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Investigating the CD4+ T cell response to infection with *Trypanosoma brucei*

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Declaration

I declare that this thesis and the results presented within it are entirely my own work, with the following exceptions: the life cycle diagram of *Trypanosoma brucei* in the introduction was taken from the CDC website (see CDC reference), and the initial *in vivo* experiment detailing CD4+ T cell activation and activation of OVA-specific T cells (found in Figures 4 and 5, Section 3.2) was carried out by Agapitos Patakas, Paul Capewell, Caroline Clucas and Andrew Platt, University of Glasgow.

No part of thesis has been previously submitted for a degree at any other institution.

Taylor-Anne Gorman

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Chapter 1: Introduction

1.1: Introduction to *Trypanosoma brucei*

African trypanosomes are a protozoan parasite with the ability to cause disease in both humans and animals, rendering them an important subject of study. The human disease is called African sleeping sickness or Human African Trypanosomiasis (HAT) and the animal disease is animal trypanosomiasis or Nagana in cattle (Matthews et al. 2015). These diseases have a vast socioeconomic impact upon the African continent (Matthews et al. 2015). Infection occurs in mammalian hosts through the bite of the primary vector, the tsetse fly, which is found in the endemic area of sub-Saharan Africa (Torr & Vale 2015).

1.1.1 Life Cycle and Phylogeny

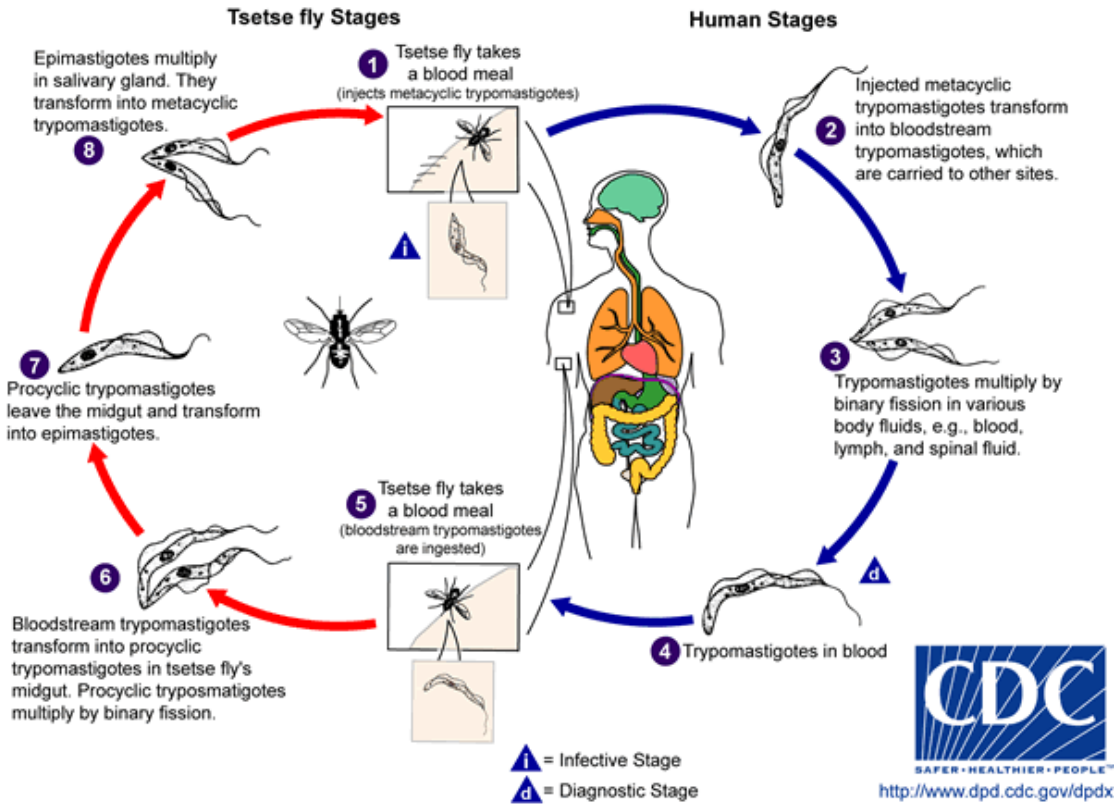

Figure 1: The life cycle of *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* (human infective species). Other species infect other mammalian hosts (CDC 2015)

The life cycle of trypanosomes involves two stages, the vector stage and the mammalian host stage (Kennedy 2013). Infection of the host begins with the injection of metacyclic parasites by the tsetse fly during a blood meal (Figure 1). These metacyclic parasites then replicate in the tissue at the site of injection – this leads to a swollen ulceration at the area known as a chancre. Following this, the parasites will transform into a long and slender bloodstream form and enter the vascular system of the host, where they continue dividing (Kennedy 2013). Some of the parasites will then become a shorter, stumpy bloodstream form of parasite, which are pre-adapted to the tsetse environment and are taken up when a tsetse fly takes a bloodmeal to further the life cycle (CDC 2015). The vector stage begins with the ingestion of stumpy bloodstream forms in the bloodmeal by the tsetse fly. These then transform into procyclic form trypanosomes within the midgut of the fly, where they replicate by binary fission (Kennedy 2013). Some parasites transform into the proventricular non-dividing forms, which migrate to the salivary glands of the vector. Here they transform into epimastigotes, which are attached to the salivary glands and undergo more replication before transforming into the host infective metacyclic form of parasite, which are pre-adapted to life in the mammalian bloodstream and are then injected into the host by the fly, completing the cycle (Kennedy 2013).

1.1.2 Vector: The Tsetse fly

African trypanosomes infect their hosts through the bite of flies of the *Glossina* genus, known as tsetse flies (Steverding 2008). These flies are found in endemic regions of sub-Saharan Africa, where trypanosomes infect both cattle and humans, leading to disease (Torr & Vale 2015). The transmission of the two human infective sub-species of trypanosomes is associated with the presence of these flies, and as such, control of these flies has been considered as a preventative measure in the control of HAT (Barrett & Croft 2012). Vector control however, has been inadequate in the eradication of the disease, as it has proven difficult to completely eliminate the tsetse fly population – while instances of infection have been reduced,

they still occur, presumably due to the existence of an infection reservoir (Steverding 2008).

1.2: Animal Trypanosomiases

1.2.1 Animal infective strains of *Trypanosoma*

There are three sub-species of *Trypanosoma brucei*, all of which are infective to non-human animals – however *Trypanosoma brucei brucei* rarely infects humans (Brun et al. 2010). Trypanosomes of this strain are lysed in human serum by trypanolytic factors, and therefore cannot infect humans (Uzureau et al. 2013). *T. b. brucei*, along with *T. congolense* and *T. vivax*, is the causative agent of animal trypanosomiasis, an infection of a wide range of mammalian hosts also known as Nagana, and causes substantial economic costs through loss of cattle (Steverding 2008). *Trypanosoma brucei brucei* is used as an animal model of human infective trypanosome infections, and is therefore key to understanding and treating both Nagana and HAT (Bucheton et al. 2011).

1.2.2 Symptoms and Treatment

Nagana is usually a chronic infection characterized by weight loss, oedema and listlessness of livestock; it can also affect the reproductive abilities of cattle, and commonly results in death of animals (Steverding 2008). The resultant loss of cattle affects farming in endemic regions, leading to economic impact on the country. Treatment of animal trypanosomiasis in Africa comes in the form of trypanocidal drugs which have been available for over 50 years – isometamidium, homidium and diminazene (Geerts et al. 2001). The availability of these drugs has led to widespread treatment of cattle, however there has also been an increase in trypanosome drug resistance due to frequent misuse of these drugs in cattle (Geerts et al. 2001). The emergence of trypanocide resistance has contributed to the resurgence of the disease (Delespaux & de Koning 2007) in both animal infections and human infections, and is therefore an important area of research.

1.3 Human African Trypanosomiasis

1.3.1 *T. b. gambiense* and *T. b. rhodesiense*

Two strains of *Trypanosoma brucei* infective to humans are *T. b. gambiense* and *T. b. rhodesiense*, which are separated geographically (Keating et al. 2015). *T. b. gambiense,* the species responsible for a larger number of cases, is found in western Africa, and accounted for 96% of the reported cases in 2006 (Brun et al. 2010). *T. b. rhodesiense* is prevalent in eastern Africa and accounts for the remaining 4% of 2006 cases, which are more acute in nature than the *T. b. gambiense* infections (Brun et al. 2010).

1.3.2 Symptoms

Both human-infective species of African trypanosomes lead to symptoms occurring in two clear stages. The primary stage of infection is characterized by the appearance of flu-like symptoms, such as fever and headaches (Kennedy 2013). The secondary stage of infection is defined by the parasite invasion of the central nervous system (CNS), and leads to more severe symptoms, such as disturbed sleep (for which human infection earns its name, "sleeping sickness"), neurological disorders (Simarro et al. 2008; Brun et al. 2010), and if left untreated, eventually death.

1.3.3 Diagnosis

Current diagnosis of *T. b. gambiense* infection relies heavily on the screening for antibodies against a commonly expressed surface antigen on trypanosomes, a test known as the Card Agglutination Test for Trypanosomes (CATT) (Barrett & Croft 2012). The CATT test does however have its disadvantages – it can vary in sensitivity, it only detects *T. b. gambiense*, and it can sometimes give false negative results (Barrett & Croft 2012; Chappuis et al. 2005). Following testing using the CATT assay, patients are diagnosed by the microscopic confirmation of parasites in the blood, lymph or CSF (Barrett & Croft 2012; Papadopoulos et al. 2004). Parasite numbers in human fluids can be low, however, and so false negatives can result in misdiagnosis, especially during infection with *T. b. gambiense* (Barrett & Croft 2012).

1.3.4 Treatment

Treatment of HAT relies on the use of four different drugs – pentamidine for the first stage of *T. b. gambiense*, and suramin for the first stage of *T. b. rhodesiense*, and melarsoprol for the second stage of both species (Babokhov et al. 2013). Suramin and pentamidine, while proven to have very effective trypanocidal qualities, are only effective while the infection is in the earlier stages – once the parasites cross into the CNS, leading to stage II infection, these drugs are ineffective (Babokhov et al. 2013). Melarsoprol, which has been in use since 1949, is effective against second stage infection of both human infective subspecies of trypanosome (Babokhov et al. 2013). However, it also results in adverse and dangerous side effects – up to 10% of treated patients can end up suffering from an array of side effects such as seizures, dizziness, vomiting and fevers, and up to 5% of treated patients will subsequently die (Babokhov et al. 2013). An additional drug, eflornithine, can be used during the second stage of *T. b. gambiense* infection (Simarro et al. 2008; Barrett & Croft 2012), however this also results in side effects such as vomiting, seizures and anaemia (Babokhov et al. 2013; Burri & Brun 2003). Even with these disadvantageous side effects, eflornithine is relatively well tolerated – and better still, since 2009, it can be given in tandem with nifurtimox in order to cure both stages of disease with even fewer side effects (Babokhov et al. 2013). Trypanosomal resistance to drug treatment remains a concern, and so looking to other, non-chemotherapy based strategies would be beneficial in eradicating the disease.

1.3.5 Asymptomatic Patient Status

It has been a widespread belief that human infection with African trypanosomes without treatment leads to death without exception, however there have been studies in recent years which would suggest that there exists asymptomatic patients. A 2012 study has shown natural asymptomatic patient status resulting in clearance in infection, and has acknowledged possible trypanotolerant patients as being important in understanding and

eradicating the disease (Jamonneau et al. 2012). In addition to this, these asymptomatic patients could act as perpetuators of disease foci, which could go someway to explaining why the eradication of HAT has been difficult so far.

1.4 The Immune System

1.4.1 The Innate Immune System

The first barriers to a long lasting infection include both physical barriers such as the skin, and the components of the innate, non-adaptive immune system (Moens & Veldhoen 2012). These primary defences work in some cases to eradicate the invading pathogen, and in others to limit the spread or development of infection before the adaptive immune system can begin to work (Moens & Veldhoen 2012).

Once a pathogen breaches the epithelia and enters the tissues of the host, it can be recognized by tissue resident phagocytes known as macrophages and dendritic cells (Malissen et al. 2014). These phagocytes can broadly recognize pathogenic motifs (PAMPs) through pathogen recognition receptors (PRRs), and can subsequently ingest and destroy many invaders (Kay et al. 2013). Recognition of pathogens by macrophages leads to phagocytosis, in tandem with the production and release of cytokines and toxic products such as antimicrobial peptides and nitric oxide (Kay et al. 2013). The release of inflammatory mediators from phagocytes results in local inflammation, which induces the migration of cells to the site of infection – cells such as neutrophils, monocytes (which then differentiate into more macrophages), eosinophils, and eventually lymphocytes (Mosser & Edwards 2008). Recognition of invading pathogens by dendritic cells (DCs), however, can result in the processing of antigen within the cells. Upon antigen recognition, DCs will ingest the pathogen, and migrate to the lymph nodes in order to initiate the adaptive immune response (Wang et al. 2015). DCs therefore act as a bridge between the innate and adaptive immune response, allowing for the specific responses required to eradicate infections (Wang et al. 2015).

1.4.2 The Adaptive Immune System

While the innate immune system does broadly recognize invading pathogens, it is limited to the destruction of pathogens expressing specific pathogenic motifs (Kay et al. 2013) . In order to effectively defend the host organism, the adaptive immune system has developed in such way that its lymphocytes can recognize and act against a vast array of antigens, while also providing long lasting defence through the generation of immunological memory (Reinherz 2015). As previously mentioned, DCs will ingest and process antigen, leading to the presentation of antigen to lymphocytes within the lymph nodes (Wang et al. 2015).

The two chief populations of lymphocytes are B and T lymphocytes, and both express antigen receptors of vast specificities (Reinherz 2015) in order to induce as specific and direct an immune response as possible. DCs present antigen to T lymphocytes in an indirect fashion; antigen is bound to cell surface expressed MHC molecules, which allows the T cells to distinguish between self-expressed host antigens and invading pathogenic antigens (Luckheeram et al. 2012). Activation of T cells can then promote the activation of B cells through the secretion of cytokines – this leads to the B cell production of antigen specific molecules known as immunoglobulins (Ig) (Luckheeram et al. 2012). It can also result in class switching of the immunoglobulin molecules – allowing the Ig molecules to maintain their specificity but alter their function, and enabling their binding to their specific antigen, targeting the antigen for degradation by the immune system (Ollila & Vihinen 2005).

1.5 T lymphocytes

1.5.1 Recognition of Antigen

T lymphocytes recognize antigen through the heterodimeric receptor complex on the cell surface, known as the T cell receptor (TCR) (Luckheeram et al. 2012). The recognition of antigen through this receptor is limited to antigens bound to major histocompatibility (MHC) molecules, allowing the T cell to distinguish between self and non-self antigens as previously noted (Eckle et al. 2013). In the case of CD4+ T cells, recognition

is restricted to antigens expressed on MHCII molecules on antigen presenting cells (APCs), while CD8+ T cells recognize antigen bound to MHCI molecules on APCs (Artyomov et al. 2010). Dendritic cells, macrophages and B cells can present antigen to T cells, with dendritic cells being considered the most important and efficient of the APCs. Binding of the TCR to the MHC-antigen complex results in a signal transduction within the T cells, leading to their activation and subsequent effector functions (Luckheeram et al. 2012).

1.5.2 Activation

Activation of CD4+ T cells occurs, as previously described, through recognition of a peptide-MHC complex on an APC. Again, as mentioned, CD4+ T cells recognize those antigens presented on MHCII molecules, to which the CD4 co-receptor binds. These APCs must then interact with naïve T cells in two further ways in order to effectively drive activation and effector functions (Elias et al. 2005).

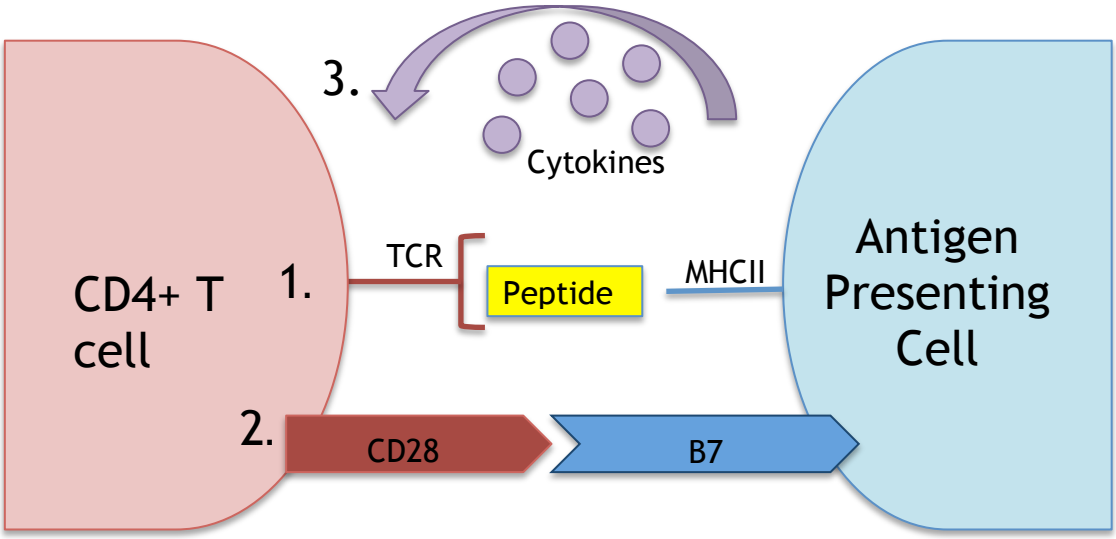

Figure 1: 3-signal activation model of CD4+ T cells by APCs. Signal 1: the recognition of antigen-MHCII complex by TCR. Signal 2: Costimulation by CD28 binding with B7. Signal 3: cytokines in extracellular environment promote effector differentiation (Elias et al. 2005)

A second, costimulatory signal is then given to the T cell by the APC – the most well known of these signals comes from the B7 molecule family (Sharpe & Freeman 2002). B7 molecules bind to CD28, a surface molecule of T cells, resulting in the appropriate clonal expansion of T cells, or if necessary, inhibition of T cell activation (Chen & Flies 2012). A third activation signal can then determine the effector function of the responding T cell – cytokines released by the presenting APC into the surrounding environment drive the T cell to differentiate into a context dependent effector cell (Cosmi et al. 2014; Yamane & Paul 2012).

1.5.3 Inhibition

Prolonged inflammation can lead to autoimmunity, and therefore it is critical that the immune system has within it systems of regulation. This occurs in T lymphocytes through the ligation of molecules to inhibitory receptors on the cell surface (Chen & Flies 2012). CTLA-4 is one of these inhibitory receptors, homologous to the co-stimulatory CD28 molecule previously described, and competitively binds to the B7 molecules on the APC (Parry et al. 2005). This ligation results in a signal transduction that will inhibit T lymphocyte activation. Abatacept, a CTLA-4 fusion with IgG, is a drug used to prevent T cell activation in the treatment of rheumatoid arthritis (Moreland et al. 2006), and has been studied as a possible therapy in a number of other autoimmunities. It binds to the B7 molecules on the APCs, halting their ability to activate T cells (Moreland et al. 2006). PD-1 is another inhibitory receptor, expressed both by T and B cells, and upon other cells of the immune system (Parry et al. 2005; Fife & Pauken 2011). Its ligands are found constitutively expressed throughout the body, and are upregulated during periods of inflammation (Merelli et al. 2014). Expression of PD-1 on T cells has also been discussed as being an indicator of lymphocyte exhaustion, and is upregulated during chronic infections (Day et al. 2006; Wherry 2011).

1.5.4 Exhaustion

During a chronic infection, the long term activation of T cells can result in the exhaustion of lymphocytes – this is characterized by diminished effector function, and shows phenotypically as a continued expression of inhibitory receptors such as PD-1 and LAG-3 (Wherry 2011). These exhausted cells can be considered a state of T cell differentiation before their deletion. Continuous exhaustion of T cells during a chronic infection can have a detrimental effect on the immune response, and can hamper the efforts to control or clear infection. Keeping this in mind, blocking of inhibitory receptors as a therapy in some chronic infections has been researched (Butler et al. 2012; Day et al. 2006), and could be useful in reawakening the immune system to fight infection.

1.5.5 CD4+ T lymphocytes

(i) Effector Functions

CD4+ T cells, or T helper cells, function primarily to help other cells of the immune system carry out their functions during an immune response – such as activating innate immune cells, or switching the class of antibody that a B cell produces (Crotty 2015). Each CD4+ T cell subset has a slightly different function and therefore different methods by which they help these other cells, to coincide with the different types of pathogens that a host organism can face (Luckheeram et al. 2012).

T_H1 effector cells are generated in order to eliminate intracellular pathogens (Luckheeram et al. 2012). They primarily produce IFN-γ – this helps the immune response by promoting further activation of macrophages and other phagocytes, enhancing their ability to degrade invaders (Cosmi et al. 2014). T_H1 cells also secrete IL-2, which induces proliferation of both CD4+ T cells and CD8+ T cells, further augmenting the efficiency of the response (Yamane & Paul 2012).

While T_H1 cells fight intracellular pathogens, T_H2 cells are made available to combat extracellular pathogens, such as parasitic helminthes, and secrete

cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13 (Cosmi et al. 2014). A T_H2 response also results in the degranulation of mast cells, and the activation of other innate immune cells such as eosinophils and basophils (Luckheeram et al. 2012). The secretion of IL-10 actively suppresses any T_H1 responses that could occur, allowing for the maintenance only of the appropriate response while limiting any damage to the host tissue (Moore et al. 2001).

Effector T Cell Subset	Cytokines Produced	Functions
T_H1	IFN-γ, IL-2	Elimination of intracellular pathogens – bacteria and protozoa
T_H2	IL-4, IL-5, IL-9, IL-10, IL-13	Destruction of extracellular parasites – e.g. parasitic helminthes. Also contribute to allergy
T_H17	IL-17A, IL-17F, IL-21, IL-22	Eradication of extracellular bacteria and fungi
T_{REG}	TGF-β, IL-10, 1L-35	Negative regulators of immune responses
CD8+	TNF-α, IFN-γ	Destroy intracellular pathogens such as viruses

Table 1: Effector T cell subsets, the cytokines they secrete and their functions within the immune response (Luckheeram et al. 2012).

As with T_H2 cells, T_H17 cells are required for the eradication of extracellular pathogens, this time fungi and other bacteria (Luckheeram et al. 2012). As their name suggests, they mainly produce cytokines from the IL-17 family along with IL-21 (Luckheeram et al. 2012). This promotes a pro-inflammatory environment by interacting with the IL-17R on multiple cell types throughout the host.

Regulatory T cells function as negative regulators of the immune system, to return the environment to homeostasis and prevent any autoinflammation. It carries this function out through the production of anti-inflammatory cytokines such as TGF-β, IL-10 and IL-35 (Luckheeram et al. 2012).

1.5.6 CD8+ T lymphocytes

(iii) Effector Functions

CD8+ T lymphocytes, or cytotoxic T cells as they are otherwise known, recognize antigen presented on MHCI molecules, which are expressed ubiquitously throughout the host, upon all nucleated cells (Wong & Pamer 2003). Similar to T_H1 cells, they work to eradicate intracellular pathogens such as viruses (Novais & Scott 2015). They can do this through the secretion of TNF-α and IFN-γ, the controlled release of cytotoxic granules into the affected cells, or through induced apoptosis of the target cells (Harty et al. 2000).

1.6 The Immune Response to Trypanosomes

1.6.1 The Innate Response to Trypanosomes

(i) Human Serum Trypanolytic Factors

Human blood serum contains within it multiprotein high density lipoprotein complexes which act as trypanolytic factors (TLF1 and TLF2) (Raper et al. 1999). Parasites must overcome destruction by these complexes in order to fully establish long-term infection. As such, these TLFs confer resistance in humans and in some primate species to most of the trypanosomes, except those which have evolved to evade killing by them – *T. b. gambiense* and *T. b. rhodesiense* (Raper et al. 1999).

Both TLFs contains apolipoproteins L-1 (apoL1) and haptoglobin-related protein (HPR), which act in tandem to allow destruction of the parasite. TLF2 also contains IgM (Raper et al. 1999). The human infective strains have developed different ways to avoid this innate immune defence – *T. b. gambiense* expresses *Trypanosoma gambiense*-specific glycoprotein (TgsGP), which was found to be essential in parasite resistance to host TLFs

(Capewell et al. 2013). TgsGP expression leads to prevention of apoL-1 function within the parasites and consequent parasite evasion of death (Uzureau et al. 2013). In the case of *T. b. rhodesiense*, human serum resistance is attributed to the expression and transcription of a gene called the serum resistance associated (SRA) gene (Capewell et al. 2014), which interferes with the apoL-1 within the trypanosome lysosome and subsequently prevents parasite death.

(ii) PAMP recognition

Pathogen-associated molecular patterns (PAMPs) in trypanosome infections can come from trypanosomal components. Recognition of these components by the PRRs of the innate immune cells is crucial in the induction of a quick and effective immune response. The glycosyl-inositol-phosphate (GIP) residue attached to soluble variable surface glycoprotein (sVSG) acts as one of these PAMPs and activates macrophages through a protein tyrosine kinase signal transduction pathway (Tachado & Gerold 1997), prompting the release of the pro-inflammatory TNF-α (Magez et al. 1998). Membrane bound VSG has also been noted to have macrophage activating capacities, through the glycophosphatidylinositol (GPI) anchor, however this stimulation results in the secretion of IL-1α and responsiveness to LPS (Magez et al. 1998). In either case, recognition of GIP or GPI moieties results in the activation of pro-inflammatory macrophages.

Detection of trypanosomal DNA can also result in the activation of the innate immune system (Harris et al. 2006) through the ligation of trypanosomal CpG DNA with macrophage Toll-like receptor (TLR) 9. This response was demonstrated to be important to host survival through an increased resistance to infection in mice following artificial stimulation through TLR9 (Harris et al. 2007). Exposure of these PAMPs to their ligands on innate immune cells, particularly macrophages, can enhance both the innate and adaptive response to infection.

(iii) Macrophage activation

The bite of a tsetse fly results in a localized inflammatory response, distinguished by invasion of the area by macrophages, granulocytes and lymphocytes as a result of the replicating parasites (Naessens & Mwangi 2003). Activation of macrophages in response to trypanosomal components, as previously described, can result in a strong T_H1 response. The production of cytokines such as IL-12, IL-6, TNF-α and IL-1 lead to a pro-inflammatory response defined by the production of macrophage-derived trypanocidal molecules, such as reactive nitrogen oxide species (RNOS) and reactive oxygen intermediates (ROIs) (Paulnock et al. 2010). These secreted molecules prove to be destructive to the trypanosomes. TNF-α in particular has been discussed as having a detrimental effect on parasite growth within the blood of a mammalian host – mice with disrupted TNF-α genes show higher levels of blood parasitemia than control mice (Magez & Radwanska 1999). The activation of these macrophages works as a positive feedback loop – degradation of parasites leads to the availability of more PAMPs, allowing for further parasite recognition and subsequently more macrophage activation, which initiates a robust adaptive immune response.

1.6.2 The Adaptive Immune Response to Trypanosomes

(i) T_H1 Response

As previously alluded to, the activation of innate cells such as macrophages and dendritic cells results in the production of IL-12 and IL-18. This can aid the activation of the adaptive immune response, and polarizes this response in favour of a T_H1 effector response (Perito et al. 1992; Mansfield & Paulnock 2005). The resultant IFN-γ from the T cells further activates the macrophages, thereby propagating a continuously activated immune response to the parasites. This IFN-γ production has been discussed as being both IL-12 dependent and also crucial to the resistance of mice to infection (Hertz et al. 1998; Barkhuizen et al. 2007), suggesting that a T_H1 effector response is important in the response to trypanosome infection.

(ii) B cells and Antibodies

B cells have been shown to be necessary to survival of trypanosome infection through the infection of B cell deficient mice (Magez et al. 2008), which could suggest the production of antibodies to be crucial to trypanosomiasis resistance. IgM antibodies have been considered to be the first humoral line of defence against parasites, with isotype switching occurring in response to specific T cell responses (Radwanska et al. 2008). The generation of antibodies in response to parasite antigens has been shown to be stage specific. During the first few days of *Trypanosoma brucei brucei* infection, immunoglobulin (Ig) isotypes IgG2a and IgG2b are prevalent – however, during later stages of infection, these give way to T-cell dependent IgG1 and IgG3 antibody responses, while maintaining constant IgM respsonses. (Radwanska et al. 2000). Similarly, infection of mice with *Trypanosoma brucei rhodesiense* results in immunoglobulin class switching to IgG1, IgG2a and IgG3, however IgA and IgE antibodies were not induced (Schopf & Filutowicz 1998). During infection however, it has been noted that B cells are depleted following a period of polyclonal activation and subsequent exhaustion (Bockstal et al. 2011). This contributes to progression of infection through a lessened host ability to generate antibodies against the trypanosomes (Bockstal et al. 2011). Studies in humans, however, suggest that B cell depletion is not as much a problem as in experimental mouse models – however the authors do note that these B cells may lack some functionality, which they could not test (Lejon et al. 2014).

1.7 Evasion of the Immune Responses by African Trypanosomes

1.7.1 Antigenic variation

Trypanosomes have had to evolve in order to evade the host immune response. One such method of defence is the variation of the variable surface glycoprotein (VSG), a homodimeric antigen which is densely present on the surface of the trypanosome (Pays et al. 2004). These antigens protect the surface of the parasite from the immune system of the host, by

hiding and preventing adaptive immune responses to the invariable surface antigens present there (Pays et al. 2004).Trypanosomes are able to undergo genetic switching in order to vary the VSG that they present – while they present one VSG at a time, they stochastically alter it through genetic switching mechanisms (Robinson et al. 1999). This allows for the persistence of infection, as while antibodies are generated against specific VSGs, other parasites within the host will already be expressing other variants, allowing for the survival of the species despite the host humoral responses. *T. b. brucei* alone has 806 VSG genes, some of which are psuedogenes used to create mosaic VSG genes and further enhancing the variablility (Berriman et al. 2005). As the VSGs are shielding the invariant genes, they have been effective in hindering the progress of trypanosome vaccine development, and allowed for persistence of disease in mammals.

1.7.2 B and T cell suppression

Following evasion of the immune system, *Trypanosoma brucei brucei* also alters the immune response, suppressing the adaptive response through alteration of macrophage function (Namangala et al. 2000). Specifically, macrophage ability to present antigens using MHCII molecules is lessened, and the macrophages produce immunosuppressive mediators such as IL-10 which can impact directly upon lymphocyte activation (Namangala et al. 2000). Further studies have also shown a mucin of *Trypanosoma cruzi* to be an inhibitor of CD4+ T cell function (Nunes et al. 2013) and this inhibition has been suggested to further disease progression. There has also been data showing a similar suppression of B cells, also during *Trypanosoma cruzi* infection, which would again further disease progression through lower numbers of antibodies generated (Zuñiga et al. 2000). Additionally, B cells have been shown to be depleted following activation and exhaustion in experimental models of *Trypanosoma brucei brucei* (Bockstal et al. 2011), and this has been discussed recently to be dependent upon IFN-γ (Cnops et al. 2015). A similar situation has been noted in chronic human malarial infection, where both B and T lymphocytes have been shown to be

functionally exhausted (Illingworth et al. 2013).

1.7.3 Alteration of antigen presenting cell functions

It has been discussed in previous studies that one aspect of immune evasion employed by trypanosomes is the alteration of APC functions. A study published in 2000 suggested that trypanosomes induce a decrease in MHCII expression in macrophages, thereby decreasing their ability to activate T cells, and lessening the chance of an effective adaptive response (Namangala et al. 2000). Further studies demonstrated that during infection, APCs had a reduced capacity for the presentation of new VSG antigen (Dagenais et al. 2009) – this study also showed that susceptible animals suffered from depletion of splenic DC populations, which had been proven to be important in generation of an effective T_H1 response.

1.8 Polyclonal Lymphocyte Activation

1.8.1 Leishmania and malaria

Polyclonal lymphocyte activation and subsequent exhaustion has been reported in a number of chronic infections (Day et al. 2006; Zajac et al. 1998; Brooks et al. 2005). This has also been noted in parasite infections. During the blood stage of malarial infections, B and T cells are polyclonally activated, before showing phenotypic characteristics of exhaustion and dying by apoptosis (Illingworth et al. 2013; Butler et al. 2012; Muxel et al. 2011). The increased expression of exhaustion markers PD-1 and LAG-3 on CD4+ T cells during *Plasmodium falciparum* infection led to further study in *in vivo* models (Butler et al. 2012). Blocking these inhibitory markers on CD4+ T cells during *Plasmodium yoelii* infection in mice resulted in the clearance of parasites from the blood of the host, suggesting that the phenotypic exhaustion of T cells aids parasite survival (Butler et al. 2012). The comparable increase in inhibitory marker expression in humans suggests that this could be an avenue of treatment for malaria through restoration of CD4+ T cell function (Butler et al. 2012). Similarly, non-specific activation of B and T lymphocytes occurs during visceral leishmaniasis, resulting in a pro-inflammatory response which is thought to contribute to the

immunopathogensis of the disease (Santos-Oliveira & Da-Cruz 2012). This is also followed by a depletion of lymphocytes during infection.

1.8.2 Trypanosomes

The polyclonal activation of both B and T lymphocytes is also a hallmark of infection with trypanosomes of the *Trypanosoma cruzi* species (Gao & Pereira 2001), and has been discussed as occurring in order to benefit parasite survival within the host. Activation of lymphocytes leads to exhaustion during chronic infection (Wherry 2011), and could contribute to the immunosuppression seen in trypanosome infections – leading also to the development of secondary infections. Studies have shown that a neuraminidase expressed by *T. cruzi* enhances both mitogenic and antigenic T cell activation (Gao & Pereira 2001), through polyclonal activation of B cells. As previously described above, similar levels of lymphocyte activation can be seen in malaria and leishmania, which could suggest that the exhaustion of T lymphocytes during infection works in favour of parasite survival. If true, this could lead to the development of new therapeutics against trypanosome infection and better understanding of the parasite, leading eventually to absolute eradication to the benefit of the African continent.

1.9 Aims of the thesis

Overall, this project aims to further characterize the CD4+ T cell response to trypanosome infection. In doing so, it is hoped that this will lead to a better understanding of how the infection progresses and eventually to the investigation of new treatment strategies. The project has two main routes of investigation: *in vivo* characterization of CD4+ cell response to infection, and the development of an *in vitro* assay to replicate this *in vivo* response.

Chapter 2: Materials and Methods

2.1 Animals

2.1.1 Infection of animals with *T.b.brucei*

Mice were used in accordance with local and Home Office regulations. Female Balb/c mice, aged 8 to 10 weeks were used for infection. Frozen stabilates of *T.b.brucei* clones were grown in Balb/c mice before initiating experimental infections. Strains of *Trypanosoma brucei* used throughout these experiments were *Trypanosoma brucei brucei* STIB 247 and TREU 927, and *Trypanosoma brucei gambiense* PA44. These donor mice were immunosuppressed by cyclophosphamide treatment (0.25µg/g, Sigma) 24 hours before intraperitoneal injection with trypanosomes. Animals were exsanguinated, and parasite number was counted using a haemocytometer. Blood was then diluted with phosphate-buffered saline (PBS) containing 1% glucose (PBS-G) to give 10^4 parasites in 200µl. 10-week old female Balb/c mice were then infected by an intra peritoneal injection of 200µl of 10^4 parasites. The infection was monitored through assessment of weight, blood parasitemia, and general health until their cull.

2.1.2 Monitoring parasite burden

Blood parasite burden was measured daily by venipuncture and parasite counts as instructed by Herbert and Lumsden 1976 (Herbert & Lumsden 1976). Health was also assessed by weights and mouse behaviour/appearance.

2.2 *In vivo* treatment of mice

2.2.1 Abatacept treatment of mice

Abatacept was provided by Bristol-Myers Squibb and was administered at a dose of 10mg/kg intraperitoneally every second day from the day before infection. A human IgG1 was used as a control fusion protein.

2.2.2 PD-L1 and LAG-3 treatment of mice

Anti-mouse LAG-3 and anti-mouse PD-L1 *in vivo* monoclonal antibodies were acquired from BioXCell. Both antibodies were administered via

intraperitoneal injection in a single volume of 200µl containing 200µg of each every 2 days from day 5 post-infection. Rat IgG was used as an isotype control.

2.3 Fluorescence activated cell-sorting analysis (FACs)

2.3.1 Preparation of spleen for FACs

Spleens were mechanically disrupted and filtered through a Falcon® 40µm cell strainer (Corning), and the resulting cell suspension transferred to 15ml tubes. These were then washed by centrifugation at 400*g* for 5 minutes, the supernatant discarded, and the pellet resuspended in the little remaining volume. 5ml of 1X red blood cell (RBC) lysis buffer (eBioscience) was added to the cell suspension and incubated on ice for 4 minutes. Cells were washed twice using PBS and transferred to FACs tubes.

2.3.2 Preparation of lymph nodes for FACs

Lymph nodes were mechanically disrupted in PBS using a nylon monofilament filter fabric (45microns, Cadisch Precision Meshes) and the cell suspension transferred to 15ml tubes, which were centrifuged at 400*g* for 5 minutes. Discarding the supernatant, the cells were then resuspended in 1ml of PBS and transferred to FACs tubes.

2.3.3 Preparation of blood for FACs

Blood was centrifuged at 3000rpm for 10 minutes. Supernatant was collected and frozen for future assays. 4ml of RBC lysis buffer was added to the cells and incubated on ice for 5 minutes. Following this, cells were washed twice with PBS, resuspended in 1ml of PBS and transferred to FACs tubes.

2.3.4 Cell Staining for FACs analysis

200µl of cells from blood, inguinal lymph nodes and spleen single cell suspensions were incubated at 4°C in 100µl Fc block with mouse serum for 20 minutes, and then with anti-mouse ICOS-, CD4-, LAG-3- and PD-1-fluorochrome-conjugated antibodies for 30 minutes. The cells were then

washed, and fixed by incubation with 1% paraformaldehyde for 20 minutes. Cell marker expression was analysed on a MACSquant (Miltenyi) and

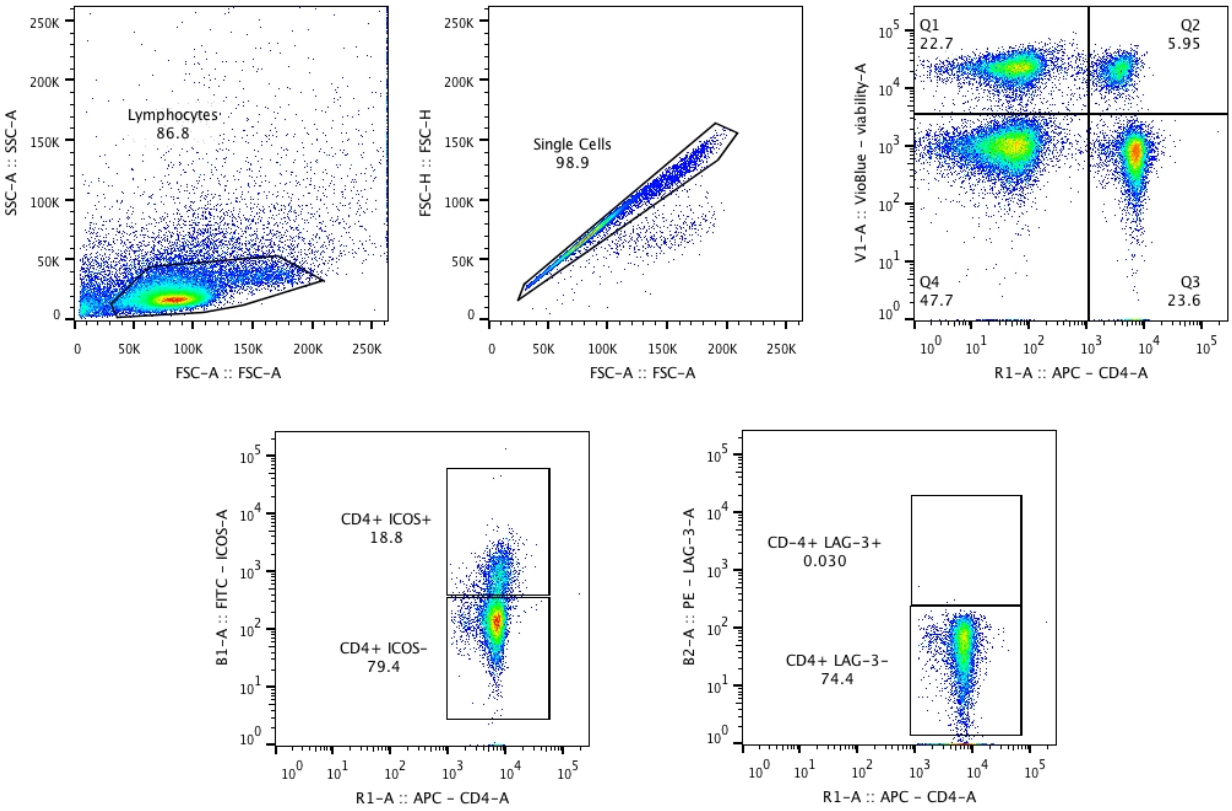

Figure 4: Example of gating strategy from *in vitro* assay. Cells were gated on a lymphocyte phenotype – small, non-granular cells. Cells were then assessed on FSC-H against FSC-A to gate upon single cells. Within this, cells were then gated on viability and CD4 expression, before being analysed for ICOS and LAG-3 expression.

subsequently analysed using FlowJo software. Unstained cells were used as controls for auto-fluoresence.

2.4 *In vitro* culture of bloodstream *T.b.brucei*

2.4.1 Culturing bloodstream *T. b. brucei*

T. brucei strain STIB 247 bloodstream form parasites were cultured in HMI-9 medium, supplemented to 20% (v/v) Serum plus. Parasites were maintained at a density of roughly 10^5-10^6 parasites per ml in a vented lid flask at 37°C, 5% CO_2 incubator. Cultures were passaged every 2-3 days to prevent from overgrowing.

2.5 Co-culture of splenocytes with parasites

2.5.1 Preparation of splenocytes for culture

Spleens were removed from naïve Balb/c mice and prepared for FACs as previously described. Cells were then counted and resuspended at 5 x 10^6 cells/ml, before being plated out on 96-well plates at 5 x 10^5 cells/well in 100µl and maintained in a 37°C, 5% CO_2 incubator.

2.5.4 Addition of Concanavalin A (ConA) for full and sub-optimal stimulation

Following plating of cells in 100µl of media to wells of a 96-well plate, 100µl of media containing experimental conditions was added. Positive control wells received an extra 100µl of media containing 5µg/ml ConA, bringing the final concentration in each well to 2.5µg.ml. Sub-optimal stimulation control wells received 100µl of media containing 0.2µg/ml ConA, bringing the final concentration to 0.1µg/ml. Trypanosome control wells received 100µl of media containing 10^5 trypanosomes. Experimental wells received an addition 100µl of media containing 0.2µg/ml ConA and 10^5 trypanosomes. Plates were incubated for up to 72 hours in a 37°C, 5% CO_2 incubator.

2.5.5 Preparation of *in vitro* co-culture for FACs

Cells and supernatant were transferred into FACs tubes and centrifuged at 400*g* for 5 minutes. They were then incubated at 4°C in Fc block with mouse serum for 20 minutes, and then with ICOS-, CD4-, LAG-3- and PD-1-fluorochrome-conjugated antibodies for 30 minutes. The cells were then washed and analysed on a MACSquant (Miltenyi) and subsequently analysed using FlowJo software.

2.6 Statistics

Data was analysed using GraphPad Prism® software. To compare the means of two or more samples, an ANOVA test was used. Where two independent variables were present, two-way ANOVA was carried out. P values < 0.05 were considered to be significant.

Chapter 3: Results – CD4+ T cell Activation During Murine *T.b.brucei* Infection

3.1 Introduction and Aims

Different methods of immune manipulation by trypanosomes have been reported and described previously in this thesis – these methods can allow the parasite to effectively evade the adaptive immune system. Prior to the immunosuppression that characterizes trypanosome infection comes a period of massive polyclonal lymphocyte activation (Aksonas & Bancroft 1984; Bockstal et al. 2011; Olsson et al. 1991), meaning that many T cells of many different TCR specificities are being activated at one time. This generates a non-antigen-specific adaptive immune response and could contribute to parasite evasion of the host human system through subsequent lymphocyte exhaustion. In addition to this, a 1991 study found a factor released during experimental African Trypanosomiasis that triggers CD8+ T cell proliferation and production of IFN-γ, which has been suggested to be beneficial to parasite growth (Olsson et al. 1991) – this gives the impression that the parasites induce this response to benefit the progression of infection. However, within the literature, the use of different mouse strains and parasite strains has led to an incomplete story – standardizing experimental protocols would allow us to characterize the response to infection more clearly, and allow for the application of this knowledge to human infection and subsequent treatment.

T cell activation during infection can lead to exhaustion, as noted in the introduction. The activation associated upregulation of inhibitory molecules such as PD-1 and LAG-3 has led to investigating blockade of these molecules in malarial infection as a therapy against blood stage malaria (Butler et al. 2012). Due to similarities in the literature with trypanosome infection, it was decided to investigate the ability of PD-1 and LAG-3 blockade on the number of trypanosomes in the blood. This blocking of T cell exhaustion could hypothetically lead to a new avenue of treatment.

This chapter aims to further characterize the CD4+ cell response to trypanosome infection. Primarily we examine T cell activation, and then aimed to establish whether it is a strain specific response. We also tried to determine whether this activation was co-stimulation dependent through the blockade of CD28, and attempted to block the inhibitory T cell markers expressed during infection to determine what effect these have on infection and/or parasite survival.

3.2 Trypanosome infection results in polyclonal CD4+ T cell activation

Infection of mice with trypanosomes results in a massive proliferation of lymphocytes followed by lymphocyte exhaustion and consequent immunosuppression within the host. Characterising the CD4+ T cell response to trypanosome infection could help us to understand this response and potentially prevent the exhaustion and immunosuppression that follows – depending on the response this could also result in an option for treatment of infection.

Unpublished work carried out in our lab by other scientists show a clear indication that experimental trypanosome infection in mice results in a polyclonal CD4+ T cell response.

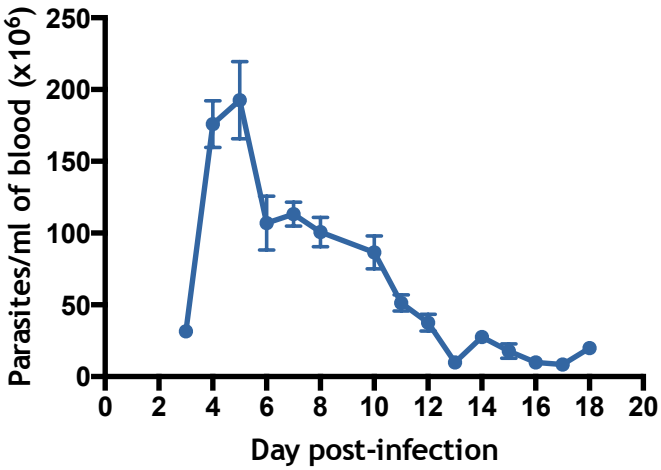

Figure 4: Number of parasites per ml of blood during infection with *Trypanosoma brucei brucei*. Mice were infected with T.b.brucei strain TREU 927, culled at day 18. Data represents mean ± SD.

Infection of mice with *Trypanosoma brucei brucei* leads to an initial peak parasitemia of ~2 x 10^8 parasites/ml of blood at day 5 (Figure 4). Following this, the parasitemia appears to decrease, until day 14 which shows a small resurgence of parasite numbers. By the end of the experiment (day 18, figure 4), it appears as though the parasite numbers are increasing in the blood again, which could suggest a pattern of successive peaks of parasitemia which are characteristic of infection (Magez et al. 2008).

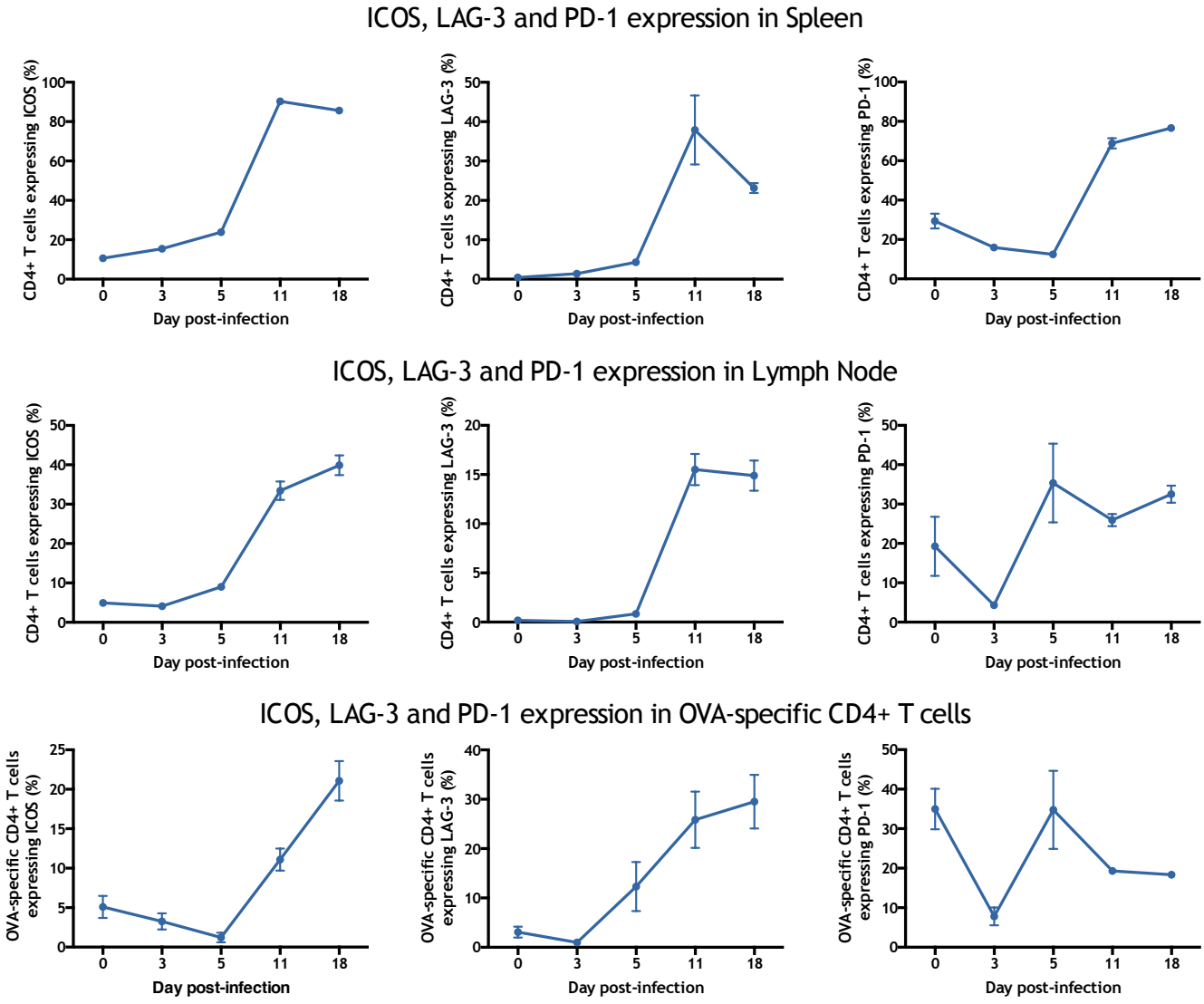

Figure 5: Infection with *Trypanosoma brucei brucei* leads to high percentages of CD4+ cells expressing activation markers such as ICOS and exhaustion markers such as PD-1 and LAG-3. Mice were infected with T.b.brucei strain TREU 927, culled at day 18, and their spleens and lymph nodes assessed for CD4+ cell activation. Mice also received intravenous injection of 1x10^6 D011.10 T cells to assess specificity of activated response. Data represents mean ± SEM.

Results show that infection with *Trypanosoma brucei brucei* results in activation of a large percentage of CD4+ T cells (Figure 5) – this is evident in the spleen where ICOS is expressed on roughly 90%, LAG-3 on 64%, and PD-1 on 80% of CD4+ T cells at day 11. CD4+ T cells in the lymph nodes also express high levels of these markers – 30-40%, 16%, and over 30% of CD4+ T cells express ICOS, LAG-3, and PD-1 respectively at day 11 post infection (Figure 5). Such high numbers of activated CD4+ T cells are suggestive of a polyclonal, non-antigen-specific immune response, and as such, $1x10^6$ transgenic T cells with a receptor specific for OVA peptide were intravenously injected into mice. As the OVA peptide is not naturally expressed in mice, nor was it being introduced by experimental conditions, these cells should not be activated during the course of infection. Analysis of these OVA-specific T cells in lymph nodes (Figure 5) showed that 22-25% expressed ICOS, 17-40% expressed LAG-3, and 16-21% expressed PD-1, suggesting an activated phenotype in the absence of their cognate antigen. This would also indicate that the large percentages of CD4+ T cells we see activated through infection are activated regardless of their TCR specificity, in a polyclonal nature.

3.3 Polyclonal CD4+ cell activation is not parasite-strain specific

Following infection of mice with *T.b.brucei* TREU 927, other parasite strains were investigated in murine models, in order to characterize the response and to standardize experimental protocols while still being able to compare them to human infection.

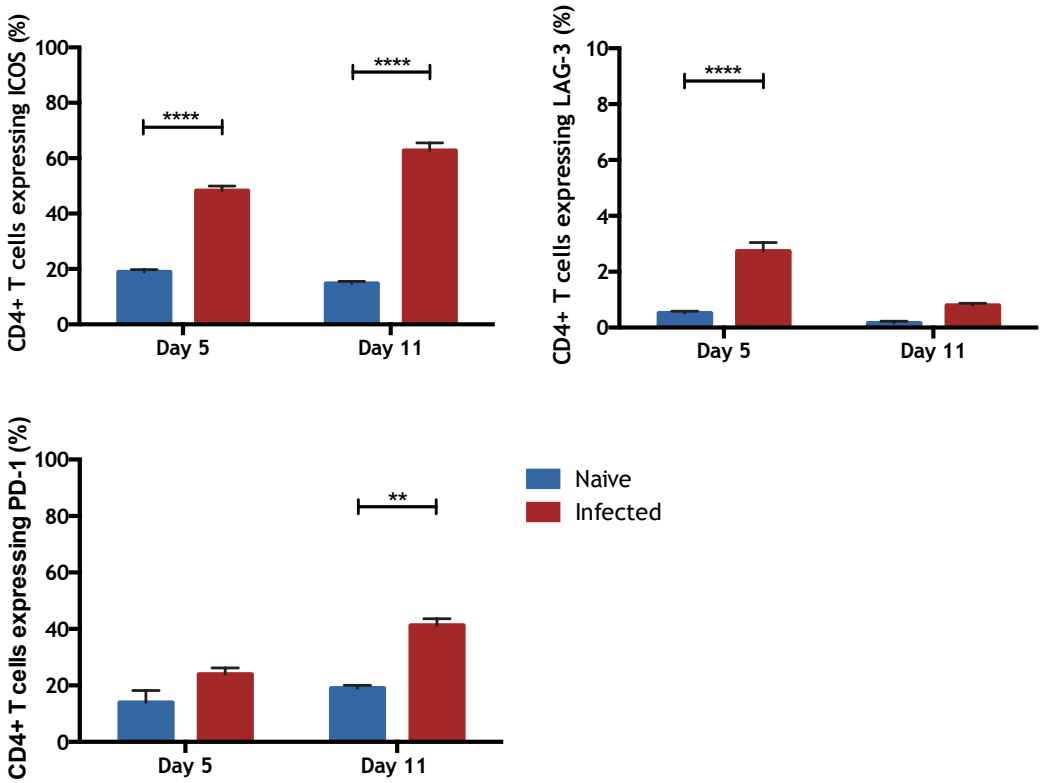

Figure 6: Infection with *T. b. brucei* strain STIB 247 results in large percentages of CD4+ cells becoming activated. Mice were infected with *T.b.brucei* 247, and culled at day 5 and day 11. Splenocytes were assessed for signs of CD4+ T cell activation through flow cytometry. Data represents mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

At day 5 post-infection with STIB 247 (figure 6), a significant increase in CD4+ cells expressing ICOS is noted in infected mice when compared with the uninfected controls – a result previously noted during infection with TREU 927. Similarly, at day 11, the number of CD4+ cells expressing ICOS is again significantly increased in infected mice relative to the uninfected negative controls.

In order to relate these results to a human infective strain of trypanosome, we decided then to investigate the CD4+ T cell response to a strain of *Trypanosoma brucei gambiense*.

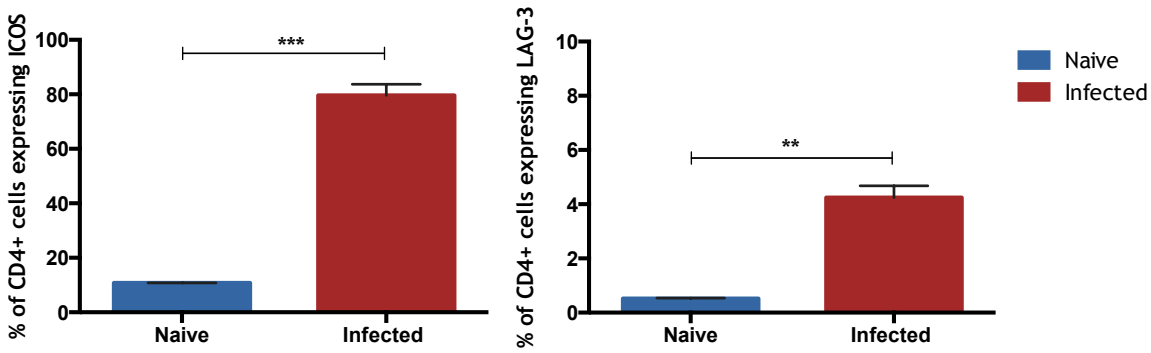

Figure 7: Infection with *T. b. gambiense* results in the activation of large percentages of CD4+ cells. Mice were infected with *T. b. gambiense* strain PA44, and culled at day 11. The splenocytes were then assessed for CD4+ cell activation through flow cytometry as before. Data represents mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

As with TREU 927 and STIB 247, infection of mice with *T. b. gambiense* leads to a large percentage of CD4+ cells being activated (Figure 7). Using an unpaired t test, it was determined that the expression of ICOS and LAG-3 on these CD4+ cells is significantly increased infected mice compared to naïve uninfected control mice. This suggests that the massive CD4+ T cell activation during infection is not specific to *T. b. brucei*, and could be found during human infection.

3.4 Trypanosome infection causes CD28-dependent T cell activation

Prolonged T lymphocyte activation has been considered as a prominent factor in the development and progression of rheumatoid arthritis, and from this theory Abatacept has been developed. A CTLA-4 fusion IgG, it binds with the B7 molecules on the APC, preventing ligation with the CD28 co-stimulatory molecule on T cell surfaces (Moreland et al. 2006). We could therefore hypothesize that use of Abatacept would determine whether CD28 signalling was important in the activation of T cells during trypanosome infection, and that this could shed some light upon the methods of immune system manipulation the parasite employs. This would help in the development of therapeutics aimed at halting this manipulation, and

allowing the immune system to mount the appropriate response to parasite infection.

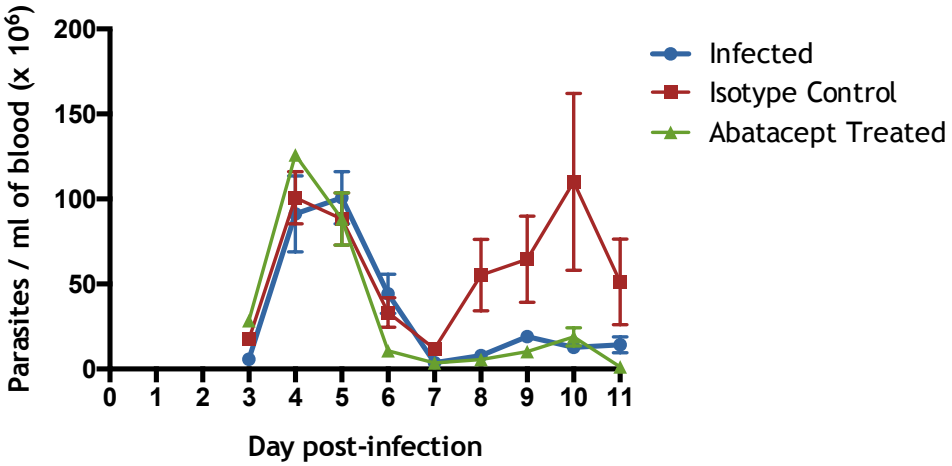

Figure 8: Number of parasites per ml of blood during infection with *Trypanosoma brucei brucei*. Mice were infected with T.b.brucei strain TREU 927, and separated into groups dependent on treatment status. Mice were intraperitoneally injected every second day from day -1 with 10mg/kg Abatacept or a control fusion protein acting as an isotype control. Mice were culled at day 11 and their spleens removed and analysed for CD4+ cell activation through flow cytometry. Data represents mean ± SD.

The number of parasites present per ml of blood primarily peaks at about day 4 in the groups of mice treated with either the isotype control fusion protein or the Abatacept (Figure 8). Untreated infected controls appear to have a peak parasitemia a day later than treatment groups, at day 5 as we saw in previous *T. b. brucei* TREU 927 infections (Figure 4). Abatacept treated mice show a low to undetectable level of blood parasitemia at day 11, which is lower than both the untreated infected mice and the isotype control treated mice – however this difference is only noticeable at day 11, and is not a significant difference. A longer experiment might result in a more significant difference in parasitemia between the groups, however the parasitemia levels in the isotype control would have to be taken into consideration, as they are noticeably higher and showed an increased secondary peak of parasitemia when compared with the other experimental groups.

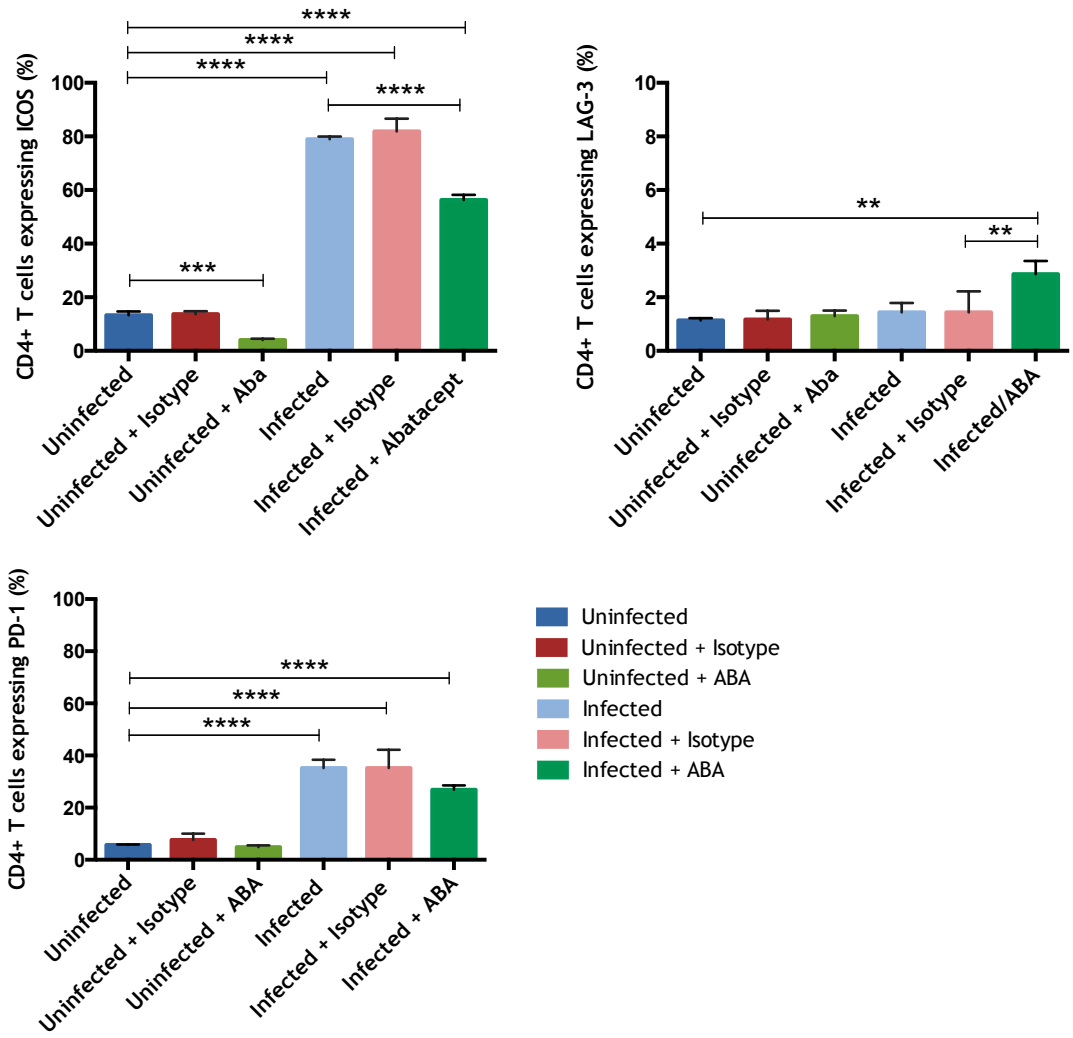

Figure 9: Infection with *Trypanosoma brucei brucei* leads to high percentages of CD4+ cells expressing activation markers such as ICOS and exhaustion markers such as PD-1 and LAG-3. Mice were separated into groups based on treatments and infection status. Treated mice were intraperitoneally injected every second day from day -1 with 10mg/kg Abatacept or a control fusion protein acting as an isotype control. Mice were infected with T.b.brucei strain TREU 927, culled at day 11, and their spleens assessed for CD4+ cell activation. Abatacept treatment lessens the expression of PD-1 and ICOS in infected mice relative to their untreated counterparts. Data represents mean ± SEM. All pairwise comparisons were tested for significance; only those with a significance of p<0.05 are annotated. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

As previously noted, infection resulted in an increased expression of ICOS on CD4+ T cells when compared with uninfected mice (Figure 9). Infected mice

treated with Abatacept showed a significant decrease in the expression of ICOS in splenic CD4+ cells when compared with the untreated infected group (Figure 9). The expression of LAG-3 appeared to be lower in infected mice than we expected and was not significantly different from the uninfected mice - except in the case of the Abatacept treated infected mice, which was significantly higher than the naïve mouse controls (Figure 9). Expression of PD-1 was increased significantly in infected mice relative to the uninfected controls. Abatacept treatment results in a decrease in the expression of PD-1 in infected mice, however this is not significant from the untreated counterpart groups (Figure 9).

3.5 Does blockade of exhaustion molecules restore CD4+ T cell functionality?

As seen in the Butler paper (Butler et al. 2012), blockade of exhaustion markers PD-1 and LAG-3 during murine infection with *Plasmodium yoelli* lead to clearance of the blood stage infection. Given that during infection with *Trypanosoma* species T cells become exhausted, we hypothesized that something similar may occur were these markers to be blocked.
Treatment groups in the *in vivo* experiment included untreated, isotype control treated, and combined anti-PD-L1 and anti-LAG-3 antibody treatment. Mice were infected with *T. b. brucei* reference strain TREU 927 and treated with every third day from day 4 post-infection. Mice were culled at day 14 and their spleens assessed for CD4+ cell activation status. They were also assessed for blood parasitemia on a daily basis.

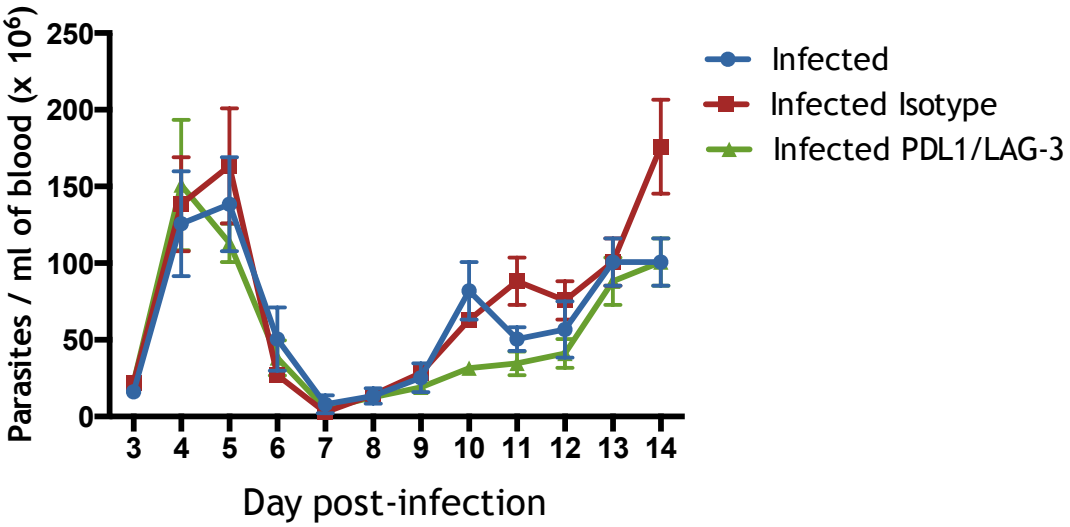

Figure 10: Treatment with anti-PD-L1 and anti-LAG-3 during *T. b. brucei* infection does not clear parasites from the blood. Mice were infected intraperitoneally with *T. b. brucei* TREU 927. Treatment was administered every 2 days from day 5 post-infection - 200µg of each anti-mouse LAG-3 and anti-mouse PD-1 were administered in 200µl via intraperitoneal injection in anti-LAG-3 and anti-PD-1 treated mice, while 200µg of Rat IgG was administered in 200µl via intraperitoneal injection in the isotype control treated mice. Mice were culled at day 14, their spleens were harvested and assessed for signs of CD4+ T cell activation by flow cytomtery. Data represents mean ± SD.

Blood parasitemia was assessed daily in each treatment group in order to ascertain what effect inhibitory marker blockade would have on the number of blood parasites. As seen in Figure 10, the first peak of parasitemia in mice occurred at days 4-5. Following the subsequent rapid decline, the parasites increase in number from day 7 onwards. Isotype control treated mice showed a higher blood parasitemia on the cull day (day 14) than either the untreated or the inhibitory blockade treated animals (Figure 10), both of which appeared to have a similar level of blood parasitemia (~100x10^6 parasites/ml of blood).

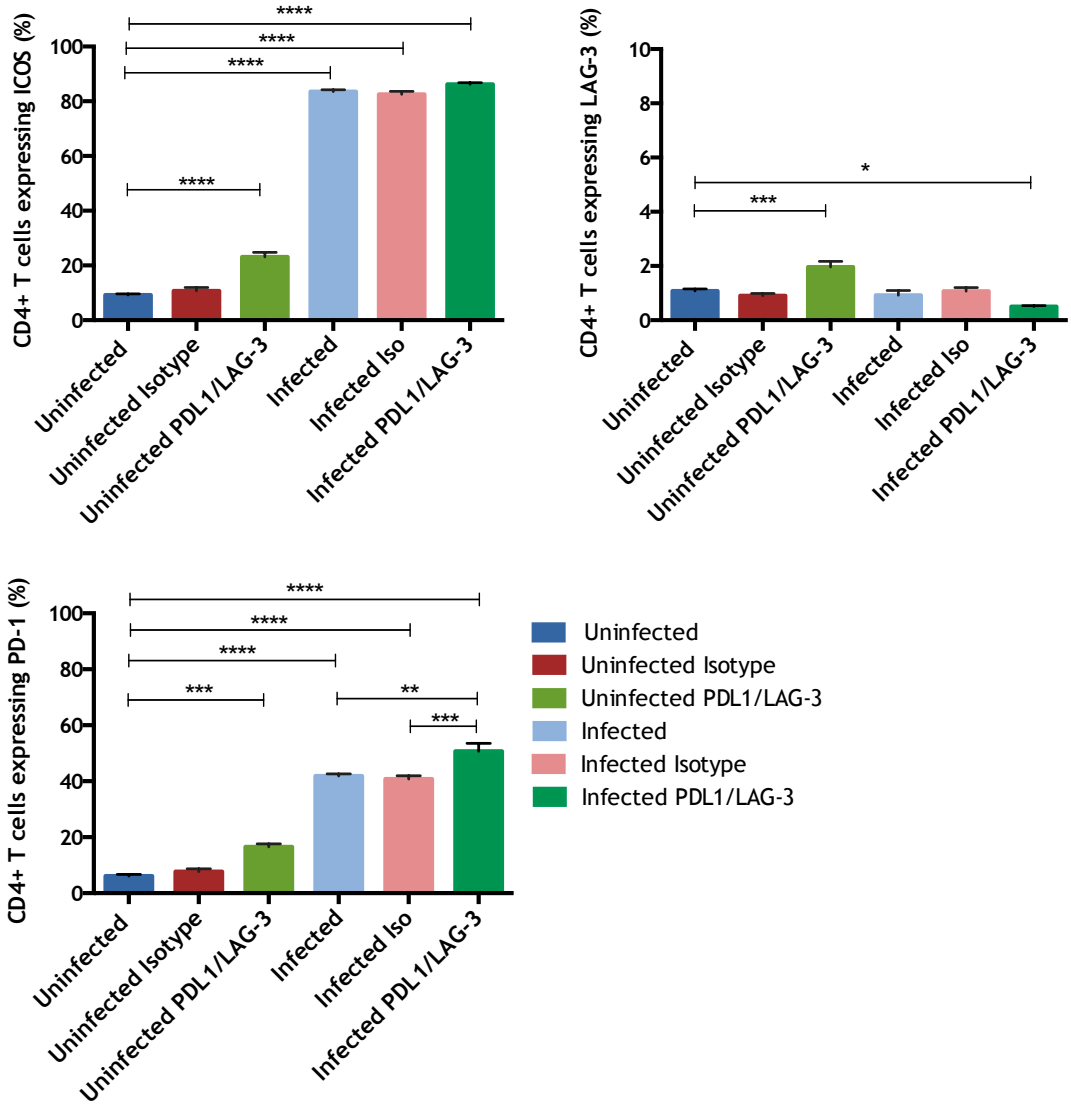

Figure 11: The effect of treatment with anti-PD-L1 and anti-LAG-3 on CD4+ T cell activation and exhaustion markers during infection. Mice were infected intraperitoneally with *T. b. brucei* TREU 927. Treatment was administered every 2 days from day 5 post-infection - 200µg of each anti-mouse LAG-3 and anti-mouse PD-1 were administered in 200µl via intraperitoneal injection in anti-LAG-3 and anti-PD-1 treated mice, while 200µg of Rat IgG was administered in 200µl via intraperitoneal injection in the isotype control treated mice. Mice were culled at day 14, their spleens were harvested and assessed for signs of CD4+ T cell activation by flow cytomtery. Data represents mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

As we have previously seen, infection with trypanosomes induces a significant increase in the expression of ICOS and PD-1, which we see here again in Figure 11. LAG-3 expression was again not significantly changed

during this infection – as we have seen it significantly increased previously, we could hypothesize that there has been a problem with staining that particular marker, or using that particular antibody.
ICOS expression on CD4+ T cells following treatment with anti-LAG-3 and anti-PD-1 remains similar to that in untreated infection, suggesting that blocking these markers does not impact upon activation status of the CD4+ T cells. PD-1 expression however, is significantly increased in treated mice compared to untreated or isotype control treated mice (Figure 11).

3.5 Discussion of Results

Using Balb/c mice and *T. b. brucei* strains, work carried out in this chapter shows that large percentages of CD4+ cells are activated during infection with these parasites (Figure 4 and Figure 5). These large percentages of activated cells suggest activation of a polyclonal nature – that is, T cells are being activated regardless of antigenic specificity. This was confirmed through use of transgenic T cells specific to OVA peptide – these cells were activated during trypanosome infection despite the absence of their cognate antigen, suggesting that the method by which trypanosomes activate CD4+ T cells is non-antigen specific, causing inflammation that could result in damage to host tissue.

In order to further study CD4+ cell response to trypanosome infection, it was important to determine whether this response was specific to one strain of parasite. This would help us to standardize experimental protocols and would also help us to relate experimental models to human infection. TREU 927 is a difficult strain to culture in laboratory conditions, and so it was decided to test the CD4+ cell response to strain STIB 247 (Figure 6). This strain, as well as being easier to culture in the lab, is easier to genetically manipulate, which would be advantageous in the elucidation of the mechanisms by which trypanosomes manipulate host immune cells. At both days 5 and 11 post-infection, ICOS expression was significantly increased ($p<0.0001$) in infected mice relative to the uninfected controls (Figure 6). This increase was also noted to be significant in LAG-3 expression at day 5

($p<0.0001$) and PD-1 expression at day 11 ($p<0.01$). As with previous data using *T. b. brucei* TREU 927, this suggests that *T. b. brucei* STIB 247 activates CD4+ T cells in large numbers, and would also indicate that this phenomenon is not parasite strain specific. This would be beneficial in future studies, both for use of parasites in *in vitro* models, and through use of genetic knock out parasites *in vivo*.

As with our STIB 247 work, assessment of the ability of *T. b. gambiense* to provoke a polyclonal CD4+ cell activation was carried out. *T. b. gambiense* has been described in this thesis as one of the two human infective trypanosomes, and so establishing its ability to activate CD4+ cells could have an effect on the investigation and treatment of human African trypanosomiasis. In figure 7, it is shown that ICOS expression and LAG-3 expression is increased significantly ($p<0.001$, $p<0.01$ respectively) in infected mice when they are compared with the uninfected control group, indicating that the human infective parasite has a similar effect as the *T. b. brucei* strains we have investigated prior to this.

Due to the multi-step nature of T cell activation discussed in the introduction, further investigation could elucidate which step(s) the trypanosomes manipulate in order to promote this response. Keeping this in mind, use of Abatacept, a drug which prevents the CD28 co-stimulatory signal occurring in T cells, has revealed that this activation is partially CD28-dependent – in the case of both ICOS and PD-1, both markers were significantly reduced in Abatacept treated mice compared to their untreated infected counterparts. This suggests that the costimulatory T cell activation signal is important in propagating this response to trypanosome infection. However, LAG-3 staining seems in this instance to have possibly been aberrant – we might have expected LAG-3 expression to be significantly higher in infected mice than uninfected mice, however this was only true of the Abatacept treated mice. It is possible that LAG-3 is not up-regulated in infection, however previous work in the lab has indicated otherwise and would therefore suggest that there was an operator error or

problem with the reagent. Through the significant increase of ICOS and PD-1 expression in CD4+ cells during trypanosome infection (both $p<0.0001$, seen in Figure 9), we can note that high percentages of CD4+ cells are activated and display exhaustion markers following trypanosome infection. It can also be observed that use of Abatacept reduces the expression of both of these markers - ICOS expression reduced significantly ($p<0.0001$), and PD-1 reduced, however not significantly. This would imply that the method of activation of CD4+ cells during trypanosome infection involves use of the CD28 co-stimulatory signaling pathway. Interestingly, the number of parasites present in the blood at day 11 following Abatacept treatment is low to undetectable (Figure 8), where the untreated and isotype control treated maintain a detectable parasite level. As previously stated, this is not a significant result, and no real conclusions can be drawn from this. However, if further investigation shows this to be a true result, this could suggest that blocking CD4+ T cell activation could result in the clearance of the parasites from the blood. A repeat of this experiment with a possible longer time line involving Abatacept treatment would be required to prove any significant impacts of Abatacept treatment on blood parasitemia. As well as this, the isotype control treated mice (Figure 8) had a much higher secondary peak of parasitemia at day 10 – this could maybe point towards an inflammatory response to the control fusion protein resulting in a higher blood parasitemia.

Finally, having read in the literature available that blockade of inhibitory receptors lead to a clearance of parasites within the blood during malarial infection (Butler et al. 2012), we hypothesized that something similar may be happening during trypanosome infection. As such, we decided to block the inhibitory receptors PD-1 and LAG-3, and assessed the blood parasite burden on a daily basis. Antibody treatment was given from day 4 post-infection, and did not seem to clear parasites from the blood (Figure 10). The second peak of parasitemia for treated mice appeared to be lower than untreated and isotype treated controls, however by the time of the cull on day 14, the treated mice had blood parasite numbers comparable to that of

the untreated mice (100 x 10^6 parasites/ml of blood). One reason behind not seeing clearance of parasites could be that the infection needs to progress further, and the mice culled at a later date. This could also apply to the CD4+ T cell functionality following treatment – the effects of anti-PD-1 and anti-LAG-3 treatment which were noted during infection with *P. yoelli* (Butler et al. 2012) were seen during a longer term infection (34 days) than the one we carried out. As such, while we cannot say now that the antibody treatment results in blood parasite clearance or affects CD4+ T cell functionality, it may well do so further along in the infection, and it may therefore be beneficial to carry out a longer-term infection model.

Chapter 4: Results – Developing an *in vitro* assay which reproduces the CD4+ T cell activation seen *in vivo*

4.1 Introduction and Aims

In order to better elucidate the mechanisms underlying the polyclonal T cell activation observed during trypanosome infection in vivo I decided to develop an in vitro assay. This reductionist approach would allow me to test and manipulate both host and parasite components under carefully controlled and relatively high throughput in vitro conditions before going on to confirm them in more complex, lengthy, and expensive in vivo systems. I initially started with splenocytes with the intention of progressing to defined T cell and APC populations.

Similarly, I aimed to optimize the system using live parasites before progressing to parasite extracts and molecules and genetically manipulated parasites. The mechanism by which trypanosomes activate T cells polyclonally is unclear, and could occur by direct interaction of parasites or parasite secretions upon the T cells, or through other indirect mechanisms. An in vitro assay will allow us to assess each possibility quickly and inexpensively.

4.2 Live, Lysed and *Ex Vivo* parasites do not induce CD4+ T cell activation *in vitro*

In order to assess the effect of live, cultured STIB 247 trypanosomes on CD4+ cells, splenocytes were cultured with trypanosomes for up to 48 hours. A range of parasite numbers was also used, in order to determine an optimal number of trypanosomes for future experiments. The T cell mitogen concanavalin A (ConA, 2.5µg/ml) was used as a positive control (Lopes & Dos Reis 1994).

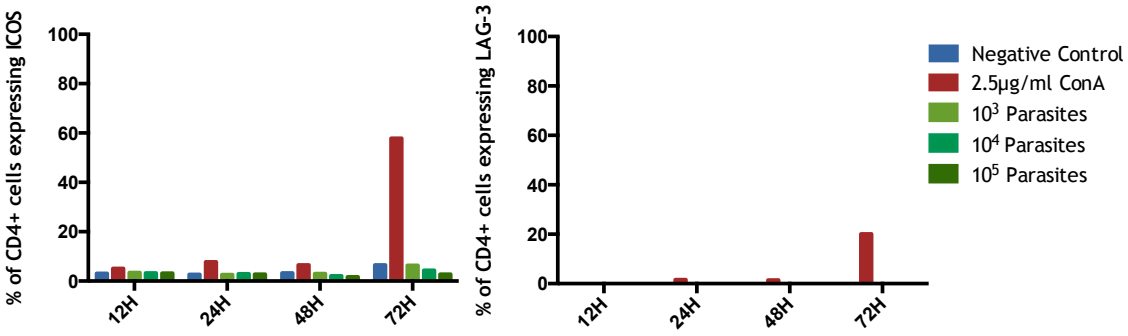

Figure 12: Live trypanosomes do not induce CD4+ lymphocyte activation. ICOS (left) and LAG-3 (right) expression following co-culture with 10^3, 10^4 and 10^5 trypanosomes was assessed on live CD4+ lymphocytes through flow cytometry.

ConA stimulation resulted in an increased expression of ICOS and LAG-3 on live CD4+ splenocytes after 72 hours co-culture, which indicates an activated phenotype. However, as seen in Figure 12, all live trypanosome co-cultures showed no increase in expression of either of these cell markers, suggesting that live trypanosomes alone do not activate CD4+ T cells. The experiment was only performed once and does not have any replicates with which to perform statistical analysis at this point. Parasites were live and appeared healthy at all time points during the experiment, as assessed by light microscopy. At 72 hours the number of splenocytes in culture seemed to be decreased, and the media had yellowed, suggesting cellular death leading to acidification of the media. This can also occur in response to overgrowth of cells leading to the depletion of nutrients within the media – however fewer splenocytes were seen at this time point, implying this was not the case. Live parasites do not appear therefore to promote proliferation of parasites *in vitro*. At this point, we decided to modify the media to allow for further cell survival and proliferation - specifically we added supplementary glucose, bringing the final concentration of the media to 4.5g/L, as trypanosomes are high metabolisers of glucose (Haanstra et al. 2014).

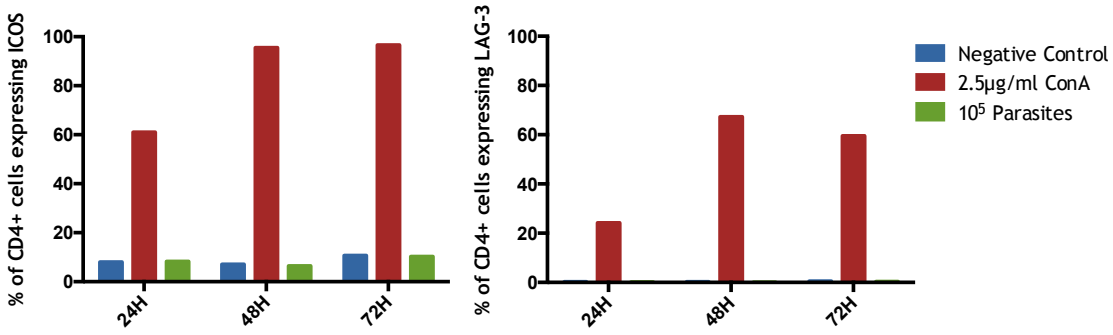

Figure 13: Lysed trypanosomes do not induce CD4+ lymphocyte actiation. Splenocytes were cultured with the lysate of 10^5 trypanosomes and cultured for up to 72 hours. Percentage of live CD4+ lymphocytes expressing ICOS (left) and LAG-3 (right) was assessed through flow cytometry. The experiment was performed once and has no replicates available for statistical analysis

As live trypanosomes did not induce CD4+ T cell activation, lysed trypanosomes were next investigated, in case the trypanosomal component involved is not secreted by the parasites, and must be released upon death or lysis. Again, ConA (2.5µg/ml) was used as a positive control, and parasites were lysed through a number of freeze-thaw cycles over a period of six hours. However, again it was noted that while ConA stimulated cells showed an increase in the expression of ICOS and LAG-3, cells cultured with the lysates of 10^5 parasites showed no significant upregulation of these markers, implying that lysed trypanosomes also do not activate CD4+ T cells (Figure 13).

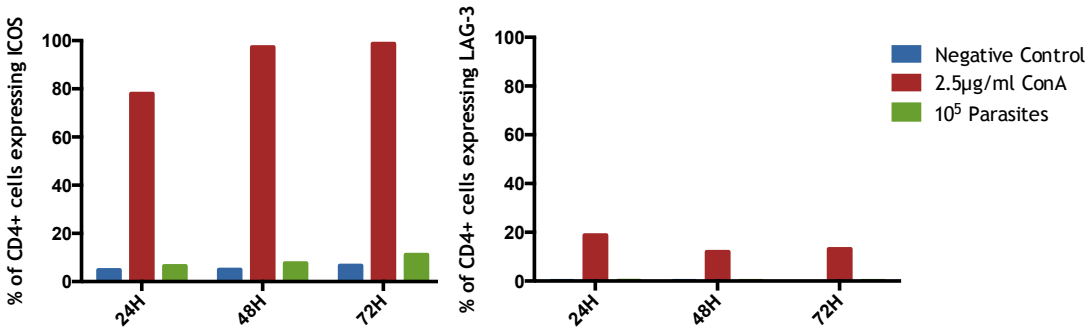

Figure 14: *Ex vivo* trypanosomes do not induce CD4+ lymphocyte activation. Trypanosomes were extracted from an infected Balb/c mouse 5 days following infection. Naïve splenocytes were then co-cultured with 10^5 trypanosomes and cultured for 72 hours. ICOS (left) and LAG-3 (right) expression following co-culture was assessed on live CD4+ lymphocytes through flow cytometry

Following the previous results, we considered the idea that the cultured parasites had adapted to culture, losing some of their ability to induce CD4+ T cell activation. Therefore, examination of the effect of *ex vivo* parasites upon CD4+ T cell activation was carried out (Figure 14). Blood was extracted from an infected Balb/c mouse, and the parasites were collected by centrifugation and added to splenocytes *in vitro* at a concentration 10^5 parasites per well. As before, 2.5µg/ml ConA was used as a positive control. As seen in previous experiments, the expression of ICOS and LAG-3 on CD4+ cells was upregulated in response to ConA stimulation, but remained similar to the negative control in the splenocyte and trypanosome co-culture wells. This, along with the previous two experiments, suggests that trypanosomes alone, whether live, lysed, or *ex vivo*, cannot induce the polyclonal CD4+ T cell activation that we have witnessed *in vivo*.

4.3 CD4+ T cell activation persists *ex vivo* for up to 72 hours

It remained possible that failure to reproduce *in vivo* CD4+ T cell activation in response to trypanosomes *in vitro* could be due to lack of an environmental factor in culture. In order to examine this possibility, splenocytes were taken from an infected mouse as opposed to a naïve, uninfected mouse, and cultured *in vitro* for 72 hours.

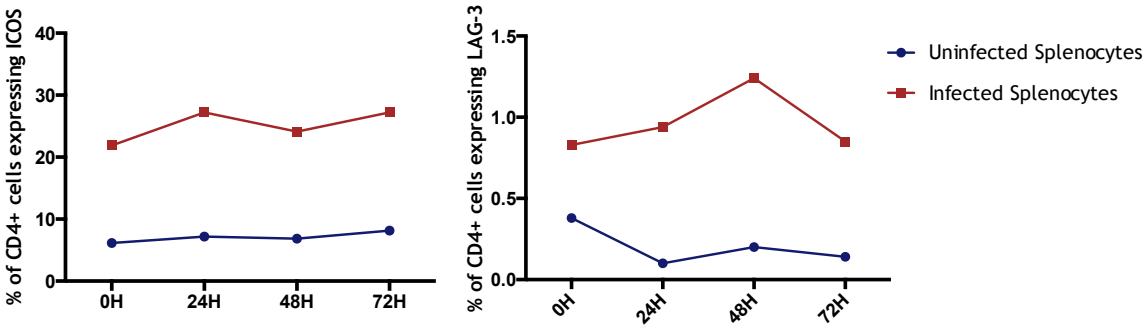

Figure 15: Activation of CD4+ lymphocytes during *T.b.brucei* infection persists *ex vivo* for up to 72 hours. Infected splenocytes were extracted 5 days post infection of Balb/c mice, and cultured *in vitro* for 72 hours. Cells were assessed for expression of ICOS (left) and LAG-3 (right) by flow cytometry at 24 hour time points.

We expected to see CD4+ cell activation at 0 hours *ex vivo*, and we hypothesized that if an environmental factor present in the live animal but not in in vitro culture was required to maintain the CD4+ T cell activation, we would see the CD4+ cell activation decrease in culture. Keeping this in mind, we noted that following extraction of splenocytes from the infected mouse, CD4+ cell activation (as measured by ICOS and LAG-3 upregulation) remained consistently and significantly higher than in the negative controls for up to 72 hours (Figure 15). This would suggest that our *in vitro* assay does not currently include a key component of the initial activation, however it does allow for sustained activation of CD4+ cells.

4.4 Trypanosomes can enhance Con A mediated activation of CD4+ T cells in vitro

As previously discussed, the ability of *ex vivo* infected splenocytes to retain CD4+ cell activation could be due to the initial activation signal having occurred *in vivo*, starting the chain of events. To test the hypothesis that an

initial signal absent from our in vitro cultures is required to work in tandem with the parasite in order to promote CD4+ T cell activation, sub-optimal levels of ConA (0.1µg/ml) were added to trypanosome and splenocyte co-cultures. These levels of ConA are sub-optimal for CD4+ T cell activation on its own, which is seen in Figure 16, but when added to splenocytes with cultured trypanosomes, an upregulation of both ICOS and LAG-3 can be seen 96 hours later, indicating CD4+ T cell activation.

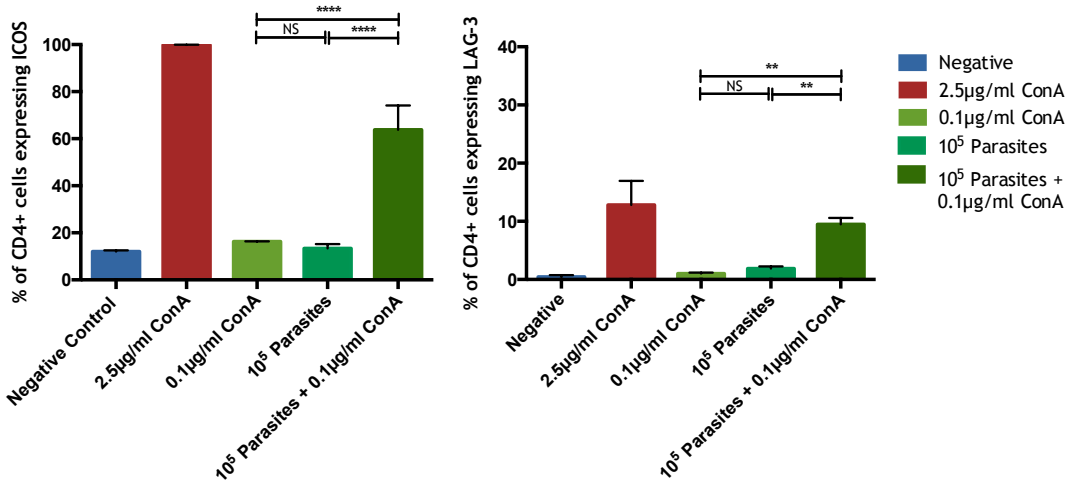

Figure 16: Presence of sub-optimal levels of ConA during trypanosome and splenocyte co-culture results in activation of CD4+ cells *in vitro* after 96 hours in culture. Naïve splenocytes were cultured in the presence of 10^5 parasites and 0.1µg/ml ConA for up to 96 hours. Cells were then assessed for levels of ICOS and LAG-3 expression by flow cytometry. Data represents mean ± SD. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

This is consistent with the result seen in a study of *Trypanosoma cruzi* infection (Gao & Pereira 2001), in which the authors noted that ConA and *Trypanosoma cruzi* parasites act in tandem to promote T cell activation and proliferation.

4.5 Discussion of Results

In this chapter I have begun to develop an *in vitro* assay to replicate the *in vivo* conditions seen in response to *T. b. brucei* infection.

From our development of an *in vitro* assay, we discovered that *T. b. brucei* parasites alone, whether they are live, lysed, or *ex vivo*, do not directly activate T cells in the polyclonal manner that we have come to expect both from the literature and from our *in vivo* observations. This hinted towards there being an indirect mechanism, or a synergistic route by which CD4+ T cells are activated *in vivo*.

Keeping this in mind, we found that parasites in the presence of a low level of mitogenic ConA results in a synergistic effect on CD4+ cells and promotes their activated phenotype. This had been shown to be the case in *T. cruzi* in a study by Gao & Pereira (Gao & Pereira 2001), however this had not been shown to be the case in *T. b. brucei* until now. Within this study, the authors also discuss a neuraminaidase expressed by *T. cruzi* that may be the potentiator of lymphocyte activation during infection. This neuraminidase, called trans-sialidase, is also expressed in *T. b. brucei*, and could therefore be the cause of lymphocyte activation during this infection. Further testing will have to be carried out in order to confirm this hypothesis. This assay could therefore be used to test for which *T. b. brucei* derived genes can activate CD4+ T cells in this manner – as noted formerly trans-sialidase would perhaps be a good place to start testing.

Discovering the mechanism by which trypanosomes activate T lymphocytes could have implications on the development of future treatments through an increased understanding of the polyclonal activation of T cells during infection, and the potential manipulation of this interaction.

Chapter 5: Results – Conclusions and Future Perspectives

In this thesis I aimed to further investigate the CD4+ T cell response to trypanosome infection. Two routes of investigation were used – the *in vivo* infection of mice and the *in vitro* assay development. Through *in vivo* work we have shown that large percentages of T cells are activated during infection with *Trypanosoma brucei brucei* and *Trypanosoma brucei gambiense*, and that this activation is polyclonal in nature, resulting in exhaustion of lymphocytes. This exhaustion partially contributes to the immunosuppression characteristic of trypanosome infection, and so understanding how it is initiated is crucial to the development of therapeutics manipulating this response in order to protect the host. With this in mind we also demonstrated through the use of Abatacept that this activation is dependent in part on CD28 signalling. The lower number of blood parasites present in mice following Abatacept treatment, if significant, could potentially indicate a link between T cell activation and the location of parasites within the host, and this could in turn explain both the presence of asymptomatic patients within the human population (Jamonneau et al. 2012) and the persistence of infection despite drug treatment and vector control. However, as previously discussed, this would need to be further investigated.

The work carried out in this thesis has also shown, through the development of an *in vitro* assay, that this activation occurs in response to trypanosomes in tandem with another activating signal, such as in the case of *T. cruzi* (Gao & Pereira 2001). The development of a working *in vitro* assay means that future work can involve testing candidate T cell activating trypanosome genes, through the use of knock out parasites and RNAi libraries, allowing for validation of theories before the expensive and time consuming *in vivo* models. This could lead to the elucidation of the genetic mechanism of trypanosome immune manipulation, and would also allow research into how to stop this effect during infection. This could lead to a more targeted therapeutic approach to the eradication of HAT and Nagana.

In this thesis, we also used antibodies to block LAG-3 and PD-1, both markers of T cell exhaustion, and hypothesized that this would have some effect on the number of parasites in the blood of the host. However, this was not the case for our experiment – there was no clearance of parasites from the host blood, and it seemed little effect was had upon CD4+ T cell functionality. It might be advantageous to see a longer-term infection, and the effect that these antibodies would have there, and to assess the presence of cytokines or chemokines associated with activation to determine lymphocyte functionality further.

This thesis has described CD4+ T cell response to trypanosome infection, both in murine infection models and *in vitro* assay development. With trypanosomes actively evading and suppressing the immune response in both humans and other mammalian infections, it is crucial to the development of new therapeutics that we seek to understand and characterize these responses.

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