss, Eichmann et al. 1988, Butler 2012, Azcarate, Marin-Garcia et al. 2014, Perez-Mazliah and Langhorne 2014). Nevertheless, there is growing evidence from human (Butler 2012, Illingworth, Butler et al. 2013) and murine studies (Xu, Wipasa et al. 2002, Chandele 2011, Butler 2012, Villegas-Mendez, Shaw et al. 2016), that malaria induces T cell exhaustion, which is characterised by decreased IFNg production, proliferation and deletion of parasite-specific T cells prior to the peak of infection. Importantly, T cell exhaustion during malaria impairs parasite clearance (Butler 2012). However, the critical molecular mechanisms through which $CD4^+$ T cell exhaustion is established during malaria are yet to be fully explored. Improvement in our understanding of T cell exhaustion during malaria will be important in informing the development of novel vaccines and treatment strategies.

The overall aim of my PhD was, therefore, to further our understanding of the mechanisms underlying $CD4^+$ T cell exhaustion during blood stage malaria.

To address this aim, I utilised a model antigen-specific $CD4^+$ T cell system to investigate the specific molecular pathways that contribute to the development of $CD4^+$ T cell exhaustion during a murine model of malaria. Importantly, by using an antigen-specific system we have excluded any effects from bystander activation. In addition, the use of a clonal system removes any differences induced by TCR stimulation strength (Corse, Gottschalk et al. 2011) and allows us to image antigen-specific T cell behaviour during infection.

T cell exhaustion is a common feature in many chronic diseases and has been extensively studied in $CD8^+$ T cells during chronic viral infections and cancer (Wherry 2011). Although less well studied, $CD4^+$ T cell responses also become exhausted during chronic infections, including malaria (Brooks, Teyton et al. 2005, Butler 2012, Jayaraman, Jacques et al. 2016). Exhausted $CD8^+$ and $CD4^+$ T cells share a similar core transcriptional signature; including the expression of Blimp1 and Eomes, however, exhausted $CD4^+$ T cells also express distinct patterns of inhibitory molecules and unique transcription factors, such as Helios, that modulate effector functions (Crawford, Angelosanto et al. 2014). This suggests that divergent regulatory pathways may induce $CD4^+$ T cell exhaustion or $CD8^+$ T cell exhaustion. Therefore, more extensive studies investigating $CD4^+$ T cell exhaustion are required.

In **Chapter 2**, we specifically examined phenotypical and functional changes, as well as the dynamic behaviour of antigen-specific $CD4^+$ T cells over the course of blood

stage malaria. Strikingly, the numbers of antigen-specific $CD4^+$ T cells rapidly decreased in the spleen following day 5 of infection. This contraction of the antigen-specific $CD4^+$ T cell response could be the result of cell death, as *Plasmodium* infections induces parasite-specific $CD4^+$ T cell deletion (Xu, Wipasa et al. 2002). Alternatively, the reduction in $CD4^+$ T cells could be the result of migration into other non-lymphoid tissues (Villegas-Mendez, Shaw et al. 2015). Nevertheless, the antigen-specific $CD4^+$ T cell response did not only collapse during infection, but rapidly developed an exhausted phenotype, illustrated by the loss of IFNg production and proliferation and upregulation of multiple inhibitory receptors. Thus, our work provides evidence for antigen-specific $CD4^+$ T cell exhaustion during malaria and is consistent with previous reports studying polyclonal $CD4^+$ T cells during malaria (Butler 2012, Illingworth, Butler et al. 2013).

Effector $CD4^+$ T cells require constant engagement with APC and TCR signalling for the maintenance of effector functions (Huppa, Gleimer et al. 2003, Corbin and Harty 2005, Obst, van Santen et al. 2005, Egen, Rothfuchs et al. 2011). However, there is evidence that *Plasmodium* infection may disrupt T cell-APC interactions, which impairs T cell activation and migration (Millington, Di Lorenzo et al. 2006, Millington, Gibson et al. 2007). Nevertheless, these studies focussed on naïve T cells therefore, it is unclear whether effector T cell-APC interactions are also disrupted during malaria and whether this leads to a reduced effector T cell response. Consequently, we investigated antigen-specific effector $CD4^+$ T cell interactions with CX_3CR1^+ APC during *P.yoelii* infection. Interestingly, although we did not confirm the exact location

of exhausted $CD4^+$ T cells, the majority of $CD4^+$ T cells appeared to be predominately located within the WP as T cell exhaustion was established during *P.yoelii* infection. This is in contrast with previous studies that found activated and exhausted $CD8^+$ T cells primarily locate within the RP during *P.berghei* infection and LCMV infection (Khanna, McNamara et al. 2007, Zinselmeyer, Heydari et al. 2013, Bayarsaikhan 2017). It is therefore possible that $CD4^+$ T cell and $CD8^+$ T cell exhaustion is directed by distinct APC subsets in different splenic compartments during infection. Importantly, the major alterations in splenic organisation we and others have demonstrated (Achtman, Khan et al. 2003, Beattie 2006) did not seem to impact the ability of $CD4^+$ T cells to co-localise and engage with APC during malaria.

As $CD4^+$ T cell exhaustion was established during *P.yoelii* infection, we observed a decrease in $CD4^+$ T cell motility, indicative of antigen-dependent arrest (Bousso and Robey 2003, Celli 2007, Egen, Rothfuchs et al. 2011). Indeed, on day 9 of infection a high proportion of $CD4^+$ T cells were arrested in contact with CX_3CR1^+ cells. This was surprising as T cell arrest is tightly associated with cytokine production and TCR signalling (Celli 2007, Egen, Rothfuchs et al. 2011, Honda 2014). Indeed, cessation of TCR stimulation during priming terminates long-lived T cell-APC interactions (Celli 2007). However, we showed that blockade of MHC II did not exacerbate the attrition of the Th1 response during *P.yoelii* infection, suggesting that peptide MHC II-TCR signalling is lost in Th1 cells during infection. It is possible therefore, that during the onset of $CD4^+$ T cell exhaustion the immunological synapse (IS) is altered, allowing inhibitory receptors to cluster around the TCR and attenuate the stimulatory signals

(Parry, Chemnitz et al. 2005, Downey 2008, Qureshi, Zheng et al. 2011, Honda 2014, Johnston, Comps-Agrar et al. 2014, 2017, Hui, Cheung et al. 2017). Indeed, CTLA-4 can modify the IS by removal of CD80 and CD86 (Qureshi, Zheng et al. 2011). TIGIT has also been implicated in modulating the IS to mediate its inhibitory effects (Stengel, Harden-Bowles et al. 2012). Moreover, ligated PD-1 forms microclusters with the TCR *in vitro* (Yokosuka, Takamatsu et al. 2012) and *in vivo* PD-L1 stabilises the IS allowing PD-1 to dephosphorylate downstream targets of the TCR (Zinselmeyer, Heydari et al. 2013). In contrast, the IS between functional effector $CD4^+$ T cells and APC may exclude inhibitory receptors, thus allowing for positive stimulation. This may also explain why highly functional Th1 cells could also express high levels of inhibitory receptors. Further studies analysing the nanoscale arrangement of inhibitory receptors and activating receptors at the IS of functional and exhausted T cells, would provide highly important mechanistic insight into $CD4^+$ T cell exhaustion.

A high proportion of exhausted Th1 cells co-expressed multiple inhibitory receptors, suggesting that $CD4^+$ T cells are regulated through co-ordinated signalling from multiple inhibitory molecules during malaria. During chronic LCMV infection, exhausted $CD8^+$ T cells also express high numbers of co-inhibitory receptors, which was associated with a greater dysfunctional phenotype (Blackburn, Shin et al. 2009). Further studies investigating the association between co-inhibitory receptor expression and $CD4^+$ T cell effector functions are required to determine whether expressing higher numbers of inhibitory receptors leads to more severe $CD4^+$ T cell

exhaustion during malaria. Importantly, targeting co-inhibitory receptors during chronic diseases restores T cell effector functions and improves pathogen and tumour clearance (Wherry and Kurachi 2015). However, the contribution of specific inhibitory receptors to development of $CD4^+$ T cell exhaustion during malaria has not been extensively explored.

In **Chapter 3** we investigated whether TIGIT and PD-1 synergistically contributed to $CD4^+$ T cell exhaustion during malaria. Despite a significant improvement of parasite control following blockade of TIGIT and PD-L1, the Th1 response was not significantly improved. Similarly, a previous study demonstrated that administration of LAG3 and PD-L1 blocking antibodies from day 9 of *P.yoelii* infection, did not significantly improve parasite clearance due to highly suppressive Treg cells (Kurup, Obeng-Adjei et al. 2017). Whereas, administration from day 14 was highly successful at recovering effector T cell responses and improving parasite control (Butler 2012, Kurup 2017). Although we observed a significant decrease in Treg cells following dual TIGIT and PD-L1 blockade, $CD4^+$ T cells are subject to negative regulation from multiple nonredundant co-inhibitory pathways and soluble mediators such as; IL-10, IL-27 and TGFβ during infection (Blackburn, Shin et al. 2009, Crompton, Moebius et al. 2014, Wherry and Kurachi 2015). Therefore, removal of TIGIT and PD-1 may not be sufficient to prevent $CD4^+$ T cell exhaustion during malaria. This therefore brings into question whether blockade of inhibitory receptors can prevent exhaustion, or just restore function once T cell exhaustion is established. Indeed, previous reports have defined a more severe exhausted phenotype in $CD8^+$ T cells genetically lacking PD-1

during chronic infection (Odorizzi, Pauken et al. 2015), suggesting compensatory mechanisms regulate T cell exhaustion during infection. Moreover, a unique sub-population of PD-1^{lo} CD8^{+} T cells, but not the exhausted T cell population, selectively proliferate following PD-1 blockade (Im, Hashimoto et al. 2016). Whether an equivalent CD4^{+} T cell population exists during malaria is currently unclear. Nevertheless, administrating TIGIT and PD-L1 blocking antibodies later in infection once T cell exhaustion is established, may significantly improve effector T cell responses and contribute to accelerated parasite clearance during malaria.

Tim3 is a co-inhibitory receptor that specifically regulates Th1 cells and contributes to the induction of CD4^{+} T cell exhaustion during chronic infection (Zhu, Anderson et al. 2005, Jayaraman, Jacques et al. 2016). However, in **Chapter 4** we showed that Tim3 was transiently expressed on Th1 cells during blood stage malaria and blockade of Tim3 in combination with PD-L1 confirmed that Tim3 did not contribute to the development of exhausted CD4^{+} T cells during infection. Although it is currently unclear why Tim3 expression was lost during malaria, early Tim3 signalling may provide positive co-stimulation during T cell activation and support the generation of effector CD4^{+} T cells, as previously seen in CD8^{+} T cells during chronic LCMV infection (Avery, Filderman et al. 2018). Further studies investigating apoptosis within the Tim3^{+} CD4^{+} T cells and perturbation studies during T cell priming are required to determine whether early Tim3 signalling modulates the CD4^{+} T cell response during malaria.

The control and resolution of blood stage malaria is mediated through carefully co-ordinated effector and regulatory responses. However, multiple immunoregulatory signals may act in concert to drive $CD4^+$ T cell exhaustion during malaria. *Plasmodium* infection induces IL-10 production in IFNg producing Th1 cells (Freitas do Rosario, Lamb et al. 2012), which has now been recognised as a sign of $CD4^+$ T cell exhaustion during chronic LCMV infection (Wherry and Kurachi 2015, Parish, Marshall et al. 2014). In agreement, $IFNg^+$ $IL\text{-}10^+$ $CD4^+$ T cells produce lower levels of IL-2 and TNF, express greater levels of TIGIT and LAG3 and have a limited capacity to populate the memory compartment compared with $IFNg^+$ $IL\text{-}10^-$ $CD4^+$ T cells during malaria (Villegas-Mendez, Shaw et al. 2015, Villegas-Mendez, Inkson et al. 2016). Collectively these studies could suggest that expression of IL-10 by $IFNg^+$ $CD4^+$ T cells may also be a marker of T cell exhaustion during malaria. However, it is currently unclear whether IL-10 promotes inhibitory receptor expression and eventual loss of IFNg and T cell exhaustion, or whether co-inhibitory receptors and IL-10 signalling are two distinct immunoregulatory pathways, that act in parallel to supress the effector T cell population during malaria.

During chronic LCMV infection IL-10 is not required for PD-1 expression (Brooks, Ha et al. 2008). Similarly, PD-1 expression was not required for IL-10 production, suggesting that PD-1 and IL-10 are distinct inhibitory mechanisms. Consistent with this, co-blockade of IL-10 and PD-L1 is significantly better at improving T cell effector functions compared to IL-10 or PD-L1 blockade alone during chronic LCMV infection (Brooks, Ha et al. 2008). Therefore, it is possible that the generation of IL-10

producing Th1 cells during *Plasmodium* infection may represent an immunoregulatory pathway that is distinct, but parallel, to the negative signalling pathways induced by co-inhibitory receptors. Although IL-10 produced from Th1 cells does not appear to work in an autocrine manner (Villegas-Mendez, Shaw et al. 2015), IL-10, in combination with TGFβ and IL-27 may promote a suppressive environment that limits APC functions and upregulates inhibitory molecules (Villegas-Mendez, de Souza et al. 2013, Brooks, Ha et al. 2008). This suppression, in combination with the upregulation of multiple inhibitory receptors that subvert positive signalling in effector $CD4^+$ T cells, could promote T cell exhaustion and parasite persistence during malaria (figure 5.1). Therefore, therapeutic blockade of IL-10 in combination with TIGIT and PD-L1 during established malaria infection may provide a novel strategy for improving the parasite-specific Th1 and Tfh cell responses to *Plasmodium* infection.

Together, data generated from my PhD have shown that dysfunctional antigen-specific $CD4^+$ T cells are able to interact with APC during *Plasmodium* infection, however, apparent subversion of MHC II-TCR signalling may be associated with loss of T cell effector functions. Moreover, the co-inhibitory receptors TIGIT and PD-1 synergistically impair protective immunity to *Plasmodium* infection.

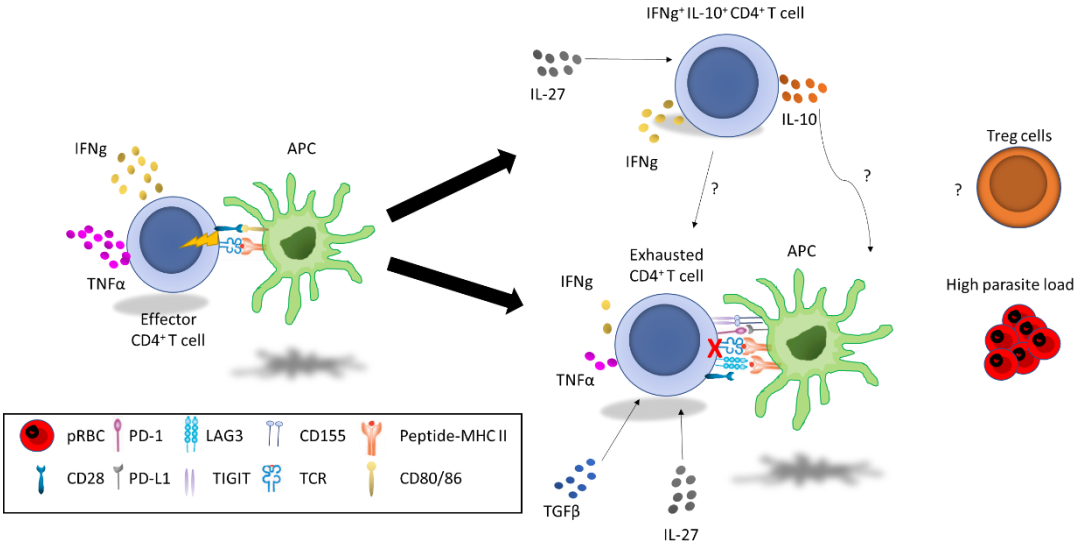

Figure 5.1 Hypothetical model of the mechanisms contributing to CD4⁺ T cell exhaustion during blood stage malaria

Stable interactions between antigen presenting cells (APC) and functional effector CD4⁺ T cells stimulates strong T cell receptor (TCR) signalling which drives IFNg and TNFα production during *Plasmodium* infection. However, as the infection progresses IL-27 signalling induces IL-10 production in IFNg⁺ Th1 cells. IL-10 may then act on APC to dampen their functions and upregulate inhibitory ligands. In parallel, the upregulation of multiple inhibitory receptors on effector CD4⁺ T cells, possibly due to high parasite load and IL-27 signalling, subverts MHC II-TCR signalling, inhibiting IFNg and TNFα production. Treg mediated suppression may also contribute to effector CD4⁺ T cell regulation.

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